Analysis of Protein Factors Involved in Transport of the Precursor of the Small Subunit of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase Into Intact Chloroplasts.

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Analysis of protein factors involved in transport of the precursor of the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase into intact chloroplasts

Pomarico, Steven Mark, Ph.D.
The Louisiana State University and Agricultural and Mechanical Col., 1992
ANALYSIS OF PROTEIN FACTORS INVOLVED IN TRANSPORT OF THE PRECURSOR OF THE SMALL SUBUNIT OF RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE INTO INTACT CHLOROPLASTS

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Biochemistry

by

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May 1992
ACKNOWLEDGEMENTS

I would like to gratefully acknowledge Dr. Anthony R. Means of the Department of Cell Biology at Baylor College of Medicine for the plasmid containing the chicken brain calmodulin cDNA. I would also like to thank Dr. Roland Douce of the Département de Recherche Fondamentale at the Centre D'Etudes Nucléaires, Physiologie Cellulaire Vegetale for the antibody against the spinach chloroplast envelope protein E30. These contributions greatly aided in the success of this research.

In addition, I would like to express my deepest gratitude to Dr. Sue G. Bartlett, affectionately known as Boss, for the friendship, patience and guidance she has contributed throughout this educational excursion. I am also thankful for the collection of "bent paper-clips" which she has attracted over the years including; Dr. Sam Landry, Dr. Tim Fawcett, Dr. Tom Moore, Carmen Dessauer, Rodney McFerrin, Mike Dzedza, Brian Zganjer, Michelle Fenchock, Braden Paul, and Micheal Carroll. This assortment of individuals have made the lab a stimulating, interesting, and above all enjoyable environment in which to work. I also am appreciative of the past and present members of the biochemistry department; faculty, students, and staff. These individuals have made my research and education not only possible but occasionally entertaining.
Aside from some of those individual already mentioned who are like a family. I would like to thank my sister, Carolyn Pomarico for the love, laughs and support she has provided and especially my mother and father, Elizabeth C. and Victor E. Pomarico, who gave me life and in so many ways showed me how to live it.
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Description of the Construction of Protein Fusions Consisting of Various Lengths of Wheat pS Fused to Calmodulin.
LIST OF ABBREVIATIONS

ACC = the mitochondrial ADP/ATP carrier
ADP = adenosine 5'-diphosphate
AMP-PCP = β,γ-methyleneadenosine 5'-triphosphate
AMP-PNP = β,γ-imidoadenosine 5'-triphosphate
ATP = adenosine 5'-triphosphate
BAM = benzamidine
bp = base pair
CaM = calmodulin
DEPC = diethyl pyrocarbonate
DHAP = dihydroxyacetone phosphate
DHFR = dihydrofolate reductase
DIDS = 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid
DTT = dithiothreitol
ε-ACA = ε-amino-n-caproic acid
EGTA = ethyleneglycol-bis(β-amino ethyl ether)-N,N'-tetraacetic acid
EPSP synthase = 5-enolpyruvylshikimate-3-phosphate synthase
ER = endoplasmic reticulum
GIP = the general insertion protein in the outer mitochondrial membrane
H₂-DIDS = 1,2-di-hydro-1,2-(4,4'-diisothiocyanato) diphenylethane-2,2'-disulfonic acid
[³H₂]DIDS = 1,2-di-tritio-1,2-(4,4'-diisothiocyanato) diphenylethane-2,2'-disulfonic acid
hsc70 = a constitutively expressed form of hsp70
hsp70 = a member of the 70 kDa heat shock protein family
L = the large subunit of RuBisCO
LE = soluble leaf extract
LHP = the light-harvesting chlorophyll a/b binding protein
MBN = mung bean nuclease
MBP = E. coli maltose-binding protein
NEM = N-ethylmaleimide
NPTII = neomycin phosphotransferase II
OAA = oxaloacetic acid
oATP = adenosine 5'-triphosphate-2',3'-dialdehyde
PBF = presequence binding factor
P_i = inorganic phosphate
pCoxIV = precursor protein of subunit IV of cytochrome c oxidase
pCoxIV-DHFR = fusion of the first 22 amino acids of pCoxIV and DHFR
pEPSP synthase = precursor to chloroplast EPSP synthase
3-PGA = 3-phosphoglycerate
pLHP = the precursor for the light-harvesting chlorophyll a/b binding protein
PLP = pyridoxal 5'-phosphate
PMSF = phenylmethylsulfonyl fluoride
pOAT = the precursor of ornithine aminotransferase
poly A+ = polyadenylated mRNA
poly A- = non-polyadenylated mRNA
poly U = polyuridylicate
pO-DHFR = the presequence of rat ornithine transcarbamylase fused to DHFR
pOTC = the precursor of rat ornithine transcarbamylase
PP_i = pyrophosphate
pro-OmpA = the precursor of outer membrane protein A
pS = the precursor to the small subunit (S) of RuBisCO
PTP = the mitochondrial phosphate transport protein
Put2 = the precursor of delta$^1$-pyrroline-5-carboxylase dehydrogenase

RuBisCO = the chloroplast enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase

S = the small subunit of RuBisCO

SITS = 4-acetamido-4'isothiocyanatostilbene-2,2'-disulfonic acid

SR = the SRP-receptor

SRP = the signal recognition particle

SSR = the signal sequence receptor

TPR = the tetratricopeptide repeat

ts = transit sequence
ABSTRACT

Two protein factors involved in the transport of precursor proteins into intact chloroplasts have been examined. The first of these was observed in experiments utilizing fusion proteins containing various lengths of wheat pS fused to CaM. Transport experiments with these fusion proteins demonstrated a requirement for transport of a pS/CaM fusion protein of a portion of mature S greater than 4 amino acids. Additionally, when the CaM portion of a fusion protein adopted a stable conformation by binding Ca\(^{2+}\), a larger portion of mature S was needed for efficient transport.

A second protein factor was examined using DIDS, and the related compounds H\(_2\)-DIDS, and SITS. Modification of intact pea chloroplasts with these reagents inhibited chloroplast protein transport. This inhibition was not due to disruption of the chloroplast envelope membrane or a non-specific effect on chloroplast metabolism.

The level of DIDS required to inhibit chloroplast protein transport correlates well with the level of DIDS which mediates inhibition of chloroplast ATP transport. While DIDS also inhibited chloroplast P\(_i\) transport, the level needed was lower than that required for inhibition of chloroplast protein transport. In addition, the presence of 3-PGA during DIDS modification did not prevent DIDS-based inhibition chloroplast protein transport.
The apparent presence of ATP inside the chloroplast and addition of ATP outside the organelle did not relieve inhibition of chloroplast protein transport caused by DIDS. These results indicate that the physical movement of ATP across the chloroplast envelope membrane may be required for chloroplast protein transport. Alternatively, a chloroplast envelope protein may function in a dual capacity to transport ATP and chloroplast precursor proteins.

Chloroplast envelope proteins labeled by \( ^{3}\text{H}_{2} \)-DIDS treatment of intact pea chloroplasts demonstrated that the chloroplast phosphate translocator is the most prominently labeled chloroplast envelope protein. However, an additional labeled species was resolved by SDS-PAGE on 12-18\% gradient gels containing 7 M urea. This additional labeled protein did not react with antibodies against the spinach chloroplast envelope protein E30 which appear to recognize the pea chloroplast phosphate translocator. This additional \( ^{3}\text{H}_{2} \)-DIDS-labeled protein may be involved in the transport of chloroplast precursor proteins.
INTRODUCTION AND LITERATURE REVIEW
The chloroplast is a complex organelle of plant cells. This organelle, like the mitochondrion, supplies metabolic energy for the cell. Two of the processes which occur in the chloroplast, photosynthetic electron transport and carbon fixation, furnish the thermodynamic base to which most other terrestrial life forms owe their existence. Though chloroplasts contain their own genetic material, they are not autonomous entities within the cell.

The interplay of two cellular protein-synthesizing systems is essential for the biogenesis and normal function of chloroplasts in higher plants. The cytoplasmic system translates nuclear-encoded polyadenylated (ploy A+) mRNA on free 80S eukaryotic ribosomes, while the chloroplast system translates mRNA derived from the chloroplast genome on organellar 70S ribosomes. Both systems are necessary, but the preponderance of chloroplast proteins are synthesized in the cytoplasm (1). The cytoplasmic synthesis of proteins which are destined for locations within various organelles or even outside of the cell is a common occurrence. In all cases where the site of protein synthesis is separate from its final destination, the obstacle of transport must be overcome.

The majority of chloroplast proteins encoded in the nucleus are synthesized as soluble precursor proteins on free cytoplasmic ribosomes (2,3). A precursor protein contains an amino-terminal extension termed the transit
peptide or transit sequence (4). After synthesis in the cytoplasm a precursor protein is post-translationally transported across the double membraned chloroplast envelope into the organelle (5,6). Proteolytic cleavage of the amino-terminal transit peptides of a precursor occurs during, or immediately after, the transport process (1).

The predominant cytoplasmically synthesized chloroplast protein is the precursor (pS) to the small subunit (S) of the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO E.C. 4.1.1.39) (6-9). This enzyme catalyzes two reactions: A) The assimilation of atmospheric CO₂ to form carbohydrates by the carboxylation of ribulose-1,5-bisphosphate to give two molecules of 3-phosphoglycerate (3-PGA), and B) the photorespiration reaction in which ribulose-1,5-bisphosphate is oxygenated to generate phosphoglycolate and 3-PGA (10). Ribulose-1,5-bisphosphate carboxylase/oxygenase probably is the most abundant protein in the world, and accounts for up to 65% of soluble leaf protein (11). In higher plants the RuBisCO holoenzyme is composed of eight cytoplasmically synthesized S subunits assembled in the chloroplast stroma with eight large subunits (L) to form the functional 18S holoenzyme (6,12). The large subunit of RuBisCO is encoded in chloroplast DNA and synthesized inside the chloroplast (13). The catalytic functions of the enzyme are ascribed to the large subunits, while a precise role for the small subunit remains unclear (10).
The process of post-translational protein import into chloroplasts has been studied for over a decade and, as yet, a precise mechanism is still unknown (8). Reconstitution of chloroplast protein transport in vitro has facilitated the study of the import process and helped in determining some of the components of the transport machinery (5,6). The in vitro synthesis of pS in a wheat germ cell-free system directed by poly A+ RNA from different species of higher plants and from the green alga Chlamydomonas reinhardtii, yields precursor proteins 4000-6000 daltons larger than mature S from the same species (5,6,8,9,12). Similar results have been obtained when cloned cDNA sequences encoding pS from either Pisum sativum or Triticum aestivum have been transcribed in vitro from plasmids containing the SP6 promoter, and then translated in vitro in a wheat germ cell-free system (14,15).

**TRANSIT PEPTIDES**

A transit peptide is required for the transport of most proteins into chloroplasts, including pS (14,16,17). Transit peptides of chloroplast precursors as well as mitochondrial precursor proteins function to direct the precursor proteins to the appropriate organelle membrane (17–20). Cytoplasmically synthesized chloroplast and mitochondria proteins which lack an amino-terminal transit peptide are hypothesized to follow a sorting pathway which
is different than that of typical precursor proteins (21-23). The ability of transit peptides to "target" or direct passenger proteins extends beyond the sorting of precursors to the correct organelle. Transit peptides may also provide information needed to direct intraorganellar targeting of newly imported proteins (18,23-27). Fusion of a transit peptide to a foreign protein can result in the targeting of the foreign protein to the organellar location directed by the transit peptide. These fusions may direct a passenger protein to an organelle instead of its usual cytoplasmic location, misdirect a protein from one organelle to another, or misdirect a protein to a different intraorganellar location (17,24,28-33). While the transit peptide is sufficient to direct the transport of a foreign protein into the chloroplast, a portion of the mature protein may be required for efficient targeting and transport (24,26,30,32,34-36). The mature portion of a precursor protein, or a portion of the passenger protein in the case of the fusion of a transit sequence to a foreign protein, is also involved in efficient targeting and transport into mitochondria (37-39). The mechanism by which the transit peptide and its associated passenger protein convey the information needed for correct and efficient targeting and transport into chloroplasts is not clear.

The structure-function relationship of chloroplast transit peptides has been investigated at the sequence
level. While "blocks of amino acid homology" have been found among some chloroplast transit peptides, common sequences are not present in all of them. Despite the lack of a homologous sequence for all chloroplast transit peptides, there are some features which they share. They are generally rich in serine and threonine, with these amino acids comprising 20-35% of the amino acid composition. Alanine and valine also are usually abundant. Chloroplast transit peptides are not rich in basic amino acids and therefore have a lower arginine/serine ratio than mitochondrial presequences. However, by virtue of a general deficiency of acidic residues, chloroplast transit peptides usually possess a net positive charge (1,40,41).

The importance of various types of amino acids for protein import into intact chloroplasts or proteolytic processing to generate mature polypeptides has been examined. In these experiments amino-acid analogs were incorporated into pea precursor proteins during in vitro synthesis by substituting one of the following analogs for the corresponding natural amino acid: azetidine-2-carboxylic acid, canavanine, S-(2-aminoethyl)-cysteine, β-hydroxynorvaline, and threo-β-DL-hydroxyleucine (substituting for proline, arginine, lysine, threonine, and leucine, respectively). The analogs to proline, arginine, and leucine all greatly inhibited the processing of pS to mature S, as well as its import into intact chloroplasts. It was not possible to determine if the effects of these
analogs were due to amino acid replacements in the transit sequence or in the mature region of the protein. However, since a variety of different passenger proteins, with potentially different secondary structures, can be targeted to the chloroplast, it seems likely that the significant changes conferred by these analogs are located in the transit peptide. The other analogs tested, S-(2-aminoethyl)-cysteine and β-hydroxynorvaline, also resulted in inhibition of import or processing, but to a lesser extent than those described above (42).

In experiments with mitochondrial precursor proteins, the substitution of various amino acids for certain arginine residues in the transit peptide resulted in varying levels of protein import (43,44). The change of a single arginine residue (position 23) to a glycine in the precursor of the mitochondrial matrix enzyme ornithine transcarbamylase, inhibits both processing and import into mitochondria in vitro. Replacement of the arginine residues at positions 14 and 15 in the transit sequence of the precursor to mitochondrial malate dehydrogenase with a variety of amino acids resulted in a range of diminished rates and relative amounts of protein import in most cases (43).

The minimal sequence homology which exists among transit peptides of various chloroplast precursor proteins indicates the involvement of secondary (or tertiary) structural features in the import process (41). Three
regions which could form structural features of transit peptides are common to some chloroplast precursors (46). These three regions are; A) an amino-terminal uncharged component, B) a non-amphiphilic central portion, and C) an amphiphilic β-sheet tract at the carboxy-terminus adjacent to the cleavage site (45,46). Deletion analysis of the transit peptide of pS has revealed that both the amino-terminal (residues 1-24) and the carboxy-terminal (residues 42-58) regions are essential for transport, while the central region (residues 26-35) has little effect on transport (47). The carboxy-terminal portion was further characterized to show that the last 10 amino acids (deletion of residues 48-56 and substitution of glycine for cysteine at residues 57) are needed for consistent processing to mature S, but are not required for efficient import of pS (48,49). Furthermore, the deletion of an additional three residues (amino acids 45-47 plus the changes noted above) resulted in decreased import efficiency, as well as a lack of processing to mature S (48).

Not all chloroplast transit peptides exhibit these regions, and in the case of precursors for thylakoid lumen proteins, an additional apolar carboxy-terminal region is present (41,45,46). The additional carboxy-terminal region corresponds to a thylakoid transfer domain and is part of a bipartite transit peptide arrangement found in precursors which are routed to the thylakoid lumen. In this bipartite
arrangement the amino-terminal portion acts as the stroma-
targeting segment of the transit peptide (24, 25, 27, 46). In
the chloroplast stroma the amino-terminus may be cleaved by
the stromal processing peptidase to generate a transport
intermediate (24, 25, 27, 50). The remaining carboxy-terminal
portion of the transit peptide is needed for thylakoid
localization and is analogous to signal peptides of
precursors for prokaryotic and eukaryotic secretory
proteins (46). A thylakoid processing peptidase removes
this extant portion of the transit peptide during, or
immediately after, transport into the thylakoid (50-52).

Targeting to the correct cellular location is
undoubtedly an important function of the chloroplast
transit peptide. The outcome of this targeting, and the
first step in the transport process, is the binding of the
precursor proteins to the chloroplast surface. The initial
demonstration that binding of precursor proteins to the
chloroplast surface and the subsequent import are separate
events was made utilizing a heterologous uptake system.
Binding of pea ps from in vitro cell-free translation of
pea poly A+ mRNA to intact spinach chloroplasts occurred in
the dark at 4°C. The bound precursor proteins were
susceptible to digestion by exogenous proteases. Upon
transfer of the chloroplasts to 25°C, the bound ps was
imported into the chloroplasts and converted to mature S,
as demonstrated by its inaccessibility to added proteases
(53). Envelope membranes isolated from pea or spinach
chloroplasts were shown to bind a number of precursor proteins generated by *in vitro* translation of poly A+ mRNA isolated from greening plants. One of the predominant proteins bound was pS. Furthermore precursor proteins were preferentially bound relative to mature proteins (54).

**ATP REQUIREMENT**

The adenosine 5'-triphosphate (ATP) requirement for binding of precursor proteins to the chloroplast surface has been demonstrated with isolated pea chloroplasts. Nonhydrolyzable ATP analogs cannot satisfy the requirement for ATP, and a protonmotive force across the chloroplast envelope is not involved in the binding of precursor proteins. Using conditions which favored the generation of ATP within the chloroplast while hydrolyzing extrachloroplastic ATP, it was ascertained that the ATP needed for precursor binding must be inside the chloroplast (55). The ATP requirement for precursor binding is not seen in the case of spinach chloroplasts where, under conditions which inhibit protein transport (0°C in the dark), binding was observed in the absence of added ATP (45). This apparent conflict may be explained by the observation that spinach chloroplasts maintain significant (approximately 600 μM) ATP levels even in the dark and in the absence of exogenous substrates (56).
In addition to the requirement of ATP for precursor binding, energy in the form of ATP is necessary for protein transport into chloroplasts (57). An ATP requirement for protein transport across cellular membranes is widespread. It is essential for post-translational protein transport into the endoplasmic reticulum (ER), mitochondria, peroxisomes, nuclei, and etioplasts in addition to chloroplasts (58-72). ATP is also required for the export of proteins out of Escherichia coli and quite possibly all Gram-negative bacteria (73-75). Among these protein transport systems, import into mitochondria and export of proteins out of Escherichia coli require more than ATP (58). In the case of mitochondrial protein import, a membrane potential across the inner membrane is required in addition to ATP (64,66). Translocation of proteins out of Escherichia coli appears to require both a membrane potential and a proton motive force across the membrane, as well as ATP. The presence of either the electrochemical, or the proton gradient in combination with ATP will support protein transport, however both are necessary for optimal protein export in Escherichia coli (73-75). Chloroplasts, on the other hand, have no requirements for either a membrane potential in general or a proton motive force to sustain protein import (76-80).

The energetics of protein transport into intact chloroplasts have obviously been extensively studied, and while the ATP requirement for import is clear, the precise
location of ATP utilization is still under debate (45,77-82). The dispute over the site of ATP usage stems from conflicting results regarding experiments in which ATP is depleted from different sides of the chloroplast inner envelope. In studies with intact spinach chloroplasts, ATP levels of intact chloroplasts were measured under various conditions (77). As stated earlier, spinach chloroplasts maintain significant ATP levels in the dark even in the absence of exogenous substrates (56). Glycerate can be transported into chloroplasts via the glycerate transporter, where it is metabolized to 3-PGA at the expense of ATP. The addition of glycerate to spinach chloroplasts incubated in the dark decreased the internal level of ATP only slightly (77). The hypothesis has been raised that the remaining ATP in the spinach chloroplast is tightly bound to the thylakoid ATP synthase (i.e. CF₁) and therefore not freely accessible. It was inferred from these results that the tightly bound ATP is not available to drive protein transport into the chloroplast. The level of spinach chloroplast ATP remains low (equivalent to control dark level), even when exogenous ATP is added to chloroplasts in the dark, so long as glycerate is present. Under these conditions of low stromal ATP level (externally added ATP, glycerate and incubation in the dark), protein transport occurs (56,77).

The ability of stromal ATP to drive protein transport was also examined in the light. In the light chloroplasts
accumulated a much higher level of ATP, and were able to actuate protein transport. In order to enzymatically remove ATP from the chloroplasts in the light, alkaline phosphatase was used. This resulted in a negligible decrease in the level of stromal ATP at a concentration of enzyme which removed externally added ATP completely. Under these conditions the import of proteins into intact chloroplasts was strongly inhibited. However, the use of other enzyme systems to remove ATP from outside the chloroplasts (phosphofructokinase/fructose 6-phosphate or hexokinase/glucose) resulted in only a 10% reduction of protein import. Based on these results it was concluded that the effect of alkaline phosphatase on protein import was due to the dephosphorylation of a component which functions in the transport process and is accessible from outside the outer chloroplast envelope (13).

An ATP analog, adenosine 5'-triphosphate-2',3'-dialdehyde (oATP) was used to inhibit the chloroplast ATP translocator and prevent the export of stromal ATP. In the presence of this analog, protein import in the light was inhibited. This result was interpreted as a demonstration that in the light, stromal ATP must be translocation out of the chloroplast to be used in the protein transport process. However, the oATP also inhibited protein transport in the dark even in the presence of added ATP (77). This seems to indicate that the oATP was inhibiting some chloroplast function which is involved in the
transport of proteins into the chloroplast under both light and dark conditions. Alternatively, the translocation of ATP may be involved in protein transport in a way other than providing ATP to the correct side of the chloroplast envelope. The oATP was not transported since only an approximate 15% decrease in CO₂-dependent O₂ evolution was seen at a concentration of 2 mM oATP, whereas, ATP analogs which are transported cause much greater levels of inhibition (77,83).

In similar experiments utilizing pea chloroplasts, other researchers concluded that the ATP used in protein transport was needed at the outer chloroplast envelope (79). This was concluded based on the inhibition of protein transport by glucose in the presence of increasing amounts of hexokinase. In these experiments ATP was generated inside the chloroplast in the dark by the addition of oxaloacetic acid (OAA), dihydroxyacetone phosphate (DHAP), and inorganic phosphate (Pᵢ). It had previously been shown that the combination of OAA/DHAP/Pᵢ could increase the level of ATP in chloroplasts in the dark (56). The inhibition of the hexokinase in this assay with glucose-6-phosphate restored protein transport completely. These results contrast with the experiments described earlier in which the combination of hexokinase and glucose resulted in only a small (10%) inhibition of protein transport (77,79). This discrepancy has not been resolved,
though several additional studies have been reported (45,78,80-83).

In one of these studies, the ability of hexokinase/glucose to inhibit protein transport was again examined. Pea chloroplasts in the dark were provided ATP for protein transport in one of two ways; direct addition of ATP to the assay, or the addition OAA/DHAP/P₄, both methods could drive protein transport. However, when the combination of hexokinase/glucose was added, protein transport driven by added ATP was dramatically inhibited, whereas, protein transport driven by ATP synthesized from the added OAA/DHAP/P₄ mixture appeared unchanged. Comparable results were observed when apyrase, an enzyme which hydrolyzes both ATP and adenosine 5'-diphosphate (ADP), was used to consume ATP instead of hexokinase/glucose. These results support the conclusion that ATP synthesized inside the chloroplast by the OAA/DHAP/P₄ system is not required outside the chloroplast for protein import (78).

The effect of these energy consuming systems also was examined in spinach chloroplasts under both dark and light conditions. Protein import was analyzed using chloroplasts in the dark with added ATP and light incubated chloroplasts without additional ATP. The combination of hexokinase/glucose diminished protein import in the dark, but showed no effect on protein import into chloroplasts in the light. Apyrase completely inhibited protein import in
the dark chloroplasts which had added ATP, however, import in the light was not reduced. This, and some of the other evidence described here, indicates that ATP is required inside the chloroplast for protein import to occur. However, the results merely show that protein import into intact chloroplasts is independent of ATP outside the outer envelope and does not exclude the possible requirement for ATP in the intermembrane space(45).

In yet another study using pea chloroplasts in the dark, the levels of ATP inside and outside the chloroplast were measured under various conditions, and the capacity for protein import was also determined. ATP for protein transport was provided as described in the aforementioned investigation (by direct addition of ATP or via synthesis from OAA/DHAP/P$_i$). Again, as in earlier studies the combination of hexokinase/glucose was employed to dissipate ATP external to the chloroplasts, and glycerate was used to attenuate ATP levels inside the chloroplasts. When no additions were made to the chloroplasts, there was very little protein import observed (<200 molecules per chloroplast after 9 min) and the ATP levels inside and outside of the chloroplasts were correspondingly low (1.82 nmol/mg chlorophyll and <0.03 nmol/mg chlorophyll, respectively). Comparable results were seen when both sides of the chloroplast envelope were depleted of ATP by addition of glycerate and the combination of hexokinase/glucose. Under conditions where the ATP level
inside the chloroplasts was high (5.71 nmol/mg chlorophyll) and external ATP was expended (<0.03 nmol/mg chlorophyll), protein import achieved the highest level of all conditions assayed (1,200 molecules per chloroplasts after 9 min). This level of protein import was even slightly higher than conditions which promoted ATP formation inside the chloroplast at the same time that ATP was added to the outside of the chloroplast, and therefore, must be considered the optimum amount of protein transport in this assay. These results supported the conclusion that the ATP needed for protein transport is utilized inside the chloroplasts (80). However, the conditions of this assay clearly demonstrate that an abundance of ATP must be present when the OAA/DHAP/P$_i$ system is used to generate ATP. While it is not surprising that there was no measurable ATP present outside of the chloroplast when no additions were made, it seems unlikely that the presence of the ATP consuming system (hexokinase/glucose), at the levels employed in the assay, would be capable of completely removing all of the ATP from outside of the chloroplasts. Since the amount of protein import increases steadily throughout the assay (3, 6, and 9 min time points) it can be assumed that the level of ATP inside the chloroplasts remains high during the assay. The rate of nucleotide transport in pea chloroplasts is 330 µmol ATP/g chlorophyll/min, at that rate, the chloroplasts in the import assay should transport approximately 6.5 nmol of
If ATP is plentiful inside the chloroplast, then it seems reasonable to assume that the chloroplast ATP/ADP translocator could provide a significant amount of ATP to the outside of the chloroplasts. This could result in an increase in ATP in the space between the inner and outer chloroplast envelope membranes. The hypothesis that the ATP utilized for protein import may be in the intermembrane space has been proposed but has not been thoroughly examined (45, 79-81).

The chloroplast outer envelope is nonspecifically permeable to all small molecules, both charged and uncharged, and has an exclusion limit for molecules between 7,000-13,000 $M_r$ (85). This should permit the free movement of ATP between the intermembrane space and the solution surrounding the chloroplast. However, a transient increase in the ATP level of the intermembrane space may be possible. An alternative explanation may be that the movement of ATP across the chloroplast envelope is required for protein import into intact chloroplast.

The rate of nucleotide transport in pea chloroplasts (330 $\mu$mol ATP/g chlorophyll/min) is more than four times greater than in spinach chloroplasts (75 $\mu$mol ATP/g chlorophyll/min) (86). This difference in the rates of ATP transport was first hypothesized in a study which showed that the stimulation of CO$_2$ fixation by ATP is higher in pea chloroplasts than in spinach chloroplasts (87). The level of stimulation by ATP of CO$_2$ fixation in pea
chloroplasts varies with the age of the plants from which the chloroplasts were isolated, with young chloroplasts exhibiting a greater degree of stimulation than older chloroplasts (88). In addition, pyrophosphate (PP\(_i\)) inhibits CO\(_2\) fixation in chloroplasts isolated from young peas (89). This inhibition is suggested to be caused by the exchange PP\(_i\) for endogenous ATP via the ATP/ADP translocator which results in a depletion of the internal ATP pool. The inhibition of CO\(_2\) fixation by PP\(_i\) was not significant for spinach chloroplasts, or pea chloroplasts isolated from older plants. This again indicates that the ATP/ADP translocator may vary in function with maturity of tissue and possibly between different species.

The rate and total amount of protein import observed in pea chloroplasts were inhibited by oATP (80). Similar results were seen with spinach chloroplasts. Two other ATP analogs, \(\beta,\gamma\)-methyleneadenosine 5'-triphosphate (AMP-PCP) and \(\beta,\gamma\)-imidoadenosine 5'-triphosphate (AMP-PNP), inhibit low levels of protein import in pea chloroplasts in the absence of exogenous ATP (78, 79). When ATP is added at the same level as the analogs (6.6 mM), the inhibition by AMP-PCP is relieved. However, exogenous ATP could not completely overcome AMP-PNP inhibition of protein import (79). Experiments with lower concentrations of the analogs (1mM versus 6.6mM) resulted in complete inhibition of protein transport by AMP-PNP, and partially inhibition by AMP-PCP with intact pea chloroplasts in the light (78).
The inhibition by AMP-PCP was alleviated by addition of an equal amount (1mM) of ATP, whereas ten times the amount (10mM) of ATP was required to overcome the inhibition of protein import caused by AMP-PNP (78).

The inhibition of protein import by AMP-PNP and AMP-PCP may be a consequence of the action of the analogs within the chloroplasts since it appears that both analogs are transported into chloroplasts by the ATP/ADP translocator (83). Inhibition of CO2 fixation by both AMP-PNP and AMP-PCP is a result of the ability of these analogs to exchange for chloroplast internal ATP and decrease the endogenous ATP levels. AMP-PNP also inhibits binding of precursor proteins to intact pea chloroplasts when added in equimolar amount with ATP (55). Inhibition of binding by AMP-PNP could be relieved by the addition of a ten fold excess of ATP, as was the case with AMP-PNP inhibition of protein import. AMP-PNP and AMP-PCP also are transported by the mitochondrial adenine nucleotide translocator (90,91). However, the effects of these analogs on protein import into intact mitochondria have not been examined.

Protein import also was inhibited by PPi in intact pea etioplasts. The addition of PPi (2mM) inhibits protein import even in the presence of added ATP (1mM). This inhibition is alleviated by the addition of pyrophosphatase (10 units) which hydrolyzes the PPi (79). In similar studies with pea chloroplasts in the light, PPi (1mM) inhibited protein transport only slightly (≈7% decrease)
This low level of inhibition was overcome by addition of ATP (1mM). An increased level of inhibition of protein import (≈20-30% decrease) occurs at higher concentrations (10mM) of PP\textsubscript{i}. The inhibition of protein import by PP\textsubscript{i} was relieved by pyrophosphatase which is in agreement with previous results. In comparison to the ATP analogs, PP\textsubscript{i} appears to exchange for ATP more slowly via the ATP/ADP translocator (83). This difference may be a result of the lower affinity of the ATP/ADP translocator for PP\textsubscript{i}.

The aforementioned analyses all were aimed at determining where the ATP requirement for protein transport into intact chloroplasts is localized. Studies also have been performed examining how ATP may be utilized in the protein transport process. One proposed mechanism involves the phosphorylation of precursor proteins or a membrane component to create a high energy intermediate in the transport process. Phosphorylation of S by an envelope-bound kinase prompted a analogous hypothesis for chloroplast protein transport (92). A corresponding proposition also has been proposed for protein transport across the ER (59). While no kinase activity has been linked to protein import in chloroplasts, an inhibitor of phosphatases, sodium fluoride, inhibits protein transport into both etioplasts and chloroplasts from pea and chloroplasts from spinach (72,77-79).
The phosphorylation of a 51 kDa outer envelope membrane protein is related to the import of proteins into intact spinach chloroplasts. Protein transport was inhibited when chloroplasts were incubated with increasing concentrations of pyridoxal 5'-phosphate (81). Pyridoxal 5'-phosphate (PLP) can form a Schiff base with free amines, such as the ε-amino group of lysine residues. The resulting Schiff base then can be irreversibly reduced, resulting in covalent modification of a protein which reacts with PLP (93).

The most obvious product of PLP-based modification is a 29 kDa chloroplast envelope protein. However, several other less abundant chloroplast envelope proteins also are targets of this reagent (93,94). The 29 kDa envelope protein which is modified by PLP is the chloroplast phosphate translocator, and the modification results in an inhibition of translocator function (81,93). The chloroplast ATP/ADP translocator is not inhibited by treatment of intact spinach chloroplasts with PLP (81). The inhibition of the chloroplast phosphate translocator by PLP can be ameliorated if phosphate is present during the PLP modification reaction (93). The inhibition of phosphate transport by PLP in the presence of phosphate shows characteristics indicative of competitive inhibition.

Under conditions which inhibit protein transport (treatment with PLP), the level of phosphorylation of the 51 kDa protein increased (93). The $K_m$ for MgATP for
phosphorylation of the 51 kDa envelope protein was determined to be approximately 5 \( \mu M \), while the apparent \( K_m \) for ATP for protein import into intact chloroplasts is roughly 0.9 mM (77,81). This difference seems to indicate that a phosphorylation/dephosphorylation cycle of the 51 kDa protein may function in the binding of precursor proteins to the chloroplast, instead of during translocation portion of import (41).

While there may still be some contention regarding the precise role ATP plays in the import of proteins into intact chloroplasts, the requirement is indisputable. ATP also is required for intrachloroplastic sorting of proteins into the correct compartment. This requirement has been demonstrated for the integration of the apoprotein of the light-harvesting chlorophyll \( a/b \) binding protein (LHP) into the thylakoid membrane (95,96). Proteins which ultimately are localized in the thylakoid lumen also require ATP to traverse the thylakoid membrane (97,98).

As outlined earlier, precursor proteins are proteolytically modified, or processed, either during the transport process or immediately afterward (1). This processing step involves cleavage of the amino-terminal transit peptide of chloroplast precursor proteins. The processing activity which converts pS to S is localized in the chloroplast stroma (99). The partially purified stromal processing protease from pea chloroplasts processes the precursors of both stromal and thylakoid proteins to
mature size (100). Furthermore, the processing of pS to S proceeds via a processing intermediate, and the same enzyme may catalyze both steps (101). An intermediate also was found in the processing of pS to S in *Chlamydomonas reinhardtii* (102). The intermediate processing form of *Chlamydomonas reinhardtii* pS also has been detected when the algal precursor is imported by isolated pea or spinach chloroplasts (16).

Proteins destined for the lumen of the thylakoids also may be processed in two steps, with the stromal processing protease cleaving the precursor to an intermediate form. An additional processing protease, located in the thylakoids, completes the processing to the mature form (24,51). The thylakoidal processing protease has been partially purified and processes the intermediate form of a thylakoid lumen precursor to the mature size (103). This thylakoid processing enzyme also can process lumen-bound precursors directly to their mature size, but at a lower rate. Neither the stromal nor the thylakoidal processing peptidase requires ATP (100,103).

**PRECURSOR UNFOLDING**

Many facets of protein transport remain to be explored and clarified. The post-translational transport of a protein through the chloroplast envelope seems to be a monumental task. Relative to the metabolites which are
transported across cellular membranes, proteins are enormous. Evidence indicates that an unfolding process is necessary for a completed precursor protein to traverse a membrane (58).

Mitochondrial precursors may be arrested during the translocation process, such that the amino terminal presequence extends into the mitochondrial matrix while a major portion of the polypeptide chain remains outside of the organelle. This is accomplished either by lowering the temperature of the transport assay or by preincubation of the precursor protein with antibodies which recognize and bind the mature portion of the protein. When protein transport is arrested with a precursor in this membrane-spanning configuration, the presequence can be processed by the matrix protease, while the external portion of the polypeptide remains susceptible to exogenous protease treatment. These results indicate that the transit sequence precedes the mature portion of a precursor into the mitochondrial matrix (104).

Evidence that the precursor protein unfolds during transport was obtained using a fusion protein comprised of a mitochondrial presequence fused to dihydrofolate reductase (DHFR). This fusion protein is imported into intact mitochondria and the presequence is removed by the mitochondrial processing protease. The DHFR portion of the precursor retained its enzymatic activity and the ability to tightly bind methotrexate, a folate antagonist.
Addition of methotrexate to the transport mixture inhibited import of the DHFR fusion protein but had no effect on the import of other precursor proteins. Moreover, methotrexate did not significantly change the binding of the fusion protein to intact mitochondria or the ability of the mitochondrial processing protease to remove the presequence. Thus it appears that binding of methotrexate to DHFR prevented unfolding of that portion of the precursor protein and thereby inhibited translocation of the fusion protein into intact mitochondria (105).

A series of experiments exploiting the DHFR fusion protein described above have shown that unfolding of precursor proteins occurs outside the mitochondrion as a part of the import process (106-108). Transport of a urea-denatured fusion precursor was more efficient and occur faster (at least 10-fold) than translocation of the native fusion precursor. In addition, transport of the urea-denatured precursor could take place at lower temperature than the native precursor. Urea-denaturation of the DHFR fusion also prevented the inhibition of transport by methotrexate, indicating that the unfolded precursor was not capable of binding the folate antagonist (106).

Several lines of evidence also demonstrate that precursor of DHFR fusion proteins bound to the mitochondrial surface prior to import exist as unfolded intermediates. These unfolded, membrane-bound intermediates could be transported in the presence of
methotrexate, could be transported at much lower temperatures than native precursors, and were sensitive to trypsin digestion (trypsin resistance is a characteristic of the DHFR portion of the undenatured precursor) (106). Several point mutations introduced into the DHFR portion of the precursor appeared to decrease stability of the folded precursor proteins as judged by increased sensitivity to trypsin treatment. Precursor proteins containing these point mutations were less susceptible to inhibition of import by methotrexate or lower temperatures (107).

Binding of the DHFR fusion precursor to the mitochondrial surface and subsequent unfolding proceeds in the absence of ATP, and appears to require only an energized membrane (107). However, the release of the bound intermediate for ensuing import requires ATP, but is independent of a membrane potential (106). Incubation of this precursor–DHFR fusion protein with vesicles rich in acidic phospholipids produced a partial unfolding of the precursor protein (108). Taken together these results suggest that a precursor must be unfolded for transport and that interaction of the precursor with the organellar membrane leads to unfolding of the precursor protein.

Results of studies with the maltose-binding protein (MBP) of *Escherichia coli* also indicate that efficient protein export requires an unfolded precursor. The precursor to MBP attains a stable tertiary conformation similar to the mature protein. In the folded conformation
MBP and its precursor are resistant to proteolysis, this characteristic permits the protein conformation to be assessed indirectly. MBP containing a point mutation in the leader sequence folded faster than the wild type precursor in vitro, and was exported more slowly than the wild type precursor in vivo. In addition, the folding kinetics of the mutant precursor (judged by proteolytic sensitivity) correlated with its ability to be transported. The effect of the mutation in the precursor to MBP could be partially overcome by a suppressor mutation (prlA4). In the presence of this suppressor mutation, the mutant MBP was exported to a greater extent and also folded more slowly. To establish that these results were not caused by the mutation in the leader sequence directly (instead of the faster folding of the mutant precursor) an experiment was performed in which the protein export apparatus was saturated. This resulted in an increased degree of folding of precursor to wild-type MBP (109). These results demonstrate that in E. coli as with mitochondria the presence of a stable tertiary conformation of a precursor protein correlates inversely with the capacity for protein translocation.

The involvement of precursor unfolding also has been proposed for transport of proteins into intact chloroplasts. The herbicide N-[phosphonomethyl]glycine (glyphosate) inhibits transport of the precursor to the chloroplast protein 5-enolpyruvylshikimate-3-phosphate
synthase (pEPSP synthase) (EC 2.5.1.19). The inhibition only occurs in the presence of the substrate, shikimate 3-phosphate, and is specific for pEPSP synthase. A mutant form of pEPSP synthase which does not bind glyphosate shows no inhibition of transport with this herbicide. These results suggest that unfolding of pEPSP synthase is inhibited by binding of the herbicide to the precursor protein and that inhibition of unfolding impairs transport (110).

**SOLUBLE FACTORS**

It seems clear from the results discussed above that precursor conformation plays a part in the translocation process. Since thermodynamic constraints will guide a protein to achieve its most stable folded conformation a mechanism for protein unfolding must be a part of the import process. As discussed above for mitochondria the membrane across which transport occurs may be involved in the unfolding of precursor proteins. Soluble protein factors also may function to maintain precursor proteins in a conformation which permits translocation. The requirement for a soluble factor(s) for post-translational transport of proteins has been demonstrated for protein export from *Escherichia coli*, as well as into the ER, mitochondria and chloroplasts (111-119).

In *Escherichia coli*, a soluble activity was required for protein export (assayed as import into inverted
vesicles). This "activity" was characterized as a 12S export factor, which was necessary for translocation but was not required for protein synthesis. It was proposed that this factor was a complex of molecules which may be related to several genetically described export factors (111). Six genes involved in protein transport have been identified by mutational analysis in *Escherichia coli*; *secA* (*prlD*), *secB*, *secD*, *secE* (*prlG*), *secF*, and *secY* (*prlA*) (112). The protein products of most of these genes have been identified and their functions in the protein export pathway partially characterized.

One of these proteins, SecB, is a soluble protein which interacts with the precursor for *E. coli* MBP. The interaction of the MBP precursor with SecB prevents premature folding of the precursor protein into a conformation which is not exportable (113). It had previously been determined that proteins which were export-defective could interfere with the export of some normal proteins in *Escherichia coli* (114). A series of proteins were constructed containing various portions of MBP, or fusions of MBP to β-galactosidase. These proteins were examined for their ability to interfere with the export of normal precursor proteins. The results of these experiments indicated that a region of mature MBP between amino acid residues 150 and 186 must be present in the export-defective protein in order for it to disrupt export of some normal *Escherichia coli* proteins. This
interference in protein export by an export-defective protein could be overcome by the overexpression of SecB. Two additional mutant MBP precursor were determined to be less sensitive to export disruption by the presence of an export-defective protein. These mutant precursor proteins were also more efficiently exported from SecB− E. coli than wild-type precursor for MBP (113). These results demonstrate that SecB binds to the precursor of MBP, in part recognizing a portion of the mature protein, and maintains the precursor in a conformation suitable for subsequent translocation.

The translocation of another Escherichia coli protein, the precursor of outer membrane protein A (pro-OmpA) also requires a soluble factor. A soluble factor in the S100 fraction of E. coli cytosol was required for the translocation of radiochemically pure [35S]-pro-OmpA into inverted membrane vesicles. The mere addition of the S100 fraction was not sufficient to render the purified pro-OmpA translocation-competent. The soluble activity had to be present during the purification step where pro-OmpA was able to re-fold. This soluble activity appears to promote the attainment of a translocation-competent form of pro-OmpA. The component of the S100 fraction which assists pro-OmpA in achieving the translocation-competent form has been termed "trigger factor" since it is thought to trigger membrane-assembly-competent folding (115).
Further characterization has shown that trigger factor is a soluble 63 kDa monomeric protein which complexes with pro-OmpA in a 1:1 ratio (116). Trigger factor also has been found associated with the 50S subunit of \textit{E. coli} ribosomes in a 1:1 ratio. This ribosomal subunit contains the exit domain for nascent polypeptides. Such an arrangement may permit trigger factor to associate with nascent polypeptides as they are synthesized. Free trigger factor can compete with the pro-OmpA-trigger factor complex for binding sites on the \textit{E. coli} membrane (117).

A model for the action of trigger factor has been devised based on these results: A) trigger factor binds pro-OmpA during or soon after completion of protein synthesis, B) trigger factor stabilizes pro-OmpA in a translocation-competent form and the complex binds to a membrane binding site, C) after binding the membrane pro-OmpA is released from trigger factor for translocation, D) trigger factor is released from the membrane binding site and is free to rebind the ribosome (117). Thus trigger factor appears to play a role in protein export from \textit{E. coli} which is analogous to that of the eukaryotic signal recognition particle (SRP) in the translocation of protein across the ER membrane. In fact, SRP also can stabilize pro-OmpA in a translocation-competent form (116,117).

In addition to SecB and trigger factor, other soluble factors may play a role in the translocation of proteins across the \textit{E. coli} membrane. Two additional proteins,
GroEL and GroES, acting in concert may assist in maintenance of an assembly- or folding-competent conformation of newly-synthesized proteins (118). GroEL is a tetradecamer with subunits arranged in two rings (7 subunits each), and GroES is comprised of 6-8 subunits in a ring structure (119-121). The \textit{groE} genes are essential for cell viability and are subject to heat shock regulation (118).

GroEL has been identified by photo-crosslinking to be a major component of a complex formed with the nascent polypeptide pre-\(\beta\)-lactamase. If pre-\(\beta\)-lactamase is incubated (and presumably allowed to fold) after translation without GroEL, then the interaction between the precursor and GroEL is not seen. In addition, the presence of GroEL increases the length of time after the completion of translation in which pre-\(\beta\)-lactamase remains translocation-competent (122). These results indicate that GroEL may bind to the nascent pre-\(\beta\)-lactamase precursor and prevent it from folding into a translocation-incompetent form.

SecB, trigger factor, and GroEL all have been implicated in maintaining precursor proteins in a translocation-competent form (123). These protein factors (and others like them) have been termed chaperones. Another heat-shock protein, DnaK, also has been added to the list of proteins connected with the facilitation of protein export in \textit{Escherichia coli} (124). Additional,
though as of yet unidentified, heat-shock proteins can functionally substitute for SecB function and also may be part of the protein export process in *E. coli* (125).

The ability of the SRP to stabilize a bacterial precursor protein (pro-OmpA) in a form competent for transport across bacterial membrane vesicles was discussed above. Related experiments examining protein translocation across ER microsomes have shown that pro-OmpA could maintain a translocation-competent form if incubated with either trigger factor or SRP (126). As with other translocation-competent precursors, pro-OmpA is more sensitive to protease treatment in the presence of SRP. This indicates that SRP may maintain pro-OmpA in a partially unfolded state.

The only presumed function of SRP related to folding is to preserve the signal sequence conformation such that it remains accessible to start the translocation process (127). The SRP is apparently not an "unfoldase", since it can only preserve the conformation of the signal sequence while it is still unfolded. Once the nascent protein attains a folded, translocation-incompetent conformation, the addition of SRP is ineffective at rendering the precursor translocation-competent (128).

Another cytosolic factor that plays a role in the translocation of proteins into the ER is a member of the 70 kDa heat shock protein (hsp70) family (129-131). Genetic studies revealed that mutations in either of the genes
coding for two constitutively expressed yeast hsp70 proteins (ssal/ssa2) resulted in a wild-type phenotype, while double mutants displayed temperature sensitive growth (132). Therefore, these two genes (ssal and ssa2) are functionally related.

Mutations in yeast which disrupt the chromosomal copies of these constitutive hsp70 genes can be rescued by placing one of the hsp70 genes on a plasmid. Repression of expression of a plasmid borne copy of ssa1 resulted in depletion of cytoplasmic hsp70 and accumulation of the mitochondrial precursor of the β-subunit of F\textsubscript{1}ATPase, and of the precursor of a secretory protein, prepro-α-factor. Partially-purified SSA1 was unable to stimulate translocation of prepro-α-factor into yeast ER in vitro unless supplemented by a yeast post-ribosomal supernatant fraction (129). The component(s) of this fraction required for protein translocation has not been identified.

The yeast hsp70 proteins involved in protein transport, SSA1 and SSA2, are isoforms of the 70 kDa heat shock proteins. The presence of these two (or possibly either) hsp70 proteins stimulated the initial in vitro rate of translocation of native prepro-α-factor into yeast microsomes by approximately eight-fold. Prepro-α-factor denatured with urea prior to translocation had an initial rate of transport 11-fold faster than the native precursor. This is another indication that protein translocation requires precursor unfolding. Addition of hsp70 to urea
denatured prepro-α-factor yielded a further stimulation of translocation relative to the denatured precursor alone. Taken together, these results suggest that hsp70 plays a role in unfolding of precursor proteins prior to translocation, by either stimulating the unfolding process or, more probably, by stabilizing the unfolded precursor so that refolding does not readily occur (130).

Hsp70 also stimulates protein translocation into ER from canine pancreas (131). A constitutively expressed form of hsp70 (hsc70) isolated from rat stimulated translocation of the precursor to M13 coat protein (procoat). The presence of hsc70 also altered the protease sensitivity of procoat, possibly indicating a direct interaction between hsc70 and the precursor (130). An additional unidentified factor from rabbit reticulocyte lysate also was required for optimal stimulation of protein transport by hsc70.

Hsp70 interacts with newly-synthesized proteins in HeLa cells. This interaction appears to occur co-translationally since hsp70 was associated with polypeptides which were released from ribosomes prior to completion of protein synthesis. In addition, hsp70 cosedimented with polysomes, presumably due to interaction of hsp70 with nascent polypeptides. Association of hsp70 with these newly synthesized proteins was transient and ATP dependent (133). This general interaction of hsp70 with nascent polypeptides may facilitate post-translational
transport of proteins into a number of organelles. If hsp70 functions to stabilize precursor proteins in an unfolded, translocation-competent form, then it may be functionally analogous to SecB or Dnak in *E. coli* (123). Significant amino-acid homology (48%) between Dnak and hsp70 lends weight to this analogy (125).

The first evidence that import of mitochondrial proteins requires a soluble factor was demonstrated in experiments studying the import of a 34 amino acid peptide. This peptide, corresponding to the presequence of the precursor of ornithine aminotransferase, was chemically synthesized and utilized for import experiments. The peptide was imported into mitochondria in an ATP-independent manner, and protein import was stimulated significantly by addition of rabbit reticulocyte lysate. Further experiments indicated that a high molecular weight (≈200 kDa) proteinaceous factor present in the reticulocyte lysate could form a complex with the presequence peptide and was required for binding of the peptide to the mitochondrial membrane (134).

Biochemical evidence was obtained following the genetic evidence (described earlier) to confirm a role for the involvement of hsp70 in protein import into mitochondria. Import of the precursor of delta¹-pyrroline-5-carboxylase dehydrogenase (Put2) into intact mitochondria *in vitro* required a factor(s) present in a yeast post-ribosomal supernatant. Fractionation of the post-ribosomal
supernatant shows that at least two cytosolic factors stimulated protein translocation. One of these factors was the pair of related hsp70 proteins. The hsp70 proteins stimulated protein import only slightly when added to the import assay in the absence of the post-ribosomal supernatant. However, addition of purified hsp70 to the post-ribosomal supernatant (which already contained hsp70 proteins) further enhanced the stimulation of protein import afforded by the post-ribosomal supernatant. Treatment of the post-ribosomal supernatant with N-ethylmaleimide (NEM) abolished protein import and addition of purified hsp70 was unable to restore protein translocation. These studies showed that an NEM sensitive factor acts synergistically with the hsp70 proteins to stimulate protein import of Put2 into intact mitochondria (135).

This postulate is reinforced by results of experiments utilizing a fusion protein comprised of the presequence of rat ornithine transcarbamylase fused to a DHFR protein from mouse. The precursor fusion protein (pO-DHFR) expressed in E. coli could be imported directly into intact mitochondria after purification under denaturing conditions. If the purified pO-DHFR was allowed to renature prior to import, then the level of import-competence decreased. However, dilution of the fusion from denaturing conditions in the presence of a cytosolic extract preserved the import-
competence. Hsp70 alone could not substitute for the cytosolic extract to preserve of import-competence (136).

While hsp70 was unable to maintain pO-DHFR in an import-competent form, it did inhibit folding of the precursor into an enzymatically active state (136). In addition, hsp70 diminished the aggregation of the precursor protein in an ATP-dependent manner. A partially purified fraction of the reticulocyte cytosolic extract was shown to the maintain translocation-competence of the precursor. This fraction had an apparent mass of 200-250 kDa which is similar to the factor found in previous experiments discussed above (134,136). Hsp70 and NEM-sensitive component were both constituents of this fraction (136).

Another factor, termed the presequence binding factor (PBF), has been isolated from a rabbit reticulocyte lysate and also from the cytosol of rat liver or heart cells. The precursor of rat ornithine transcarbamylase (pOTC) binds PBF whereas the mature form of this protein does not. Purified PBF is composed of a single 50 kDa protein which forms a 5.5S complex. When this complex is incubated with pOTC the two proteins (PBF and pOTC) co-sediment at 7.1S (137). The possibility that the 5.5S complex is related to the ≈200 kDa factor referred to above remains unexplored.

Import of pOTC into intact mitochondria is stimulated by PBF, but the rate of import supported by PBF is lower than that sustained by addition of rabbit reticulocyte lysate. The depletion of PBF from reticulocyte lysate
eliminates the ability of the lysate to stimulate protein import, and readdition of PBF restores the stimulatory function (137). While hsp70 was unable to promote protein import by itself, it enhanced the level of pOTC import stimulated by PBF (135-137). This reiterates the fact that hsp70 acts in conjunction with other cytoplasmic factors (ie., PBF) to increase the efficiency of protein import into intact mitochondria (135-137).

A 28 kDa protein isolated from rabbit reticulocyte lysate also may be essential for protein import into mitochondria. This protein was isolated by affinity chromatography using a synthetic peptide which corresponds to the presequence of ornithine aminotransferase (138). This synthetic peptide was discussed earlier in experiments which demonstrated the requirement of a cytosolic factor for import into mitochondria (134). Antibodies to the 28 kDa protein inhibited import of the precursor of ornithine aminotransferase and another protein, the precursor of sulfite oxidase. This inhibition of import seemed to occur at the level of precursor binding to the mitochondrion and so the 28 kDa protein was termed "targeting factor" (138).

Cytosolic factors also are required for protein transport into isolated chloroplasts. The precursor for the light-harvesting chlorophyll a/b binding protein (pLHP) was expressed in E. coli and purified. Denatured pLHP was not import-competent even if no time was allowed for protein renaturation (139). These results are in
contrast to the situation for protein import into mitochondria (described above), where denatured precursor proteins maintained their import-competence unless permitted time to renature (106,130). When a soluble leaf extract (LE) was incubated with purified pLHP prior to addition to intact chloroplasts, the precursor was transported (139).

Stimulation of transport of pLHP was obtained only if LE was present during renaturation of the precursor. The translocation-competent form of pLHP was more sensitive to exogenous proteases than the translocation-incompetent form. As with protein translocation into either ER or mitochondria, hsp70 appears to play a role in the transport of pLHP into intact chloroplasts. Hsc70 is a component of LE, but does not act alone. The addition of hsp70 to pLHP only partially promoted translocation, whereas the combination of LE and supplemental hsp70 enhanced the level of import provided by LE alone (139).

MEMBRANE FACTORS

The precursor protein itself, the energy source which drives protein translocation (ATP and/or an electrochemical/proton gradient), and the soluble factors which facilitate transport all are components of the protein translocation process. An additional component is
the membrane through which the precursor proteins are transported.

In Escherichia coli, four of the six genetically identified sec genes involved in protein transport code for membrane proteins; secY (prlA), secD, secE (prlG), and secF (112,140-142). Genetic analysis demonstrated that the integral membrane proteins SecY and SecE interact directly during the protein transport process. Additional biochemical evidence indicated that the precursor protein contacts SecE prior to interaction with SecY. A model derived from these results hypothesizes that the precursor protein first binds to SecE, followed by the interaction between SecE and SecY to form a complex necessary for protein translocation (141).

The genes both for SecY and SecE have been cloned and sequenced. The products predicted from the cDNA sequences indicate that SecY is a 49 kDa protein with the potential for several membrane spanning regions, whereas SecE is a 13.6 kDa protein (143,144). In experiments where the cDNAs for these proteins were expressed in E. coli from a plasmid using a high efficiency promoter, the overproduction of SecY was SecE-dependent. Both of these proteins were present in equimolar amounts in the cytoplasmic membrane where they are normally found (145). The reconstitution of purified SecE and SecY into proteoliposomes was used to demonstrate that both proteins are required for protein translocation in E. coli. SecA and ATP also are necessary
for the reconstitution of protein translocation in vesicles (146-148).

Two other *E. coli* integral membrane proteins, SecD and SecF, also are required for protein export. The genes for these proteins are located together in the same operon. SecD and SecF appear to have large periplasmic domains which may indicate that these proteins function late in the protein translocation process. This premise is supported by the fact that no suppressor mutations for signal sequence mutants have been found for either SecD or SecF (142).

Involvement of a membrane receptor protein was hypothesized early in the study of protein translocation across the ER (149). Trypsin treatment of salt-stripped microsomes demonstrated that at least one integral membrane protein functions in the process of protein transport. This membrane protein was hypothesized to be a recognition site for either the precursor signal sequence or the ribosome or possibly both (150). Initial characterization of this protein component confirmed the involvement of an integral membrane protein with an apparent molecular weight of 72,000 in the protein translocation process. The term "docking protein" was suggested since this protein appeared to direct the complex of ribosome-nascent peptide-SRP to the correct membrane location for subsequent protein translocation (151). When the SRP binds to the docking protein, the SRP-mediated arrest of protein elongation is
abrogated and permits translation of the nascent polypeptide (151,152). The docking protein also is termed the SRP-receptor based on its affinity for SRP and its possible function in protein translocation (152).

The SRP-receptor protein has a large ($M_r=60,000$) cytosolic domain which can be removed by protease treatment and subsequent salt washes. Both the cytosolic and membrane domains are required for protein transport into the ER (153). The cDNA for the SRP-receptor was cloned and the deduced protein has a predicted apparent molecular weight of 69,684. Analysis of the predicted protein sequence confirms a large cytoplasmic domain of 52 kDa and an amino-terminal membrane anchor of 17.5 kDa (154).

Further experiments show that the SRP-receptor is composed of two subunits. The protein originally termed the docking protein now is designated the $\alpha$ subunit of the SRP-receptor (SR$\alpha$). SR$\beta$ is a 30 kDa integral membrane protein with an affinity for the SRP. SR$\beta$ forms a stable, equimolar complex with SR$\alpha$ in the rough ER (155).

While the SRP-receptor is important for targeting the ribosome-nascent peptide-SRP complex to the ER, neither the SRP nor its receptor are directly involved in the ensuing interaction between the ribosome and the ER membrane. The SRP-receptor relieves the SRP-mediated block of translation by displacing the SRP from the ribosome (156). The SRP-receptor also mediates displacement of the signal sequence from the SRP in a GTP-dependent manner (157,158).
After release from the SRP and the SRP-receptor, the signal sequence interacts with an abundant glycosylated, integral membrane protein of the rough ER. This protein of \( \approx 34-35 \) kDa has been designated the signal sequence receptor (SSR)\(^{(159-161)}\). Additional analysis showed that the signal sequence receptor is a tightly bound heterodimer composed of the previously described 34-35 kDa protein, now designated \( \alpha \text{SSR} \), and 22 kDa integral membrane glycoprotein termed \( \beta \text{SSR} \)\(^{(162)}\).

Additional membrane proteins also may be involved in transport of precursor proteins across the ER membrane. Some of these proteins may be responsible for ribosome binding during the translation/translocation process, while other proteins may form a proteinaceous channel through which nascent proteins pass. Two membrane proteins suggested to be involved in ribosome binding are ribophorins I and II. These integral membrane proteins of 65 kDa (ribophorin I) and 63 kDa (ribophorin II) are associated with rough ER, but not with the smooth ER\(^{(163)}\). Results of crosslinking experiments suggested that ribophorins I and II are in close proximity to membrane-bound ribosomes\(^{(164)}\). Ribophorins I and II segregate with ER membranes which contain bound ribosomes, and the molar ratio of ribophorins/ribosomes is approximately one. These results prompted the hypothesis that the ribophorins mediated binding of ribosomes to the ER, or were part of the translocation mechanism\(^{(165)}\).
Limited protease digestion of ER membrane proteins which resulted in total loss of ribosome binding left 85% of ribophorin I intact and had no apparent effect on ribophorin II (166). The disposition of the ribophorin proteins in the ER membrane indicates that they may not be directly involved in ribosome binding. Each protein has a large part of its sequence sequestered in the ER lumen and a comparatively small cytoplasmic domain. Ribophorin I has 150 amino acid residues exposed to the cytoplasm, while ribophorin II has only 70 of its amino acids cytoplasmically situated (167,168).

Proteoliposomes reconstituted from detergent solubilized ER microsomes can co-translationally translocate precursor proteins. If the ER glycoproteins (including ribophorins) were depleted from the solubilized microsomes before reconstitution of the vesicles, then protein translocation no longer occurred. However, these depleted vesicles were still functional in the targeting and binding of precursor proteins (169). While these results do not rule out involvement of the ribophorins (or other ER glycoproteins) in ribosome binding, it adds weight to the supposition that they are not required for targeting and subsequent binding of the SRP-arrested protein-ribosome complex. Nonetheless, incubation of microsomes with polyclonal antibodies that recognize the cytoplasmic epitopes of ribophorins I and II disrupted ribosome binding. Thus, the ribophorins appear to be in close
spatial proximity to membrane factors responsible for binding of active ribosomes (170).

Another integral membrane protein proposed as an alternative to the ribophorins for the role of ribosome receptor in the rough ER has an apparent molecular weight of 180,000 and a large \( (M_r \approx 160,000) \) cytoplasmic domain. Limited proteolytic digestion of salt-washed ER membranes liberates the large cytoplasmic domain along with a variety of other proteins. The soluble products of this digestion inhibited binding of ribosomes to the ER and a fraction enriched with the 160K fragment had high inhibitory activity. Liposomes containing the purified intact 180k membrane protein bound ribosomes in a manner consistent with its being a ribosome receptor (171).

Contradictory results were obtained when differential detergent extraction of ER membranes was used to prepare vesicles which either contained or lacked the 180 kDa protein. Both types of vesicles bound ribosomes with similar affinities. On the other hand crosslinking experiments from the same study demonstrated that the 180 kDa protein is the most abundant ER protein crosslinked to ribosomes engaged in translation/translocation of proteins. Taken together, these results indicate that the 180 kDa protein, like the ribophorins, is situated in close spatial proximity to active ER-bound ribosomes (172). Currently, there is no direct evidence for the involvement of the 180 kDa protein in the translocation process.
Mitochondria also may transport proteins cotranslationally. Comparison of mitochondria from yeast spheroplasts with the mitochondria from starved spheroplasts show less ribosomes on the surface of mitochondria from the starved spheroplasts. These bound ribosomes were mainly polysomes and remained attached to the mitochondrial surface during isolation of the organelles. This ribosome attachment to the mitochondrial surface is non-random and appears to occur specifically at contact sites between the inner and outer mitochondrial membranes (173).

The majority (80 to 85%) of proteins translated on polysomes bound to mitochondrial remained associated with the organelles. Most of these proteins also were protected from digestion by exogenous proteases unless the mitochondrial membranes were disrupted. By comparison, when free cytoplasmic polysomes were incubated with isolated mitochondria, only 20% of their translation products associated with the mitochondria and the majority of the translation proteins were susceptible to protease digestion regardless of the state of the mitochondrial membranes (174).

Examination of the translational distribution (free or bound polysomes) of several mitochondrial proteins demonstrated that the distribution varies and, while many were enriched in the bound polysome fraction some showed an enrichment in the free polysome fraction (175). Clearly
mitochondrial protein import may occur either co-
translationally or post-translationally. However, in the
case of co-translational transport a ribosome receptor may
or may not be required.

The process of post-translational transport of
proteins into intact mitochondria is thought to require
some type of membrane receptor (176). The presence of a
proteinaceous factor on the surface of the mitochondrion
which mediates binding of precursor proteins was first
demonstrated in experiments where protease treatment of
mitochondria inhibited binding and import of the precursor
of ornithine transcarbamylase (177). Similarly, binding
and import of the precursors for cytochrome b$_2$, citrate
synthase, and the $\beta$ subunit of the F$_1$-ATPase were inhibited
when the mitochondria were treated with trypsin. The
degree of inhibition of binding and import by trypsin
treatment varied among the different precursor proteins. A
subset of mitochondrial membrane proteins solubilized by
non-ionic detergent also can bind of the precursor of
cytochrome b$_2$ (178).

In further experiments either trypsin or elastase
treatment inhibited mitochondrial binding of the precursor
of the ATP/ADP carrier and porin bound for the
mitochondrion. However, binding and import of two other
precursor proteins, the precursors of the mitochondrial
H$^+/ATPase subunits 2 and 9, were only slightly inhibited by
treatment with elastase. These results suggest that there
are at least two different protein factors involved in precursor protein binding on the surface of the mitochondrion (179).

Antibodies against a total mitochondrial outer membrane fraction or a fraction of electrophoretically-purified 45 kDa outer membrane proteins inhibited import of several mitochondrial precursor proteins. Antibodies against other mitochondrial membrane proteins did not inhibit or only partially inhibited protein import. Pretreatment of mitochondria with a low level of trypsin greatly increased the inhibition of import actuated by antibodies against the 45 kDa proteins. However, trypsin treatment alone inhibited protein import only slightly. These antibodies against the 45 kDa outer membrane proteins were not specific, but recognized a population of proteins (180).

Results of subsequent studies showed that a protein of the mitochondrial outer membrane with a relative molecular weight of 42,000 (ISP42) is a component of the protein translocation machinery. This protein was identified by using a precursor protein which becomes stuck in the membrane during transport. This stuck precursor protein was photo-crosslinked to ISP42 and the crosslinked complex could be immunoprecipitated with the previously described anti-45 kDa antibodies. Affinity purification of the anti-45 kDa antibodies showed that the 42 kDa protein was the critical protein recognized since the affinity purified
anti-42 kDa inhibited protein import into mitochondria whereas the purified anti-45 fraction did not (181).

The gene for this 42 kDa outer mitochondria membrane protein, ISP42, was cloned and sequenced. The sequence predicted a 41,983 molecular weight protein which lacked any obvious transmembrane segments. Disruption of one of the two yeast genes for ISP42 resulted in a decrease spore viability by 50%, indicating that the gene for ISP42 is essential for survival. To examine the function of ISP42, both genes in the yeast chromosome were disrupted and complemented by a plasmid borne copy of the ISP42 gene controlled by a galactose inducible/glucose repressible promoter. Depletion of ISP42 resulting from repression of the plasmid copy of the ISP42 gene arrested cell growth. Depletion of ISP42 also resulted in accumulation of several precursors to mitochondrial proteins including the precursor for the α and β subunits of the $F_1$-ATPase, citrate synthase, and Mn-superoxide dismutase. These results indicate that ISP42 functions in the mitochondrial protein import process and is essential for cell viability (182).

A homolog to the yeast mitochondrial protein ISP42 is present in the outer mitochondrial membrane of Neurospora crassa. The cDNA for the $N$. crassa protein was cloned and sequenced and found to code for a protein with an apparent molecular weight of 38,108 termed MOM38. Analysis of the deduced amino acid sequence showed one potential membrane
spanning region and no clear mitochondrial targeting sequence. The lack of a precursor protein for MOM38 was confirmed when in vitro translation of MOM38 yielded a protein of the same apparent size as the mature protein. The deduced amino acid sequences of MOM38 and ISP42 show 40% identity and an additional 38% of the amino acids represent isofunctional substitutions (183).

In *N. crassa* MOM38 is part of a receptor complex involved in the recognition, membrane insertion, and translocation of mitochondrial precursor proteins. MOM38 and two other proteins (MOM72 and MOM22) were identified as part of a complex which was immunoprecipitated with antibodies directed against a 19 kDa protein (MOM19) identified as a general mitochondrial protein import receptor. The proteins which coprecipitate with MOM19 (MOM22, MOM38, and MOM72) also cofractionated with MOM19 when solubilized membrane proteins were separated by gel filtration (183).

The interaction between MOM19 and MOM38 appears to be abolished by a mild trypsin treatment which cleaves MOM19. Complexes comprised of MOM38/MOM19/MOM72 are as abundant as those with only MOM19 and MOM38. This interaction between MOM72 and MOM38/MOM19 is susceptible to elastase treatment which degrades MOM72, whereas the MOM19/MOM38 complex is resistant to treatment with elastase (183).

MOM19 was first shown to be an import receptor for mitochondrial precursor proteins in experiments which were
similar to those described for ISP42. Antibodies against a number of *Neurospora crassa* mitochondria outer membrane proteins were prepared, and antibodies against MOM19 inhibited transport of certain precursor proteins into mitochondria. Import of the precursor proteins for porin, cytochrome $c_1$, the Fe/S protein of the cytochrome $b/c_1$ complex, subunit 9 of the ATPase $F_0$ component, and the $\beta$ subunit of the ATPase $F_1$ component was inhibited by antibodies against MOM19. In contrast, antibodies to MOM19 did not inhibit the import of the precursor proteins for the mitochondrial ADP/ATP carrier and cytochrome $c$ (184).

Treatment of *N. crassa* mitochondria with the protease elastase initially generates an 18 kDa fragment from MOM19 and further digestion results in the appearance of a 17 kDa product. This degradation of MOM19 and the 18 kDa fragment results in inhibition of import of most precursor proteins which appear to use MOM19 as an import receptor. However, the precursor for the $\beta$ subunit of the ATPase $F_1$ component apparently can utilize the 17 kDa fragment of MOM19 as an import receptor, as well as the larger 18 kDa fragment and the full length protein (184). These results help explain earlier results (discussed above) which showed that inhibition of protein import induced by protease treatment of the mitochondria varied depending on the precursor protein examined.

While MOM19 is the receptor for many mitochondrial precursor proteins, it appears not to be involved in import
of the mitochondrial ADP/ATP carrier (184). Antibodies to MOM72 inhibited import of the precursor of the mitochondrial ADP/ATP carrier (ACC) into mitochondria, without inhibiting the import of other mitochondrial proteins. This indicates that the receptor for ACC is MOM72 (185). In addition to its interaction with MOM72, ACC also is associated with MOM38 while ACC is still associated with the general insertion protein (GIP) in the outer mitochondrial membrane. GIP interacts with directly with mitochondrial import receptors and serves as a general insertion site in the outer mitochondrial membrane for precursor proteins. Degradation of MOM38 by proteinase K correlates well with the sensitivity of the GIP/ACC interaction. These results indicate that MOM38 may be GIP, or at least a part of a functional complex with GIP (183).

As with the case of MOM38/ISP42, the *N. crassa* protein MOM72 appears to have a yeast homolog termed MAS70. In yeast, this protein is involved in the import of many precursor proteins, which indicates a difference in function compared to its counterpart in *Neurospora crassa*. The virtual removal of MAS70 by antibody binding, by protease digestion or by the creation of MAS70 null mutations greatly slows the import of several mitochondrial proteins, but has little effect of porin import. Therefore, while MAS70 greatly enhances the import of many mitochondrial precursors in yeast, it is not essential for import function (186).
The cDNAs for both the *Neurospora crassa* MOM72 protein and MAS70 from yeast have been cloned and sequenced. The derived amino acid sequences predict proteins of 68,839 and 70,216 molecular weight for the *N. crassa* and yeast proteins, respectively (187,188). Analysis of the amino acid sequences showed a 33.0% amino acid identity and an additional 12.9% isofunctional exchange of amino acids between the two proteins. In addition, both proteins have several repeats of a 34 amino acid repeat, termed the tetratricopeptide repeat (TPR) motif (188). The TPR motif has been found in a number of proteins which are involved in the regulation of RNA synthesis and mitosis (189). The significance of the presence of these TPR motifs in MOM72 and MAS70 is not currently understood (188).

Results of further recent experiments studying import of the precursor for ACC into intact *Neurospora crassa* mitochondria contradict earlier findings (discussed above) which demonstrated that MOM19 was not involved in the import of ACC. In this study the results indicate that both MOM72 and MOM19 can act as a receptor for the import of ACC into *N. crassa* mitochondria. In addition, it seems that both receptors are independent of each other and that MOM72 can direct the import of ACC with a higher efficiency than MOM19 (188).

In experiments utilizing a synthetic peptide comprising the first 27 amino acids of the precursor protein for ornithine transcarbamylase, another potential
protein receptor was identified in the outer membrane of rat heart mitochondria. Crosslinking studies with this synthetic peptide demonstrated specific binding of this peptide to a 30 kDa mitochondrial membrane protein. The binding of the synthetic peptide was reversible and saturable, which are characteristics typically seen in a receptor/ligand interaction. Protease treatment of the mitochondria eliminated the ability of the peptide to be specifically crosslinked to the 30 kDa protein, as well as the capacity of the mitochondria to import the precursor to ornithine transcarbamylase (190).

Using a similar approach, a mitochondrial protein from rat liver was identified as a putative receptor for the import of the precursor of ornithine aminotransferase (pOAT). A peptide corresponding to the first 53 amino acids, the 34 amino acid transit peptide and the first 19 amino acids of the mature protein (plus a cysteine residue for chemical coupling to an agarose matrix) of pOAT was synthesized and used to affinity purify a 29 kDa protein from solubilized rat liver mitochondria (191). The 29 kDa protein bound both the 54 amino acid peptide and a 34 amino acid synthetic peptide corresponding to only the pOAT signal sequence that can be imported into mitochondria in an energy-dependent manner (134,191). Involvement of the 29 kDa protein in the mitochondrial import process was confirmed by experiments in which import of pOAT into intact rat liver mitochondria was inhibited by Fab
fragments derived from antibodies against the 29 kDa mitochondrial membrane protein (191).

Anti-idiotypic antibodies generated using a synthetic peptide corresponding to the signal sequence of the precursor protein of subunit IV of cytochrome c oxidase (pCoxIV) identified a yeast mitochondrial integral membrane protein with an apparent molecular weight of 32,000 termed p32. Mitochondrial import of a fusion protein formed from the first 22 amino acids of pCoxIV fused in frame to mouse dihydrofolate reductase (pCoxIV-DHFR) was inhibited by Fab fragments derived from either the anti-idiotypic antibodies or antibodies against purified p32. In addition, antibodies against p32 immunoprecipitated pCoxIV-DHFR from a reaction mixture in which solubilized mitochondrial membrane proteins were incubated together with pCoxIV-DHFR. These results were interpreted as a demonstration that p32 forms a complex with pCoxIV-DHFR and is a receptor for protein import into mitochondria (192). It is possible that p32 from yeast is a homolog of the 30 kDa protein in rat heart mitochondria and/or the 29 kDa protein of mitochondria from rat liver though this homology has not yet been demonstrated.

The yeast gene which codes for p32 has been cloned and designated MIR1. Analysis of the sequence predicts a protein product with a molecular weight of 32,800 (193). The amino acid sequence of MIR1 has 40% identity with the mitochondrial phosphate carrier protein from either bovine
heart or rat liver (193-195). When isofunctional substitutions are considered there is a greater than 70% homology between these proteins. Two of these proteins, the phosphate carriers from rat liver mitochondria and bovine heart mitochondria, also have a degree of similarity to the ADP/ATP translocator of bovine heart mitochondria (194,195). Recently, the gene for the mitochondrial phosphate transport protein (PTP) from yeast has been cloned and characterized. This gene for yeast PTP was isolated using oligonucleotides which were based on the sequence of three CNBr peptide fragments generated from purified PTP. The sequence of the PTP gene was virtually identical to the gene for the MIR1 protein (196). The significance of these findings is not yet clear.

Disruption of the MIR1 gene in haploid yeast cells resulted in their inability to grow on a non-fermentable carbon source while growth on glucose still occurred. These results indicate that the MIR1 gene is essential for some yeast mitochondrial function. In light of the identity of MIR1 with the PTP protein, phosphate transport may be the crucial function which is disrupted. However, some mitochondrial functions involved in the metabolism of amino acids and lipids must be retained, since loss of these functions would be lethal. Complimentation of the disrupted MIR1 gene by a plasmid borne copy of the MIR1 gene was apparently able to restore mitochondrial function (193).
The import of several mitochondria precursor proteins was examined in a \textit{MIR1}^- haploid yeast strain. Porin and Put2 import levels were not influenced by disruption of the \textit{MIR1} gene. However, the levels of import for the precursor proteins of the $\gamma$ subunit of F$_1$-ATPase and cytochrome $c$ oxidase subunit IV were reduced by 74\% and 73\%, respectively. These results reinforce the notion that more than one pathway exists for the import of mitochondrial precursor proteins (193).

In addition to a reduction in the level of import of some mitochondrial proteins, the protein product of a mitochondrial gene, subunit II of cytochrome $c$ oxidase, also was reduced by 94\% in \textit{MIR1}^- mutants. This was not due to loss of the mitochondrial DNA in the \textit{MIR1}^- petite-like phenotype since the mutation could be complimented by a plasmid borne copy of \textit{MIR1}. This reduction in a mitochondrially synthesized protein was interpreted as indicating that the \textit{MIR1} gene product may be required for the import of a mitochondrial protein involved in either mitochondrial protein synthesis, or the assembly of cytochrome $c$ oxidase (193). This explanation may be correct since organelle protein complexes which require both cytoplasmically-produced and organellar-synthesized subunits may exhibit tight coordination of protein synthesis and/or stability of the protein subunits. Alternatively, the loss of PTP function may prevent the synthesis of some mitochondrial proteins. The effect of
disruption of MIR1 on phosphate transport into the mitochondria has, unfortunately, not yet been examined.

The distribution of sites in the mitochondrial membranes (inner and outer) where protein transport occurs also has been studied. In experiments discussed earlier where import of mitochondrial precursor proteins was arrested during the translocation process, the precursor proteins spanned both mitochondrial membranes during the transport process suggesting that protein import into mitochondria occurs at contact sites between the inner and outer membranes (104). The distribution of ribosomes attached to the mitochondrial surface also appears to occur specifically at the mitochondrial membrane contact sites (173).

Involvement of contact sites in the transport process also was examined using mitochondria which had the majority of their outer membrane removed (mitoplasts). Mitochondrial precursor proteins could form translocation intermediates in mitoplast membranes which were equivalent to those formed in mitochondria. Mitoplasts also transported a mitochondrial precursor protein so long as the inner membrane was energized. Mitoplasts treated with 50mM KCl lost the ability to form translocation intermediates and to import proteins whereas the formation of translocation intermediates and subsequent protein import in intact mitochondria was not affected by salt treatment even up to 300mM KCl. The salt extractable
mitoplast factor appeared to be proteinaceous in nature and could be added back to the salt-extracted mitoplasts to restore protein import function. These results indicate that a peripheral inner mitochondrial membrane protein which resides in the inter-membrane space may be associated with the protein transport process (197).

Mitochondria with precursor proteins stopped in the translocation process were used to isolate membrane vesicles enriched for mitochondrial protein translocation sites. In one case a translocation intermediate was formed when the precursor protein was bound to mitochondria depleted of ATP. The translocation intermediate of this type could be internalized into the mitochondrion upon addition of ATP. The other translocation intermediate was formed using a tripartite chimeric precursor which becomes irreversibly stuck in the membrane during the transport process. Both types of translocation intermediates cofractionated with membrane vesicles with a buoyant density intermediate to that of inner membrane or outer membrane vesicles (198).

In agreement with previous results, membrane attached cytoplasmic ribosomes cofractionated with the intermediate density vesicles. Two putative contact site proteins, CSP-1 and CSP-2, were enriched in the intermediate density vesicles. CSP-1 and CSP-2 have apparent molecular weights of 100 kDa and 64 kDa, respectively. Immunoelectron microscopy and western blot analysis using antibodies
against CSP-1 and CSP-2 demonstrated that these proteins are distributed primarily in the intermediate density vesicles and at the contact sites of intact mitochondria (198).

Immunoelectron microscopy also showed that the *Neurospora crassa* protein MAS70 and its yeast homolog MOM72, both putative import receptors, are enriched at the mitochondrial contact sites. However, substantial amounts of both of these proteins also were detected in unappressed regions of the outer mitochondrial membrane (185,186). The 29 kDa putative import receptor from rat liver cofractionated with inner mitochondrial membrane vesicles, but in intact mitochondria this protein is sensitive to trypsin treatment. This combination of results was taken as indirect evidence that the 29 kDa protein is located at the contact sites between the inner and outer mitochondrial membranes (191). A large percentage of the p32 receptor protein of yeast was localized using either the anti-idiotypic antibodies or antibodies against p32 at mitochondrial contact sites and cofractionated with mitochondrial membrane vesicles of intermediate density (192).

A proteinaceous receptor on the outer surface of the chloroplast also has been proposed to be a component of the protein translocation process for import of proteins (5,199). The metalloprotease thermolysin is a suitable probe for outer envelope proteins of isolated intact
chloroplasts. Numerous outer envelope proteins are targets of thermolysin treatment, whereas, proteins of the inner envelope are not affected (200, 201). Pretreatment of chloroplasts with this protease inhibits binding of precursor proteins to the organelle, as well as import of proteins into the treated chloroplasts (76).

As discussed earlier, isolated chloroplast envelope membranes from pea or spinach bind a number of chloroplast precursor proteins (54). This binding capability was examined by detergent extraction of chloroplast proteins. A decrease in the amount of some envelope proteins was concomitant with a reduction in the capability of the envelopes to bind precursor proteins. Envelope vesicles reconstituted by removal of the detergent had their binding capacity for precursor proteins restored (202, 203). These results indicate that an envelope protein involved in binding precursor proteins may be extracted by detergent and reconstituted into vesicles.

In experiments designed to identify chloroplast envelope proteins which interact with precursor proteins, in vitro translation products from pea mRNA were bound to isolated chloroplast envelopes and photocrosslinked. Five envelope proteins of 14, 24, 31, 35, and 42 kDa were specifically photocrosslinked to the in vitro translation products. Pretreatment of the envelopes with trypsin, which prevents precursor binding, also prevented photocrosslinking of translation products to the envelopes.
(54,204). At least two of the proteins identified by photocrosslinking, the 31 and 35 kDa proteins, were susceptible to trypsin treatment (204).

Other evidence supporting the involvement of an envelope protein in transport of proteins into intact chloroplasts comes from experiments in which chloroplasts were treated with PLP (81). These experiments were described earlier when discussing the ATP requirement for protein transport into intact chloroplasts. Import of pS into chloroplasts was inhibited when chloroplast surface proteins were covalently modified by treatment with PLP (81). This treatment modifies a number of chloroplast envelope proteins, the most abundant of which is the 29 kDa, phosphate translocator (93,94).

The most direct inference from these results is that one of the PLP-modified proteins of the chloroplast envelope is involved in the import of precursor proteins into the chloroplast. In addition, it is likely that treatment of chloroplasts with PLP results in a secondary modification, namely increased phosphorylation of the 51 kDa envelope protein (81). A role for this protein as a receptor for precursor proteins has been suggested based on a similarity between the $K_m$ of ATP for phosphorylation of the 51 kDa protein and the $K_m$ of ATP for the binding of precursor proteins (41).

Another possible receptor for protein import into chloroplasts is a 30 kDa polypeptide of the chloroplast
inner envelope membrane (205). This protein was identified using an anti-idiotypic antibody approach similar to that used for identification of a mitochondrial import receptor (192,205). A synthetic peptide representing the last 30 amino acids of the transit peptide of pea pS was used to generate anti-idiotypic antibodies. The resulting antibodies cross-reacted with two chloroplast proteins with apparent molecular weights of 30 kDa and 52 kDa. The majority of the 52 kDa polypeptide was located in the stroma and the protein subsequently was identified as the large subunit of RuBisCO. The 30 kDa protein is an integral membrane protein which co-purified with chloroplast envelope membranes (205).

The addition of anti-idiotypic antibodies to a suspension of isolated chloroplasts resulted in aggregation of the chloroplasts, indicating that the antibodies recognized an epitope present on the chloroplast surface. Import of pS into isolated pea chloroplasts was inhibited by Fab fragments generated from the anti-idiotypic antibodies. These results suggest that the anti-idiotypic antibodies are reacting with a receptor for protein import into chloroplasts (205). The prospect that the 30 kDa protein identified by the anti-idiotypic antibody technique is, in fact, the chloroplast phosphate translocator has been raised since the major 30 kDa protein of the chloroplast envelope has been identified as the phosphate translocator (206).
A radioactive, photoactivatable synthetic peptide which corresponds to the first 24 residues of the transit sequence of wheat pS also has been used to identify a 30 kDa polypeptide in the inner membrane of the chloroplast envelope. The 30 kDa protein along with a 52 kDa protein, which is associated with both the inner and outer envelope membranes, are the major proteins labeled by the synthetic peptide. The 52 kDa polypeptide was tentatively identified as the large subunit of RuBisCO, while the labeled 30 kDa protein was identified as the chloroplast phosphate translocator. These results, as well as those discussed above, raise the possibility that a single 30 kDa envelope polypeptide may function as both the transporter of a metabolite (phosphate) and a component of the protein import mechanism (207).

The gene for the 30 kDa chloroplast envelope protein, designated as an import receptor protein by the anti-idiotypic antibody approach, was identified using a 61 base pair oligonucleotide probe corresponding to sequence information derived from the purified 30 kDa protein and resulting CNBr peptide fragments. The amino acid sequence deduced from the cDNA predicted a precursor polypeptide of 43,654 Da consisting of 402 amino acids and a 36 kDa mature protein (p36) with eight potential transmembrane regions based on corresponding regions of hydrophobicity. Monospecific antibodies against p36 were used to generate Fab fragments which inhibited import of pS into intact pea
chloroplasts (208). This result is similar to result described above using anti-idiotypic antibodies (205). Anti-p36 Fab fragments inhibited import at the level of precursor binding to the chloroplast (208).

In chloroplasts, as in mitochondria, contact sites between inner and outer envelope membranes may be sites of protein translocation (8). Immunofluorescence microscopy and immunoelectron microscopy using either the anti-idiotypic antibodies or monospecific antibodies against p36 demonstrated that the putative protein import receptor was located in contact zones between the inner and outer envelope membrane of the chloroplast and was distributed in patches on the chloroplast surface (205, 208). Synthetic transit peptides which inhibit binding of precursor proteins to the chloroplast were localized by immunofluorescence at the contact sites between the inner and outer envelope membranes (209).

Detergent solubilization of chloroplasts with pS bound to the surface liberated a complex containing pS and p36 which could be immunoprecipitated by anti-p36 antibodies. Immunoprecipitation of the pS-p36 complex was blocked when a synthetic peptide (SC30), corresponding to the last 30 amino acids of transit sequence of pea pS, was included in the reaction mixture. These results suggested that SC30 blocked binding of pS to the chloroplast envelope and therefore, the formation of the pS-p36 complex (208). Additional experiments demonstrated that the SC30 peptide
exhibits the ability to block import of pS at the level of precursor binding to the chloroplast (209).

Recently, a full length cDNA for the chloroplast phosphate translocator was isolated from a spinach library. The deduced amino acid sequence has a high degree of hydrophobicity and the predicted secondary structure exhibits seven potential membrane-spanning regions. The phosphate translocator cDNA codes for a 404 amino acid polypeptide which has a molecular weight of 44,234 (82). Comparison of the amino acid sequences of the pea chloroplast import receptor, p36, and the phosphate translocator from spinach shows a high degree of identity (84%) in the mature portion of the proteins, with the most differences (17/22) residing in the first 22 residues whereas the proposed transit sequences of these two proteins have only 44% homology. This high homology suggests that the 30 kDa chloroplast envelope protein which functions in protein import in pea and the phosphate translocator from spinach are homologues. The protein import receptor appeared to fractionate differently on hydroxylapatite than the spinach phosphate translocator indicating that it was unlikely that p36 was the pea chloroplast phosphate translocator (208). However, the experiment demonstrating the binding characteristics of p36 to hydroxylapatite used conditions which were significantly different than those used in the purification of the spinach chloroplast phosphate translocator (208,210,211).
Recently, the cDNA sequence coding for the pea phosphate translocator from pea was cloned and sequenced. The deduced amino acid sequence is identical to p36, the pea chloroplast envelope protein identified as a protein import receptor (212). This strengthens the argument that p36 is the pea chloroplast phosphate translocator. A reexamination of the binding of the spinach chloroplast phosphate translocator to hydroxylapatite showed that if the experimental conditions (4°C) are identical to those used for the initial isolation of this protein, then there is no binding of the phosphate translocator to hydroxylapatite. Whereas, if the reaction conditions reflected those used to conclude that p36 is not the phosphate translocator, then the spinach chloroplast phosphate translocator binds to hydroxylapatite (211).

A disulfonic acid stilbene, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), is a potent inhibitor of the chloroplast phosphate translocator (213). A labeled analog of DIDS, 1,2-di-tritio-1,2-(4,4'-diisothiocyanato) diphenylethane-2,2'-disulfonic acid ([3H2]-DIDS), labeled only a 28- to 30-kDa protein of chloroplasts from either C3 and C4 plants. The chloroplast protein labeled by [3H2]-DIDS in these experiments was proposed to be the phosphate translocator (94). This hypothesis was confirmed by results which demonstrated that a 29 kDa spinach chloroplast envelope protein labeled by [3H2]-DIDS was purified by the procedure used to purify the
spinach chloroplast phosphate translocator. However, an additional higher molecular weight polypeptide also was labeled by $[^3H_2]^{-}$-DIDS at higher concentrations. The identity of the labeled 29 kDa protein as the chloroplast phosphate translocator was verified by results which demonstrated that amino acid sequence information derived from a 26.4 kDa tryptic fragment of the labeled protein matched a deduced sequence of amino acid residues from both the pea and spinach phosphate translocators (82,211,212).

In addition to inhibition of phosphate transport, DIDS treatment also inhibits transport of pS into intact chloroplasts. However, the degree of inhibition of protein import is less than for phosphate transport at equivalent DIDS concentrations. Pretreatment of intact chloroplasts with PLP (with no subsequent reduction of the Schiff base) prior to treatment with either DIDS or $[^3H_2]^{-}$-DIDS, followed by removal of the PLP, resulted in a reduction of DIDS inhibition of phosphate transport and a decrease in the incorporation of label into the 29 kDa phosphate translocator without significantly altering the inhibition of pS import. It was concluded from these results that inhibition of protein import by DIDS does not involve the phosphate translocator, and that the 29 kDa spinach chloroplast phosphate translocator (and by inference the 30 kDa pea chloroplast homolog) is not involved in transport of precursor proteins into the chloroplast (211).
Additional evidence which indicates that the phosphate translocator is not a protein import receptor comes from experiments which demonstrate that its location in the chloroplast envelope and its protease sensitivity are different relative to a precursor binding activity. The chloroplast phosphate translocator is present in the inner chloroplast envelope membrane, and while some binding of pS to inner membrane vesicles was detected, a five fold greater (100% versus 20%) increase in relative binding of pS was found with outer envelope membrane vesicles (211). Thermolysin treatment of chloroplasts inhibits binding of precursor proteins and ensuing protein import (discussed earlier), but, the 29 kDa chloroplast phosphate translocator is resistant to thermolysin treatment (76,211). In agreement with these results, binding of pS to chloroplast outer envelope membrane vesicles also is sensitive to thermolysin treatment.

The number of precursor binding sites on the chloroplast surface has been estimated to be in the range of 1,500 to 3,500 (54,214). Based on these numbers, the chloroplast protein import receptor should comprise approximately 0.03-0.04% of total chloroplast envelope protein (211). These estimates are based on in vitro results and may not accurately represent the exact number of chloroplast protein import receptors. The phosphate translocator, by contrast, is a major envelope protein
representing between 15-20% of the envelope protein (206,210).

Taken together, the results demonstrate that the chloroplast phosphate translocator is identical to the p36 protein identified as a chloroplast protein import receptor. The hypothesis that this single protein functions in a dual capacity for protein and metabolite import seems unlikely based on differences in the characteristics (membrane location, DIDS labeling/inhibition, protease sensitivity, number of membrane sites) of the two functions. The inhibition of protein import by both the anti-idiotypic antibody and the monospecific antibodies against p36 may be explained in two ways: the protein import receptor and the phosphate translocator, both being membrane proteins, may share a common epitope (211). Alternatively, the phosphate translocator (or at least a portion of the translocator population) is at the inner and outer envelope contact site and in close proximity to the protein import receptor. This arrangement could result in steric hindrance of the import receptor by antibodies recognizing the phosphate translocator.

While it seems clear that membrane proteins play an important role in the transport of proteins, the lipid component of the membrane also may be involved, either directly or indirectly, in the transport process. In the bacterial transport system evidence suggests that the lipid
component of the bacterial membrane may be involved in the initial stage of the export of some proteins. The interaction of the signal peptide of the *E. coli* λ phage receptor (LamB) with a phospholipid monolayer results in an ordered conformational changes of the signal peptide. These changes induce an \( \alpha \) helix conformation which favors insertion of the signal peptide into the lipid phase (215).

The signal peptide of an export-defective LamB mutant was found to have a greatly decreased tendency to form an \( \alpha \) helix relative to the wild-type signal peptide (216). In addition, the interaction of the mutant peptide with a lipid monolayer was much less than either the wild-type signal peptide or a signal peptides of pseudorevertants which have export competence restored. The changes in the signal peptide of the pseudorevertants have been predicted to restore the \( \alpha \) helix forming tendency of the peptides (217). These results indicate that the ability of the signal peptide of LamB to form a lipid-induced \( \alpha \) helix is related to its transport potential.

Involvement of phosphatidylglycerol, the major anionic membrane phospholipid of *E. coli*, in transport of proteins was demonstrated in mutants which are defective in synthesis of this lipid. These mutants have decreased rates of protein translocation relative to wild-type. Since protein transport in these mutants was not completely inhibited a requirement for phosphatidylglycerol in the protein transport process is not absolute, and other
membrane lipids may substitute for phosphatidylglycerol (218).

A synthetic signal peptide of an *E. coli* outer membrane protein (PhoE) inhibited translocation of the PhoE precursor into inverted *E. coli* vesicles in a concentration dependent manner. This signal peptide also associates with the inner membrane vesicles. Additional peptides, known to have an affinity for acidic phospholipids, also caused a strong concentration dependent inhibition of transport of the PhoE precursor (219). Taken together, these results suggest a role for the interaction of the signal peptide of *E. coli* precursor protein with the acidic phospholipids of the bacterial membrane.

Similar results have been found for the mitochondrial protein import system. Anionic phospholipids induced a conformational change in a synthetic peptide which corresponds to the first 27 amino acids of pOTC. This synthetic peptide became significantly more α-helical in the presence of anionic phospholipids (220). Liposomes prepared from mitochondrial phospholipids bound mitochondrial precursor proteins, whereas liposomes derived from microsomal membranes did not bind the precursor proteins. When various phospholipids were used to prepare liposomes, binding of a mitochondrial precursor protein to the liposomes increased with the increase of cardiolipin content of the liposomes. Mutations in the signal peptide of the precursor for mitochondrial cytochrome P-450 which
changed positively charged amino acids (lysine and arginine) to neutral amino acids resulted in an inability of the mutant precursors to bind liposomes containing cardiolipin as well as an inability to be transported into mitochondria in vitro (221,222).

Binding of mitochondrial precursors to membrane phospholipids has been suggested to be involved in a conformational change of the precursors which is required for import (108). This hypothesis was supported by results using adriamycin, a drug which binds to cardiolipin and other anionic phospholipids. Import of pCoxIV-DHFR into mitochondria was inhibited by this drug. If the precursor was denatured by urea first the inhibition was abolished (223). The pCoxIV-DHFR precursor protein was found to undergo a conformational change when bound to phospholipid vesicles containing cardiolipin. This conformational change was dependent on the signal sequence and was different from the total unfolding of the precursor (224). It appears that in mitochondria, as with the bacterial protein transport system, the anionic phospholipids of the mitochondrial membrane interact with the signal sequence as part of the protein import process.

As of yet, no direct role for membrane lipids has been demonstrated for protein transport across the endoplasmic reticulum or into the chloroplast. However, the lipid composition of the chloroplast envelope is clearly distinct from that of other cellular membranes (225). Based on the
phospholipid composition, the surface of the chloroplast is thought to have a negative charge at physiological pH. This negative charge has been proposed to permit interaction with a transit sequence possessing a net positive charge (225,226).

It is clear that a tremendous amount of research has been carried out in an effort to discern the mechanism by which proteins traverse membranes. While an increasing number of components and their apparent functions have been uncovered for bacterial, mitochondrial, and endoplasmic reticulum protein transport, the process for chloroplasts is still a black (or at best a dimly lit) box. It was the general goal of my research to study the transport of proteins into chloroplasts with a hope of illuminating some of the components involved. The specific aims of my research were as follows:

1. To determine if there is a chloroplast component which functions in the uptake mechanism.
2. To ascertain the biochemical nature of this component towards an understanding of its role in the uptake process.
3. To examine some of the features of pS which may have a role in the uptake process.

In attempting to realize these goals I also hope to gain a glimpse at a potential import mechanism.
CHAPTER 1
Import of a Precursor of the Small Subunit of RuBisCO Fused to Calmodulin. Requirement for a Portion of the Mature Small Subunit for the Transport of a Fusion Protein with Highly Stable Tertiary Conformation.
INTRODUCTION

The importance of the transit sequence in the transport of precursor proteins into intact chloroplasts has been amply demonstrated. A transit sequence is required for transport of most proteins into the chloroplast, including pS (14,16,17). The transit sequence of a chloroplast precursor, as well as the presequence of a mitochondrial precursor protein, functions to direct the precursor protein to the appropriate organelle membrane (17-20). This ability of transit peptides to "target" or direct passenger proteins extends beyond the sorting of precursors to the correct organelle. Transit peptides also may provide information needed to direct intraorganellar targeting of newly imported proteins (18,23-27).

The structure-function relationships of chloroplast transit peptides have been investigated at the sequence level and while "blocks of amino acid homology" have been found among some chloroplast transit peptides, common sequences are not present in all of them (1,40,41). The minimal sequence homology which exists among transit peptides of various chloroplast precursor proteins is indicative of the involvement of structural features (secondary or tertiary) of the precursor protein in the import process (41). Three regions which could form transit peptide structural features are common to some chloroplast precursors (46). Deletion analysis of the
transit peptide of pS has revealed that both the amino-terminal (residues 1-24) and the carboxy-terminal (residues 42-58) regions are essential for transport, while the central region (residues 26-35) has little effect on protein transport (47). The carboxy-terminal portion was further characterized to show that the last 10 amino acids (deletion of residues 48-56 and substitution of glycine for cysteine at residues 57) are needed for consistent processing of pea pS to mature S, but are not required for efficient import of pS (48,49). Furthermore, deletion of an additional three residues (amino acids 45-47 plus the changes noted above) resulted in decreased import efficiency as well as a lack of processing to mature S (48).

While the information encoded in the chloroplast transit sequence is sufficient to direct the transport of a foreign protein into the chloroplast, a portion of the mature protein may be required for efficient targeting and transport (24,26,30,32,34-36). A similar arrangement exists in mitochondria, where efficient targeting and transport of precursor proteins involves the mature portion of a precursor protein (or a portion of the passenger protein in the case of the fusion of a transit sequence to a foreign protein) (37-39). The efficiency of co-translational import of a chimeric protein into the ER also is influenced by the amino acid sequence beyond the cleavage site. In this case the sequence differences
influence the interaction of the precursor protein with components of the ER protein translocation system (227).

The transit sequence of pea pS can target a non-chloroplast fusion protein to the chloroplast. The chimeric protein expressed from a portion of a pea pS gene coding for the transit peptide linked to the bacterial gene for neomycin phosphotransferase II (NPTII) is localized to the chloroplast stroma both in vivo and in vitro (17). Similar results were obtained in vivo with a transit sequence-NPTII fusion which contained the first 22 amino acid of mature S between the transit sequence and NPTII (28). However, the efficiency with which the pS transit sequence was able to direct the fusion protein to the chloroplast relative to the normally high efficiency of pS transport was not examined for either of these fusion proteins.

Efficiency of protein transport into intact chloroplasts in vitro was compared for two chimeric proteins. One fusion protein comprised of the transit sequence of soybean pS and mature S from pea, and the second a tripartite fusion protein incorporating the transit sequence of soybean pS, the first 13 amino acids of pea S, and all but the first three amino acids of a cytoplasmic heat shock protein from soybean. Both protein constructs were imported into the chloroplast whereas the soybean heat shock protein without the pS transit sequence was not, reconfirming the ability of a transit sequence to
direct protein translocation into the chloroplast. The chimeric protein containing the transit sequence of soybean pS and the heat shock protein was imported less efficiently than the fusion comprised of the soybean transit sequence and pea mature S. These results raised the possibility that a portion of the transported protein other than the transit sequence may affect the efficiency of import (34).

Gene sequence constructs for chimeric proteins made up of portions of pea pS and NPTII (similar to those described above) were prepared to determine the importance of protein sequences other than the transit sequence in the transport process. A chimeric protein containing the transit sequence and the first 23 amino acids of mature S from pea connected via a linker to NPTII was synthesized in vitro and transported into intact chloroplasts. The efficiency of transport of this fusion protein was equal to that of pea pS. However, a similar chimeric protein consisting of the transit sequence joined by a linker directly (no mature S sequence) to NPTII was transported with an estimated efficiency of 10% or less relative to either pS or the chimera containing the region of mature S. These results indicate that the information, either sequence or structural (or both), within the first 23 amino acids of mature S is needed for efficient transport of these proteins into intact chloroplasts (30).

Efficiency of transport of chimeric proteins either containing or lacking a portion of mature S between the
transit sequence and NPTII was measured in vivo and it was determined (based on NPTII enzymatic activity) that the chimeric construct containing a region of mature S accumulated in chloroplasts to a lesser degree than the construct which lacked the portion of mature S. However, assessment of in vivo protein import based on NPTII enzymatic activity may be inaccurate because expression of the two chimeric proteins or their enzymatic activity may not be equivalent in vivo. A possible higher rate of cytoplasmic degradation of the fusion containing the region of mature S also could explain the lower chloroplastic level of the chimera containing the region of S (31). While these in vivo results appear to contradict the in vitro results, many alternative explanations also are available.

The sequence of S required for the efficient transport of a transit sequence-NPTII chimeric protein in vitro has been suggested to serve merely as a spacer between the transit sequence and the NPTII sequence which allows the transit sequence to interact in the transport process (30). This hypothesis was examined using protein fusions containing the transit sequence of pS from soybean fused to bacterial 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (EC 2.5.1.19). The transit sequence was fused either directly to EPSP or with a 24 amino acid region between the transit sequence and the EPSP amino acid sequence. This 24 amino acid region was comprised of
either the first 24 amino acid of mature S from pea or a 24 amino acid alternative (non-S) sequence. The fusion protein consisting of only the transit sequence and EPSP synthase was not transported into chloroplasts either in vivo or in vitro. In confirmation of the earlier studies with NPTII fusion proteins, the chimeric protein containing the 24 amino acids of S from pea was transported both in vivo and in vitro. However, in vivo experiments with the chimeric protein containing the alternative 24 amino acid sequence revealed no chloroplast import of this fusion protein. These results indicate that a portion of mature S is required for the efficient import of the fusion protein and that an alternative 24 amino acid region cannot substitute for this region of S (35). A similar experiment demonstrated the import of a fusion protein containing the transit sequence and a portion (amino acids 1-27) of the mature petunia chloroplast EPSP synthase fused to a mutant form of bacterial EPSP synthase into Latuca sativa chloroplasts. This fusion protein was imported as efficiently as the precursor for petunia chloroplast EPSP synthase (228).

In addition to the need for a portion of the mature protein for efficient transport of precursor proteins into chloroplasts, a requirement for precursor unfolding in the protein transport process also has been proposed (110). This hypothesis was tested using the precursor to chloroplast EPSP synthase (pEPSP synthase). This
chloroplast precursor protein is enzymatically active and can catalyze the condensation reaction resulting in the transfer of the carboxyvinyl moiety of phosphoenolpyruvate to shikimate-3-phosphate. The enzymatic activity of pEPSP synthase also can be inhibited by the broad spectrum herbicide N-[phosphonomethyl]glycine (glyphosate) and has a sensitivity to this herbicide which is essentially the same as the mature chloroplast enzyme. These results suggest that the catalytic site of pEPSP can fold into a three-dimensional conformation equivalent to that of the mature protein (229).

Transport of pEPSP synthase into intact chloroplasts also can be inhibited by glyphosate under conditions known to result in the inhibition of the enzymatic activity (the presence of the substrate, shikimate 3-phosphate). The inhibition of chloroplast protein import by glyphosate is specific for pEPSP synthase and a mutant form of pEPSP synthase which does not bind glyphosate shows no inhibition of protein transport in the presence of this herbicide. These results indicate that the unfolding of pEPSP synthase is required for protein translocation, and this unfolding is inhibited by the binding of the herbicide (110).

Evidence also has accumulated which indicates a requirement for precursor unfolding for protein transport into mitochondria (58). Precursor protein import by mitochondria could be arrested during the translocation process, such that the amino terminal presequence extends
into the mitochondrial matrix while a major portion of the polypeptide chain remains outside of the organelle. This blockage in protein translocation was accomplished by two separate techniques; lowering the temperature of the transport assay, or transport of a precursor protein after preincubation with antibodies which recognize and bind to the mature portion of the precursor protein. The arrest of the protein transport process in this membrane-spanning configuration permitted the processing of the presequence by the matrix protease, while the external portion of the polypeptide remained susceptible to exogenous protease treatment. These results indicate that the transit sequence precedes the mature portion of a precursor into the mitochondrial matrix (104).

Further evidence demonstrating a requirement for precursor unfolding during the transport process in mitochondria was obtained using a chimeric protein comprised of the presequence of a mitochondrial protein fused to DHFR. This fusion protein is imported into intact mitochondria and the presequence removed by the mitochondrial processing protease. The DHFR portion of this fusion protein retained its enzymatic activity and the ability to tightly bind methotrexate, a folate antagonist. Results similar to those described above for pEPSP synthase-glyphosate demonstrated that methotrexate was able to inhibit the import of the DHFR fusion protein without affecting the import of other precursor proteins. These
results prompted the conclusion that the binding of methotrexate to the DHFR portion of the fusion protein prevented the unfolding of that portion of the chimeric protein and thereby inhibited its translocation into intact mitochondria (105).

A series of experiments exploiting the DHFR fusion protein described above have shown that the unfolding of precursor proteins occurs outside of the mitochondria. This unfolding is part of the import process and precursors bound to the mitochondrial surface prior to import exist as unfolded intermediates. Point mutations introduced into the DHFR portion of the precursor fusion which appeared to decrease the stability of the folded chimeric precursor protein resulted in a reduced inhibition of mitochondrial import of the fusion by methotrexate (106,107).

The folding of an *Escherichia coli* precursor protein also has been correlated with the ability of the precursor to be transported. The precursor to maltose-binding protein (MBP) can attain a stable tertiary conformation similar to the mature protein. In this folded conformation MBP and its precursor are resistant to proteolysis. A mutant form of MBP which folds faster than the wild-type precursor was exported more slowly than the wild-type precursor and the kinetics of folding of the mutant precursor (judged by proteolytic sensitivity) correlated inversely with its ability to be transported. Thus the presence of a stable tertiary protein conformation of a
precursor correlates with a decreased capacity for protein translocation in *E. coli* (109).

In order to study the requirement for a portion of mature S in addition to the pS transit sequence in the transport of a foreign protein, as well as to examine the effect protein conformation may play in the transport of proteins into intact chloroplasts, we constructed protein fusions consisting of various lengths of pS from wheat fused to calmodulin (CaM) from chicken brain (230,231). CaM is a highly conserved, small (*M*<sub>r</sub> = 16,700) Ca<sup>2+</sup>-binding protein present in most, if not all, eukaryotes. Functionally, CaM has been implicated in the regulation of a broad spectrum of intracellular enzymes and biochemical processes (232). The crystal structure of CaM in the presence of Ca<sup>2+</sup> has been examined and the structure has been established to have an overall shape, consisting of two globular lobes connected by a long central helical tether with a small bend or flexible area in the center (233,234). The crystal structure with Ca<sup>2+</sup> bound also confirmed the presence of four E-F hand structures similar to those originally proposed for the Ca<sup>2+</sup> binding sites of parvalbumin (233-235).

The binding of four Ca<sup>2+</sup> ions to CaM appears to be an ordered process which induces marked conformational changes in the protein (232,236). The four Ca<sup>2+</sup> binding sites differ slightly in their affinity for Ca<sup>2+</sup> with binding constants between 67 nM and 900 nM (236). As a result of
the high affinity of CaM for Ca$^{+2}$ ions, the protein conformation assumed by CaM in the presence of Ca$^{+2}$ is very stable. This characteristic is illustrated by the observation that Ca$^{+2}$-dependent changes in the electrophoretic mobility of CaM can be seen even in the presence of 0.1% SDS (237). We have exploited the Ca$^{+2}$-induced protein conformational change of CaM to examine the ability various lengths of pS from wheat to transport a CaM fusion into isolated intact chloroplasts.

MATERIALS AND METHODS

Plant Material. Plant tissue was obtained from light grown wheat seedlings (Triticum aestivum var. Coker 976). Seeds were surface sterilized and washed for 5 min in a mixture of 10% v/v household bleach and $\approx$1% v/v Liqui-Nox (Alconox, Inc.). Seeds were then rinsed thoroughly under running water, and planted in expanded vermiculite. Tissue for chloroplast isolation was taken from plants grown for 6-10 days under restricted light (only light exposure to check progress of growth and to water) in an EGC growth chamber. When average plant height was $\approx8$ centimeters, plants were given full light for 12-24 hrs, and leaves were collected for chloroplast isolation.
**Chloroplast Isolation.** Isolation of intact chloroplasts for protein transport studies was as described by Bartlett, et al. (238). Plant material was collected into a 2 liter beaker containing ≈1.5 liters of cold GR buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-KOH, pH 8.0/0.33 M sorbitol/5.0 mM Na ascorbate/2.0 mM EDTA-NaOH, pH 7.4/1 mM Mg$_2$Cl$_2$/1 mM Mn$_2$Cl$_2$). Plant material was homogenized with a Polytron PT 35K (Brinkman) probe at a setting of 6. The resulting homogenate was filtered through four layers of Miracloth (Calbiochem) and the filtrate centrifuged in a GS-3 rotor by acceleration up to ≈4250 x g (max) and immediately breaking to a stop. The resulting supernatant was removed, the pellets resuspended in GR buffer and loaded onto 10 to 80 % Percoll (Pharmacia) density gradients (Table 1).

Percoll gradients overlaid with the resuspended pellets containing intact chloroplasts were centrifuged at 14,600 x g (max) in a HB-4 swinging bucket rotor (Sorvall) for 10 min. This centrifugation results in the separation of two green bands in the gradient. The upper band contains predominantly broken chloroplasts while the lower band contains intact chloroplasts. The lower band was collected and diluted 5 to 10 fold with cold GR buffer and centrifuged up to 5211 x g (max) and after 10 sec stopped. The pellets containing intact chloroplasts were resuspended in cold S-H buffer (0.33 M sorbitol/50 mM HEPES-KOH, pH 8.0), pooled together and centrifuged up to 3618 x g (max)
Table of solutions for preparing Percoll gradients.

<table>
<thead>
<tr>
<th>PBF-Percoll solution</th>
<th>Amount</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG (polyethylene glycol) molecular weight 6000</td>
<td>1.58 g</td>
<td>2.98% w/v</td>
</tr>
<tr>
<td>BSA</td>
<td>0.53 g</td>
<td>1.0% w/v</td>
</tr>
<tr>
<td>Ficoll</td>
<td>0.53 g</td>
<td>1.0% w/v</td>
</tr>
<tr>
<td>Percoll to</td>
<td>53.0 ml</td>
<td>-------</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>10%</th>
<th>80%</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBF-Percoll stock</td>
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</tr>
<tr>
<td>0.2 M EDTA-KOH, pH 8.0</td>
<td>525 µl</td>
</tr>
<tr>
<td>1 M MgCl₂</td>
<td>52.5 µl</td>
</tr>
<tr>
<td>1 M MnCl₂</td>
<td>52.5 µl</td>
</tr>
<tr>
<td>1 M HEPES-KOH, pH 8.0</td>
<td>2.63 ml</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>3.15 g</td>
</tr>
<tr>
<td>Na ascorbate</td>
<td>46.5 mg</td>
</tr>
<tr>
<td>Glutathione</td>
<td>9.0 mg</td>
</tr>
<tr>
<td>Sterile Distilled Water to</td>
<td>52.5 ml</td>
</tr>
</tbody>
</table>

12.5 ml of each solution (10% and 80%) is used for each gradient. The above solutions will make enough for 4 Percoll gradients.

and immediately stopped. The resulting chloroplast pellet was resuspended in cold S-H buffer and the chlorophyll concentration measured.

Preparation of Wheat Germ Extracts. A wheat germ cell free extract for in vitro protein synthesis was prepared by the method of Grossman et al. (239) with the following
modifications: the extraction and column buffers each contained 3 mM DTT. The centrifugations were carried out at 12,000 x g for 15 min. Four batches of extract supernatant were combined (40 ml), passed through a Sephadex G-25 (Pharmacia) column and a 40 ml turbid fraction from the void volume was collected and stored at -70° C.

**Plasmid Constructions.** A series of DNA plasmids were constructed containing increasing lengths of pS from wheat fused in frame to the coding sequence of calmodulin. A complete description of all wheat pS derived CaM fusions is presented in the Appendix. Standard molecular biological techniques were used in the construction of these plasmids. All restriction endonuclease reactions were performed as per the manufacturer's suggested conditions and where necessary DNA fragments were purified prior to ligation by electrophoresis in low-melting agarose gels. Ligation of agarose gel purified DNA fragments was as described by Struhl (240). Ligated plasmids were transformed into *E. coli* strain DH5α and mini-preparation of plasmid DNA from the resulting transformants was prepared by the method of Zhou et al. (241) with the addition of a phenol-chloroform, chloroform extraction sequence prior to the initial ethanol precipitation of nucleic acids. The resulting plasmid DNAs were screened for the presence of desired DNA constructs by restriction endonuclease digestion analysis.
The initial plasmid constructs used to produce all subsequent constructs were: pSn5 and pCAM23. pSn5 contains the full coding sequence (525 bp) of pS from wheat with 24 bp of upstream and 197 bp of downstream sequence was cloned into the Eco RI/Pst I sites of a pSP65 (Promega) vector which had the vector Sph I site deleted (see ref. 15 for details). The second plasmid, pCAM23 (see references 237 and 238 for a description of this plasmid) contains a full length cDNA clone for calmodulin (CaM) from chicken brain. The sequence around the CaM initiator ATG was altered to create an Nco I restriction site in pCAM23.

The gene fusions composed of various lengths of pS fused in frame to CaM were created utilizing restriction endonuclease sites within the mature portion of wheat pS (see figure 1). The smallest of these fusion proteins, BCaM, was constructed by digestion of pSn5 with restriction endonucleases Bal I (New England Biolabs) and Pst I (BRL) generating a vector fragment containing the transit sequence and upstream region of wheat pS to the Bal I site, 12 bp (4 amino acids) into mature S. Plasmid pCAM23 was restriction endonuclease digested with Nco I (BRL) and the resulting 5' overhang was converted to a blunt end with mung bean nuclease (New England Biolabs). The linearized vector was then digested with Pst I generating a 313 bp insert fragment containing the amino terminal portion of the CaM coding sequence extending from 4 base pairs downstream of the initiation start site to the Pst I site.
at position 318. The restriction endonuclease digestions were subjected to agarose gel electrophoresis. The appropriate fragment from pCAM23 and the vector portion from pSn5 were ligated to create an intermediate construct which was digested with Hind III (BRL) thus generating a vector fragment containing all of the aforementioned wheat pS sequence and the first 49 bp of the CaM coding sequence. Plasmid pCAM23 was digested with Hind III generating an 891 bp fragment containing the carboxy-portion of the CaM coding sequence and 488 bp of downstream sequence. The fragment and vector were gel purified, ligated and the resulting vector was named pBCaM.

Similar cloning strategies were used to construct the larger fusion proteins in the series containing increasing lengths of pS from wheat fused to CaM. In addition to the fusions, the CaM gene alone also was cloned into the modified pSP65 transcription vector.

**In Vitro Transcription of Plasmid Constructs.** Plasmid DNA containing the coding sequence for wheat pS or one of the pS derived CaM fusions or CaM alone was linearized with a restriction endonuclease which recognizes a site downstream of the CaM coding sequence. The linearized DNA was then transcribed by the method of Krieg and Melton (242) and Melton et al. (243) with the following modifications: RNAsin (Promega) was used at 25.6 U/ml; BSA final concentration was 70 μg/ml; ATP, CTP, and UTP were
used at a final concentration of 500 μM; GTP final concentration was 50 μM; 500 μM diguanosine triphosphate was included in the reaction; and SP6 RNA polymerase (BRL) was used at 600 U/ml final concentration. The transcription reactions were incubated at 40°C for 90 min. Following transcription, the nucleic acids were extracted with phenol:chloroform, then chloroform alone, and then precipitated with ethanol. The precipitated nucleic acids were collected by centrifugation and resuspended in diethyl pyrocarbonate (DEPC) 0.1% v/v treated water. The DNA template was digested at 37°C for 5 min with 20 U/ml RNase-free DNase (BRL) in the presence of 100 μM DTT and RNAsin at 800 U/ml. After another extraction with phenol:chloroform and chloroform, the transcripts were precipitated with two volumes of 100% ethanol. The in vitro transcripts were collected by centrifugation, resuspended in water and either used immediately for in vitro protein synthesis or stored in water until needed at -80°C.

**In Vitro Protein Synthesis.** Radioactively labeled chloroplast precursor proteins were synthesized in vitro using a wheat germ cell free translation system. The translation reaction contained the following: 50 mM (HEPES)-KOH, pH 7.6, 100 mM K+ acetate, 124 μM spermine neutralized with HCl, 2.1 mM Mg+2 acetate, 1.2 mM ATP, 80 μM GTP, 9.6 mM phosphocreatine, 20 μM each of the amino
acids (minus methionine), 0.4 volume wheat germ extract, approximately 400 μCi/ml [³⁵S]methionine (New England Nuclear, specific activity approximately 1000 Ci/mmol), ≈60 μg/ml of in vitro RNA transcripts, 64 μg/ml creatine phosphokinase (EC 2.7.3.2), and 100 μM S-adenosylmethionine (Boehringer Mannheim). The reagents were mixed together and incubated at 26° C for 90 min. Following translation, the ribosomes were pelleted by centrifugation (at 4° C) for 1 hr. at 142,822 x g (max) and the post-ribosomal supernatant was used for chloroplast protein transport experiments.

Transport of In Vitro Translation Products into Intact Chloroplast. Conditions for transport of in vitro synthesized translation products into intact chloroplasts were according to Bartlett et al. (238). Briefly, chloroplasts were combined with in vitro translation products and the volume adjusted so the final chlorophyll concentration is 660 μg/ml. A typical 300 μl incubation contained 50 μl of chloroplasts at 4 mg/ml, 100 μl of in vitro translation products, 15 μl of 1.0 M HEPES-KOH, pH 7.6, 40 μl of 2.0 M Sorbitol, 10 μl of 250 mM methionine and water to the appropriate volume. The chloroplasts were added last. Any additions to the transport reactions are noted in figure legends.

The transport reactions were incubated in the light for 1 hr at room temperature with gentle shaking. (see
chloroplast isolation procedure) and centrifuged up to 3618 X g (max) and immediately stopped. The resulting chloroplast pellets were resuspended in 500 \( \mu l \) of cold S-H buffer and 6.25 \( \mu g \) each of trypsin and chymotrypsin were added and the mixtures incubated on ice for 30 min. After proteolysis the chloroplasts were diluted with 10 ml of cold S-H buffer containing the following protease inhibitors: 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine (BAM), 5 mM \( \varepsilon \)-amino-\( \eta \)-caproic acid (ACA), and 100 \( \mu g/ml \) soybean trypsin inhibitor. The diluted chloroplasts were pelleted by centrifugation up to 3618 X g (max) and immediately stopped.

The protease treated chloroplast were lysed by addition of 125 \( \mu l \) of 50 mM HEPES-KOH, pH 8.0 containing: 1 mM PMSF, 1 mM BAM, 5 mM ACA, and 100 \( \mu g/ml \) soybean trypsin inhibitor. The chloroplasts were vortexed for 10 to 15 sec. and then centrifuged at 7092 X g (max) for 10 min to pellet the chloroplast membrane fraction. The stroma and membrane fractions were then analyzed by SDS-PAGE.

Polyacrylamide Gel Electrophoresis. Proteins were analyzed by SDS-PAGE on 10-15% gradient gels. Sample preparation and polyacrylamide gel procedures were as described by Piccioni, et al. (244). Calcium containing gels were used to demonstrate the presence of calmodulin. Calcium binding proteins like calmodulin exhibit a shift in electrophoretic
mobility in the presence of calcium. All procedures were the same as for standard SDS-PAGE except that 1 mM CaCl₂ was included in the gel as well as the lower reservoir buffer. Low range SDS-PAGE molecular weight standards (Bio-Rad) were used to determine protein positions in SDS-PAGE analysis. After electrophoresis, gels were fixed, stained, dried and subjected to autoradiography.

RESULTS AND DISCUSSION

We have utilized several restriction endonuclease sites within the mature S portion of the coding sequence of wheat pS (Fig. 1) to construct a series of fusion proteins consisting of increasing lengths of pS linked in frame to CaM. The coding sequences for these fusion proteins (Fig. 2) in addition to the unfused wheat pS and CaM sequences were cloned downstream of the SP6 promoter. This promoter was used for subsequent in vitro transcription with SP6 RNA polymerase.

The resulting in vitro transcripts were translated in vitro in a wheat germ cell-free lysate. The resulting translation products were analyzed by SDS-PAGE and autoradiography. Bands corresponding to proteins of the expected molecular weights were evident for all constructs (Fig. 3, panel b). In each lane the band representing the
Figure 1. Schematic representation of pS cDNA from wheat. The transit sequence (ts) and the restriction endonuclease site used to construct the pS/CaM fusions are indicated.
Figure 2. Schematic representation of wheat pS/CaM fusions: a) BCaM fusion, b) E0CaM fusion, c) AvaCaM fusion, d) SmaCaM fusion, and e) pSCaM fusion. The thick bar represents the coding region starting with the transit sequence (ts) and increasing lengths of mature S fused inframe to the calmodulin sequence. Where necessary a linker was used to maintain the correct reading frame.
Figure 3. Autoradiographs of SDS-PAGE examining the in vitro translation products generated from in vitro transcripts. Proteins represented in individual lanes of both panels a) and b) are as follows: lane 1 wheat pS, lane 2 CaM, lane 3 the precursor to BCaM, lane 4 the precursor to EOCaM, lane 5 the precursor to AvaCaM, lane 6 the precursor to SmaCaM, lane 7 the precursor to pSCaM. Electrophoresis of gel depicted in panel a) was performed in the presence of 1 mM CaCl₂ (see Material and Methods). Control gel depicted in panel b) was electrophoresed under standard conditions. The darkest upper band in each lane represents the protein product with the expected molecular weight. > indicates the band of interest in each lane.
protein of highest molecular weight is the expected full length product. In addition, bands corresponding to proteins of lower molecular weight also were present in all lanes containing in vitro products synthesized from transcripts which code for wheat pS or a portion of pS fused with CaM.

The lower molecular weight proteins represented by these bands increase in size as the corresponding predicted product increases in size. These lower bands may represent the product of proteolytic processing of the primary in vitro translation products. Protease activities have been reported in wheat germ lysates and cleavage may result in the presence of the lower molecular weight proteins. However, the lower molecular weight bands observed do not appear to be the result of non-specific degradation (245). It is likely that since both the in vitro translation products and the translating system are derived from wheat that the processing which appears to occur results from the stromal processing protease. While wheat germ clearly does not contain developed chloroplasts it does contain proplastids, the progenitors of chloroplasts. Alternatively, these bands could result from translation initiation at the internal methionine present at the transit sequence cleavage site.

The ability of the CaM portion of the fusion proteins to bind Ca$^{+2}$ was demonstrated by SDS-PAGE in the presence of 1 mM CaCl$_2$ (Fig. 3, panel a). The in vitro translation
products containing CaM, either alone or present as a fusion with a portion of wheat pS, exhibited a change in electrophoretic mobility in the presence of Ca$^{+2}$ (Fig. 3, compare panels a and b, lanes 2-7). The translation products corresponding to wheat pS alone showed no change in electrophoretic mobility when Ca$^{+2}$ was present (Fig 3, compare panels a and b, lane 1). This shift in electrophoretic mobility in the presence of Ca$^{+2}$ has been previously used to identify CaM in crude protein preparations and appears to be unique to CaM (237). In the presence of Ca$^{+2}$, CaM conformation is very stable and x-ray crystallography (Fig. 4) reveals a "dumbbell" shape (233). The presence of this stable, specific conformation may be electrophoretic mobility change in the presence of Ca$^{+2}$ (231, 237). The precise reason for the shift in electrophoretic mobility of CaM in the presence of Ca$^{+2}$ is unclear, but it may be the result of conformational changes in the protein or a change in overall charge due to the Ca$^{+2}$ ions, or a combination of both.

The effect of this stable protein conformation on the transport of fusion precursor proteins was examined by performing in vitro transport into wheat chloroplasts in the presence of Ca$^{+2}$. BCaM and EOCaM, consisting of the transit sequence of wheat pS, and 4 amino acids or 25 amino acids of mature S, respectively, fused to CaM demonstrated partially responsible for the shift in electrophoretic mobility in the presence of Ca$^{+2}$. However, troponin C has
Figure 4. Drawing of the α-carbon backbone of CaM depicting the protein tertiary structure determined by x-ray crystallography (238). • indicates bound Ca^{2+} ions.
an analogous shape, but does not demonstrate the
differential transport competence (Fig. 5). No transport
of EOCaM is seen under control conditions or when the
transport reaction contains 1 mM CaCl₂ (Fig. 5, lanes 8 and
9). However, when 10 mM ethyleneglycol-bis (β-amino ethyl
ether)-N,N'-tetraacetic acid (EGTA) was included in the
transport mixture, either in the presence or absence of 1 mM
CaCl₂, EOCaM was transported and processed to a "mature"
size (Fig. 5, lanes 10 and 11). The presence of any
combination of 1 mM CaCl₂ and/or 10 mM EGTA had no effect
on the transport of wheat pS (Fig. 5, compare lane 4-7).
These results demonstrate that the presence of Ca²⁺ inhibits transport of a fusion protein containing CaM into
intact chloroplasts. We believe that enough free Ca²⁺ is
present in the control transport reaction to result in an
inhibition of import similar to that seen when 1 mM CaCl₂
was present (Fig. 5, compare lanes 8 and 9).

In the case of BCaM, no transport is seen regardless of
the uptake conditions (Fig. 5, lanes 12-15). The protein
bands evident in the BCaM post-transport stroma (Fig. 5
lanes 12 and 13) represent a product of proteolysis which is
resistant to further degradation when Ca²⁺ is bound to the
protein. The transport-incompetence of the BCaM fusion
protein is not the result of a Ca²⁺-based conformation
change since no transport is observed even in the presence
of 10 mM EGTA (Fig. 5, lanes 14 and 15). We feel that the
import-incompetence of BCaM is a result of an inadequate
Figure 5. Autoradiograph of SDS-PAGE of in vitro transport of translation products generated from in vitro transcripts of pSn5, pEOCaM, and pBCaM. Lanes 1-3 contain in vitro translation products containing the precursor of S, EOCaM, and BCaM respectively. Lanes 4-15 contain chloroplast stromal fractions after import of in vitro translation products. Lanes 4-7 stromal fractions following import of pS, lanes 8-11 stromal fractions following import of the precursor of EOCaM, and lanes 12-15 stromal fractions following import of the precursor of BCaM. The conditions for transport are as follows: Lanes 4, 8, and 12 control conditions which contain no additions, lanes 5, 9, and 13 transport in the presence of 1 mM CaCl$_2$, lanes 6, 10, and 14 transport in the presence of 1 mM CaCl$_2$/10 mM EGTA, and lanes 7, 11, and 15 transport in the presence of 10 mM EGTA. > indicates the location of the precursor proteins, * indicates the imported, processed proteins, and ▲ indicates a protease resistant breakdown product.
portion of mature S (only 4 amino acids) in this fusion protein. If this is the case then these results demonstrate that a portion of mature S longer than 4 amino acids (but, possibly less than 24 amino acids) is required in addition to the transit sequence for the transport of the CaM fusion proteins into intact wheat chloroplasts.

Transport of SmaCaM, consisting of the transit sequence of wheat pS and 57 amino acids of mature S fused to CaM also was inhibited by Ca\(^{+2}\) present in the control reaction mixture, as well as by added 1 mM CaCl\(_2\) (Fig. 6, lanes 7 and 8). However, the degree of inhibition was not as great as was seen with EOCaM in the presence of 1 mM CaCl\(_2\). Inhibition of transport of SmaCaM by Ca\(^{+2}\) was relieved by the addition of 10 mM EGTA. These results indicate that if a larger portion of mature S is present between the transit sequence and the CaM portion of the fusion, then the ability of the conformational stability of CaM to inhibit transport is partially alleviated.

In the presence of Ca\(^{+2}\) CaM and the CaM portions of the fusion proteins which have not been transported are resistant to proteolytic degradation by the exogenous proteases (trypsin and chymotrypsin) added to the intact chloroplasts after the completion of the transport incubation (Fig. 6, lanes 7, 8, 10, and 11). However, addition of EGTA permits the degradation of this protease resistant species (Fig. 6, lanes 12 and 15). The presence of a protease resistant species outside of the chloroplasts
Figure 6. Autoradiograph of SDS-PAGE of in vitro transport of translation products generated from in vitro transcripts of pSn5, pSmaCaM, and pCaM65. Lanes 1-3 contain in vitro translation products with containing the precursor of S, and SmaCaM and CaM with no amino-terminal pS sequence, respectively. Lanes 4-15 contain the stromal fractions from chloroplasts after import of in vitro translation products. Lanes 4-6 stromal fractions following import of pS, lanes 7-9 stromal fractions following import of the precursor of SmaCaM, lanes 10-15 stromal fractions following import of CaM. The conditions for transport are as follows: Lanes 4, 7, 10, and 13 are control conditions which contain no additions, lanes 5, 8, 11, and 14 transport in the presence of 1 mM CaCl₂, lanes 6, 9, 12, and 15 transport in the presence of 1 mM CaCl₂/10 mM EGTA. Stromal fractions in lanes 13-15 are derived from chloroplasts which were treated with 1% v/v Triton X-100 prior to the post-transport protease treatment. > indicates the location of the precursor proteins, * indicates the imported, processed proteins, and ▲ indicates a protease resistant breakdown product.
also can be seen for EO CaM and BCaM when Ca\(^{2+}\) is present (Fig. 5, lanes 8, 9, 12, and 13). If detergent (1% Triton X-100) is added prior to proteolytic digestion, then the protease resistance which results from Ca\(^{2+}\) availability in the control transport mixture is overcome (Fig. 6, compare lanes 10 and 13). It is possible that the detergent interferes with the calcium binding and therefore diminishes the protease resistance. However, the addition of 1 mM CaCl\(_2\) maintains the protease resistance even in the presence of detergent (Fig. 6, compare lanes 11 and 14).

To confirm the results which indicated that different levels of transport-competence exist for the fusions containing various lengths of mature S between the transit sequence and the CaM moiety, we transported the series of fusions in the presence of either 1 mM CaCl\(_2\) and 10 mM EGTA or with calcium alone (Fig. 7). In the presence of CaCl\(_2\)/EGTA only BCaM is not imported and processed (Fig. 7, calcium/EGTA lanes 3-7). Whereas, with the addition of 1 mM CaCl\(_2\) alone, BCaM is still not imported and import of EO CaM is severely inhibited (Fig. 7, calcium lanes 8 and 9). The remaining fusion proteins AvaCaM, SmaCaM, and pSCaM show increasing transport-competence (relative to transport with calcium/EGTA) which correlates with the increasing amounts of mature S present in the fusions (Fig. 7, calcium lanes 10-12 compared to calcium/EGTA lanes 5-7).

These results demonstrate that three levels of transport-competence exists with increasing lengths of wheat
<1 mM CaCl₂/10 mM EGTA> <1 mM CaCl₂>

Figure 7. Autoradiograph of SDS-PAGE of in vitro transport of translation products generated from in vitro transcripts of pSn5, pCaM65, pBCaM, pEOCaM, pAvaCaM, pSmaCaM, and ppSCaM. Lanes 1-7 transport in the presence of 1 mM CaCl₂/10 mM EGTA, lanes 8-12 transport in the presence of 1 mM CaCl₂. In vitro translation products present in the transport reactions were as follows: Lane 1 wheat ps, lane 2 CaM, lanes 3 and 8 the precursor to BCaM, lanes 4 and 9 the precursor to EOCaM, lanes 5 and 10 the precursor to AvaCaM, lanes 6 and 11 the precursor to SmaCaM, lanes 7 and 12 the precursor to pSCaM. > indicates the location of the imported, processed proteins.
pS fused to CaM. When only a small portion of mature S (4 amino acids) is present between the transit sequence and CaM, the fusion protein is transport-incompetent. If a longer segment of S connects the transit sequence and CaM then transport-competence is achieved, so long as the CaM portion of the protein is not in a conformation stabilized by Ca$^{+2}$. As the region of mature S joining the transit sequence to CaM becomes progressively longer (41 amino acid to full length pS) the transport-competence of the fusion proteins becomes less influenced by Ca$^{+2}$ based conformational changes of the CaM portion of the fusion proteins.

One possible explanation for the existence of different levels of transport-competence is that a minimum amount of mature S (greater than 4 amino acids) is required for the transport of a CaM fusion protein. In the case of an intermediate length segment (25 amino acids) of mature S joining the transit sequence and the CaM portion of the fusion protein, the transport-competence seems to be a function of the conformational state of CaM. A correlation of CaM conformational state and the transport-competence of a CaM fusion protein may be a result of the folded CaM portion of the protein interfering with an interaction of the transit sequence and the transport machinery. Alternatively, if a portion of the mature protein must reach the chloroplast stroma for the transport process to continue, then the 25 amino acids of this intermediate
construct may be of insufficient length to provide a functional spanning of the chloroplast envelope membranes with CaM in a folded conformation. Either of these possibilities allows for the decrease in influence that CaM conformation has on the transport-competence of CaM fusion proteins containing longer portions (41 amino acids and longer) mature S.
CHAPTER 2
Modification of Intact Pea Chloroplasts with DIDS Inhibits Transport of Precursor Proteins.
INTRODUCTION

The interplay of two cellular protein-synthesizing systems is essential for the biogenesis and normal function of the chloroplast in higher plants. The cytoplasmic system translates nuclear-encoded poly A+ mRNA on 80S eukaryotic ribosomes and the chloroplast system translates mRNA derived from the chloroplast genome on organellar 70S ribosomes. While both systems are necessary, the preponderance of chloroplast proteins are synthesized in the cytoplasm (1). Most of the chloroplast proteins which are encoded in the nucleus are made as precursor proteins which are slightly larger than the mature proteins found in the chloroplast (2,3). After synthesis in the cytoplasm the precursor proteins are post-translationally transported across the double-membraned chloroplast envelope into the organelle (5,6).

The predominant cytoplasmically synthesized chloroplast protein is the precursor to the small subunit of RuBisCO (6-9). This precursor protein is transported into the chloroplast, proteolytically processed to mature S and assembles in the chloroplast stroma with the large subunits into an L8S8 holoenzyme (5). In addition to pS, the precursor to the chlorophyll a/b binding light-harvesting protein (pLHP) is cytoplasmically synthesized (246,247). LHP is the most predominant protein of the chloroplast thylakoid membranes and is the apoprotein of
the chlorophyll a/b light-harvesting complex (248). The precursor to LHP is post-translationally transported into the chloroplast, proteolytically processed to mature LHP and inserted into the thylakoid membrane (246,247). LHP also binds chlorophyll after transport into the chloroplast however it is currently unclear when during the process this binding occurs.

The process of post-translational protein import into chloroplasts has been studied for over a decade and, as yet, a precise mechanism is still unknown (8). Reconstitution of chloroplast protein transport in vitro has facilitated the study of the import process and helped to determine some of the components of the transport machinery (5,6). One component is the transit sequence of the precursor protein which is required for the transport of most proteins into chloroplasts, including pS (14,16,17). The transit sequence appears to direct or "target" the precursor proteins to the appropriate organelle membrane and also may provide information needed to direct intraorganellar targeting of newly imported proteins (17,24-27).

Energy in the form of ATP is another component required for protein transport into chloroplasts. ATP is required for precursor binding to the chloroplast and the subsequent translocation of the bound precursor proteins (54,55,57,77-82). ATP also is required for intrachloroplasmic sorting of proteins into the correct
compartment. The integration of LHP into the thylakoid membrane requires ATP; and proteins that are ultimately localized in the thylakoid lumen also require ATP to traverse the thylakoid membrane (95-98). Isolated intact thylakoid vesicles have an ATP requirement for the translocation of either a full length or a partially processed intermediate form of a protein destined for the thylakoid lumen (97,98).

Precursor proteins are proteolytically modified, or processed, either during the transport process or immediately afterward (1). This processing step involves cleavage of the amino-terminal transit peptide of a chloroplast precursor protein. The processing activity which converts pS to S is localized in the chloroplast stroma (99). The partially purified stromal processing protease from pea chloroplasts processes the precursors of both stromal and thylakoid proteins to mature size (100). Proteins destined for the lumen of the thylakoids also may be processed in two steps, with the stromal processing protease cleaving the precursor to an intermediate form. An additional processing protease, located in the thylakoids, completes the processing to the mature form (24,51). The thylakoidal processing protease has been partially purified and has been shown to process the intermediate form of a thylakoid lumen precursor to the mature size (103). This thylakoid processing enzyme also can process lumen-bound precursors directly to their
mature size, but at a lower rate. Neither the stromal nor the thylakoidal processing peptidase requires ATP (100,103).

The ATP requirement for import is clear. However chloroplasts have no requirement for either a membrane potential in general or a proton motive force to sustain protein import or binding of precursor proteins (55,76-80). While the energetics of protein transport into intact chloroplasts have been extensively studied, the precise site (or sites) and the mechanism of ATP utilization in the process of protein import are still under debate (45,77-82). The dispute over the site of ATP usage stems from conflicting results regarding experiments in which ATP is depleted from different sides of the double membraned chloroplast envelope.

An important factor contributing to the lack of consensus in the assessment of the location of ATP utilization is the design of the chloroplast envelope. The chloroplast outer envelope is nonspecifically permeable to all small molecules, both charged and uncharged, and has a molecular exclusion limit between 7,000-13,000 Mr (85). This should permit the free movement of ATP between the intermembrane space and the solution surrounding the chloroplast. While the outer envelope may be considered porous, the chloroplast inner envelope acts as a selectively permeable barrier in regard to metabolite traffic between organelle and cytosol. The movement of ATP
and ADP across the inner envelope is facilitated by a specific transporter (86). This ability of ATP to move across the chloroplast envelope serves to complicate experiments designed to remove ATP from either outside or inside of the chloroplast, and allows only indirect removal of ATP from the intermembrane space.

Another proposed component of the protein translocation process for import of chloroplast proteins is a proteinaceous receptor on the outer surface of the chloroplast (5,199). Evidence for the involvement of a chloroplast surface protein was obtained through protease treatment of chloroplasts prior to import. Pretreatment of chloroplasts with the metalloprotease thermolysin inhibits binding of precursor proteins to the organelle as well as import of proteins into the treated chloroplasts (76). Thermolysin is a suitable probe for outer envelope proteins of isolated intact chloroplasts, whereas proteins of the inner envelope are not cleaved by this protease (200,201).

Several chloroplast envelope proteins have been proposed to play a role in the process of protein import (81,204,205,207,208). Anti-idiotypic antibodies generated using a synthetic peptide representing the last 30 amino acids of the transit peptide of pea pS and monospecific antibodies against a purified envelope protein, p36, have both been used to generate Fab fragments which inhibit import of pS into intact chloroplasts. A 30 kDa polypeptide in the inner membrane of the chloroplast
envelope recognized by both antibodies was identified as a chloroplast protein import receptor (205,208).

The chloroplast phosphate translocator is a 29-30 kDa protein which is the most abundant chloroplast envelope protein (93,94,206). The possibility that the 30 kDa protein identified as the chloroplast protein import receptor is the phosphate translocator was at first ignored and then denied (205,208). However, it has recently been demonstrated that, p36, the protein identified as a chloroplast protein import receptor is clearly the chloroplast phosphate translocator and not a receptor for protein import (210,211).

This evidence indicates that a single protein, the chloroplast phosphate translocator, may function in a dual capacity for protein and metabolite import. Import of pS into chloroplasts is inhibited when chloroplast surface proteins are covalently modified by treatment with PLP (81). This treatment modifies a number of chloroplast envelope proteins, the most abundant of which is the 29 kDa phosphate translocator (93,94). Modification of the chloroplast phosphate translocator by PLP also inhibits the transport of phosphate across the chloroplast envelope, but the concentration of PLP needed to affect phosphate transport is approximately two times greater than the amount of PLP which inhibits chloroplast protein translocation (81).
A disulfonic acid stilbene, DIDS, also is a potent inhibitor of the chloroplast phosphate translocator (213). A labeled analog of DIDS, $[^3H_2]$-DIDS, labeled only a 28- to 30-kDa protein of chloroplasts from either C$_3$ and C$_4$ plants. The chloroplast protein labeled by $[^3H_2]$-DIDS in these experiments was proposed to be the phosphate translocator (94). This hypothesis was confirmed by results which demonstrated that a 29 kDa spinach chloroplast envelope protein labeled by $[^3H_2]$-DIDS was purified by the procedure used to purify the spinach chloroplast phosphate translocator. However, an additional higher molecular weight polypeptide also was labeled by $[^3H_2]$-DIDS at higher concentrations (211). The identity of the labeled 29 kDa protein as the chloroplast phosphate translocator was verified by amino acid sequence information derived from a 26.4 kDa tryptic fragment of the labeled protein which matched the deduced amino acid sequence from both the pea and spinach phosphate translocators (82,211,212).

In addition to the inhibition of phosphate transport, DIDS treatment also inhibits the transport of pS into intact chloroplasts. However, the degree of inhibition of protein import is less than for phosphate transport at equivalent DIDS concentrations. Pretreatment of intact chloroplasts with PLP (with no subsequent reduction of the Schiff base) prior to treatment with either DIDS or $[^3H_2]$-DIDS, and followed by removal of the PLP, resulted in a
reduction of DIDS inhibition of phosphate transport and a decrease in the incorporation of label into the 29 kDa phosphate translocator without significantly altering the inhibition of pS import (211). These results indicate that non-covalent PLP binding prevents modification and inhibition of the phosphate translocator by DIDS while still permitting DIDS based inhibition of protein import.

Additional evidence which indicates that the phosphate translocator is not a protein import receptor comes from experiments which demonstrate that both the location in the chloroplast envelope and protease sensitivity of the phosphate translocator are different relative to a precursor binding activity. The chloroplast phosphate translocator is present in the inner chloroplast envelope membrane, and while some binding of pS to inner membrane vesicles was detected, a five fold (100% versus 20%) increase in relative binding of pS occurred with outer envelope membrane vesicles (211). Thermolysin treatment of chloroplasts inhibited binding of precursor proteins and ensuing protein import, but the 29 kDa chloroplast phosphate translocator is resistant to thermolysin treatment (76,211). In agreement with those results, the binding of pS to chloroplast outer envelope membrane vesicles also is sensitive to thermolysin treatment (211).

Another characteristic which appears to differ between the chloroplast phosphate translocator and a potential import receptor is the number of protein sites on the
chloroplast surface. The number of precursor binding sites on the chloroplast surface has been estimated at being in the range of 1,500 to 3,500 (54,214). These numbers were calculated from kinetic experiments of precursor binding and may reflect only a portion of the possible binding sites. However, based on those numbers, the chloroplast protein import receptor should comprise approximately 0.03-0.04% of total chloroplast envelope protein (211). The phosphate translocator, by contrast, is a major envelope protein representing between 15-20% of the total envelope protein (206,210).

Based on differences in the functional characteristics (membrane location, DIDS labeling/inhibition, protease sensitivity, number of membrane sites) of the chloroplast phosphate translocator and a potential chloroplast protein import receptor the hypothesis that the phosphate translocator functions in a dual capacity for protein and metabolite import seems unlikely. The inhibition of protein import by both the anti-idiotypic antibody and the monospecific antibodies against p36 may be explained in two ways: the protein import receptor and the phosphate translocator, both being membrane proteins, may share a common epitope recognized by these antibodies (211). Alternatively, the phosphate translocator (or at least a portion of the translocator population) is at the inner and outer envelope contact site and in close proximity to the protein import receptor. This arrangement could result in
steric hindrance of the import receptor by antibodies recognizing the phosphate translocator.

To determine if there are any possible correlations between the function of the phosphate translocator and the import of precursor protein into intact chloroplasts, we have examined the ability of DIDS and two additional related compounds, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS) and 1,2-dihydro-1,2-(4,4′-diisothiocyanato)-diphenylethane 2,2′-disulfonic acid (H₂-DIDS), to inhibit the transport of pea pS into intact pea chloroplasts. In addition we examined the effect of modification by these compounds on the function of the chloroplast phosphate translocator and the chloroplast ATP/ADP translocator. A possible relationship between DIDS inhibition of protein transport and inhibition of ATP/ADP translocator function is demonstrated.

MATERIALS AND METHODS

Plant Material. Plant tissue was obtained from light grown pea seedlings (Pisum sativum var. Little Marvel). Seeds were surface sterilized for 5 min in 10% v/v household bleach, rinsed thoroughly, allowed to imbibe overnight under running water, and planted in expanded vermiculite. Tissue for mRNA extraction was taken from 7 to 9 day old plants (at the time of expansion of the first leaves).
Tissue for chloroplast isolation was taken from 10 to 14 day old plants.

Chloroplast Isolation. Isolation of intact chloroplasts for protein transport studies was as described by Bartlett, et al. (238) and as outlined in chapter one.

Isolation of Poly A+ mRNA. Poly A+ mRNA isolation was as described by Grossman et al. (239) with the following modifications: tissue was frozen by immersion in liquid N$_2$ after harvesting, and then homogenized with a Polytron homogenizer (PT 35K probe) in 1 liter of extraction buffer containing 100 mM Tris-HCl, pH 9.0/20 mM NaCl/10 mM Mg$^{+2}$ acetate/250 mM sucrose/5 mM DTT. To accommodate larger extraction volumes, all centrifugations were performed in a Sorvall GSA rotor. Two phenol-chloroform-isoamyl alcohol extractions were performed, followed by a third extraction with chloroform alone. The poly A+ mRNA was isolated by the following affinity chromatography procedure: total plant RNA was ethanol precipitated and collected by centrifugation. The resulting pellet was dried under a stream of dry N$_2$ and the dried RNA pellet was dissolved in 10 mM Tris-HCl, pH 7.4/5 mM EDTA-NaOH/0.2% sodium dodecyl sulfate (SDS). The resulting RNA solution was adjusted to 0.4 M NaCl and chromatographed on a polyuridylicate (Poly U) Sepharose 4B (Pharmacia) column which was pre-equilibrated with Wash Buffer (0.4 M NaCl/10 mM Tris-HCl, pH 7.4/5 mM
EDTA/0.2% SDS). The column was washed until all non-polyadenylated (poly A-) RNA detected by UV-monitor had been eluted. The poly A+ mRNA was then eluted from the column with Elution Buffer (90% formamide/10 mM Tris-HCl, pH 7.4/5mM EDTA-NaOH/0.2% SDS). The eluted poly A+ mRNA fraction was diluted 1:1 with water and precipitated twice with ethanol. The final mRNA pellet was dried under a stream of dry N₂, dissolved in water and stored at -70° C.

**Preparation of Wheat Germ Extracts.** A wheat germ cell free extract for in vitro protein synthesis was prepared by the method of Grossman et al. (239) with the following modifications: the extraction and column buffers each contained 3 mM DTT. The centrifugations were carried out at 12,000 x g for 15 min. Four batches of extract supernatant were combined (40 ml), and passed through a Sephadex G-25 (Pharmacia) column. A 40 ml turbid fraction from the void volume was collected, and stored at -70° C.

**In Vitro Protein Synthesis.** Radioactively labeled chloroplast precursor proteins were synthesized in vitro using a wheat germ cell free translation system. The translation reaction contained the following; 50 mM HEPES-KOH, pH 7.6, 100 mM K⁺ acetate, 124 μM spermine neutralized with HCl, 2.1 mM Mg⁺² acetate, 1.2 mM ATP, 80 μM GTP, 9.6 mM phosphocreatine, 20 μM each of the amino acids (minus methionine), 0.4 volume wheat germ extract, approximately
400 μCi/ml [\textsuperscript{35}S]methionine (specific activity approximately 1000 Ci/mmol; New England Nuclear), 40 μg/ml of poly A+ RNA from pea (1 A\textsubscript{260}/ml), and 64 μg/ml creatine phosphokinase (EC 2.7.3.2). The reagents were mixed together and incubated at 26\degree C for 90 min. Following the incubation, the ribosomes were pelleted by centrifugation (at 4\degree C) for 1 hr. at 142,822 \times g (max). The post-ribosomal supernatant was used for chloroplast protein transport experiments.

**Transport of In Vitro Translation Products into Intact Chloroplasts.** Conditions for transport of in vitro synthesized translation products into intact chloroplasts were according to Bartlett et al. (238) and as outlined in chapter one. Any additions to the transport reactions are noted in figure legends.

**Polyacrylamide Gel Electrophoresis.** Proteins were analyzed by SDS-PAGE on 10-15% gradient gels or 12-18% gradient gels containing 7 M urea. Sample preparation and SDS-PAGE procedures were as described by Piccioni, et al. (244). Low range SDS-PAGE molecular weight standards (Bio-Rad) and prestained molecular weight standards (Bio-Rad) were used to determine positions of radiolabeled proteins in the gels and on subsequent electro-bLOTS to nitrocellulose membranes. After electrophoresis, gels were fixed, stained, dried and subjected to autoradiography.
Modification of Intact Chloroplasts with DIDS, H₂-DIDS, or SITS. Isolated intact pea chloroplasts were modified prior to protein transport by the following procedure: chloroplasts equivalent to 200 µg of chlorophyll were treated with various concentrations of modifying reagent (DIDS, H₂-DIDS, or SITS). The modifying reagents were prepared fresh prior to use as 9 mM stock solutions in 50 mM HEPES-KOH, pH 8.0/0.33 M sorbitol and added directly to the chloroplasts to give a final reaction volume of 200 µl. The modifying reaction was incubated for 30 min at 23°C with gentle shaking. After incubation, 100 µl of a solution containing 5 µg/µl BSA in 50 mM HEPES-KOH, pH 8.0/0.33 M sorbitol was added and the reaction incubated for 5 min. The total mixture was then diluted with 5 ml of 50 mM HEPES-KOH, pH 8.0/0.33 M sorbitol and the chloroplasts collected by centrifugation. The chloroplasts were resuspended in 50 µl 50 mM HEPES-KOH, pH 8.0/0.33 M sorbitol and used directly for the transport of in vitro translation products.

Transport of Metabolites into Intact Chloroplasts. The chloroplast modification reactions prior to metabolite transport were similar in all respects to the procedure described above except that after the modification reaction the chloroplasts were diluted to 2 ml with 50 mM HEPES-KOH, pH 8.0/0.33 M sorbitol and used directly for the transport
of metabolites into intact chloroplasts. Phosphate transport and ATP transport were measured by a modification of the silicon-layer centrifugation method of Heldt and Rapley (249). This procedure utilizes elongated 400 μl microcentrifuge tubes (Brinkman) for the transport reaction. The tubes contain 30 μl of 10% w/v perchloric acid overlaid by 170-200 μl of a 99:1 mixture of silicon fluid AR 200 and silicon fluid AR 20 (Wacker). The metabolite transport reactions were initiated by addition of intact chloroplasts to the metabolite uptake reaction layered on top of the silicon fluid mixture. After a two min incubation at 23°C the microcentrifuge tubes were spun and the chloroplasts pelleted through the silicon layer into the 10% perchloric acid terminating metabolite transport.

Metabolite transport reaction mixtures contained the following: isolated intact pea chloroplast were either unmodified or were pretreated with various concentrations of DIDS, H₂-DIDS, or SITS for 15 min at 23°C. Chloroplasts equivalent to 100 μg of chlorophyll were then used for each metabolite transport reaction. The substrate to be transported, either Pᵢ or ATP, was present as a radiolabeled form and each reaction mixture contained 10 μCi. The labeled metabolites used for these transport experiments were 285 mCi/μg H₃[³²P]O₄ (ICN Radiochemicals) and 3 Ci/μmol [α-³²P]ATP (ICN Radiochemicals). The transport reaction mixtures were buffered with 50 mM HEPES-
KOH, pH 8.0 and contained 0.33 M sorbitol as an osmoticum. The total volume of a transport reaction mixture was 200 μl.

After the transport reactions were terminated a sample of the aqueous reaction mixture which remained on top of the silicon fluid layer was taken and the amount of radioactivity present measured to confirm that the isotopically labeled substrates had not become limiting during the transport reaction (greater than 90% of the total radioactivity remaining in the top layer). Next the top layer was removed and the tubes sliced with a razor blade just above the 10% w/v perchloric acid layer at the bottom of the tube. The tube bottom containing the perchloric acid layer and the chloroplast pellet was transferred to a larger tube and 300 μl of 0.5 N NaOH/0.1% v/v Triton X-100 was added. The perchloric acid layer and chloroplast pellet were resuspended by vortexing and a sample was assayed to determine the amount of radioactivity present. Each metabolite transport reaction was repeated in triplicate within a given experiment and the average and standard deviation for each treatment were calculated.

In addition to the transport reactions using P₁ or ATP, reactions using either [³H]₂O (ICN Radiochemicals) or [U-¹⁴C] D-sorbitol (ICN Radiochemicals) also were performed to confirm that the chloroplast modification reactions did not significantly alter the chloroplast volume or unspecific permeability. The reaction mixtures for these
control reactions were the same as those described for the transport reactions except that the substrate was omitted and the reactions contained either 50 μCi [\(^3\)H]₂O or 0.25 μCi [U-\(^{14}\)C] D-sorbitol. The specific activity for the [U-\(^{14}\)C] D-sorbitol was 315 μCi/μmol. As with the transport reactions all control reactions were performed in triplicate within a given experiment and the average and standard deviation of the results were calculated.

**Labeling of Isolated Intact Chloroplasts with \([\^{3}\)H₂]-DIDS.**

Isolated intact pea chloroplasts equivalent to 5 mg chlorophyll were labeled by incubation with \([\^{3}\)H₂]-DIDS with a specific activity of 200 mCi/mmol (HSC Research and Development). The 5 ml incubation mixture also contained 50 mM HEPES-KOH, pH 8.0 and 0.33 M sorbitol. After addition of 33.3 to 100 μCi of \([\^{3}\)H₂]-DIDS the reaction was incubated for 15 min at 23°C with gentle shaking. The reaction mixture was then adjusted to 0.1% w/v BSA and the intact chloroplasts were pelleted through 10 ml of a 25% v/v Percoll (Pharmacia) solution containing 50 mM HEPES-KOH, pH 8.0/0.33 M sorbitol/0.1% w/v BSA by centrifugation at 700 x g (max) for 10 min. The pelleted chloroplasts were resuspended and washed twice with 5 ml of 50 mM HEPES-KOH, pH 8.0/0.33 M sorbitol.

The washed, labeled chloroplasts were then used to isolate chloroplast envelope membranes by the method of Cline et al. (250) with the following modifications: all
envelope solutions contained 10 mM Tricine-KOH, pH 8.0/2 mM EDTA. After the chloroplasts were lysed by the freeze-thaw cycle the thylakoid membranes and stromal fractions were separated by centrifugation at ≈14,500 x g (max) for 15 min. The supernatant containing the stromal fraction was adjusted to 1.3 M sucrose for further envelope isolated. The thylakoid membrane pellet was resuspended in 1.3 M sucrose envelope solution and also was used for further envelope isolation. After the flotation centrifugation procedure the envelope layer was collected and diluted ≈5-fold with 10 mM Tricine-KOH, pH 8.0/2 mM EDTA and pelleted by centrifugation at 285,000 x g (max) for 2 hr at 4°C in a Beckman SW40 rotor. Envelope pellets were collected and prepared for SDS-PAGE.

Isolated envelope proteins were separated by SDS-PAGE through either 10-15% gradient gels or 12-18% gradient gels containing 7 M urea and were electro-transferred to nitrocellulose membrane by the method of Burnette (251). The efficacy of protein transfer was assessed by visualization of prestained SDS-PAGE molecular weight standards and by staining of proteins on the nitrocellulose membrane with 0.5% w/v Ponceau S (C.I. 27195) in 1.0% v/v acetic acid. The nitrocellulose membrane was then fluorographed using the procedure developed by Bonner and Laskey for fluorography of SDS-PAGE gels except that scintillation grade toluene was used instead of dimethyl sulfoxide throughout the procedure (252). Fluorographed
nitrocellulose membranes were then visualized by exposure to Kodak XAR-5 film at -70°C for 30 to 45 days.

Western Blot Analysis of Pea Chloroplast Envelopes. The protein from isolated chloroplast envelope membranes prepared as described above were separated by SDS-PAGE and 7 M urea SDS-PAGE. The electrophoretically-separated proteins were electro-transferred to nitrocellulose membrane by the method of Burnette (251) and the protein transfer was assessed by the procedure described earlier. Antibodies against spinach chloroplast envelope polypeptide E30 (253) were used to probe nitrocellulose membranes containing the chloroplast envelope proteins by the procedure of Burnette (251). E30 antibodies bound to the envelope proteins on the nitrocellulose membrane were detected by subsequent binding of radioiodinated *Staphylococcus aureus* protein A, followed by autoradiography.

Radioiodination of *S.aureus* protein A. Lyophilized *Staphylococcus aureus* protein A (Sigma) was resuspended to 2.5 mg/ml in 50 mM Tris-HCl, pH 7.4/150 mM NaCl. 100 μg of protein A solution and 1 mCi of carrier free Na\(^{125}\text{I}\) in 0.01 N NaOH were mixed together and diluted to 180 μl with 50 mM Tris-HCl, pH 7.4/150 mM NaCl. Three Iodobeads (Pierce), which contain immobilized N-chlorobenzenesulfonamide on nonporous polystyrene beads, were
added as the oxidizing agent for the iodination reaction and the mixture was incubated for 15 min on ice. The reaction was terminated by addition of 50 μl of a 25 mg/ml sodium metabisulfite solution in 50 mM Tris-HCl, pH 7.4/150 mM NaCl and 50 μl of 0.5 M NaI. The labeled protein A was separated from the free Na$^{125}$I by size exclusion chromatography on a Sephadex G-50 (Pharmacia) column which had been equilibrated with 50 mM Tris-HCl, pH 7.4/150 mM NaCl. Radiodinated protein A prepared by this method typically generated 1.5-2 x 10$^8$ cpm of acid precipitable protein.

RESULTS AND DISCUSSION

Transport of in vitro synthesized ps into intact pea chloroplasts is inhibited when chloroplasts are modified by increasing concentrations of DIDS (Fig. 8, lanes 4-9). The amounts of DIDS used for the chloroplast modification in this experiment were 100 nmol, 200 nmol, 300 nmol, 400 nmol, and 500 nmol per mg equivalent of chlorophyll. The import of a second unidentified, higher molecular weight, stromal protein also is apparently inhibited by DIDS modification of chloroplasts prior to protein import (Fig. 8, lanes 4-9). In addition to the inhibition of import of stromal precursor proteins, chloroplast modification by
Figure 8. Autoradiograph of SDS-PAGE of chloroplast stromal fractions following in vitro protein transport of precursor protein generated by in vitro translation of pea poly A+ mRNA. Lane 1 in vitro translation products used for protein transport, lanes 2-9 post-transport stromal fractions of chloroplasts which received the following treatments: lane 2 control chloroplasts with no pretreatment, lane 3 chloroplasts treated with the BSA wash and pellet, lanes 4 chloroplasts diluted with only 0.33 M sorbitol/50 mM HEPES-KOH, pH 8.0 during the modification reaction (dilution control), lane 5 chloroplasts modified with 100 nmol DIDS per mg equivalent of chlorophyll, lane 6 chloroplasts modified with 200 nmol DIDS per mg equivalent of chlorophyll, lane 7 chloroplasts modified with 300 nmol DIDS per mg equivalent of chlorophyll, lane 8 chloroplasts modified with 400 nmol DIDS per mg equivalent of chlorophyll, and lane 9 chloroplasts modified with 500 nmol per mg equivalent of chlorophyll. The position of pS, pLHP, and S are indicated.
increasing concentrations of DIDS also inhibit the import of pLHP (fig 9, lanes 4-9). DIDS treatment also leads to increasing amounts of pS present in the membrane fraction following protein import (Fig. 9, lanes 4-9). This may indicate that, while DIDS treatment of chloroplasts inhibits the transport of precursor proteins, binding of precursors to the chloroplast envelope may still occur and lead to partial protection of the bound precursor from post-transport protease treatment of the chloroplasts. The presence of an additional lower molecular weight protein also is pointed out (Fig. 9, lanes 6-9) and correlates with increasing concentration of DIDS pretreatment of chloroplasts prior to protein transport. The identity of this protein is not known but it may represent a proteolytic degradation product of a precursor bound to the chloroplast surface or a partially processed precursor protein trapped in the chloroplast envelope.

The chloroplast phosphate translocator is the major chloroplast envelope protein modified by DIDS treatment of chloroplasts (94,211). Therefore the possibility that DIDS modification of the chloroplast phosphate translocator was responsible for the inhibition of protein transport into intact chloroplasts was examined. Pre-incubation of chloroplasts with 3-PGA or other metabolites transported by the chloroplast phosphate translocator protected chloroplasts against DIDS mediated inhibition of the chloroplast phosphate translocator (213). However, the
Figure 9. Autoradiograph of SDS-PAGE of chloroplast membrane fractions following in vitro protein transport of precursor protein generated by in vitro translation of pea poly A+ mRNA. Lane 1 in vitro translation products used for protein transport, lanes 2-9 post-transport membrane fractions of chloroplasts which received the following treatments: lane 2 control chloroplasts with no pretreatment, lane 3 chloroplasts treated with the BSA wash and pellet, lanes 4 chloroplasts diluted with only 0.33 M sorbitol/50 mM HEPES-KOH, pH 8.0 during the modification reaction (dilution control), lane 5 chloroplasts modified with 100 nmol DIDS per mg equivalent of chlorophyll, lane 6 chloroplasts modified with 200 nmol DIDS per mg equivalent of chlorophyll, lane 7 chloroplasts modified with 300 nmol DIDS per mg equivalent of chlorophyll, lane 8 chloroplasts modified with 400 nmol DIDS per mg equivalent of chlorophyll, and lane 9 chloroplasts modified with 500 nmol per mg equivalent of chlorophyll. The position of pS, pLHP, and LHP are indicated.
inhibition of chloroplast protein transport by DIDS modification was not affected by pre-incubation of the chloroplasts with 10 mM 3-PGA (fig 10, panel a, lanes 2 and 3, and panel b, lanes 2-4). The 3-PGA pre-incubation of chloroplasts, as well as the presence of 3-PGA in the transport reaction, had no significant inhibitory effect on chloroplast protein transport (fig 10, panel a, lane 2, and panel b, compare lanes 2 and 3). This is indirect evidence that the modification of the chloroplast phosphate translocator by DIDS is not related to the inhibition of protein transport which results from DIDS treatment of chloroplasts.

Since ATP is a critical component of the chloroplast protein transport mechanism, the possibility that DIDS modification of the chloroplasts affected ATP availability was examined. Pre-incubation of chloroplasts with 10 mM Mg2+/ATP prior to the DIDS modification reaction did not prevent the inhibition of protein transport (fig 10, panel a, lanes 4 and 5, and panel b, lanes 4 and 5). As with the 3-PGA results, the presence of the Mg2+/ATP alone had no significant effect on chloroplast protein transport (fig 10, panel a, lane 4, and panel b, compare lanes 2 and 5). In addition to examining the possibility that pre-incubation with Mg2+/ATP would prevent the DIDS inhibition of protein transport, the possibility that the addition of Mg2+/ATP after DIDS modification would relieve the inhibition of chloroplast protein transport also was
Figure 10. Autoradiograph of SDS-PAGE of chloroplast stromal fractions, panel a) and membrane fractions, panel b) following in vitro protein transport of precursor protein generated by in vitro translation of pea poly A+ mRNA. Lane 1 in both panels a) and b) in vitro translation products used for protein transport. Panel a) lanes 2-7 are stromal fractions and panel b) lanes 2-8 membrane fractions after in vitro protein transport in which chloroplasts received the following treatments: panel b) lane 2, control chloroplasts with no pretreatment, panel a) lane 2 and panel b) lane 3, chloroplasts incubated with 10 mM 3-PGA, panel a) lane 3 and panel b) lane 4 chloroplasts modified with 500 nmol DIDS per mg equivalent of chlorophyll in the presence of 10 mM 3-PGA, panel a) lane 4 and panel b) lane 5, chloroplasts incubated with 10 mM Mg$^{2+}$/ATP, panel a) lane 5 and panel b) lane 6, chloroplasts modified with 500 nmol DIDS per mg equivalent of chlorophyll in the presence of 10 mM Mg$^{2+}$/ATP, panel a) lane 6 and panel b) lane 7, control chloroplasts with no pretreatment with 10 mM Mg$^{2+}$/ATP added to the protein transport reaction, panel a) lane 7 and panel b) lane 8, chloroplasts modified with 500 nmol DIDS per mg equivalent of chlorophyll with 10 mM Mg$^{2+}$/ATP added to the protein transport reaction. The positions of pS, pLHP, S and LHP are indicated.
explored. The addition of 10 mM Mg\(^{2+}\)/ATP to the protein transport reaction mixture was unable to stimulate protein transport in chloroplasts which had been modified by DIDS (fig 10, panel a, lanes 6 and 7, and panel b, lanes 7 and 8).

If the DIDS modification treatment of the chloroplasts disrupts the integrity of the chloroplast envelope, then the post-transport protease treatment of the chloroplasts should result in the proteolytic cleavage of a number of stromal and thylakoid membrane proteins. Since there was no apparent decrease in the amount of Coomassie blue stained proteins present in the stromal fractions when analyzed by SDS-PAGE (data not shown) the inhibition of chloroplast protein transport by DIDS was not caused by a disruption of the chloroplast envelope membrane. As an additional test of integrity of the organelle chloroplast protein synthesis was assayed as an indicator of chloroplast function. Since DIDS is a nonpenetrating agent it should not permeate the chloroplast inner envelope, and the effects of DIDS modification should be only on the chloroplast surface (213,254). Chloroplasts modified with a wide range of DIDS (12.5 to 750 nmol DIDS per mg equivalent of chlorophyll) exhibit equal levels of acid precipitable \(^{35}\text{S}\)methionine incorporation as unmodified chloroplasts (data not shown). This is an indication that DIDS modification of chloroplasts does not result in a general non-specific inhibition of chloroplast metabolism.
Since protein synthesis is a complex metabolic process which requires significant ATP utilization, these results also are indirect evidence that chloroplast ATP levels are not reduced by DIDS modification.

The effect of DIDS modification on the chloroplast transport of $P_i$ and ATP was examined to determine if the DIDS modification of pea chloroplasts resulted in a correlation between the inhibition of the phosphate translocator and the inhibition of chloroplast protein transport. The modification of pea chloroplasts with DIDS at 12.5 nmol, 25 nmol and 37.5 nmol per mg equivalent of chlorophyll all resulted in an extensive inhibition of $P_i$ transport into intact pea chloroplasts (fig 11, treatments 2-4). In addition to examining the inhibition of the chloroplast phosphate translocator by DIDS the effect of two other related compounds, $H_2$-DIDS and SITS (Fig. 12), on the chloroplast phosphate translocator was also tested. Treatment of intact pea chloroplasts with either of these compounds at the same level as DIDS treatment (12.5 nmol, 25 nmol, and 37.5 nmol per mg equivalent of chlorophyll) all resulted in similar levels of inhibition of the chloroplast phosphate translocator (fig 11, treatments 5-7 and 8-10 for $H_2$-DIDS and SITS, respectively).

The effect of these compounds on the transport of ATP was also examined. Inhibition of ATP transport was seen when chloroplasts were modified by all three compounds (Fig. 13 treatments 2-4, 5-7, and 8-10, for DIDS, $H_2$-DIDS,
Figure 11. P$_i$ transport into unmodified and modified chloroplasts measured by transport of $[^{32}\text{P}]$P$_i$. Treatments to chloroplasts prior to the P$_i$ transport reactions were as follows: treatment 1, unmodified control chloroplasts, treatment 2, chloroplasts modified with 12.5 nmol DIDS per mg equivalent of chlorophyll, treatment 3, chloroplasts modified with 25 nmol DIDS per mg equivalent of chlorophyll, treatment 4, chloroplasts modified with 37.5 nmol DIDS per mg equivalent of chlorophyll, treatment 5, chloroplasts modified with 12.5 nmol H$_2$-DIDS per mg equivalent of chlorophyll, treatment 6, chloroplasts modified with 25 nmol H$_2$-DIDS per mg equivalent of chlorophyll, treatment 7, chloroplasts modified with 37.5 nmol H$_2$-DIDS per mg equivalent of chlorophyll, treatment 8, chloroplasts modified with 12.5 nmol SITS per mg equivalent of chlorophyll, treatment 9, chloroplasts modified with 25 nmol SITS per mg equivalent of chlorophyll, and treatment 10, chloroplasts modified with 37.5 nmol SITS per mg equivalent of chlorophyll. The radioactivity present in a 20 $\mu$l sample of the resuspended 10% perchloric acid layer containing the chloroplast pellets was determined by liquid scintillation counting. The mean of three replicates for each treatment was calculated and is shown above. The error bars correspond to the standard deviation of the calculated mean.
Figure 12. Chemical structure of DIDS, \( \text{H}_2\text{-DIDS} \), and SITS.
Figure 13. ATP transport into unmodified and modified chloroplasts measured by transport of $[\alpha^{-32}P]ATP$. Treatments to chloroplasts prior to the ATP transport reactions were as follows: treatment 1, unmodified control chloroplasts, treatment 2, chloroplasts modified with 12.5 nmol DIDS per mg equivalent of chlorophyll, treatment 3, chloroplasts modified with 25 nmol DIDS per mg equivalent of chlorophyll, treatment 4, chloroplasts modified with 37.5 nmol DIDS per mg equivalent of chlorophyll, treatment 5, chloroplasts modified with 12.5 nmol $H_2$-DIDS per mg equivalent of chlorophyll, treatment 6, chloroplasts modified with 25 nmol $H_2$-DIDS per mg equivalent of chlorophyll, treatment 7, chloroplasts modified with 37.5 nmol $H_2$-DIDS per mg equivalent of chlorophyll, treatment 8, chloroplasts modified with 12.5 nmol SITS per mg equivalent of chlorophyll, treatment 9, chloroplasts modified with 25 nmol SITS per mg equivalent of chlorophyll, and treatment 10, chloroplasts modified with 37.5 nmol SITS per mg equivalent of chlorophyll. The radioactivity present in a 20 µl sample of the resuspended 10% perchloric acid layer containing the chloroplast pellets was determined by liquid scintillation counting. The mean of three replicates for each treatment was calculated and is shown above. The error bars correspond to the standard deviation of the calculated mean.
and SITS, respectively). The effect that increased levels of DIDS had on the transport of ATP into intact chloroplasts also was examined. With higher levels of DIDS (up to 750 nmol per mg equivalent chlorophyll), the transport of ATP into chloroplasts was inhibited significantly (Fig. 14).

A comparison of the amounts of DIDS needed to mediate inhibition of chloroplast protein transport (100 to 500 nmol per mg equivalent of chlorophyll) and the amount to DIDS needed to abolish phosphate translocator function make it clear that DIDS inhibition of protein transport does not correlate with DIDS inhibition of the chloroplast phosphate translocator. However, the levels of DIDS which mediate significant inhibition of ATP transport (125 to 750 nmol per mg equivalent of chlorophyll) correlate well with the levels of DIDS required for inhibition of protein transport. Taken together these result indicate that DIDS inhibition of chloroplast protein transport involves a modification of a chloroplast protein which also functions in the transport of ATP.

The chloroplast envelope proteins labeled by treatment of intact chloroplasts with $[^3H_2]$-DIDS was examined. A single 30 kDa band was detected after SDS-PAGE and fluorography of electro-blotted proteins (Fig. 15). To determine if this single labeled species represented two (or more) co-migrating proteins 7 M urea SDS-PAGE also was used to analyze the chloroplast envelope proteins labeled
Figure 14. ATP transport into unmodified and modified chloroplasts measured by transport of \([\alpha-^32P]ATP\). Treatments to chloroplasts prior to the ATP transport reactions were as follows: treatment 1, unmodified control chloroplasts, treatment 2, chloroplasts modified with 12.5 nmol DIDS per mg equivalent of chlorophyll, treatment 3, chloroplasts modified with 25 nmol DIDS per mg equivalent of chlorophyll, treatment 4, chloroplasts modified with 37.5 nmol DIDS per mg equivalent of chlorophyll, treatment 5, chloroplasts modified with 50 nmol DIDS per mg equivalent of chlorophyll, treatment 6, chloroplasts modified with 125 nmol DIDS per mg equivalent of chlorophyll, treatment 7, chloroplasts modified with 250 nmol DIDS per mg equivalent of chlorophyll, treatment 8, chloroplasts modified with 375 nmol DIDS per mg equivalent of chlorophyll, treatment 9, chloroplasts modified with 500 nmol DIDS per mg equivalent of chlorophyll, and treatment 10, chloroplasts modified with 750 nmol DIDS per mg equivalent of chlorophyll. The radioactivity present in a 20 μl sample of the resuspended 10% perchloric acid layer containing the chloroplast pellets was determined by liquid scintillation counting. The mean of three replicates for each treatment was calculated and is shown above. The error bars correspond to the standard deviation of the calculated mean.
Figure 15. Fluorograph of a nitrocellulose membrane containing electro-botted chloroplast envelope proteins which were separated on a 10-15% gradient SDS-PAGE. Proteins were labeled by treatment of intact chloroplasts with $[^3]H_2$-DIDS. Envelopes from two separate experiments were run on the gel. The relative positions of low range SDS-PAGE molecular weight standards and prestained SDS-PAGE molecular weight standards are indicated.

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by $[^3\text{H}_2]$-DIDS (Fig. 16). A labeled protein was detected with an apparent molecular weight of 24 to 25 kDa and an additional slower migrating labeled protein also was detected. This second labeled species migrated as a diffuse band with an apparent molecular weight ranging between 26 and 32 kDa (Fig. 16). However, the determination of precise molecular weights of the proteins separated by 7 M urea SDS-PAGE is not possible since the presence of urea can dramatically influence the rate of protein migration during electrophoresis, and therefore, alter the relative migration of molecular weight standards (244). These results indicate that (at least) two proteins may be labeled by $[^3\text{H}_2]$-DIDS. The $[^3\text{H}_2]$-DIDS labeling of the chloroplast envelope proteins could be prevented by pretreatment of the chloroplasts with either DIDS or unlabeled H$_2$-DIDS. This indicates that the proteins labeled by $[^3\text{H}_2]$-DIDS are the same as those modified by DIDS, H$_2$-DIDS, and most probably SITS.

To determine which of the chloroplast envelope proteins labeled by $[^3\text{H}_2]$-DIDS is the chloroplast phosphate translocator Western blot analysis was performed on isolated unlabeled chloroplast envelope proteins separated by either SDS-PAGE or 7 M urea SDS-PAGE (Fig. 17, panels a and b). The antibodies used for this analysis were raised against a spinach chloroplast envelope protein, E30. The E30 used to generate these antibodies was purified by 8 M urea SDS-PAGE separation of proteins extracted by
Figure 16. Fluorograph of a nitrocellulose membrane containing electro-blotted chloroplast envelope proteins which were separated on a 12-18% gradient SDS-PAGE containing 7 M urea. Proteins were labeled by treatment of intact chloroplasts with $[^3H_2]$-DIDS. Envelopes from two separate experiments were run on the gel. The relative positions of low range SDS-PAGE molecular weight standards and prestained SDS-PAGE molecular weight standards are indicated.
Figure 17. Autoradiographs of Western blots probed using antibodies against spinach envelope protein E30. Panel a) nitrocellulose membrane containing electro-blotted chloroplast envelope proteins separated on a 10-15% gradient SDS-PAGE, and panel b) nitrocellulose membrane containing electro-blotted chloroplast envelope proteins separated on a 12-18% gradient SDS-PAGE containing 7 M urea.
chloroform/methanol (2:1, v/v) from isolated chloroplast envelopes. The chloroplast envelope protein recognized by antibodies against E30 is the spinach chloroplast phosphate translocator, a hydrophobic, integral membrane protein (253). The presence of antibody bound to the nitrocellulose membrane was detected by binding of $^{125}\text{I}]$-protein A. In both the SDS-PAGE blot (fig 17, panel a) and the 7 M urea SDS-PAGE blot (Fig. 17, panel b) a single antigenic species was detected. The antigenic protein detected by Western blot analysis in both types of gels co-migrated with the most prominent $[^3\text{H}_2]$-DIDS labeled chloroplast envelope protein. These results confirm earlier results which demonstrated that the chloroplast phosphate translocator is a target of DIDS modification of the chloroplast surface (94,211,213). However, the slower migrating $[^3\text{H}_2]$-DIDS labeled chloroplast envelope protein observed by 7 M urea SDS-PAGE analysis (Fig. 16) was not detected by Western blot analysis with antibodies against E30.

Taken together these results demonstrate that a chloroplast envelope protein which can be modified by DIDS, or the related compounds H$_2$-DIDS and SITS, is involved in chloroplast protein transport. The modification procedure does not affect the integrity of the chloroplast envelope membrane or significantly alter the chloroplast volume. In addition, DIDS modified chloroplasts maintain the ability to synthesize proteins at the same level as unmodified
chloroplasts. This indicates that DIDS modification is not non-specifically affecting chloroplast function and that the interchloroplast pool of ATP remains high enough to drive protein synthesis.

While the chloroplast phosphate translocator is modified by DIDS, the level of DIDS needed to inhibit translocator function does not correlate with the levels of DIDS required to mediate inhibition of chloroplast protein transport. These results are in agreement with those which demonstrated that the presence of non-covalently bound PLP could protect the chloroplast phosphate translocator from DIDS based inhibition and \([^3\text{H}_2]\)-DIDS labeling, while still permitting the inhibition of protein transport by DIDS (211). While earlier results showed that the inhibition of the chloroplast phosphate translocator by DIDS could be diminished by the presence of the substrates for the phosphate translocator, including 3-PGA (213), the presence of 3-PGA could not protect against the DIDS-induced inhibition of chloroplast protein transport. These results cannot rule out the possibility that two separate functional sites exist and that these site have different thresholds for functional inhibition by DIDS.

In addition to the inhibition of the chloroplast phosphate translocator, DIDS modification of intact chloroplasts also inhibited the transport of ATP. The level of DIDS needed to inhibit ATP transport correlates well with the level of DIDS required for inhibition of
chloroplast protein transport. The presence of the translocator substrate (ATP) could not prevent the DIDS mediated inhibition of chloroplast protein transport and addition of ATP after DIDS modification could not relieve the inhibition of chloroplast protein transport. These results, and those inferred from the chloroplast protein synthesis experiment, indicate that even though ATP is present on both sides of the chloroplast membrane, protein transport is still inhibited by DIDS modification. This evidence strongly support the conclusion that the DIDS modification of a chloroplast envelope protein which results in the inhibition of ATP transport also result in the inhibition of chloroplast protein transport. If the availability of ATP of either side of the chloroplast envelope membrane is not the cause of the inhibition of protein transport, then it is possible that the physical movement of ATP through the chloroplast membrane is required for the transport of chloroplast precursor proteins. Alternatively, it is possible that a protein involved in chloroplast protein transport has a dual function and is also involved in the transport of ATP.

While the chloroplast phosphate translocator is the most prominently \(^{3}H_2\)-DIDS labeled chloroplast envelope protein, a second labeled species was also detected. This second protein (or proteins) was not recognized by antibodies against E30. A high molecular weight spinach chloroplast envelope protein also is labeled by a much
higher concentration (5-fold higher) of $[^3\text{H}_2]$-DIDS (211). Therefore, it is possible that another protein unrelated to ATP transport also is modified by DIDS and results in the inhibition of chloroplast protein transport.
SUMMARY AND CONCLUSIONS
Two protein factors involved in the transport of precursor proteins into intact chloroplasts have been examined. The first of these was observed in experiments utilizing a series of fusion proteins containing various lengths of wheat pS fused to CaM. Transport experiments with these proteins demonstrated a requirement for a portion of mature S greater than 4 amino acids for transport of a pS/CaM fusion protein. In addition, when the CaM portion of a fusion protein adopted a stable secondary conformation by binding Ca\(^{2+}\), a larger portion of mature S was needed for efficient transport.

A second protein factor was examined using the protein modifying reagent DIDS, and the related compounds H\(_2\)DIDS, and SITS. Modification of intact pea chloroplasts with these reagents resulted in the inhibition of chloroplast protein transport. This inhibition of chloroplast protein transport was not due to a disruption of the integrity the chloroplast envelope membrane or the result of a non-specific effect on chloroplast metabolism.

The level of DIDS required to cause the inhibition of chloroplast protein transport correlates well with the level of DIDS which mediates inhibition of ATP transport into intact chloroplasts. While DIDS also inhibited the transport of P\(_i\) into intact chloroplasts, the level needed was much lower than that required for the inhibition of chloroplast protein transport. In addition, the presence of 3-PGA during the DIDS modification reaction did not
prevent the DIDS-based inhibition chloroplast protein transport.

While chloroplast ATP transport was inhibited, the apparent presence of ATP inside the chloroplast and the addition of ATP outside the organelle did not relieve the inhibition of chloroplast protein transport caused by DIDS. These results indicate that the physical movement of ATP across the chloroplast envelope membrane may be required for chloroplast protein transport. Alternatively, a chloroplast envelope protein may function in a dual capacity to transport ATP and chloroplast precursor proteins. It also is possible that another protein unrelated to ATP transport also is modified by DIDS at the same level which inhibits ATP transport and this results in the inhibition of chloroplast protein transport.

Chloroplast envelope proteins labeled by $[^3\text{H}_2]$-DIDS treatment of intact pea chloroplast demonstrate that the chloroplast phosphate translocator is the most prominently labeled chloroplast envelope protein. However, an additional labeled species was resolved by SDS-PAGE on 12-18% gradient gels containing 7 M urea. These additional labeled protein (or proteins) did not react with antibodies against the spinach chloroplast envelope protein E30. The E30 antibodies did appear to recognize the pea chloroplast phosphate translocator. This additional protein labeled by $[^3\text{H}_2]$-DIDS may be involved in the transport of chloroplast precursor proteins.
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APPENDIX
Description of the Construction of Protein Fusions Consisting of Various Lengths of Wheat pS Fused to Calmodulin
Construction of pBCaM

STARTING MATERIAL

pSn5 containing the full coding sequence (525 bp) of pS from wheat including 24 bp upstream and 197 bp downstream of the coding region was cloned into the Eco RI/Pst I sites of a pSP65 (Promega) vector which had the vector Sph I site deleted (see ref. 15 for details). The region upstream of the wheat pS sequence in this vector has a portion of a pT7/T3 (BRL) polylinker from an intermediate cloning step.

pCAM23 was obtained from A.R. Means (see references 237 and 238 for a description of this plasmid). This plasmid contains a full length cDNA clone for calmodulin (CaM) from chicken brain. The sequence around the calmodulin initiator ATG in pCAM23 was altered to create an Nco I site in pCAM23.

CONSTRUCTION

pSn5 was digested with restriction endonucleases Bal I (New England Biolabs) and Pst I generating a vector fragment containing the transit sequence and upstream region of pS from wheat to the Bal I site, 12 bp (4 amino acids) into mature S. Plasmid pCAM23 was restriction endonuclease digested with Nco I (BRL) and the resulting 5' overhang was converted to a blunt end with mung bean nuclease (New England Biolabs). The linearized vector was digested with Pst I generating a 313 bp insert fragment containing the first third of CaM extending from 4 base
pairs downstream of the initiation start site to the Pst I site at position 313. The restriction endonuclease digestions were subjected to agarose gel electrophoresis and the appropriate fragment from pCAM23 and the vector portion from pSn5 were ligated to create an intermediate construct. This intermediate construct was digested with Hind III (BRL), thus generating a vector fragment containing all of the aforementioned wheat ps sequence and the first 49 bp of the CaM coding sequence. Plasmid pCAM23 also was digested with Hind III generating an 891 bp fragment containing the carboxy-portion of the CaM coding sequence and 488 bp of downstream sequence. The last 8 bp of this fragment, including the 3' Hind III site, are from a pKK-233 (Pharmacia LKB Biotechnology) vector used to produce pCAM23. The fragment and vector were gel purified, ligated and the resulting vector was named pBCaM.

Figure 18. Schematic depiction of some of the important features of pBCaM
Construction of pEOCaM

STARTING MATERIAL

Plasmids pSn5 and pCAM23 were used as the starting material for this construction. These plasmids are described under the construction of pBCaM.

CONSTRUCTION

pSn5 was digested with the restriction endonuclease EcoO109 (BRL) and the resulting 5' overhang was converted to a blunt end with mung bean nuclease (New England Biolabs). The linearized vector was then digested with restriction endonuclease Pst I generating a vector fragment containing the transit sequence and upstream region of wheat pS to the EcoO109 site, 72 bp (24 amino acids) into mature S. Plasmid pCAM23 was restriction endonuclease digested with Nco I and the resulting 5' overhang was converted to a blunt end with mung bean nuclease (New England Biolabs). The linearized vector was then digested with Pst I generating a 313 bp insert fragment containing the first third of CaM extending from 4 base pairs downstream of the initiation start site to the Pst I site at position 313. The restriction endonuclease digestions were subjected to agarose gel electrophoresis and the appropriate fragment from pCAM23 and the vector portion from pSn5 were ligated to create an intermediate construct. This intermediate construct was digested with restriction endonuclease Hind III generating a vector fragment containing all of the aforementioned wheat pS sequence and
the first 49 bp of the CaM coding sequence. Plasmid pCAM23 also was digested with Hind III generating an 891 bp fragment containing the carboxy-portion of the CaM coding sequence and 488 bp of downstream sequence. The last 8 bp of this fragment, including the 3' Hind III site, are from a pKK-233 (Pharmacia LKB Biotechnology) vector used to produce pCAM23. The fragment and vector were gel purified, ligated and the resulting plasmid was named pEOCaM.

Figure 19. Schematic depiction of some of the important features of pEOCaM
Construction of pAvaCaM

STARTING MATERIAL

An intermediate plasmid, pSn4CaM, was designed as the starting material for the construction of pAvaCaM and ppSCaM. pSn4CaM was assembled from two plasmids: A) pSn4, containing the full coding sequence (525 bp) of pS from wheat including 24 bp upstream and 197 bp downstream of the coding region cloned into the Eco RI/Pst I sites of a pSP65 (Promega) vector which had the vector Sph I site deleted (see ref. 15 for details). The region upstream of the wheat pS sequence has a portion of a pT7/T3 (BRL) polylinker from an earlier cloning step. B) pCAM23, see the construction of pBCaM for a description of this plasmid.

pSn4 was digested with restriction endonuclease Stu I (BRL) generating a vector fragment containing the transit sequence and upstream region of wheat pS to the Stu I site, 379 bp (126 amino acids) into mature S. The Stu I site at the end of mature S disrupts the reading frame in the last codon leaving one extra base. A 10 bp Bam HI linker (Pharmacia LKB Biotechnology) was added to the Stu I blunt ends and the linearized plasmid was then digested with restriction endonucleases Pst I and Bam HI. This digestion removes the 5' end of the linearized plasmid and converts the Bam HI linker remaining at the 3' end to a cohesive end. Plasmid pCAM23 was digested with Bam HI and Pst I generating a 317 bp insert fragment containing the first
third of the CaM sequence beginning 4 bp (-4 bp) upstream from the initiation site to the Pst I site at position 313. The restriction endonuclease digestions were subjected to agarose gel electrophoresis and the appropriate fragment from pCAM23 and the vector portion from pSn4 were ligated. This ligation should have placed the CaM gene in frame with pS, but it did not. This intermediate construct was restriction endonuclease digested with Hind III generating a vector fragment containing all of the aforementioned wheat pS sequence and the first 49 bp of the CaM coding sequence. Plasmid pCAM23 also was digested with Hind III generating an 891 bp fragment containing the carboxy-portion of the CaM coding sequence and 488 bp of downstream sequence. The last 8 bp of this fragment, including the 3' Hind III site, are from a pKK-233 vector used to produce pCAM23. The fragment and vector were gel purified, ligated and the resulting vector was named pSn4CaM.

Plasmid pSn4CaM was digested with restriction endonucleases Ava I (BRL) and Xba I (BRL) generating a vector fragment containing the transit sequence and upstream region of pS from wheat to the Ava I site, 121 bp (40 amino acids) into mature S. The other end (5' end) of the vector fragment has the last 171 bp of the downstream region of CaM. The last 8 bp of this downstream region are from a pKK-233 vector used to produce pCAM23. Plasmid pSn4CaM was digested with Nco I and the resulting
linearized vector was treated with MBN to generate blunt ends. A 10 bp Xho I linker (New England Biolabs) was ligated onto the blunt ends and the DNA was digested with restriction endonucleases Xho I and Xba I generating a 770 bp fragment which contained 5 bp of Xho I linker upstream of the calmodulin gene in addition to the entire calmodulin coding sequence and the first 317 bp of the downstream region. The vector, containing the portion of the wheat pS sequence and the downstream sequence of CaM, and the insert, containing the CaM coding region and a portion of the downstream sequence, were gel purified and ligated. The resulting plasmid had the calmodulin gene out of frame behind the portion of wheat pS. To correct this the plasmid was digested with Xho I, treated with MBN, and ligated. This reduced the spacer region between the truncated S and the calmodulin gene by 4 bp and restored the correct reading frame. The resulting plasmid was named pAvaCaM.

Figure 20. Schematic depiction of some of the important features of pAvaCaM
**Construction of pSmaCaM**

**STARTING MATERIAL**

Plasmids pSn5 and pCAM23 were used as the starting material for this construction. These plasmids are described under the construction of pBCaM.

**CONSTRUCTION**

pSn5 was digested with the restriction endonucleases Sma I (BRL) and Pst I generating a vector fragment containing the transit sequence and upstream region of wheat pS to the Sma I site, 171 bp (57 amino acids) into mature S. Plasmid pCAM23 was digested with restriction endonuclease Nco I and the resulting 5' overhang was converted to a blunt end with mung bean nuclease (New England Biolabs). The linearized vector was then digested with Pst I generating a 313 bp insert fragment containing the first third of CaM extending from 4 base pairs downstream of the initiation start site to the Pst I site at position 313. The restriction endonuclease digestions were subjected to agarose gel electrophoresis and the appropriate fragment from pCAM23 and the vector portion from pSn5 were ligated to create an intermediate construct. This intermediate construct was digested with Hind III generating a vector fragment containing all of the aforementioned wheat pS sequence and the first 49 bp of the CaM coding sequence. Plasmid pCAM23 also was restriction endonuclease digested with Hind III generating an 891 bp fragment containing the carboxy-portion of the CaM coding sequence.
sequence and 488 bp of downstream sequence. The last 8 bp of this fragment, including the 3' Hind III site, are from a pKK-233 vector used to produce pCAM23. The fragment and vector were gel purified, ligated and the resulting vector was named \textit{pSmaCaM}. 

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{psmacam.png}
\caption{Schematic depiction of some of the important features of \textit{pSmaCaM}}
\end{figure}
Construction of ppSCaM

STARTING MATERIAL

The intermediate plasmid construct, pSn4CaM, described in the construction of pAvaCaM was used to create ppSCaM.

CONSTRUCTION

Plasmid pSn4CaM contains the full length (minus 2 bp of the last codon) wheat pS gene fused out of frame via a Bam HI linker to CaM. To correct this problem pSn4CaM was digested with Bam HI, treated with MBN, then religated. This generated a vector in which the space between the wheat pS sequence and CaM was reduced by 4 bp. This restored the correct reading frame and the resulting vector was named ppSCaM.

Figure 22. Schematic depiction of some of the important features of ppSCaM
VITA
Steven M. Pomarico was born in Pittsburgh, Pennsylvania on October 28, 1956. He moved with his parents in 1966 to Naples, Florida. He attended Naples Senior High School and graduated in June of 1975. He then attended the University of South Florida in Tampa, Florida and graduated in June of 1980 with 2 degrees, a B.A. in Biology and a B.A. in Chemistry. In August of 1980 he began pursuit of graduate degree in biochemistry at Louisiana State University in Baton Rouge, Louisiana and pending the completion of his degree requirements will receive a Ph.D. in Biochemistry. He has been a member of the Southern Section of the American Society of Plant Physiologists for several years and was awarded the Outstanding Graduate Student Presentation in 1989.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Steven M. Pomarico

Major Field: Biochemistry

Title of Dissertation: Analysis of Protein Factors Involved in Transport of the Precursor of the Small Subunit of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase into Intact Chloroplasts

Approved:

[Signatures]

Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination: December 13, 1991