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The Effects of Cyclodextrin Mediated Lipid Depletion of the Chloroplast Outer Envelope on the Import of the Small Subunit of Ribulose-1, 5-Bisphosphate Carboxylase/Oxygenase

Robert George Mynatt

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I am submitting herewith a thesis written by Robert George Mynatt entitled "The Effects of Cyclodextrin Mediated Lipid Depletion of the Chloroplast Outer Envelope on the Import of the Small Subunit of Ribulose-1, 5-Bisphosphate Carboxylase/Oxygenase." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Life Sciences.

Barry D. Bruce, Major Professor

We have read this thesis and recommend its acceptance:

Jeffrey Becker & Wesley Wicks

Accepted for the Council:

Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)
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Accepted by the Council:

[Signature]
Associate Vice Chancellor and Dean of the Graduate School
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Chloroplast Outer Envelope on the Import of the Small Subunit for
Ribulose-1,5-Bisphosphate Carboxylase / Oxygenase

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Robert G. Mynatt
August 1998
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Abstract

Chloroplasts have a genome that encodes a minority of proteins necessary for organelle function. The rest are encoded in the plant cell nucleus, translated in the cytosol, and post-translationally imported into the chloroplast. Nuclear encoded chloroplast precursor proteins are made as a precursor which contains an amino-terminal extension known as the transit peptide. Transit peptides are believed to be necessary and sufficient for proper targeting. Several studies have suggested that the initial chloroplast binding interaction is between the transit peptide and the lipids of the chloroplast outer chloroplast envelope. In this study, cyclodextrins are utilized to examine the role of the outer membrane lipid constituency on import of the nuclear encoded precursor, prSSU.

Cyclodextrin mediated lipid depletion affects binding and import of prSSU. Cyclodextrins cause only slight lysis, and treated plastids appear normal by electron microscopy and their ability to evolve oxygen. CD pretreatment enhances the ATP-dependent binding of prSSU, yet has no effect on non-specific binding. Cyclodextrins attenuated in vitro import of prSSU into both pea and spinach chloroplasts in a dose dependent fashion. In addition, in vitro protein import decreased as the time length of CD pretreatment increases. As the temperature of pretreatment increases from 0 to 20°C, the effect of cyclodextrin α lessens, but cyclodextrin β enhances
import inhibition. Treatment with cyclodextrins did not alter the proteinaceous character of the outer membrane. Electron micrographs show no change in chloroplast ultrastructure with CD treatment and photosynthetic activity is not diminished. Density gradient centrifugation of purified envelopes shows an increase in the density of mixed envelopes after CD treatment indicating a decrease in the lipid to protein ratio. TLC shows that cyclodextrin α preferentially removes polar lipids, but not MGDG while β removes galactolipids, SQDG, and PC, but not PG from the outer membrane at the conditions tested. By treating the chloroplasts with cyclodextrins, we have changed the properties of the chloroplast envelopes in such a way that compromises the protein translocation process. Whether this inhibition is a result of a change in lipid to protein ratio, fluidity of the membrane, or bilayer to non-bilayer forming lipid ratio will require further study.
# Table of Contents

Chapter 1  
Introduction.................................................................................................1

Part I. Plastid Structure, Function, and Genetics..............................................1

Part II. Plastid Protein Transport...................................................................2

Part III. Outer Envelope Characteristics.........................................................4

Part IV. The Lipid Polymorphism Model..........................................................6

Part V. Evidence for Lipid : Transit Peptide Interactions...............................8

Part VI. Peptide Insertion Model.....................................................................11

Part VII. Limitations of Lipid Manipulation......................................................12

Part VIII. Cyclodextrin Structure and Function...............................................13

Part IX. Selectivity of Lipid Removal...............................................................14

Part X. Specific Goals......................................................................................15

Chapter 2  
Materials and Methods..................................................................................16

Part I. Chloroplast and Envelope Preparation..................................................16

  a. Plant growth.........................................................................................16

  b. Chloroplast Isolation and Purification..................................................16

  c. Overexpression and Purification of prSSU..........................................17

  d. Binding and Import Assays.................................................................17

  e. Preparation of Inner and Outer Chloroplast Membrane.....................18
Part II. Protein Separation, Probing, and Visualization...............................18
   a. Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis
      (SDS-PAGE)..............................................................................18
   b. Staining of SDS-PAGE................................................................18
   c. Western Blot Assays....................................................................19
   d. Fluorography of Dried Gels.............................................................19
   e. Scanning Densitometry....................................................................20
   f. InstantImaging..................................................................................20
Part III. Cyclodextrin Treatment of Chloroplasts........................................20
Part IV. Lipid Extraction............................................................................21
Part V. Thin Layer Chromatography............................................................22
Part VI. Measurements of Oxygen Evolution.............................................23
Part VII. Electron Microscopy....................................................................23
Part VIII. Statistical Analysis.....................................................................24
Chapter 3 Results......................................................................................25
Part I. Lytic Effects of Cyclodextrins α and β.............................................25
Part II. Effect of Lipid Depletion on Chloroplast Membrane Associated
         Proteins......................................................................................29
Part III. Effects of Cyclodextrins on Protein to Lipid Ratio in
         Membranes..................................................................................32
Part IV. Photosynthesis in Cyclodextrin Treated Chloroplasts.................35
Part V. Ultrastructural Effects of Cyclodextrin Treatment on Chloroplasts

Part VI. Lipid Depletion by Cyclodextrins Affects prSSU Import

a. Dose-dependent import inhibition by cyclodextrin α

b. Dose-dependent import inhibition by cyclodextrin β

c. Import inhibition is dependent on the CD / chlorophyll ratio

d. PrSSU import inhibition after cyclodextrin α incubation time course

e. PrSSU import inhibition after cyclodextrin β incubation time course

f. PrSSU import inhibition by CD treatment at different temperatures

Part VII. Effects of Lipid Depletion by Cyclodextrins on prSSU Binding

a. Cyclodextrin α treatment effects prSSU binding

b. Cyclodextrin β treatment effects prSSU binding

Part VIII. Specific Lipid Depletion by Cyclodextrins α and β

Chapter 4 Discussion

Chapter 5 Future Studies

Part I. Rescue of import with re-addition of lipids
Part II. Lipid biosynthesis mutations.............................................93
Part III. PrSSU import assays in CD-treated plastids........................93
Part IV. Investigating an outer membrane "island" model.................93
Part V. Envelope membrane protein transport after depletion...........95
Bibliography ......................................................................................96
Appendix ............................................................................................104
Vita ......................................................................................................107
List of Figures

Figure 1. Model for chloroplast precursor import.................................3
Figure 2. Molecular shape analysis of representative lipids and their phase
preference..................................................................................................7
Figure 3. Stick model of β cyclodextrin structure........................................26
Figure 4. Lytic effects of cyclodextrins on chloroplasts.............................28
Figure 5. Western blot of cyclodextrin-treated chloroplasts
and soluble supernatant...........................................................................30
Figure 6. Step-gradients of mixed envelopes from chloroplasts
separated by density centrifugation............................................................33
Figure 7. Oxygen measurements of cyclodextrin-treated
chloroplasts..................................................................................................36
Figure 8. Electron micrograph of control chloroplasts at 10,000 x
magnification.............................................................................................39
Figure 9. Electron micrograph of cyclodextrin α treated-chloroplasts at
10,000 x magnification...............................................................................40
Figure 10. Electron micrograph of cyclodextrin β treated-chloroplasts at
10,000 x magnification..............................................................................41
Figure 11. Autoradiography and scintillation data of imported prSSU after cyclodextrin $\alpha$ treatment of pea chloroplasts.................................43

Figure 12. Scintillation data from imported prSSU after cyclodextrin treatment of spinach chloroplasts.........................................................45

Figure 13. Autoradiography and scintillation data of imported prSSU after cyclodextrin $\beta$ treatment of pea chloroplasts...............................46

Figure 14. Scintillation data from imported prSSU after range of cyclodextrin treatment of chloroplasts at two chlorophyll concentrations.................................................................49

Figure 15. Autoradiography and scintillation data of imported prSSU after a cyclodextrin $\alpha$ treatment time course of pea chloroplasts..............51

Figure 16. Autoradiography and scintillation data of imported prSSU after a cyclodextrin $\beta$ treatment time course of pea chloroplasts..............53

Figure 17. Autoradiography and scintillation data of imported prSSU after cyclodextrin $\alpha$ treatment of pea chloroplasts at different temperatures...........................................................................55

Figure 18. Autoradiography and scintillation data of imported prSSU after cyclodextrin $\beta$ treatment of pea chloroplasts at different temperatures...........................................................................57

Figure 19. PrSSU binding of chloroplasts after cyclodextrin treatment........59
Figure 20. Two-dimensional thin layer chromatography of lipids in chloroplasts outer envelopes........................................................................62

Figure 21. Two-dimensional thin layer chromatography of outer envelope lipids removed from untreated chloroplasts..........................64

Figure 22. Two-dimensional thin layer chromatography of outer envelope lipids removed by cyclodextrin α treatment of chloroplasts........65

Figure 23. Two-dimensional thin layer chromatography of outer envelope lipids removed by cyclodextrin β treatment of chloroplasts......66

Figure 24. Two-step lipid removal model..................................................................................70

Figure 25. Lipids stabilize membrane protein conformation.................................................92

Figure 26. Outer envelope island model for prSSU import inhibition...............95
List of Abbreviations

α CD - cyclodextrin alpha
ATP - adenosine triphosphate
β CD - cyclodextrin beta
DGDG - digalactosyldiacylglycerol
MGDG - monogalactosyldiacylglycerol
PC - phosphatidylcholine
PG - phosphatidylglycerol
PI - phosphatidylinositol
prSSU - small subunit of ribulose 1,5-bisphosphate carboxylase / oxygenase
SQDG - sulfoquinovodiacylglycerol
TLC - thin layer chromatography
Chapter 1

Introduction

Part I. Plastid Structure, Function, and Genetics

Plastids are defined by two distinct membranes, collectively called the envelope. Chloroplasts, a developmental subset of plastids, contain an additional internal membrane called the thylakoid. The outer and inner envelope membranes are separated by the inter-membrane space. The interior which contains the thylakoid stacks, or grana, is the stroma. The organelle is the site of photosynthesis, as well as plant metabolism reactions and biosynthesis of the thousands of compounds required for cellular and whole plant function. The plastid maintains a 150 to 200 kilobase genome organized as a circular megaplasmid that encodes proteins required for proper functioning of the plastid (Palmer, 1985). The rest of the machinery is encoded in the nucleus and thus, must be imported back into the chloroplast through the various membranes. An amino-terminal presequence termed the "transit peptide" is both necessary and sufficient for transport as well as posttranslational import of nuclear encoded proteins to the chloroplast (Bruce et al., 1994). Over 600 transit peptides have been studied, and shown to have no real sequence homology (von Heijne et al., 1989, von Heijne et al., 1991). However, most are rich in hydroxylated amino acids compared to presequences and signal sequences (Dobberstein et al., 1996) and slightly
positively charged at their C-terminus (Keegstra et al., 1989). These shared physical characteristics possibly mediate the initial interactions between precursors and the chloroplast surface. They may also provide specificity for a diverse group of presequences destined for a unique localization. To date no chloroplast specific import factors have been observed in the cytosol. This fact distinguishes it from the other cellular organelles. At the surface of the chloroplast, however, import has been characterized to the extent that several of the outer and inner membrane proteins and intermembrane space constituents thought to be associated with a translocation apparatus have been identified. Though the structure and function of these proteins are still unclear, several models have been proposed (Hirsch et al., 1994, Kessler et al., 1994, Perry et al., 1991).

Part II. Plastid Protein Transport

Hypotheses about chloroplast import have been influenced by studying other systems; primarily the mitochondria, but also the endoplasmic reticulum and nucleus. These arguments draw strength from the fact that though the organelles are diverse in structure and function, the pathways traveled by nascent polypeptides to these locations use similar machinery.

Dobberstein and Schatz describe five universal requirements for protein targeting and translocation into an organelle: 1) An amino-terminus containing targeting information for the transported protein, 2) a targeting system on the cis side of the membrane, 3) a transmembrane protein
conducting channel, 4) ATP-dependent translocation machinery that is peripherally attached to the trans side of the membrane, and 5) components on the trans side of the membrane that promote folding of the transported protein (Dobberstein et al., 1996).

Chloroplast precursor import models satisfy four out of five of these requirements (see Figure 1). Precursors utilize a transit peptide that is necessary and sufficient for transport. Outer envelope proteins toc86 (Hirsch et al., 1994, Perry et al., 1994) and toc34 (Waegemann et al., 1991, Millman et al., 1997) are believed to act as a general import receptor for docking the precursor. However, the mechanism used for cis-targeting specifically to the chloroplast envelope is still poorly characterized. Toc75 (Perry et al. 1994) is

Figure 1. Model for chloroplast precursor import
thought to be a part of the translocation channel. Tic97 (Row and Gray, unpublished) may play a part in the translocation channel within the inner membrane. Micromolar levels of ATP/GTP in the inter-membrane space are required for precursor binding (Olsen et al., 1989), while millimolar levels of stromal ATP are required within the stroma of the chloroplast for import (Theg et al., 1989). HSP70’s have been found to be associated with the translocation complex and may be responsible for the ATP requirement. Either Com70 (Kourtz et al., 1997) or CSS1 (Ivey and Bruce, unpublished) may ratchet precursors through the channel or act to refold the precursor in the chloroplast stroma.

Part III. Outer Envelope Characteristics

The role of the unique outer membrane character adds an extra dimension of complexity to the process of chloroplast precursor import unaccounted for by the previous model. This may help account for the poorly defined cis targeting system that seems to be required in other protein transport systems. The membrane has certain novel properties that may elucidate steps in the translocation process. To begin with, the outer envelope has a very high lipid to protein ratio. It is made up of more than 70% lipids and 30% protein (Joyard et al., 1991). This suggests that the lipid content plays a large role in defining cytoplasmic surface of the envelope. Not only is the outer envelope unusual in the quantity of lipids present, but the quality of those lipids. The outer envelope contains the lipid classes
Table 1 Lipid composition of plant cell membranes
This table summarizes data on lipid composition of spinach chloroplast membranes. The polar lipid composition of plastid membranes was obtained after thermolysin treatment of intact organelles.

<table>
<thead>
<tr>
<th>Membrane</th>
<th>MGDG</th>
<th>DGDG</th>
<th>SQDG</th>
<th>PC</th>
<th>PG</th>
<th>PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer Env.</td>
<td>17</td>
<td>29</td>
<td>6</td>
<td>32</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Inner Env.</td>
<td>55</td>
<td>29</td>
<td>5</td>
<td>0</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>Thylakoid</td>
<td>53</td>
<td>27</td>
<td>7</td>
<td>3</td>
<td>7</td>
<td>2</td>
</tr>
</tbody>
</table>


outlined in Table 1. Also detailed are the inner envelope and thylakoid lipid contents. The chloroplast is the only known site of the lipids MGDG, DGDG, and SQDG in the plant cell. These lipids have been implicated in the import process (van’t Hof et al., 1991, de Kruijff, 1994, Bulychev et al., 1994, Rietveld et al., 1995, Pinnadu wage et al., 1996). Relative concentrations of outer envelope lipids may change from species to species (Joyard et al., 1991). The major lipid constituent of the outer membrane, PC, is polar, favors the lamellar phase, regulating membrane fluidity, and may stabilize interactions with intrinsic membrane proteins (Kerber et al., 1992). Other polar lipids that favor the lamellar phase are DGDG and PI. The outer envelope contains the anionic lipids PG and SQDG. SQDG maintains a bilayer forming
conformation, and PG probably does as well. The major non-bilayer forming lipid in the envelope is MGDG. The outer envelope is unique because of a high galactolipid content. DGDG and MGDG make up 50% of the lipids in the membrane. The high MGDG content suggests the potential for phase transitions within the membrane, but the lipid class character and saturation states of the fatty acyl groups keep the envelope in a lamellar phase. The level of MGDG content can be regulated by the coordinate activities of DGDG and MGDG synthase (Dorne et al., 1982, Shimojima et al., 1997). The extent of lipid desaturation can be manipulated by fatty acid desaturases (Browse and Somerville, 1992). These may be responsive to changes in temperature, ionic concentration, or physical stress (Gounaris et al., 1983). Though the structure of the outer envelope clearly favors a bilayer (Cline et al., 1985), exogenous factors may produce localized phase transitions and may even produce lipidic particles (Jacob and Miller, 1986).

Part IV. The Lipid Polymorphism Model

The ability of the outer envelope to undergo a localized phase transition from lamellar to hexagonal II may be involved during import of precursors into chloroplasts. This phenomenon can be explained by a lipid polymorphism model (de Kruijff et al., 1997) (see Figure 2). Lipids can be polymorphic in their aggregate state depending on the volumes filled by their head groups and acyl groups. If the total volume taken up by the fatty acids is less than that taken up by head group and associated ions or water molecules,
the lipid forms a cone shape with the acyl groups as point and the head group as the arc of the cone. These lipids tend to form micelles, or may be present in bends in membranes. Examples of these in figure 2 are detergents and lysophospholipids. If the total volume taken up by the fatty acids is equal to that taken up by head group and associated ions or water molecules, the lipid forms a rectangular shape and dictates a lamellar or bilayer phase. Examples of these lipids in chloroplast membranes are DGDG, PI, and PC. Finally, if the

Figure 2. Molecular shape analysis of representative lipids and their phase preference.

total volume taken up by the fatty acids is greater than that taken up by head
group and associated ions or water molecules, the lipid forms a cone shape
with the head group as point and the fatty acyl groups as the arc of the cone.
This structure pushes membranes towards a hexagonal II conformation at
proper conditions. These lipids are known as non-bilayer forming lipids.
MGDG is while DGDG is not a member of this subset. When a galactose is
added to the head group of MGDG, it is converted to DGDG and now is a
bilayer-preferring lipid. Cardiolipid and PE are non-bilayer lipids of
importance in other membranes (Figure 2). These lipids can create either
inverted micelles or inverted barrel structures within the membrane where
the fatty acid groups face the exterior of the inverted structure. The presence
of polyunsaturated fatty acids in the lipids may enhance this structural affect.
Studies show the presence of these lipids is required for protein transport
across the membranes of bacteria (Rietveld et al., 1995). They are also required
for binding interactions with the surface of chloroplasts (van’t Hof et al., 1993,
Pinnaduwage et al., 1996) and may be required for protein translocation there
as well.

Part V. Evidence for Lipid : Transit Peptide Interactions

Traditionally, binding and import of precursors have been examined in
terms of proteinaceous components on the surface of the chloroplast.
Mounting evidence points towards the role of membrane lipids in binding
and the initial steps of chloroplast protein import. Precursor proteins may
bind the lipid surface via the transit peptide. Transit peptides alone have been shown to interact with the outer membrane of the chloroplast (van’t Hof et al., 1995). In fact, they have a dose dependent lytic effect in the context of the precursor or by itself upon the plastid as well as liposomes made from the lipid constituents of the outer membrane of the chloroplast (Pinnaduwage et al, 1995). This suggests a possible mechanism where there is a direct association of precursors with the chloroplast envelope lipids. The step may represent the initial interaction of the precursor with the membrane leading to a docking of the rest of the polypeptide with the translocation apparatus. Transit peptides have no sequence homology, but have the ability to correctly target a large population of proteins to a single, specific location. This implied specificity may be due to the shared physical qualities of transit peptides studied to date, and the unique lipid character of the chloroplast outer envelope.

Interactions between the transit peptide and the unique lipid classes in the chloroplast outer envelope may mediate binding and translocation of precursor (de Kruijff et al., 1994). Anionic lipids, PG and SQDG mediate transit peptide-membrane interactions on the chloroplast surface (de Kruijff, 1994). The interaction is hypothesized as electrostatic between the anionic lipids and the positively charged amino acids near the transit peptide C-terminus. Transit peptides are characteristically rich in hydroxylated amino acids, small hydrophobic amino acids, and have a slightly positive charge
Interaction with negatively charged lipids is possibly mediated by the high number of hydroxylated amino acids (16 in the TP of prSSU) and positive charge. So, transit peptide binding seems to involve anionic lipids and galactolipids. The transit peptide of prSSU contains 16 hydroxylated of 60 total amino acids with which the galactose group of galactolipids may form hydrogen bonds (van't Hof et al., 1995). MGDG is required for *in vitro* binding of transit peptide to liposomes made up of outer envelope lipids in the same relative concentrations (Pinnaduwage et al., 1996), so it is likely to be required for precursor binding *in vivo*.

Non-bilayer lipids appear to be involved in the insertion of precursor transit peptides into chloroplast outer envelopes. These membranes have a high population of non-bilayer forming lipids made up of MGDG (Joyard et al., 1991). The transit peptide may displace the hydration sphere around the polar head groups of lipids with large fatty acid group volumes to turn bilayer formers into non-bilayer forming lipids, thus enhancing the transitional effect of non-bilayer formers. The combination may induce an HII phase, possibly an inverted micelle or barrel structure through the membrane. Electrophysiological studies show that the transit peptide for ferredoxin induces a reduction in electrochemical resistance of the chloroplast outer membrane (Bulychev et al., 1994). This may be due to opening of localized channels created by the TP-mediated phase transition. The altered character
of the membrane may facilitate transport of chloroplast precursors by inserting the transit peptide into the membrane (de Kruijff, 1994). Peptide fragments representing portions of the transit peptide for prSSU were used in monolayer binding studies to show their ability to insert in the presence of various lipid constituencies (van't Hof et al., 1991). Hydroxylated fragments showed the ability to insert into bilayers containing MGDG, SQDG, and PG. Therefore, the transit peptide is likely to insert in the presence of these lipids in the outer membrane.

**Part VI. Peptide Insertion Model**

The mechanism of insertion may be elucidated by studies of presequences utilized in mitochondrial protein import and signal peptides in bacterial protein export. Presequences interact with non-bilayer forming lipids, CL and PE (Rietveld et al., 1995), concomittant with localized hexagonal II phase transitions in bacterial membranes (Killian et al., 1990) which somehow lead to peptide insertion. A localized phase transition would create inverted barrels or micelles into which the peptide might insert in a process mediated by non-bilayer forming lipids. These phase transitions can be explained by the polymorphism model previously mentioned. Since transit peptides cause phase transitions in liposomes made up of the same lipid constituency as the chloroplast outer envelope (Pinnaduwanage et al., 1996), and they have the ability to insert into monolayers consisting of envelope lipids (van't Hof et al., 1993), they probably induce phase transitions in chloroplast
outer envelopes and insert by the mechanism modeled in bacterial protein export. This suggests one possible mechanism for insertion of the precursor into the translocation channel. Thus, manipulating the lipid constituency is useful for investigation of the mechanisms for precursor binding and insertion into the membrane and translocation apparatus.

Part VII. Limitations of Lipid Manipulation

Previous methods of lipid manipulation employed to study the effects membrane processes were hampered by a number of problems. Enzyme lipase treatments used (Siegenthaler et al., 1986) may decrease a particular class of lipids, but they also leave catalytic products in the membrane. Detergents tend to remove both lipids and proteins. They also remain within the membrane replacing the lipids removed (Murata et al., 1990). Liposomal lipid enrichment presents problems in directional equilibria of lipids and proteins into or out of the membranes treated (Millner et al., 1983). Catalytic hydrogenation tends to contaminate the membranes with catalyst (Vigh et al., 1985). In sum, all of these techniques tend to either leave unnatural products in or remove protein from the membrane. Cyclodextrins avoid these problems by simply removing lipids from the membrane without changing the lipids present, removing proteins, or remaining in treated membranes, themselves.
Part VIII. Cyclodextrin Structure and Function

Cyclodextrins were employed to alter the membrane characteristics by semi-selective removal of lipids from the surface of the chloroplast. They have been used on spinach thylakoid membranes (Rawyler et al., 1991) and human erythrocytes (Ohtani et al., 1989) to remove plant and RBC glycolipids, phospholipids, and cholesterol not to mention numerous biomedical applications in drug delivery (Pitha et al., 1983, Uekama et al., 1987). Cyclodextrins are rings of covalently attached glucose units in an helical array, their hydroxyls faced outward giving them an hydrophylic exterior and an hydrophobic core. There are four types of cyclodextrin called α, β, γ, and δ each corresponding to the number of glucose units in the ring from six to nine, respectively. The smallest cyclodextrin, α, has a diameter of about 8.8 angstroms and a longitudinal length of about 4.4 angstroms. They have a unique ability to carry “guest” molecules within their core to and from lipid bilayers. Lipid removal from membranes has been characterized as a two-step process (Fauvelle et al., 1997) with an initial binding of the cyclodextrin to the head group of the lipid. This slightly disrupts the membrane and pulls the lipid out so that additional cyclodextrins may insert an hydrophobic fatty acyl group into their core. Once the acy groups have been inserted, the lipid is freed from the membrane into the soluble surroundings. These molecules may be used to free lipids from the bilayer of the chloroplast surface.
Part IX. Selectivity of Lipid Removal

Selectivity of removal depends on the structure of the cyclodextrin used and the physical qualities of the specific lipid classes. The cyclodextrins used in this study vary by the length of the polysaccharide chain. They form a helical structure with a hydrophobic interior. The longer the chain, the more hydrophobic the interior. In lipid depletion of thylakoid membranes, the cyclodextrins used showed a preference of SQDG (7% in the membrane) > PG (7% in the membrane) > MGDG (53% in the membrane) > DGDG (27% in the membrane) in removal order. In addition, the cyclodextrins preferred saturated sixteen carbon acyl chains and removal was more stable at lower temperatures (Rawyler et al., 1995). Hydrophobic nature is probably important for insertion into the cyclodextrin core, but hydrogen bonding interactions between head groups and the exterior hydroxyls of the cyclodextrins dictate the initial interaction. Though polar lipids probably have the greatest affinity for cyclodextrins, lipid class selectivity must be determined empirically.

Cyclodextrin mediated lipid removal from thylakoids may differ slightly from outer envelopes in the classes removed. Thylakoid lipid constituency differs from the outer envelope by relative percentages of MGDG and PC. Thylakoids have 53% MGDG while the outer envelope has 17% (Joyard et al., 1991). This may correlate with stacking of the grana or stabilizing the multitude of thylakoid membrane proteins. However, the
outer envelope has 32% PC to 3% in the thylakoids (Joyard et al., 1991). The selectivity of the cyclodextrins for galactolipids may reflect the relative percentages of the thylakoid lipids. The selectivity between galactolipids may change in outer envelope lipid depletion due to different relative percentages, but the greater preference for SQDG and PG is probably maintained.

Cyclodextrins might disrupt import by altering the ability of the transit peptide to interact with the membrane by changes in the lipid constituency. Alternately, these changes might affect the stability of the translocation apparatus, peptide binding or insertion, depending on the quality and quantity of the lipids removed. These changes might occur more distinctly with selective removal of lipid classes, but with non-selective removal to a lesser degree.

Part X. Specific Goals

This current study attempts to elucidate the role of unique chloroplast outer envelope lipids in prSSU import. It will show that: 1) lipids are removed by cyclodextrin treatment of chloroplasts, 2) controlled lipid depletion by these molecules does not lyse or alter function of chloroplasts, 3) binding of precursor is enhanced, but import compromised by treatment, and 4) cyclodextrins have removed specific lipids. The effects of that removal may suggest their function in precursor import.
Chapter 2
Materials and Methods

Part I. Chloroplast Preparation and Precursor Import Competition

a. Plant Growth Dwarf pea seedlings were grown in an EGC TC-30 growth chamber at 17.5°C with 165 mE/m²/s of cool white incandescent light (Sylvania). Plants were grown for 12-16 days before harvesting for experiments.

b. Chloroplast Isolation and Purification Chloroplast were prepared from *Pea sativum* or Spinach leaves by the method of Bruce et al, 1993. Sixteen day old pea plants were harvested, homogenized with a polytron in grinding buffer (see Appendix for buffers) and filtered with cheese and Mira cloth. The plastids were spun down, resuspended in import buffer, and then layered onto 50% preformed continuous Percoll gradients in import buffer. A fraction of the supernatant can be taken at this point, centrifuged again at high speed to remove debris, and saved as Pea plant cytosol. After centrifugation of the cushions, the lower bands (intact chloroplasts) were removed and combined. These were then diluted three-fold in import buffer and pelleted to remove the Percoll. They were then resuspended in import buffer to make ~1.5 to 2.0 mg/ml chlorophyll. Absorbances at 663 and 645nm
of chloroplasts in 80% Acetone were used to measure the exact chlorophyll concentration.

The concentration of chlorophyll can be quantified using this equation:

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

*note - chloroplast number can be quantified by counting a diluted volume of this solution in a hemocytometer, and then comparing to total protein per mg/ml chlorophyll in later assays

c. Overexpression and Purification of prSSU  Overexpressed prSSU was isolated from the BL21 strain of Eschericia coli (Klein et al., 1992).

Radiolabeled prSSU was obtained by growing the cells for 3 hours in methionine/ cysteine-deficient media, followed by incubation with Tran$^{35}$S-label metabolic labeling reagent from ICN. The inclusion bodies were solubilized in 8M urea.

d. Binding and Import Assays  For import assays, 100 nM $^{35}$S-labeled prSSU was incubated either in the presence of 50 μM ATP in darkness for binding, or 2 mM ATP for binding and import, and fresh chloroplasts at 75 μg chlorophyll in 1 X import buffer for 15-30 minutes at room temperature. The chloroplasts were then centrifuged over 40% Percoll in 1x Import Buffer to separate them from lysed chloroplasts and the soluble fraction of the assay. After centrifugation, 50 μl was removed for BCA assays to calculate protein concentration. Import reaction samples containing equal amounts of protein
representing equal amounts of chloroplasts were electrophoresed by SDS-PAGE. The gels were dried down and used for either fluorography and scintillation or by the Packard InstantImager™ to quantitate binding and import due to the presence of the radiolabeled precursor \((^{35}S\text{-prSSU})\) and a cleaved, mature form \((^{35}S\text{-mSSU})\).

e. Preparation of Inner and Outer Chloroplast Membrane  Chloroplasts at 1.5 mg / ml chlorophyll were resuspended in a hypotonic lysis in 12.5 mM MOPS (pH 7.0) and 2.5 mM Magnesium Acetate (to stack thylakoids), left on ice for 10 minutes, and passed through a Ten Broek homogenizer. Thylakoids are centrifuged at 1,500 \(x\) g for 5 minutes, then at 2,500 \(x\) g for 5 minutes. The supernatants from both spins containing the mixed outer and inner membranes are saved. These lysate supernatants are loaded onto 0.46/1.0 M sucrose step gradients and centrifuged at 75,000 \(x\) g overnight in an SW27 rotor so that the membranes stop at the 1.0/0.46 M sucrose interphase while the chloroplast stroma remains above the sucrose. Cyclodextrin-treated mixed membranes were prepared in this manner.

Part II. Protein Separation, Probing, and Visualization

a. Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis (SDS-PAGE)  SDS-PAGE was performed by the method of Laemli et al., 1970.

b. Staining of SDS-PAGE  All SDS-PAGE gels were either stained using Coomassie Brilliant Blue (R-250) solution (1 gram/liter C.B.B. in 50% MeOH, 10% glacial acetic acid, 40% ddH2O) for two hours to overnight, then destained
(50% MEOH, 10% glacial acetic acid, 40% ddH20) for two to four hours, or using the Susan's Silver Stain protocol: a modification of an earlier publication (Heukshoren and Dernick, 1985). This method was employed for gels containing up to 50 fold lower amounts of protein than could be seen with the coomassie stain.

c. Western Blot Assays Proteins were separated by SDS-PAGE in gradient gels for 10%-20% polyacrylamide and then electroblotted to polyvinylidiflouride (PVDF) paper (Immobilon). The blots were probed with the appropriate primary antibodies and then visualized by either chemiluminescence using a goat anti-rabbit antibody coupled to horse raddish peroxidase in the Amersham's ECL system or a goat anti-rabbit antibody coupled to alkaline phosphatase in the Lumigen system.

d. Fluorography of Dried Gels After separation of radiolabelled proteins by SDS-PAGE, the gels were equilibrated into dimethylsulfoxide (DMSO) for 30 minutes, in DMSO with 10% 2,5-Diphenyloxazole (PPO) for another 30 minutes, then rinsed in running deionized water for 15 minutes. The gels were dried onto Whatman #3 paper using a Biorad gel dryer under vacuum at 80°C for two hours. The dried gels were exposed to autoradiography film for a period of time to localize the bands on the dried gel. Rectangles with uniform dimensions were drawn on the back of the Whatman paper over the areas of the gel where the autoradiography film showed bands. The rectangles were then cut out with a razor blade, the paper peeled off, and
placed into carefully labeled scintillation tubes with 10 milliliters of 20% H₂O₂. The tubes were incubated at 80°C overnight, 10 milliliters of scintillation cocktail were added the next morning, and radioactivity was counted.

d. Scanning Densitometry Autoradiographs were analysed by a Molecular Dynamics Computing Densitometer for comparison of bands within the film. The data was given in terms of the volumes that each band within the film represented. Rectangles with equal dimensions were placed around the bands on the autoradiograph to give equal areas. The intensity of each band represented the third dimension. After a background subtraction using an equal area with no sample on the autoradiograph, the volumes could then be compared to a control for a relative band size within each individual autoradiograph. These data could be closely compared to scintillation data for results.

e. Instant Imaging the Packard system for imaging radioactivity directly from dried gels was used to quantitate gross counts, cpm, or dpm of bands representing prSSU and mSSU from import assays. Equal sized rectangles were placed around the radioactive bands and an equally sized rectangle with no band was used for a background subtraction.

Part III. Cyclodextrin Treatment of Chloroplasts

Chloroplasts from pea plants were freshly prepared and resuspended at one mg/ml chlorophyll in import buffer. For lipid depletion, the cyclodextrins α
and β (Fluka) were added to 40 μmoles per milligram chlorophyll from 0°C to 30°C and incubated for a defined length of time. The absolute concentrations range from 0 to 15.4 mM CD and 0.385 mg/ml chlorophyll in removal reactions. The treated chloroplasts were then centrifuged over 40% Percoll to separate the intact from the lysed plastids and cyclodextrin-solubilized lipids, which remained at the top of the cushion. The chloroplasts were resuspended to the original volume and either subjected to a chlorophyll assay or BCA protein assay, if lysis was to be quantitated, or prepared for binding assays, import assays, western analysis, electron microscopy, lipid extraction, or oxygen measurements.

**Part IV. Lipid Extraction**

Chloroplasts treated or not treated with cyclodextrins were centrifuged and pelleted. The pellets were hypotonically lysed in MOPS/MgOAc and the outer/inner envelopes were isolated by step gradient centrifugation, as stated in the Methods. The supernatants from the cyclodextrin treatment containing solubilized lipids were removed and centrifuged at 100,000 g for one hour. The supernatant from this spin, approximately 60 mls, was removed for lipid extraction while the pellet was discarded. The samples was added to chloroform and methanol to a final ratio of 2 : 1 : 0.8 chloroform : methanol : sample for phase separation in a separatory funnel (Bligh and Dryer, 1959). The organic phase was removed for drying in a Buchi
Rotavapor R110. Another 30 ml of chloroform was added to the aqueous phase to extract additional lipids near the interface of the two phases. The dried organic phase containing the lipids was resuspended into 6 ml of chloroform for thin layer chromatography.

**Part V. Thin Layer Chromatography**

Aliquots (200 μl) from the 6 ml of extracted lipids from cyclodextrin-treated chloroplasts were dried onto thin layer chromatography plates from Whatman made from K6 Silica Gel, 5 x 20 cm, 250 μm thick (catalog number 4860-620, lot number 005835). Two-dimensional TLC was performed. The first dimension solvent system was made up of chloroform : methanol : water (65 : 25 : 4) and the second dimension solvent of chloroform : methanol : isoproplamine : saturated ammonium hydroxide (65 : 35 : 0.5 : 5). Three hundred μl of each test sample was dropped 10 μl at a time onto the origin of a TLC plate (1 inch by 1 inch on the lower left corner). Migration distances and Rf values were measured for each lipid. The separated lipids were visualized by a mixture of 10 ml 0.006% Rhodamine and 10 ml 2N NaOH that was evenly sprayed onto the TLC plate or in equilibrated iodine. The plate was then dried under nitrogen gas and exposed to short wave ultraviolet light in the dark to photograph or documented by flat bed scanner for integration into the text.
Part VI. Measurements of Oxygen Evolution

Oxygen evolution and CO₂ uptake by cyclodextrin-treated and control chloroplasts were measured using a Clark electrode (YSI Model 5300 Biological Oxygen Monitor) connected to a chart reader. Three samples, a control, and the two cyclodextrin treatments at 27.5 µmoles/mg chlorophyll, were resuspended at about 20 µg chlorophyll/ml in import buffer with 10 mM NaHCO₃. Four ml of the samples were loaded to the electrode chamber. The samples were equilibrated at 20°C in the dark for about 5 minutes to form a stable baseline, then the lights were turned on (a 60 watt incandescent and the room lights) and the baseline changed. Tangents were drawn to the pre-light and post light baselines for calculation of rates. There was a one to two minute induction period before oxygen evolution occurred. The change in oxygen on the chart recorder was calculated from calibrations at 100% (shaken and sprayed from squeeze bottle) and 0% (water boiled 45 minutes) water saturation. Totally saturated water contains 6.51 µl O₂ / ml or 2.93 nmoles O₂ / ml at 20°C. Therefore, a 1% change on the chart corresponds to a change of 1% of the 2.93 nmoles O₂ / ml. The chart recorded was set on one cm per minute. The rates could then be measured as the change in oxygen concentration per minute on the chart. Chlorophyll concentration from each sample was measured by chlorophyll assay (see Methods). Rates are recorded as µmoles / mg chlorophyll / hour.
sample was measured by chlorophyll assay (see Methods). Rates are recorded as μmoles / mg chlorophyll / hour.

**Part VII. Electron Microscopy**

Chloroplasts treated with cyclodextrins at 27.5 μmoles/mg chlorophyll for comparison to control samples were electron micrographed at the Electron Microscopy Facility in the U.T.K. Biology Division by John Dunlap. Plastids were fixed, thin-sectioned, stained and micrographed. Membranes appear negatively stained.

**Part VI. Statistical Analysis**

Data were analyzed using the StatView® SE + Graphics application for McIntosh. Data were paired and subject to mean, and standard error calculation then graphed using either Kaleidograph, or Cricket Graph.
Chapter 3

Results

Part I. Lytic Effects of Cyclodextrins $\alpha$ and $\beta$

Cyclodextrins are a class of molecules made up of oligosaccharides in an helical array. They can be used to deliver or remove “guest” molecules to or from biological membranes. These guest molecules must have a longitudinal hydrophobic domain that can sit within the binding pocket, the hydrophobic interior of the helix. There are four kinds of cyclodextrins identified by the number of glucose residues they contain. The $\alpha$ cyclodextrin, cyclohexaose, is made up of six, the $\beta$ cyclodextrin, cycloheptaose, seven, the $\gamma$ cyclodextrin, cyclooctaose, eight, and the $\delta$ cyclodextrin, cyclononaose, nine $\alpha$-D-glucose residues connected by $\alpha$ (1→4) bonds. Figure 3 shows a wireframe model of $\beta$ cyclodextrin with a diameter of 8.8 angstroms and a longitudinal distance of 4.4 angstroms. The model image was downloaded from the internet as a PBD file and spatially adjusted using Rasmol 2.6 for MacIntosh. As the unit number increases from 6 to 9 the diameter and long axis length increase. This might indicate a varying specificity for different classes of lipids due to cyclodextrin type. Cyclodextrins, therefore, are good tools to preferentially remove lipids from the membranes of organelles.
Figure 3. Stick model of β cyclodextrin structure
A-cross sectional and B-transverse views of β cyclodextrin structure downloaded as a PBD file from the Internet and spatially adjusted with Rasmol 2.6 showing diameter of 8.8 angstroms and axis length of 4.4 angstroms
Cyclodextrins α and β were used to remove lipids from the outer membranes of both pea and spinach chloroplasts to examine their effects on the stability of the chloroplasts. Chloroplasts were freshly isolated and resuspended at 1.0 mg / ml chlorophyll to use for treatments. To allow for controlled lipid removal, all depletion reactions took place in ice water for 30 minutes. The chloroplasts were re-isolated by centrifugation through a 40% percoll cushion, which separates intact from broken plastids. They were then resuspended to their original volume, and assayed for chlorophyll or protein concentration. Data from seven experiments with pea chloroplast were averaged and standard error was calculated by the StatView program for MacIntosh. Only one experiment was performed with spinach chloroplasts. The data are represented as chloroplast recovery (% of Control), calculated by chlorophyll or bicinchoninic acid (BCA) assay after treatment with cyclodextrin (Figure 4a). Protein and chlorophyll concentration are constant values representing total chloroplast number. As the cyclodextrin α to chlorophyll ratio increased from 0 to 27.5 μM CD / mg chlorophyll in the treated samples, the recovery of chloroplasts was stable. Plastid recovery then decreased from 27.5 to 40 μM CD /mg chlorophyll. The pipetting, centrifugation, and resuspension account for some lysis which is controlled for by the mock treatment. Cyclodextrin β treatments showed little change in recovery from 0 to 25 μM / mg chlorophyll but then dropped more slightly.
Figure 4. Lytic effects of cyclodextrins on chloroplasts
A-pea, and B-spinach chloroplast recovery after treatment with increasing concentrations of CD’s. Seven different data sets are included in the pea graph standard error, while the spinach experiment was only preformed once. %Recovery measured by chlorophyll assay or BCA assay for protein concentration, which correlates with total chloroplast number. Data are then compared to the mock control as described in the Methods.
than cyclodextrin $\alpha$, which was more lytic to the pea chloroplasts. Spinach chloroplasts were treated by the two cyclodextrins and recovered in the same manner. Recovered chloroplasts were assayed for protein concentration by BCA (Figure 4b). Both cyclodextrins $\alpha$ and $\beta$ showed little lytic effect until 20 $\mu$M / mg chlorophyll. Cyclodextrin $\alpha$ treated chloroplasts showed increasing lysis from 22.5 to 40 $\mu$moles CD / mg chlorophyll, while cyclodextrin $\beta$ treated chloroplasts started to lyse at 20 and were completely lysed at 25 $\mu$moles CD / mg chlorophyll. As opposed to pea recovery data, cyclodextrin $\beta$ appeared more lytic in spinach.

**Part II. Effect of Lipid Depletion on Chloroplast Membrane Associated Proteins**

Cyclodextrins were employed to investigate the consequences of lipid depletion from the outer envelope on protein import. The loss of outer envelope proteins, specifically those involved in precursor import, toc86 and toc75, would mask any import loss due to altered lipid involvement. Therefore, the ability of cyclodextrins to remove these proteins was examined (Figure 5). Chloroplasts treated with 20 $\mu$M CD / mg chlorophyll, or an absolute concentration of 7.7 mM cyclodextrin $\alpha$, $\beta$, and a no-treatment control were isolated over a 40% percoll cushion. Samples were taken from the pellet and the supernatant (solubilized proteins). The pelleted chloroplasts were solubilized, and along with supernatant samples were
Control  α CD  β CD
[7.7 mM]  [7.7 mM]

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A. 78 kDa

B. 78 kDa

C. 202 kDa
  109 kDa
  78 kDa

46.7 kDa

34.5 kDa

28.8 kDa

20.5 kDa

7.4 kDa

Figure 5. Western blot of cyclodextrin treated chloroplasts and soluble supernatant Control and treated chloroplasts pellets (P) and supernatants (S), representing the lipid solubilized fraction, were solubilized and separated by SDS-PAGE, electroblotted and probed with A-anti-Toc 86, B-anti-Toc75, and C-Ernst antibody against outer envelope proteins. Each P lane represents equal protein loaded, and each S lane equal volume.
separated by SDS-PAGE, and electroblotted. The blots were probed with antibodies against the toc86 and toc75, and Ernst, a polyclonal antibody against proteins from the outer envelope fraction. Bands on blots were visualized by a colorimetric assay facilitated by alkaline phosphatase which was conjugated to the secondary antibody used in the western assay. The blots were then scanned with a flat-bed scanner.

The blot probed with the toc86 antibody showed the 86 kDa in all treated chloroplasts (pellet (P) lanes, Figure 5a), and in the control (P lane, Figure 5a). In addition, the solubilized fractions all contain a small amount of the 86 kDa protein, including the non-treated control ((S) supernatant lanes, Figure 5a). This co-fractionation of toc86 in a soluble form has been previously noted in the supernatant of high speed centrifugations of chloroplast (Ivey and Pinnaduwage, unpublished). No alternative forms of toc86 are seen in the treated samples in comparison to the non-treated control, with the exception of a 53 kDa breakdown product present in all of the pellet lanes, but not in the supernatant lanes (data not shown).

The blot probed with the toc75 antibody showed a 75 kDa protein with a few cross-reactive bands (data not shown) below for all treated samples, and also the non-treated control (P lanes, Figure 5b). Some toc75 was seen in all of the soluble fractions, including the non-treated control (S lanes, Figure 5b). This is probably due to lysed chloroplast debris that is not easily centrifuged into the pellet. Finally, no alternative forms of toc75 are seen in the treated
samples in comparison to the non-treated control.

The blot probed with the Ernst antibody, like anti-toc86, and anti-toc75, showed no change in protein binding patterns by the antibody. BioRad broad range prestained molecular weight markers (cat#161-0318, control#79774) are shown to the left of the blot. Eleven bands, possibly 13, are present in all treated samples and also the non-treated control (lanes, Figure 5c). These bands correspond to the small number of outer envelope associated proteins that have been reported. No bands are seen in the soluble fractions. There seems to be some loss of approximately 14 kDa and 9 kDa bands in the cyclodextrin α and β treated pellets. Finally, no alternative forms are seen in the treated samples in comparison to the non-treated control.

**Part III. Effects of Cyclodextrins on Protein to Lipid Ratio in Membranes**

The density of cyclodextrin treated mixed envelopes was investigated to see if a change in protein to lipid ratio had occurred. Protein loss due to cyclodextrin treatment was evaluated to be minimal so that any change in density was due to lipid loss resulting in an increased protein to lipid ratio. In other words, mixed membranes from chloroplasts treated with cyclodextrins exhibiting increased density are evidence for substantial lipid removal.

Mixed membranes representing outer and inner envelopes from 16 day old pea chloroplasts treated with 27.5 μM cyclodextrin/ mg chlorophyll were separated by step-gradient centrifugation and compared to an untreated control as detailed in the Methods section. The photograph in figure 6 shows
Figure 6. Step-gradients of mixed envelopes from chloroplasts separated by density centrifugation Equal amounts of chloroplasts were treated with 27.5 μmoles CD/mg chlorophyll and compared to a no-treatment control. Mixed outer and inner envelopes were separated by a density gradient as described in the Methods. The gradient is made up of 12 mLs 1.0 M sucrose, 8 mLs 0.46 M sucrose, and 10 mLs of sample. Upper bands represent carotenoids, middle band mixed envelopes, and the pellet thylakoids. Control tube not shown. Tubes A and B represent the a gradients containing mixed envelopes from CD β and α treated chloroplasts, respectively. Arrows to the right of the window point out the mixed envelope band migrations in tubes A and B, and the control not shown.
two tubes. Tubes A and B contain migrated envelopes from cyclodextrin β and α-treated chloroplasts, respectively. Arrows on the right side of the figure indicate migration distances of control (not shown), cyclodextrins b and α treated envelopes both gradient tubes. Also shown are arrows indicating migration distances of carotenoid and thylakoid bands for the gradient tubes. The membranes treated with cyclodextrin β had an increased migration through the gradient, as compared with control membranes (not pictured) indicating an increased density. Those membranes treated with cyclodextrin α had a yet greater increase in mobility. The control gradient was dropped during photography but showed that the mixed membrane band migrated to a height slightly higher in the gradient than the band in the cyclodextrin β treatment gradient. Also, some spillage in the cyclodextrin α treatment gradient occurred as the control tube was dropped. The same volume of each solution was added and the tubes were balanced before centrifugation. The mixed membrane bands in the experimental tubes were centrifuged through identical conditions to reach their migration point in the gradients. Total pigmentation remains roughly constant, but the treated bands are not as resolved as the control. The gradient was designed for the interface to be isopycnic to the mixed membranes. The treated bands have not yet reached their isopycnic density in the lower solution. These results suggest a change in protein to lipid ratio in the mixed membranes increasing from the control.
to β to α cyclodextrin treated samples by a change in membrane density (Figure 6).

Part IV. Photosynthesis in Cyclodextrin Treated Chloroplasts

The ability of cyclodextrin-treated chloroplasts to undergo photosynthetic reactions was examined to check the intactness of the envelopes. Lysed, or partially lysed plastids lose the ability to evolve oxygen (Walker, 1980). Also, electron transport is inhibited in thylakoids exposed to CD’s (Rawyler et al., 1993). If the envelopes are compromised, the cyclodextrins can deplete the thylakoid membranes. Also, if CD’s pass through the envelopes this same effect might occur. Therefore, the ability of CD-treated chloroplasts to evolve oxygen reflects the ability of the CD’s to either lyse the plastid or pass through the envelope.

Intact chloroplasts were treated with β and α cyclodextrins at 27.5 μM / mg chlorophyll and tested for O₂ production to see if the plastids could still undergo photosynthesis. Chloroplast samples were equilibrated in the electrode chamber at 20°C in the dark until a constant downward slope was seen on the chart reader, representing chloroplast respiration. At which point the lights were turned on, an induction phase occurred, and the curve sloped upwards as oxygen was given off (Figure 7a). Tangents were drawn to pre-light and post-light slopes to extrapolate the rate of O₂ evolution. The untreated control and the two treated samples were assayed at equal temperature and light conditions then adjusted for total chlorophyll to see
Figure 7. Oxygen measurements of cyclodextrin treated chloroplasts Chloroplast samples treated with 27.5 μmoles cyclodextrin/mg chlorophyll were compared to control plastids by their ability to evolve O₂. Control, α, and β treated samples were measured at 88μg, 140μg, and 132μg chlorophyll, respectively. See Methods for electrode use and calculation of rates. Data are shown as A- a schematic of the primary data, and B- rates of O₂ evolution for each sample corrected for chlorophyll. The curves in A differ largely by their sample chlorophyll concentration, which were normalized for in B. These measurements were made once for each treatment, therefore, no standard error was calculated.
the amount of oxygen evolved in response to light (Figure 7b). The control sample gave off 121 μmoles O₂ / mg chlorophyll / hour while the α and β CD treated sample produced 96 μmoles O₂ / mg chlorophyll / hour and 106 μmoles O₂ / mg chlorophyll / hour, respectively. The measurements are sensitive to temperature, age of plastids, unique character of the plastid preparation, barometric pressure, and probe performance. Only one measurement was made for each treatment so no standard error was calculated. Therefore, no conclusion can be made about relative loss of photosynthetic ability. However, the values measured are reasonably similar (within 20%). Measurements made at the same time with samples from the same source are comparable, but a different preparation may give results that are relative to the experiment, but of different magnitude than another sample set. Oxygen evolution in intact spinach chloroplasts has been reported at 81 μmoles/mg chlorophyll/ hour with 4mM bicarbonate as the electron donor (Kraus et al., 1976). A higher level of bicarbonate (10 mM) is used in the current study, but the rates appear comparable. Other rates are evident in the literature that fluctuate around 100 μmoles/mg chlorophyll/ hour for intact plastids. As previously stated, these rates depend largely on the absolute intactness of the chloroplasts in the preparation.

Part V. Ultrastructural Effects of Cyclodextrin Treatment on Chloroplasts

To see if any large-scale structural changes could be detected after cyclodextrin treatment, the plastids were visualized by electron microscopy. Chloroplasts
that had been treated with 27.5 μM/mg chlorophyll α or β cyclodextrin and a
no-treatment control were fixed with glutaraldehyde, embedded, thin-
sectioned, stained, and electron micrographed at the University of Tennessee
Electron Microscopy Facility by John Dunlap, PhD. These pictures show the
changes due to cyclodextrin treatment at an ultrastructural level (Figure 8-10).
Figures 8 shows a control chloroplast with intact membranes, and thylakoid
granae and lamellae. Negatively stained particles represent starch granules
while the darkly stained particles are plastoglobules, the sites of
plastoquinone synthesis. Figures 9 and 10 show chloroplasts treated with
cyclodextrins α and β, respectively. Three centimeters of actual length in the
micrographs represent 1.0 μm distance. Thylakoids and matrix material of
treated chloroplasts stroma seem to be intact within the membranes in figures
9 and 10. No structural changes can be seen. Due to negative staining of the
membranes, no lipid polymorphism or vesiculation in the outer envelope is
easily identifiable.

Part VI. Lipid Depletion by Cyclodextrins Affects prSSU Import

a. Dose-dependent import inhibition by cyclodextrin α

The effects of cyclodextrins on chloroplast import competence were examined
by in vitro import studies using radiolabeled prSSU. The mature form of
the small subunit of ribulose 1,5 bisphosphate carboxylase/oxygenase
(rubisco), mSSU, is the processed, imported form. Any 35S-labeled mSSU
present in electrophoresed samples of repurified chloroplasts after the assay
Figure 8. Electron micrograph of control chloroplasts at 10,000 x magnification. Pea chloroplasts were isolated, fixed, thin-sectioned and stained for electron microscopy. Membranes are negatively stained, but thylakoid grana and lamellae, matrix, negatively stained starch granules and darkly stained plastoglobules are evident (3 cm actual length represents 1.0 μm).
Figure 9. Electron micrograph of cyclodextrin α treated chloroplasts at 10,000 x magnification. Pea chloroplasts were treated with 27.5 μmoles CD α/ mg chlorophyll, re-isolated, fixed, thin-sectioned and stained for electron microscopy. Membranes are negatively stained, but thylakoid grana and lamellae, matrix, negatively stained starch granules and darkly stained plastoglobules are evident (3 cm represents 1.0 μm).
Figure 10. Electron micrograph of cycloextrin β treated chloroplasts at 10,000 x magnification Pea chloroplasts were treated with 27.5 μmoles CD β/ mg chlorophyll, re-isolated, fixed, thin-sectioned and stained for electron microscopy. Membranes are negatively stained, but thylakoid grana and lamellae, matrix, negatively stained starch granules and darkly stained plastoglobules are evident (3 cm represents 1.0 μm).
represents imported precursor. The assay data was quantified by flourography of samples separated by SDS-PAGE followed by liquid scintillation. In some experiments, the samples separated by SDS-PAGE are dried, and mSSU bands counted directly by a Packard InstantImager®. Pea chloroplasts were treated with a range of cyclodextrin concentrations represented by μmoles CD/ mg chlorophyll, re-isolated, and used in prSSU import assays. In Figure 11b, import data is represented by the amount of mSSU quantified as percent of mSSU repurified with chloroplasts with no cyclodextrin α treatment. An autoradiograph of an import assay with lanes corresponding to the columns on figure 11b shows representative primary data (Figure 11a). A known amount of prSSU representing 10% of the label added to the import reaction is seen in the outside left lane. Each sample has been adjusted to allow equal protein concentration representing equal total chloroplasts. They represent, on the high end, one third of the total assay volume. An equal amount of marker is added to all of the subsequent import experiments with the same loading considerations. The prSSU bands above the mSSU bands in the autoradiograph are considered non-productive binding and not quantified in this or any of the following import assays. A processing intermediate of the import reaction is evident between the prSSU and mSSU bands in lanes 0 to 25 μmoles CD/ mg chlorophyll. The band disappears with increasing cyclodextrin treatment, but is present in the control. At import levels of ATP, 2 mM, the amount of import decreases with increasing cyclodextrin α
Figure 11. Autoradiography and scintillation data of imported prSSU after cyclodextrin α treatment of pea chloroplasts. Pea chloroplasts were treated with increasing cyclodextrin concentrations and incubated with prSSU at 2 mM ATP. Amount of prSSU import as represented by A- mSSU in autoradiograph, and B- % of no treatment control in graph with data from 3 experiments corresponding to scintillation counts or densitometry of mSSU bands in the autoradiograph.
pretreatment from 0 to 40 μM / mg chlorophyll in a sigmoidal manner as demonstrated by the curve in figure 11b. Figure 11b shows the data as percent of the untreated samples. Three experimental data sets are used for average values and standard error calculated by the StatView® program for MacIntosh. Furthermore, one experiment identical to the pea chloroplast treatment and import was performed in spinach chloroplasts with cyclodextrin α (Figure 12a). These data also show decreased chloroplast import competence with increased cyclodextrin α treatment, although the spinach plastids appear more sensitive to treatment.

b. Dose-dependent import inhibition by cyclodextrin β

The same import reactions following CD treatment were performed with cyclodextrin β. Pea chloroplasts were treated with a range of cyclodextrin concentrations represented by μmoles CD/ mg chlorophyll, re-isolated, and used in prSSU import assays. The import assays at 2 mM ATP showed similar decreases in import with increasing cyclodextrin β pretreatment. An autoradiograph of an import assay with lanes corresponding to the columns on figure 13b shows representative primary data (Figure 13a). A processing intermediate of the import reaction is evident between the prSSU and mSSU bands in lanes 0 to 20 μmoles CD/ mg chlorophyll. The band disappears with increasing cyclodextrin treatment, but is present in the control. Data was averaged from four sets of import reactions and the standard error was
Figure 12. Scintillation data from imported prSSU after cyclodextrin treatment of spinach chloroplasts. Spinach chloroplasts were treated with increasing cyclodextrin concentrations and incubated with prSSU at 2 mM ATP. Amount of prSSU import in A- cyclodextrin α, and B- cyclodextrin β treated chloroplasts, data collected by direct dpm of prSSU bands from dried gel counted by a Packard InstantImager and expressed as % of control. These experiments were only performed once, therefore no standard error was calculated.
Pea chloroplasts were treated with increasing concentrations of cyclodextrin and incubated with prSSU at 2 mM ATP. Amount of prSSU import as represented by A- mSSU in autoradiograph, and B- % of no treatment control in graph with data from 4 experiments corresponding to scintillation counts or densitometry of mSSU bands in the autoradiograph.
calculated using the StatView® program for MacIntosh. Like the experiments using cyclodextrin α, the assay data was quantified by fluorography of samples separated by SDS-PAGE followed by liquid scintillation or the samples are separated by SDS-PAGE, dried, and mSSU bands counted directly by a Packard InstantImager®. In Figure 13 as in Figure 11, import data are represented by the amount of mSSU quantified as percent of mSSU repurified with chloroplasts with no cyclodextrin treatment. Similar to the cyclodextrin α-treated samples, the amount of mSSU imported in the presence of 2 mM ATP decreases with increasing cyclodextrin β pretreatment from 0 to 40 \(\mu\)M / mg chlorophyll in a sigmoidal manner. The data is fitted using the Kaleidograph application for Macintosh. These import data are from liquid scintillation, direct radioactivity counts from dried gels, or densitometry of autoradiographs. Spinach chloroplasts were treated with cyclodextrin β and used in import assays as in (Figure 12b). These data show decreased chloroplast import competence with increased cyclodextrin β treatment. Though the range is only from 0 to 22.5 \(\mu\)moles CD/ mg chlorophyll compared to 0 through 40 \(\mu\)moles / mg chlorophyll for cyclodextrin α, cyclodextrin β is less effective in inhibiting import than cyclodextrin α.

c. Import inhibition is dependent on the CD / chlorophyll ratio

Import assays were performed on chloroplasts treated at the same cyclodextrin
concentration, yet at two different chlorophyll concentrations; 0.385 mg/ml and 0.096 mg/ml. Import data was gathered for the lower chlorophyll concentration samples in the same manner as stated for the higher chlorophyll concentration samples in the previous paragraphs. The data for the 386 μg/ml samples was converted from μmoles/mg chlorophyll in figures 11 and 12 to mM cyclodextrin for comparison. The import data at the lower chlorophyll concentration showed a decreased import competence as compared with the higher chlorophyll concentration for each cyclodextrin treatment. Figure 14a shows the influence of a higher molar ratio of cyclodextrin α/chlorophyll on import inhibition, while figure 14b shows the effect of a higher cyclodextrin β/chlorophyll ratio. Both cyclodextrin α and β data sets show increasing import inhibition with higher CD to chloroplast ratios. This means that as number of CD molecules per membrane lipid increases, less import occurs. This is intuitively due to increased lipid removal. So as more lipids are removed, less prSSU import occurs. To conclude, the higher the mole ratio of cyclodextrins to chlorophyll in the pretreatment, the higher the prSSU import inhibition.

d. PrSSU import inhibition after cyclodextrin α incubation time course

A time course of cyclodextrin α pretreatment ranging from 0 to 60 minutes showed the time dependent effects of lipid depletion on prSSU import. The
Figure 14. Scintillation data of imported prSSU after range of cyclodextrin treatment of chloroplasts at two chlorophyll concentrations. Data from figures 11 and 12 were re-plotted in terms of absolute cyclodextrin concentration and compared to data from the same cyclodextrin concentration range but with a different chlorophyll concentration. Data are expressed in ultimate cyclodextrin concentrations instead of CD/chlorophyll for prSSU import into cyclodextrin A-α, and B-β treated chloroplasts. New data from direct counts from mSSU bands on dried gels.
pretreatments were performed at 0°C with 27.5 μM cyclodextrin α / mg chlorophyll. The import reactions were performed under import conditions at 2 mM ATP. An autoradiograph of an import assay with lanes corresponding to the columns on figure 15b shows representative primary data (Figure 15a). Data were averaged from four sets of import reactions and the standard errors were calculated using the StatView program for MacIntosh. Import data are represented by the amount of mSSU quantified as percent of mSSU repurified with chloroplasts with no cyclodextrin treatment. Figure 15b shows a general trend of decreasing mSSU bound from 100% to 65% of the 0 minute control with increased cyclodextrin α pretreatment from 0 to 60 minutes. Lipid depletion data from thylakoid membranes are shown in figure 15b at time points corresponding to the import data. Original thylakoid lipid content data from Siegenthaler et al., 1993, and prSSU import data in figure 15b are expressed in terms of μmoles cyclodextrin/ mg chlorophyll. The cyclodextrin per surface area treatment can be calculated for comparison of the two sets of data by ultrastructural information from Joyard et al., 1991 stating that the envelope area is 0.064 m² / mg chlorophyll and the thylkoid area is roughly 1.28 m² / mg chlorophyll. Therefore the amount of cyclodextrin / surface area is easily calculated. Thylakoids are treated at 234 μmoles cyclodextrin / m² membrane area while the outer membranes of the total chloroplast treatment for import reactions were treated at 430 μmoles / m². The zero time point in the import reactions was exposed to cyclodextrin
Figure 15. Autoradiography and scintillation data of imported prSSU after a cyclodextrin α treatment time course of pea chloroplasts. Pea chloroplasts were treated with 27.5 μmoles cyclodextrin / mg chlorophyll from 0 to 60 minutes on ice, in the dark, and incubated with prSSU at 2 mM ATP. Amount of prSSU import as represented by A- mSSU in autoradiograph, and B- % of no treatment control in line graph with data from four duplicate experiments corresponding to scintillation counts or densitometry of mSSU bands in the autoradiograph. Lipid content in B from cyclodextrin a treatment of thylakoids at 234 μmoles/m² compared with prSSU import after envelope treatment at 430 μmoles/m² over a 60 minute time period.

α for up to five minutes during manipulation of the samples. This handling delay may mean that a fast equilibrium has been reached and the zero-time value, to which all other reactions are normalized, is already showing loss of import activity. This would mean that the import diminution curve reflects only some of the CD effect and the ultimate import amount tabulated as percent of control is artificially high. Therefore the import values and curve would probably approach the thylakoid data shown below in figure 15b where the zero time point drops quickly from 100% and then levels off.

e. PrSSU import inhibition after cyclodextrin β incubation time course

The CD β incubation time course followed by import assays also showed decreasing chloroplast import competence over time (Figure 16). The experimental conditions for Figure 16 were exactly the same as those in Figure 15. An autoradiograph of an import assay with lanes corresponding to the columns on figure 16b shows representative primary data (Figure 16a). For this figure, two sets of data were used to calculate average values and standard error. The curve in Figure 16b shows a general trend of decreasing mSSU bound from 100% to 65% of the 0 minute control with increased cyclodextrin β pretreatment from 0 to 60 minutes. This is evidence for a rapid equilibrium during the first 5 minutes of incubation. Previous data show a 75% decrease in import after 30 minutes. This experiment shows a 20% decrease from the control for the same incubation. The import value used for
Figure 16. Autoradiography and scintillation data of imported prSSU after a cyclodextrin β treatment time course of pea chloroplasts. Pea chloroplasts were treated with 27.5 μmoles cyclodextrin / mg chlorophyll from 0 to 60 minutes on ice, in the dark, and incubated with prSSU at 2 mM ATP. Amount of prSSU import as represented by A- mSSU in autoradiograph, and B- % of no treatment control in line graph with data from 2 experiments corresponding to scintillation counts or densitometry of mSSU bands in the autoradiograph (90 minute point withheld).
the zero-time does not represent a zero-treatment, but probably a six minutes treatment, therefore, the other data points are artificially high. So what we see as a 20% decrease probably is ~ 75%. In conclusion, both cyclodextrins diminished chloroplast import competence from 100 to 65% over 0 to 60 minutes, but the realistic percent of import may be lower due to normalization of the data to a zero time control that could have already reached removal equilibrium. Experimental manipulation of the samples before centrifugation step after the CD incubation is about six minutes. Equilibrium is reached very quickly after 5 minutes in lipid removal from thylakoids, so may be reached in outer envelopes, as well.

\textit{f. PrSSU import inhibition by CD treatment at different temperatures}

The effects of temperature on lipid depletion of the outer membrane by cyclodextrins were examined by import assays. As the temperature increased chloroplasts treated with cyclodextrin α exhibited increased import competence from 0°C to 15°C with a decrease from 15°C to 20°C (Figure 17). The 30°C data point was dropped due to experimental error. An autoradiograph of an import assay with lanes corresponding to the columns on figure 17b shows representative primary data (Figure 17a). This experiment was only performed once to find the optimal treatment conditions, so no standard error has been calculated for the data. Sigenthaler’s group showed decreased lipid depletion from thylakoid
Figure 17. Autoradiography and scintillation data of imported prSSU after a cyclodextrin α treatment of pea chloroplasts at different temperatures. Pea chloroplasts were treated with 27.5 μmoles cyclodextrin/mg chlorophyll for 15 minutes at varying temperatures in the dark, and incubated with prSSU at 2 mM ATP. Amount of prSSU import as represented by A- mSSU in autoradiograph, and B- % of no treatment control in bar graph with data corresponding to scintillation counts or densitometry of mSSU bands in the autoradiograph (30°C point dropped due to experimental error). This experiment was only performed once, therefore no standard error was calculated.
membranes at elevated temperatures with cyclodextrin α from 0°C to 20°C. They also showed the enhanced ability of cyclodextrin β to deplete in the presence of MgCl₂. On the other hand, chloroplasts treated with cyclodextrin β showed decreased import competence as the temperature of treatment increased from 0°C to 30°C (Figure 18). Once again, the autoradiograph of an import assay with lanes corresponding to the columns on figure 16b shows representative primary data (Figure 18a). The 20°C data point in this experiment was also dropped due to experimental error. Chloroplast import competence alteration by cyclodextrins α and β have different effects due to the temperatures of treatment. This may reflect the differences in lipids removed by the two cyclodextrins.

Part VII. Effects of Lipid Depletion by Cyclodextrins on prSSU Binding

a. Cyclodextrin α treatment effects prSSU binding

Precursor binding is hypothesized to involve not only outer envelope protein receptors, but lipids. Lipid depletion might enhance binding by enriching the protein concentration on the surface, or alter binding by changing the concentration of lipid components necessary for surface interaction. Binding is thought to occur via two processes. Productive binding resulting in protein import is ATP-dependent, but non-specific binding is not. Changes in binding at low levels of ATP possibly reflect alterations in the ATP-dependent process, while changes in binding at higher levels of ATP may reflect
Figure 18. Autoradiography and scintillation data of imported prSSU after a cyclodextrin \( \beta \) treatment of pea chloroplasts at different temperatures. Pea chloroplasts were treated with 27.5 \( \mu \)moles cyclodextrin/mg chlorophyll for 15 minutes at varying temperatures in the dark, and incubated with prSSU at 2 mM ATP. Amount of prSSU import as represented by A- mSSU in autoradiograph, and B- % of no treatment control in bar graph with data corresponding to scintillation counts or densitometry of mSSU bands in the autoradiograph (20\( ^\circ \text{C} \) point dropped due to experimental error). This experiment was only performed once, therefore no standard error was calculated.
alterations in the non-specific process. At high ATP, 2mM, in \textit{in vitro} precursor import reactions, all precursor associated with the productive import pathway of the envelopes are thought to be imported. Those left bound are considered non-specifically bound. With these considerations, changes in prSSU binding at the two ATP levels after cyclodextrin treatment provide information concerning the role of lipids removed in the binding process.

The effects of cyclodextrin \(\alpha\) on binding of chloroplast protein precursors was examined at binding and import concentrations of ATP (Figure 19). Binding of chloroplast precursors can be quantified by examining the amount of \(^{35}\text{S}-\text{labeled prSSU}, the nuclear-encoded precursor to the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), that repurifies with chloroplasts and is seen in the electrophoresed chloroplast binding assay sample. The prSSU is a higher molecular weight form of SSU, containing the transit peptide, and is seen above the lower weight mSSU on SDS-PAGE. Chloroplasts were treated with varying amounts of cyclodextrin \(\alpha\) and subjected to binding assays in the presence of either 50 \(\mu\text{M}\) or 2 mM ATP (Figure 19a). Binding data are represented by the amount of prSSU quantified as percent of prSSU repurified with chloroplasts with no cyclodextrin treatment. At binding conditions, 50 \(\mu\text{M}\) ATP, the amount of prSSU bound increases from 0 to 15 \(\mu\text{M}\) cyclodextrin \(\alpha\) / mg chlorophyll then
Figure 19. PrSSU binding of chloroplasts after cyclodextrin treatment Chloroplasts were incubated with prSSU to show binding after a range of A-α, and B-β cyclodextrin treatments at 50 μM ATP (dark red or blue bars, respectively) or 2 mM ATP (light red or blue bars, respectively), data collected from scintillation counts of prSSU bands cut from a dried gel that correlate to prSSU bands on an autoradiograph, expressed as % of control. CD α experiment only performed once, so no error is shown, but CD β graph includes two data sets in the error bar calculations.
decreases from 15 to 30 μM cyclodextrin α / mg chlorophyll. In fact, prSSU only drops below the untreated level between 25 and 30 μM cyclodextrin α / mg chlorophyll. Under import conditions at 2 mM ATP, the amount of prSSU bound varies little. The unmodified scintillation data shows significantly higher amounts of prSSU present in the 50 μM ATP samples but with the same trends seen in the Figure 19 graph for both ATP concentrations (data not shown).

b. Cyclodextrin β treatment effects prSSU binding

The same experiments were performed using cyclodextrin β in Figure 19b. Chloroplasts were treated with varying amounts of cyclodextrin β and subjected to binding assays in the presence of either 50 μM or 2 mM ATP. Binding data is represented by the amount of prSSU quantified as percent of prSSU repurified with chloroplasts not treated with cyclodextrin. Data were averaged from two identical sets of binding reactions and the standard errors were calculated using the StatView program for MacIntosh. At binding conditions, 50 μM ATP, the amount of prSSU bound increases from 0 to 20 μM cyclodextrin β / mg chlorophyll then decreases from 20 to 30 μM cyclodextrin β / mg chlorophyll. However, at import conditions with 2 mM ATP, binding decreases from 0 to 20 μM cyclodextrin β / mg chlorophyll, then increases to 25 and decreases to 30 μM cyclodextrin β / mg chlorophyll. There
appears to be an enhancement in binding at the 50 µM ATP concentration. This binding is thought to be ATP-dependent and may be mediated by proteinaceous components of the envelope. Considering the error for each sample in the 2 mM ATP concentration, there may be no real change in binding brought about by cyclodextrin β, and though there are no error bars for α, no change was observed for that cyclodextrin.

Part VIII. Specific Lipid Depletion by Cyclodextrins α and β

Commercial lipid standards (MGDG, DGDG, PG, and PC from Avanti Polar Lipids) and lipids from whole pea chloroplast extraction, representing about 99% thylakoid lipids, were separated by thin layer chromatography to evaluate the migration patterns of the five major lipid classes in chloroplast membranes (parts B and A, Figure 20). Migration distances and Rf values are shown in part C. These data were coupled with the migration patterns of the same lipid classes in the same solvent systems (Allen et al., 1971) from which the TLC method came, to identify the chloroplast lipids in current experiments. The test samples came from lipids removed from chloroplast outer membranes by cyclodextrin treatment. The lipid-cyclodextrin soluble complexes in the supernatant from the cyclodextrin treatments and control used to isolate mixed membranes were extracted and dried by the method of Bligh and Dryer (1959). The lipids were resuspended in a small volume of chloroform and an equal volume of each sample was dotted onto a thin-layer chromatography plate for separation (Figures 21-23). The control
A. MG DG
B. MG DG

C. | Avg. X | Rfx  | Avg. Y | Rfy  |
---|-------|------|-------|------|
MGDG | 5.42  | 0.788| 5.63  | 0.818|
DGDG | 2.16  | 0.314| 3.82  | 0.555|
SL   | 3.21  | 0.467| 2.96  | 0.430|
PC   | 2.41  | 0.350| 2.60  | 0.378|
PG   | 4.42  | 0.642| 3.00  | 0.436|
PI   | 1.88  | 0.273| 2.25  | 0.327|

Figure 20. Two-dimensional thin layer chromatography of lipids in chloroplasts outer envelopes. Lipids from A-chloroform solubilized pea chloroplasts representing ~99% thylakoids, and B-known standards were separated by 2-D TLC. See Methods for solvent and plate details. The lipid migration distances (inches) and Rf values are shown in C for the individual lipids (MGDG: monogalactosyldiacylglycerol, DGDG: digalactosyldiacylglycerol, SL: sulfoquinovosyldiacylglycerol, PC: phosphatidylcholine, PG: phosphatidylglycerol, PI: phosphatidylinositol).
sample showed no lipids present (Figure 21) but had two spots (A and B) that are artifacts of the extraction process. These two spots are seen in every experimental plate (Figure 21, 22, and 23) where samples have undergone the extraction process described in the Methods, but are not seen in standard plates (Figure 20, a and b). In addition, they do not match published Rf values for chloroplast lipids (Allen et al., 1971). Therefore, they must be contaminants of the extraction of cyclodextrin-solubilized lipids. The α cyclodextrin treated sample showed the presence of the polar lipids, DGDG, SQDG, PC, PI, and PG, but lacked the non-polar MGDG (Figure 22). The β cyclodextrin-depleted lipids contained MGDG, DGDG, SQDG, and PC but lacked PG (Figure 23). The relative quantities shown in Table 2 indicate tentative preference of α cyclodextrin for polar lipids, while β cyclodextrin specificity remains unclear. In addition, cyclodextrin α seems to deplete a higher amount of lipids than cyclodextrin β at 0°C, in the dark. In cyclodextrin treatment TLC plates, all of the chloroplast outer envelope lipids classes are present in one or the other, so no lipids are lost as a result of the extraction conditions. Siegenthaler’s group show a lipid removal preference for cyclodextrin α of SQDG > PG > MGDG > DGDG.
Figure 21. Two-dimensional thin layer chromatography of outer envelope lipids removed from untreated chloroplasts
Solubilized lipids from chloroplasts not treated with cyclodextrins were separated by TLC. The solubilized lipids were extracted and dried down. See methods for extraction conditions. After resuspension, 300 μL of the untreated sample was separated by two-dimensional TLC.
Figure 22. Two-dimensional thin layer chromatography of outer envelope lipids removed by cyclodextrin α treatment of chloroplasts. Chloroplasts were treated with 27.5 μmoles cyclodextrin/mg chlorophyll. The solubilized lipids were extracted and dried down. See methods for extraction conditions. After resuspension, 300 μL of the α.CD treated sample was separated by two-dimensional TLC.
Figure 23. Two-dimensional thin layer chromatography of outer envelope lipids removed by cyclodextrin β treatment of chloroplasts. Chloroplasts were treated with 27.5 μmoles cyclodextrin β/mg chlorophyll. The solubilized lipids were extracted and dried down. See methods for extraction conditions. After resuspension, 300 μL of the β CD treated sample was separated by two-dimensional TLC.
Table 2
A comparison of the removabilities of lipid classes in thylakoids and outer envelopes by cyclodextrins α and β suggesting class preference per CD.

<table>
<thead>
<tr>
<th>CD</th>
<th>Membrane</th>
<th>Lipid removal (mol % (T) and spot size (E))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MGDG</td>
</tr>
<tr>
<td>α</td>
<td>T</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>E</td>
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<tr>
<td>DM-β</td>
<td>T</td>
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<td>β</td>
<td>E</td>
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Data from thylakoid lipid removal by CDs was compared to visually inspected lipid removal from outer envelopes. Thylakoids (T) were treated at 100 μmoles/mg chlorophyll while total chloroplasts (E) were treated at 27.5 μmoles/mg chlorophyll. All reactions at 0°C for 30 min. The amount of each lipid class removed from thylakoids (data from Rawyler et al., 1996) is expressed as percentage of the corresponding lipid class content of control membranes, while the amounts removed from envelopes (our data) are expressed as plusses or minusses depending on the size of the lipid class spots solubilized by cyclodextrins, extracted, concentrated, and separated by TLC.


Our results are in agreement for both classes tested with the exception of MGDG for α and PG for β. Cyclodextrins α and β do seem to have a lipid class removal preference from chloroplast outer envelopes according to these results.
facilitate transport of signal sequences across bacterial membranes (Reitveldt et al., 1995), binding of trFd to lipid vesicles (van't Hof et al., 1995), and prSSU transit peptide insertion into monolayers (van't Hof et al., 1991). In fact, in the presence of non-bilayer forming lipids, the transit peptide induces localized changes in the phase of the membrane from lamellar to hexagonal II characterized by inverted micelles or barrels (Pinnaduwage et al., 1996). This alteration is supported by the release of a fluorescent calcein dye from liposomes made up of outer envelope lipids as they are exposed to the transit peptide of prSSU.

Cyclodextrins are a class of oligosaccharides that are capable of specifically removing lipids from biological membranes (Rawyler et al., 1996, Ohtani et al., 1989). Due to the unique physical characteristics of the hydrophylic exteriors and hydrophobic interiors of cyclodextrins and the varying head groups as well as saturation levels of fatty acids of lipids, there should be a preferential removal of different lipid classes. In fact, cyclodextrins preferentially deplete anionic lipids over galactolipids in thylakoids (Rawyler et al., 1996). Considering the similarity of lipid constituents, they should preferentially deplete from the outer envelope of chloroplasts in a like manner. Upon incubation of cyclodextrins with chloroplasts at 0°C, little lysis occurs indicating a controlled lipid depletion. Western blots of outer envelope fractions showed little change in membrane protein occurrence from treated to untreated plastids. Therefore, the main
effect these molecules have upon chloroplast seems to be the selective removal of lipids. Fauvelle et al. present a “two-step” model for lipid removal based on NMR studies in human erythrocytes (Figure 24). First there is an interaction between polar or charged lipid head groups and the exterior hydroxyls of a cyclodextrin molecule which slightly pulls the lipid out of the bilayer creating a localized increase in fluidity. At this point, the fatty acids are more exposed to the aqueous exterior and vulnerable to insertion into the hydrophobic core of the cyclodextrins. Upon insertion, the lipid is solubilized and removal is complete. Hypothetically, class specific lipid depletion could change the

Figure 24. Two-step lipid removal model

ratio of bilayer to non-bilayer forming lipids. Since the majority of lipids in
the outer membrane are bilayer formers, usually ~80%, (32% PC, 39% other),
even a non-specific removal may change the ratio. The outer envelope
normally maintains a lamellar phase (Cline et al, 1985) but can be pushed
towards localized hexagonal II arrangements by changes in temperature,
electrostatic interactions with ions or water, saturation states of fatty acids,
and physical stress. (CURVATURE STRESS). So any change in the lipid
constituency will disrupt the regulated balance possibly pushing the envelope
from lamellar to hexagonal II phase in local domains.

The outer membrane may be tightly regulated to maintain a lamellar
phase in close equilibrium with hexagonal tendencies by the concentration of
MGDG and possibly saturation state. Interestingly, the highest level of MGDG
that can be stably incorporated into small unilaminar vesicles is 20%, the
same as the outer envelope membrane (Pinnaduwage et al., 1996). One level
of regulation may be through the enzymes that control these processes.
DGDG is synthesized from MGDG on the inner leaflet of the envelopes via
DGDG synthase (Cline et al., 1983). The genes responsible for DGDG synthesis
as well as MGDG synthase or the enzymes themselves may be regulated to
maintain membrane phase and fluidity by adjusting the amount of MGDG in
the membrane (Williams et al., 1984). In addition membrane desaturase
activity may be adjusted to maintain characteristic fluidity of the envelopes.
Higher levels of desaturation of fatty acids increase the volume taken up by
those groups. Increasing the number of double bonds would modulate the non-bilayer-bilayer forming lipid ratio in the membrane. Regulation of these enzymatic processes could be due to the effects of physical stresses mediated by the membrane. The curvature stress model implies that a stress placed on membrane proteins by adjacent lipids may alter their activity. The activities of several membrane proteins studied increase as membranes move toward the $H_{II}$ phase, then decrease after phase transition. This effect has been seen in chloroplast ATPase (Navarro et al., 1984), protein kinase C (Epands et al., 1991), and non-bilayer forming lipid reconstitution studies in liposomes for mannosyl transferase II (Jensen et al., 1984), cytochrome C oxidase (Vik et al., 1981), cytidylyl transferase (Cornell et al., 1996), and mitochondrial adenine nucleotide translocator (Streicher-Scott et al., 1994). Activity changes in these proteins have been hypothesized as due to a changes in three dimensional conformation of the protein in response to the influence of the membrane. Lateral pressures at the surface and membrane interior against membrane proteins have been hypothesized in response to x-ray diffraction and NMR studies of the pivotal plane of curvature stressed monolayers (Lewis et al., 1994, and Fenske et al., 1990). As a membrane approaches $H_{II}$, within the hydrophobic region pressures push against the membrane protein, but at the aqueous interface, pressures pull against the protein. These pressures could potentially alter the protein conformation in such a way as to enhance or inhibit activity. This may imply a possible mechanism for autoregulation of
the MGDG and DGDG synthase or saturase activity. In fact, membrane
bound fatty acid desturases have been shown to be regulated in vivo by just
this manner in Acholeplasma laidlawii (Wieslander et al., 1997). DGDG
synthase has been shown to be associated with the outer envelope (Cline et
al., 1983), and is a good candidate for this model. The membrane appears to be
regulated as a lamellar phase with a specific lipid constituency for
physiological functions beyond compartmentalization. Thus, changing the
lipid constituency of the outer envelopes due to lipid depletion should alter
its ability to maintain these functions. The most basic outer membrane
function is compartmentalization of chloroplast inter-membrane space from
the exterior cytosol. Therefore, the first level of examination is intactness of
the membrane. Chloroplasts are considered intact if the envelopes are in
place and the stroma is maintained in a separate compartment. Lysis of the
envelopes is a logical consequence of exaggerated lipid depletion, so intactness
of treated plastids must be evaluated.

The percent of chloroplast recovery after cyclodextrin treatment was
examined to understand the balance between lipid depletion and lysis.
Cyclodextrins α and β lysed chloroplasts little more than controlled samples
with the same physical handling up to 25 μmoles CD / mg chlorophyll at
which point lysis increases to 40 μmoles CD / mg chlorophyll. Cyclodextrin α
has a more pronounced lytic effect than cyclodextrin β upon pea chloroplasts.
Thin layer chromatography shows higher levels of lipid removal for CD α as compared to CD β. Cyclodextrin a treatment decreases the pool of bilayer forming lipids, thus destabilizing the membrane and allowing for increased lysis upon physical manipulation. Cyclodextrin α preferentially depletes polar lipids from the outer membrane, but does not remove MGDG. Thus the ratio of non-bilayer to bilayer forming lipids is increased and possibly the phase, but definitely the fluidity of the membrane is altered. Regardless, the plastids retain their integrity up to intermediate levels of cyclodextrin treatment. On the basis of the ability to migrate through a density gradient, controlled lipid depletion does not lyse chloroplasts.

To see if any large-scale structural changes could be detected after cyclodextrin treatment, the plastids were visualized by transmission electron microscopy. Cyclodextrins had no visible ultrastructural affects upon pea chloroplasts. Plastids treated with cyclodextrins α and β and a no-treatment control were micrographed to show any changes that occured in thylakoid, matrix, and membrane morphology. At a magnification so that only individual chloroplasts could be seen, no changes in membrane morphology, or obvious ultrastructural affects could be identified between the treated and non-treated samples. Clumping, or fusion of adjacent chloroplasts could not be seen. The plastids maintained their shape and general characteristics. Thylakoids displayed the same stacking patterns and were intact. No
increased vesiculation was evident at the outer membranes in comparison to the control. Unfortunately, the individual envelope membranes were not well stained so their structure and organization was not visible. In the context of the electron micrographs, no discernible difference could be detected between cyclodextrin-treated and control chloroplasts. Cyclodextrin-treated chloroplasts appear to be intact by their ability to migrate through a density gradient, and their appearance as compared to control plastids in electron micrographs.

Further evidence for intactness of lipid-depleted chloroplasts is demonstrated by their ability to undergo photosynthetic reactions. Lysed, or partially lysed plastids lose the ability to evolve oxygen (Walker, 1980). Lysed plastids lose stromal enzymes and metabolites necessary to run CO₂-dependent oxygen evolution. Also, electron transport is inhibited in thylakoids exposed to CD's (Rawyler et al., 1993). If the envelopes are compromised, the cyclodextrins can deplete the thylakoid membranes. Also, if CD's pass through the envelopes this same effect might occur. Therefore, the ability of CD-treated chloroplasts to evolve oxygen reflects the ability of the CD's to either lyse the plastid or pass through the envelope. Oxygen evolution was measured in treated and untreated samples. An intact electron transport chain is necessary for release of O₂ from H₂O by the oxygen evolving complex of photosystem II. Thylakoid membranes must be intact. Any interruption of the lipid nature of the membrane will disrupt the ability of
plastocyanin to pass from photosystem II to photosystem I within the electron transport chain, and will eliminate oxygen evolution. This effect is seen in the context of lipid depletion of thylakoid membranes. Cyclodextrin-mediated lipid depletion inhibited electron transport in thylakoid membranes (Rawyler et al., 1996). Therefore, if the thylakoid membranes are exposed to the cyclodextrins, the photosynthetic reactions will be inhibited. Oxygen measurements show that both α and β do not significantly change the chloroplasts ability to undergo photosynthesis. Oxygen evolution measured was both substrate dependent and CO₂-dependent. An exogenous electron acceptor was not added to the assays. Therefore, the assays test for inhibition of O₂ evolution on the basis of an intact plastocyanin activity or stroma. Therefore, thylakoids are not exposed to cyclodextrins. The molecules do not cross the outer and inner envelopes. Treated chloroplasts appear to be intact by their ability to migrate through a density gradient, their appearance as compared to control plastids in electron micrographs, the integrity of their thylakoid membranes, and intactness of their stroma. Cyclodextrins have no effect within the inner membrane.

Cyclodextrins were employed to investigate the consequences of lipid depletion from the outer envelope on protein import. The loss of outer envelope proteins, specifically those involved in precursor import, toc86 and toc75, would mask any import loss due to altered lipid involvement. Therefore, the ability of cyclodextrins to remove these proteins was examined.
Chloroplasts are intact after cyclodextrin treatment. Lipids are removed, but the proteinaceous character of the outer membrane does not change.

Chloroplasts were treated with two classes of cyclodextrin: \( \alpha \) and \( \beta \) at 20 \( \mu \)moles cyclodextrin / mg chlorophyll. This concentration of treatment does not affect chloroplast stability, therefore any alteration or loss of binding or import machinery might occur due to a specific removal of the components of the protein translocation apparatus. This possibility was tested via western blotting. The chloroplasts of experimental samples were solubilized, separated by SDS-PAGE, electroblotted, and probed with antibodies against outer membrane proteins. Three antibodies were used to probe for changes in membrane distribution, anti-toc86, anti-toc75, and Ernst, which is a polyclonal antibody against all proteins on the outer envelope. Using the Ernst antibody as a probe, which shows the presence of all the outer membrane associated proteins, little change was seen in outer envelope protein distribution. Slight loss of the smaller proteins OM14 and OM9 occurred as a result of cyclodextrin treatment. These proteins have not been shown to be associated with protein transport. No new intermediates were induced, and no preexisting components associated with precursor targeting and import were lost in any of the cyclodextrin treated chloroplasts.

Toc86 has been shown to be a receptor for chloroplast precursors that is associated with binding or docking the chloroplast associated precursors with the translocation channel. Loss of toc86 by thermolysin treatment is
correlated with decreased import, and possibly decreased binding (Hirsch et al., 1994). Therefore loss due to cyclodextrin treatment would have deleterious results on these processes. Probing with anti-toc86 shows released, soluble toc86 and processed 53 kDa forms of the 86 kDa protein due to general perturbation of chloroplasts for all treatments including the no treatment control (Hirsch et al., 1994). There is little loss due to any treatment as compared with the control. Therefore, cyclodextrin treatment does not release toc86 from the membrane. Changes in binding patterns do not reflect loss of toc86 function.

In addition to toc86, the presence of toc75 was examined in cyclodextrin-treated chloroplasts. Toc75 is an intrinsic membrane protein and thought to be the major component of the translocation channel in the outer envelope (Perry et al., 1994). Loss of toc75 by CD treatment would result in a loss of import, but have no effect on precursor binding. When the electroblotted CD-treated chloroplast samples were probed with anti-toc75, no loss of toc75 was seen, and novel cross reactive bands did not occur in the treated samples. Therefore, any loss of precursor import seen in treated plastids is not due to loss of toc75. To conclude, the outer envelope proteins investigated are not depleted as a result of cyclodextrin treatment. Changes in import dynamics and chloroplast stability that occur as a result of cyclodextrin mediated lipid depletion are not due to loss of binding and import machinery from the outer membrane.
Controlled lipid depletion of outer envelopes with cyclodextrins does not lyse or compromise plastid integrity, does not remove the protein machinery necessary for precursor targeting, yet somehow inhibits in vitro import of prSSU. As cyclodextrin pretreatment concentration rises, chloroplasts import less prSSU. All lipid removal reactions reach equilibrium during the 30 minute pretreatment. The amount of precursor import with 2 mM ATP is shown by the presence of mSSU. For chloroplasts pretreated with increasing amounts of cyclodextrin α, import decreases to ~10% of the untreated value at the highest CD concentration. The amount of prSSU imported is ~35% of the untreated value at 27.5 μmoles α CD / mg chlorophyll, the treatment concentration for following experiments. At this treatment, chloroplasts are >95% intact, but import inhibition is significant. Therefore, loss of import competence is not due to breakage of the plastids. An import time course after increasing cyclodextrin α incubation times also shows a general decrease in import of prSSU. Data from a time course of lipid removal from thylakoids with cyclodextrin α at 100 μmoles CD/ mg chlorophyll shows as much as 50% lipid removal at concomitant time points to chloroplasts treated at 27.5 μmoles CD/ mg chlorophyll. The extent of removal suggests that enough lipid is removed to have considerable macrostructural consequences on the envelope system as well as the previously mentioned microconsiderations on fluidity, phase, and function.
Removal treatment of the two membranes can be compared by normalizing the ratio of cyclodextrins per surface area. Thylakoids have roughly 20 times the surface area of outer envelopes (Joyard et al., 1991). So the treatments can be corrected to 420 μmoles CD / m² of outer envelope and 234 μmoles CD / m² thylakoid. The molar ratio can be further adjust by considering the lipid to protein ratio for the two membranes. The thylakoid is made up of 50% and the envelope 30% protein by mass. If we assume a roughly equal surface area to mass ratio, then the cyclodextrin per lipid surface area can be further adjusted to 614 μmoles / m² envelope and 468 μmoles / m² thylakoid. These two membranes in spinach thylakoids and pea outer envelopes are comparable because they are composed of the same lipid classes, but in slightly different proportions. The main differences lie in the amount of galactolipids, 80% in thylakoids but only 46% in outer envelopes, and PC, 32% in outer envelopes and <3% in thylakoids. A comparison of the data makes two things apparent. First, up to 50% of lipid is removed from the thylakoid membranes for the treatment concentration compared to a 35% reduction of import competence in the total chloroplast treatment for an almost 50% higher treatment concentration over time. Second, the equilibrium of lipid removal is fast, probably within the first five minutes of treatment. The zero time point in the import reactions was exposed to cyclodextrin α for up to five minutes during manipulation of the samples. This may mean that the fast equilibrium has been reached and the control reaction value, to which all
other reactions are normalized, is already significantly depressed. Therefore, prSSU import (as % of control) for every time point but the zero-time may be exaggerated in comparison to its actual value. The import curve would more realistically look like the lipid content curve where the zero-time drops quickly from 100% with time due to rapid removal equilibrium and levels off at around 5 minutes. Intuitively, the more lipid removed, the greater the decrease of import efficiency.

The same concentration effect is seen with cyclodextrin β at 2 mM ATP under import conditions. PrSSU import decreases to 10% of the control value at the highest CD treatment, as with the cyclodextrin α data. Increasing cyclodextrin β incubation times also decreases in vitro import of prSSU. The same equilibrium considerations occur for the cyclodextrin β import data. Siegenthaler’s group states that depletion of thylakoid membranes reaches equilibrium in 2 to 5 minutes. Therefore, equilibrium between the cyclodextrins and the chloroplast outer envelope lipids may be reached very quickly. The results show a gradual decrease in import competency with increased import time. At the zero time point, cyclodextrin is added, as to the other time points, and then processing begins. The control may be exposed to CD’s for up to five minutes, therefore removal equilibrium may already be reached in this sample. All data is compared to a control, the 0 minute time point, and expressed as percent of control. Therefore, the gradual decrease in
chloroplast import competence seen in the data may mask an initial rapid loss and exaggerated prSSU import.

The ratio of cyclodextrin concentration to membrane surface area or chlorophyll dictates the activity of lipid removal. The higher the ratio, the more lipid is removed from the membrane. Rawyler and Siegenthaler showed this in their work with thylakoids using the molecule cyclodextrin DM-β. They showed that at a range of cyclodextrin concentrations in mM, two different lipid removal curves occurred at different chlorophyll concentrations. The lower the chlorophyll, the more lipid was removed for each cyclodextrin concentration. Thus, as the molar ratio increases, more lipid is removed. The same effect is seen in prSSU import. Two different amounts of chloroplasts, in μg/mL chlorophyll, were treated over the same range of cyclodextrin concentrations. The samples with lower chlorophyll concentration showed increased import inhibition compared with the more concentrated chloroplast sample. This shows that as the ratio of CD/chlorophyll increases, so the prSSU import competence decreases. As more lipid is removed at a higher ratio, import is increasingly compromised.

The ability of cyclodextrins α and β to inhibit in vitro prSSU import differs with temperature. Cyclodextrin α depletes fewer lipids at temperatures elevated from 0°C to 20°C but the chloroplasts are destabilized at 30°C and import is inhibited. This data is supported by Siegenthaler’s work which
shows decreased lipid removal from thylakoid membranes by cyclodextrin $\alpha$ treatment as the temperature increases from 0°C to 20°C (Siegenthaler et al., 1993). Therefore, $\alpha$-treated chloroplast import inhibition is less at higher treatment temperatures because fewer lipids are removed. Elevated temperatures must disrupt fatty acyl insertion into $\alpha$ cyclodextrin's hydrophobic core. Cyclodextrin $\beta$, however, removes more lipids as the temperature increases from 0°C to 30°C. PrSSU import is decreased as the treatment temperature elevates. The import inhibition by cyclodextrin pretreatment shows that the removal of normal lipid constituents disrupts the lipid mediated mechanisms involved precursor targeting. Since binding experiments show a direct dependence upon the lipid constituency, and import decreases with increasing treatment, the level of inhibition may be at transit peptide insertion into the translocation apparatus. At import levels of ATP, precursor binding seems largely unaffected by cyclodextrin treatment, but import drops off. So the precursor can interact with receptor proteins and the lipid bilayer, but cannot transfer to the import channel. Phospholipase C treatment of chloroplasts had similar effects on in vitro translocation of prSSU (Kerber et al., 1992). Phosphatidylcholine is the major bilayer forming phospholipid in the outer envelope, and is only localized in that chloroplast membrane (Joyard et al., 1991). Phospholipase C cleaves phosphatidylcholine to phosphocholine and membrane bound diacylglycerol. PrSSU binding is
slightly enhanced, but import eliminated by the loss of PC. With phospholipase treatment, the chloroplast bound precursor was unable to transfer into the translocation apparatus. Soll’s group hypothesizes that perhaps the lateral diffusion of receptors is inhibited by PC hydrolysis, or PC is essential for functional translocation complex conformation, or that PC is required for precursor surface interactions. They conclude that as a result of the enzymatic cleavage, the precursor is unable to transfer into the import apparatus. Cyclodextrin α may inhibit import in the same manner by removal of PC from the membrane. The relative increase in non-bilayer forming lipids might have one of three effects here: 1) increased fluidity of the membrane allowing for greater mobility of protein complexes laterally across the membrane, 2) the stabilization of transit peptide-lipid interactions, and 3) possible disruption of normal intrinsic membrane protein-lipid interactions in lamellar phase which might preclude precursor insertion into the channel. Import inhibition may be due to the third mechanism, while the second and third would suggest that CD treatment might increase binding.

Cyclodextrin treatment had unusual effects on binding of the precursor, prSSU. Binding was examined at traditional binding (50μM) and import (2mM) concentrations of ATP. At binding concentrations of ATP, cyclodextrin α, which had been shown to have a more linear effect on lysis by proposed bilayer forming lipid depletion, enhanced binding at low
pretreatment concentrations, as might be expected by the proposed increase in non-bilayer to bilayer forming lipid ratio. Initial precursor interactions with the chloroplast probably occur between transit peptides and non-bilayer forming lipids, so as their concentration rises in the membrane, so would the affinity for transit peptide. This same affect was seen after phospholipase treatment of chloroplasts followed by binding assays (Kerber et al., 1992). Phospholipase cleaves phosphatidylcholine into diacylglycerol (DAG), a non-bilayer forming lipid, and soluble phosphocholine. The increase in DAG concentration in the outer membrane results in an enhanced binding of prSSU as might be expected by the increased concentration of non-bilayer forming lipid. This is identical to the affect of cyclodextrin \(\alpha\). As cyclodextrin treatments reached high concentration, the binding dropped off, probably due to the unavoidable increasing destabilization caused by loss of lipids and increased protein to lipid ratio.

At import concentrations of ATP, cyclodextrin \(\alpha\) had little effect on precursor binding. Binding studies at 50 \(\mu\)M ATP and at 2 mM showed similar changes in prSSU binding after cyclodextrin \(\beta\) treatment that occurred after treatment with cyclodextrin \(\alpha\). At higher levels of cyclodextrin treatment, import is almost completely shut down, therefore this increase in the amount of precursor bound probably reflects the shift from importing pool of prSSU to the bound pool. It is clear that removal of lipids has a
unique effect on binding of the precursor to the surface of the chloroplast which may be due to the specificity of interaction between the transit peptide and different classes of lipids present (Pinnaduwage et al., 1996). Precursor binding seems to require the presence of particular lipids for not only interaction between the transit peptide of the precursor and membrane lipids, but insertion of the peptide into the membrane and finally the import apparatus. These interactions can be examined by utilization of the cyclodextrins to lipid deplete the outer membranes and then follow import dynamics.

So what are the direct affects of cyclodextrin mediated lipid depletion on the chloroplast? Mixed inner and outer envelopes were isolated from chloroplasts treated with cyclodextrins α and β and a no-treatment control. The membranes were centrifuged over a step-gradient and found to have different densities. The order of density went from control < cyclodextrin β < cyclodextrin α treated chloroplasts. Equal amounts of protein separated by SDS-PAGE showed no change in proteins present from control to treated samples (data not shown). Lipid depletion by the cyclodextrins caused an increased protein to lipid ratio resulting in increased density. For these experimental conditions, cyclodextrin α more actively removed lipids than cyclodextrin β, so lipid removal from pea chloroplast outer envelopes agrees with that from spinach thylakoids (Rawyler et al., 1993).
To elucidate which lipids are removed by which cyclodextrin, solubilized fractions of cyclodextrin-treated chloroplasts were extracted, concentrated, and separated by two-dimensional thin layer chromatography. Cyclodextrin α showed a preference for more polar lipids, removing DGDG, PC, PI, SQDG, and PG. Though DGDG was the darkest spot, the other spots were present in almost equal intensity. The relative concentrations of the above stated lipids in the outer envelope are 29%, 32%, 5%, 6%, and 10%, respectively. The preferences for cyclodextrin α might then be surmised as PI = SQDG > PG > DGDG = PC. However, these numbers are derived by visual inspection of spot size compared to relative lipid class concentration in untreated spinach chloroplasts, so the preferences are rough at best. Even so, Siegenthaler concludes that the preference of cyclodextrin α and β in thylakoid lipids is SQDG > PG > MGDG > DG. The preference we observe is similar. The discrepancy in MGDG may be related to the high MGDG content in the thylakoid membrane (53%) compared to the outer envelope (17%).

Using the above stated assumptions and limitations, cyclodextrin β showed a somewhat different lipid preference, removing DGDG, MGDG, PC, and SQDG but no PG. The preferences for cyclodextrin β might then be SQDG > MGDG > PC = DGDG. This also is similar to Siegenthaler’s data. Interestingly, cyclodextrin β does not seem to remove PG or PI. This may reflect those lipids’ lower relative concentration in the membrane, and the lesser ability of
cyclodextrin β to remove lipids seen in the density centrifugation experiment. Cyclodextrin β as opposed to α is able to remove MGDG. However, Siegenthaler’s group shows that cyclodextrin α can remove MGDG from thylakoids. Cyclodextrin α has a smaller hydrophobic core and a shorter axis. The initial interactions between the less hydrophylic exterior and smaller hydrophobic interior of cyclodextrin α may not energetically be able to pull MGDG from a lamellar phase membrane. However, MGDG molecules in the thylakoid membranes are enriched in the grana may stabilize the stacking interactions in the appressed regions (Gounaris et al., 1986). These membranes are unique in that they are highly stacked and contain regions of tight curvature that are rich in MGDG. These lipids most likely experience a greater curvature stress and removal may be more energetically favorable. Also, the fatty acyl groups of MGDG within the thylakoids may have a different saturation state therefore decreasing lipid packing interactions and making them more accessible to CD’s. Cyclodextrin α removal of MGDG may require a destabilized non-lamellar membrane system that exists between the granal stacks, but not in the outer membrane. Though cyclodextrins deplete lipids only semi-specifically, they do appear to have a rough preference.

This CD removal preference delineates possible mechanisms for prSSU import inhibition. Cyclodextrin α removes polar bilayer formers such as PC
and probably inhibits prSSU import by blocking precursor association or insertion into the translocation apparatus. By removal of PG and SQDG, cyclodextrin may also inhibit initial transit peptide interaction with the envelope via ionic interactions. Cyclodextrin β seems to show less lipid class preference and less ability for lipid removal. It depleted SQDG, MGDG, and PC so it may inhibit prSSU import by decreasing binding, insertion, and translocon association of the precursor.

In summary, both cyclodextrins had only slight lytic effects at lower concentrations. Cyclodextrin α-mediated lysis occurs at a lower concentration perhaps due to removal of bilayer formers which may destabilize the outer envelopes. Cyclodextrin treatment had no effect on protein constituency of the membrane, so import inhibition was not a result of a loss of translocation machinery. Pretreatment of chloroplasts with cyclodextrins had a slight stimulatory effect on ATP-dependent precursor binding. Precursor import decreased with increasing concentrations of cyclodextrin pretreatment and increasing incubation time. The ability of each cyclodextrin to deplete lipids appears to be temperature sensitive, each behaving differently. This could be due to the different strengths of interaction between the CDs and lipid head groups and the abilities of fatty acids to insert into the different core diameters with different hydrophobicities. Binding still occurs via the lipid and receptor interactions, while import diminishes so inhibition is either at transit peptide insertion into the bilayer or at the function of the translocator. Therefore,
depletion of non-bilayer formers, MGDG by cyclodextrin β and PG by cyclodextrin α, necessary for transit peptide insertion into the membrane, or of bilayer formers, PC by cyclodextrin α, necessary for precursor association with the translocation channel seem to be the cause of prSSU import inhibition. To conclude then, specific outer envelope lipids are required in three phases of transport at the chloroplast surface: 1) initial precursor interactions with anionic lipids and galactolipids, 2) transit peptide insertion into the bilayer mediated by non-bilayer forming lipids, and 3) precursor insertion into the import channel necessitating specific bilayer forming lipid to membrane protein interactions.
Chapter 5
Future Studies

Part I. Rescue of import with re-addition of lipids

An experiment that could elucidate the requirement of each lipid in the import process involves the reconstitution of the lipids with the treated chloroplasts piecemeal. Empty cyclodextrins can be brought into equilibrium with saturating amounts of each of the classes of envelope lipids. Each lipid could then be added back to the previously treated chloroplasts to see which one rescues import and to what extent. Proof of lipid addition can be gathered by using $^{14}$C labeled lipids and later scintillation of lipid extracts. These rescues might be due to any of the hypothesized mechanisms of precursor lipid interaction outlined in the introduction. They may be due to a "curvature stress" model where physical stresses force modulates membrane proteins' toc86 or toc75 conformation. Further, unique lipids may stabilize membrane protein conformation within the membrane by their particular molecular shape (see Figure 2). Fatty acid and head group interaction with the protein folds could, in effect, chaperone the folding of the membrane protein. This idea has been hypothesized for lactose permease in *Eschericia coli* (Bogdanov et al., 1996). This stabilization of translocation proteins has been hypothesized for PC, and may also be true for non-bilayer formers that correctly fit membrane exposed domains (Figure 25). One such protein that
may modulate import by its function is inhibited as membrane fluidity decreases. The ATP translocator located on the inner membrane is down-regulated at low temperatures (4°C) (Theg et al., 1989). Changes in fluidity due to lipid depletion may stress the three-dimensional conformation of the translocator and inhibit its function just as cold temperatures does.

Radiolabeled nucleotide could be added to the treated chloroplasts followed by a wash step. The plastids could then be counted by liquid scintillation to see any change in incorporation of the molecule. Chloroplasts require a stromal source of ATP for protein import. If no label is imported, this could be another mechanism for precursor import inhibition. Further, the outer
envelope lipids could be re-added to see if rescue of translocator function occurs.

**Part II. Lipid biosynthesis mutations**

The laboratory is collaborating on two *Arabidopsis* mutants for MGDG and DGDG synthase, respectively. These knock-out mutants were made by the addition of complementary mRNAs. The DGDG mutant is slightly leaky, exhibiting a background expression of 15% of the wild type DGDG synthase activity. Other isoforms may exist. Chloroplasts will be isolated from these plants to study prSSU import without the presence of MGDG or DGDG.

**Part III. PrSSU import assays in CD treated plastids**

Imports after CD treatment performed at 0°C, would show how the change in fluidity (temperature change) of the membrane affects protein import with lipids removed. Import can occur at low temperatures, but levels are lower than at normal assay temperatures (20°C). Import is inhibited by lipid removal, but still occurs at a lower level in the presence of ATP. By changing the temperature of the in vitro import, we may see if the lipid removal further decreases fluidity of the membrane resulting in lowered imported prSSU levels. By changing light conditions, we may see if light-regulated functions affect membrane structural characteristics.

**Part IV. Investigating an outer membrane “island” model**

If 50% of outer envelope lipid are removed by cyclodextrin treatment, then the envelope structure might be envisioned as a series of double envelope
patches, or islands, within a larger context of exposed inner membrane. This view can be tested by various methods. Chloroplasts treated with cyclodextrins can be treated with thermolysin, which will non-specifically, proteolytically cleave exposed protein domains. Using an antibody to a known major protein constituent of the inner membrane, IM90, quantitation of relative amounts of intact and cleaved IM90 may be possible. This might allow for an interpretation of the percent of exposed inner membrane surface. Further evidence can be collected by using the same antibody to fluorescently tag the envelopes of whole chloroplasts and view them under a with fluorescence optics. If the inner envelope protein is exposed, then fluorescent spots will become obvious under visual scrutiny. These experiments would provide evidence to create an interpretive model for the inhibition of protein import by lipid removal (Figure 26). If the outer membrane exists as islands, as seen in Figure 26, then outer membrane translocation channels might be isolated from inner membrane translocation channels and \textit{in vitro} prSSU import would not be allowed. However, if the inserted transit peptide can make contact with the inner membrane channel, either the outer channel, or the inner channel will move to facilitate a two membrane contact site where the translocation channels come together, therefore, import occurs. The existence of such island structures might be evident with freeze-etch electron microscopy, though efforts in the current study did not resolve them due to staining difficulties.
Part V. Envelope membrane protein transport after depletion

Envelope proteins toc75, toc 86, and tic97 are able to import independently of the translocation channels studied (Tranel et al., 1995). They may possibly insert directly into the outer membrane. It would be interesting to see if cyclodextrin treatment could also inhibit their import. Import assays could be performed just as with prSSU with these envelope precursors. Insertion of toc75 and toc 86 may not be affected. Insertion of tic97 may even be enhanced as a result of exposed inner membrane regions. If insertion of the outer membrane or inner membrane proteins is inhibited, re-addition of lipids in the context of cyclodextrins could be used to identify lipids necessary for insertion as stated in the previous section.
Bibliography


Appendix
Buffers

**TBS** (Tris-Buffered Saline)

25 mM tris (pH 8.0), 3mM KCl, 137 mM NaCl

**MOPS**

12.5 mM MOPS (pH 7.0), 2.5 mM Mg-Acetate

**1M Sucrose**

1 M Sucrose, 25 mM MOPS (pH 7.0)

**0.46 M Sucrose**

0.46 M Sucrose, 25 mM MOPS (pH 7.0)

**Running Gel Buffer**

300 mM tris (pH 8.8), 0.1% Sodium dodecyl sulfate (SDS)

**Stacking Gel Buffer**

125 mM tris (pH 6.8), 0.1% SDS

**Sample Buffer (for SDS-PAGE)**

100 mM Dithiothreitol (DTT), 10 mM tris (pH 8.3), 2.5% glycerol, 1% SDS, 0.1% Bromophenol blue

**SDS-PAGE Running Buffer**

25 mM tris (pH 8.3), 0.1% SDS, 275 mM Glycine

**Transfer (electroblot buffer for Western blotting) buffer:**

50 mM tris, 380 mM glycine
**SDS-PAGE Coomassie Brilliant Blue**

0.1% (w/v) coomassie brilliant blue, 50% methanol, 10% glacial acetic acid, 40% double-distilled H₂O

**SDS-PAGE Destain**

40% methanol, 10% glacial acetic acid, 50% double-distilled H₂O

**Import Buffer (for chloroplast manipulations)**

50 mM HEPES-KOH (pH 8.0), 330 mM Sorbitol

**Grinding Buffer (for chloroplast purification)**

50 mM HEPES-KOH (pH 7.3), 330 mM Sorbitol, 0.1% bovine serum albumin, fraction V (United States Biochemicals, Cleveland), 1 mM MgCl₂, 1 mM MnCl₂, 2 mM Na₂EDTA
Vita

Robert George Mynatt was born in Knoxville, Tennessee March 27, 1971. He lived in Carol Stream, Illinois and then Olney, Maryland before moving back to Oak Ridge, Tennessee in 1980. Robert graduated from Oak Ridge High School and then matriculated at Vanderbilt University where he was awarded an University Scholarship. During college Robert worked in a medical school library, as a Head Resident Advisor for various dormitories, and co-directed an overnight hosting program for perspective freshmen. He conducted undergraduate research in the Department of Molecular Biology under the supervision of Dr. Robley C. Williams, Jr. He graduated in May 1993 with a Bachelor of Arts Degree in Molecular Biology. After participating in a summer fellowship with the Howard Hughes Medical Institute at Vanderbilt, Robert began a masters program in Cellular, Molecular, and Developmental Biology at the University of Tennessee, Knoxville under the direction of Dr. Barry D. Bruce. He held a graduate teaching assistantship in Biology from Fall 1994 to Fall 1996. Part of the way through his degree, he was married to Margaret Tippens and moved to Memphis for a two year project in the laboratory of Dr. Lorraine M. Kraus, where he published his data as an abstract in the Journal of the American Society of Nephrology. After which, he returned to finish his thesis work and was awarded a Master of Science Degree in Life Science in August 1998.