PROTEIN TARGETING TO THE THYLAKOID LUMEN.

Ruth M. Mould

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Department of Biological Sciences
University of Warwick
Coventry
U.K.

SUMMARY.

Chloroplast biogenesis involves the activities of two genetic systems, the nuclear and chloroplast genomes. The majority of chloroplast proteins are synthesised on cytosolic ribosomes as precursors with N-terminal extensions known as presequences. Presequences are involved in the posttranslational targeting of such proteins to the appropriate location within the chloroplast. The targeting of thylakoid lumen (TL) proteins is particularly complex since it involves transport across the chloroplast envelope membranes, through the stroma and across the thylakoid membrane. The presequences of nuclear-encoded TL proteins have a bipartite nature, the first (most N-terminal) domain directs transport across the chloroplast envelope and is (probably) removed by stromal processing peptidase (SPP) in the stroma to yield an intermediate size protein. The second domain directs transport across the thylakoid membrane and is removed by thylakoidal processing peptidase (TPP) in the lumen yielding the mature size protein.

When this Ph.D. project commenced, very little was known about the mechanism by which proteins are transported across the thylakoid membrane. Import assays using isolated chloroplasts had led to advances in the study of protein transport across the chloroplast envelope but were of limited use for the study of the import of nuclear-encoded TL proteins since transport across the thylakoid membrane could not be studied in isolation. Kirwin *et al*. (1989) achieved import of a TL protein into isolated thylakoids, thus enabling study of transport across the thylakoid membrane in isolation from that across the envelope membranes. During the course of this project, the assay system of Kirwin *et al*. (1989) has been improved, in terms of the efficiency of protein import by isolated thylakoids achieved, and the import of another TL protein demonstrated. This improved assay system has since been used to study the import of several other thylakoid proteins by other workers. Work presented in this thesis describes the use of this assay and the intact chloroplast assay to study the energy requirements for the import of two TL proteins, 33kDa and 23kDa components of the oxygen evolving complex. The efficient transport of both proteins across the thylakoid membrane requires a transthylakoidal proton gradient and that of 33K may also require ATP. The mechanism by which a proton gradient drives transport has yet to be determined. The ability of the thylakoid transport machinery to import the precursor form of 23K (work presented in this thesis and by J. Shackleton, University of Warwick) and artificial intermediate forms of 23K and 33K has been demonstrated. The import of 23K into isolated thylakoids can occur without the addition of any stromal factors whereas the import of 33K requires at least one stromal factor other than SPP. The requirements for the transport of several other TL and thylakoid membrane proteins have recently been studied by other workers; these studies in conjunction with the results presented in this thesis, reveal that the requirements for transport into or across the thylakoid membrane varies depending on the protein in question. Whether these proteins have a common, extremely flexible, import pathway or whether more than one import pathway for thylakoid proteins operates within the chloroplast, has yet to be determined.

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DECLARATION.

The work presented in this thesis is original work conducted by myself in the Department of Biological Sciences at the University of Warwick, U.K., under the supervision of Dr. Colin Robinson. This research was funded by an SERC studentship.

All sources of information are acknowledged by means of reference. None of the work presented in this thesis has been used in any previous application for a degree.

Dr. J. Meadows (University of Warwick) provided clones encoding wheat p33K in pGEM-4Z and in pKK233-2. H. James (University of Warwick) provided a clone encoding wheat p23K in pGEM-4Z. Dr. P. Weisbeek (University of Utrecht) provided a clone encoding Silene pratensis pre-plastocyanin in pGEM-4Z.

Some of the work presented in this thesis has been included in the following publications:-

ATP-Dependent Import of a Lumenal Protein by Isolated Thylakoids.

Kirwin, P. M., Meadows, J. W., Shackleton, J. B., Musgrove, J. E., Elderfield, P. D., Mould, R. M., Hay, N. A. and Robinson, C. (1989)

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A Proton Gradient is Required for the Transport of Two Lumenal Oxygen-Evolving Proteins across the Thylakoid Membrane.
Mould, R. M. and Robinson, C. (1991)
J. Biol. Chem. 266:12189-12193.

Transport of Proteins into Chloroplasts.
Requirements for the Efficient Import of Two Lumenal Oxygen-Evolving Complex Proteins into Isolated Thylakoids.
Mould, R. M., Shackleton, J. B. and Robinson, C. (1991)
J. Biol. Chem. 266:17286-17289.

Transport of Proteins into Chloroplasts.
Delineation of Envelope "Transit" and Thylakoid "Transfer" Signals within the Pre-sequences of Three Imported Thylakoid Lumen Proteins.
Bassham, D. C., Bartling, D., Mould, R. M., Dunbar, B., Weisbeek, P., Herrmann, R. G. and Robinson, C. (1991)
J. Biol. Chem. 266:23606-23610.

LIST OF ABBREVIATIONS.

Ai23K	artificial intermediate form of 23K
Ai33K	artificial intermediate form of 33K
Ala	alanine
ATP	adenosine 5'-triphosphate
bp	base pairs
BSA	bovine serum albumin
cDNA	complementary DNA
CF_oII	CF_o subunit II of the thylakoidal ATP synthase
CIP	calf intestinal phosphatase
CTP	cytidine 5'-triphosphate
Cys	cysteine
cyt	cytochrome
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphoshate
dTTP	2'-deoxythymidine 5'-triphosphate
ddATP	2',3'-dideoxyadenosine 5'-triphosphate
ddCTP	2',3'-dideoxycytidine 5'-triphosphate
ddGTP	2',3'-dideoxyguanoine 5'-triphosphate
ddTTP	2',3'-dideoxythymidine 5'-triphosphate
DCMU	3(3, 4-dichlorophenyl)-1,1-dimethylurea
DHAP	dihydroxyacetone phosphate
DHFR	dihydrofolate reductase
DNA	deoxyribonucleic acid
dNTPs	2'-deoxynucleotide triphosphates
DTT	dithiothreitol
E.coli	Escherichia coli
EDTA	ethyl diamine tetra-acetic acid
EGTA	ethyleneglycol-bis (-amino ethyl ether) N,N'-tetra acetic acid
EPSP	5'-enolpyruvylshikimate-3-phosphate
ER	endoplasmic reticulum
ETD	envelope transfer domain
Fd	ferredoxin
g	unit of gravitational field
GIP	general insertion protein

GTP	guanosine triphosphate
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HPLC	high performance liquid chromatography
hsp	heat shock protein
IgG	immunoglobulin G
Ile	isoleucine
IM	inner membrane
IMP1	inner membrane protease 1
IMS	intermembrane space
iPC	intermediate form of plastocyanin
IPTG	isopropyl-B-D-thio-galactoside
i23K	intermediate form of the 23kDa protein of the PSII oxygen-evolving complex
i33K	intermediate form of the 33kDa protein of the PSII oxygen-evolving complex
kb	kilo bases
kDa	kilo Dalton
LEP	leader peptidase
LHCP	chlorophyll a/b binding protein
M	molar
mA	milliampere
mM	millimolar
MOM	mitochondrial outer membrane protein
MPP	matrix processing peptidase
mRNA	messenger ribonucleic acid
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidised)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NEM	N-ethylmaleimide
NTP	nucleotide triphosphate
OD	optical density
OEC	oxygen-evolving complex
OEP	outer envelope protein
OM	outer membrane
pACP	precursor form of acyl carrier protein
PC	plastocyanin

PEG	polyethylene glycol
PEP	processing enhancing peptidase
pLHCP	precursor form of LHCP
pmf	proton motive force
PMSF	phenylmethylsulphonyl fluoride
PQ	plastoquinone
pPC	precursor form of plastocyanin
psi	pounds per square inch
PSI	photosystem I
PSII	photosystem II
pSSU	precursor form of RUBISCO small subunit
p17K	precursor form of the 17kDa protein of the PSII oxygen-evolving complex
p23K	precursor form of the 23kDa protein of the PSII oxygen-evolving complex
p33K	precursor form of the 33kDa protein of the PSII oxygen-evolving complex
P680	reaction centre of PSII
P680*	reaction centre of PSII (excited state)
P700	reaction centre of PSI
P700*	reaction centre of PSI (excited state)
RBP	ribosome binding protein
RF	replicative form
RNA	ribonucleic acid
RNase	ribonuclease
RNAsin	ribonuclease inhibitor
RUBISCO	ribulose-1,5'-bisphosphate carboxylase-oxygenase
SDS	sodium dodecylsulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SDW	sterile distilled water
SIM	sucrose isolation medium
SPP	stromal processing peptidase
SRM	sorbitol resuspension medium
SRP	signal recognition particle
SRPR	signal recognition particle receptor
SSR	signal sequence receptor
SSU	small subunit of RUBISCO
^{35}[S]	^{35}sulphur labelled

TEMED	N,N,N',N'-tetramethylethylenediamine
TL	thylakoid lumen
TPC	translocation pore complex
TPP	thylakoid processing peptidase
Tris	2'-amino-2(hydroxymethyl)-1,3-propane diol
Triton	polyoxyethylene p-t-octyl phenol
tRNA	transfer ribonucleic acid
TTD	thylakoid transfer domain
U	unit
UTP	uridine triphosphate
U.V.	ultraviolet
V	volts
Val	valine
v/v	volume/volume
w/v	weight/volume
X-GAL	5-bromo-4-chloro-3-indoly-B-D-galactoside
17K	17kDa protein of the PSII oxygen-evolving complex
23K	23kDa protein of the PSII oxygen-evolving complex
33K	33kDa protein of the PSII oxygen-evolving complex

CHAPTER 1. LITERATURE REVIEW.

1.1 INTRODUCTION.

Eukaryotic cells consist of many distinct, membrane-bound compartments. Each compartment carries out a characteristic set of functions and therefore has a characteristic protein composition. Although two such compartments, chloroplasts and mitochondria, encode a small proportion of their constituent proteins, which are synthesised within the organelle, the majority of eukaryotic cell proteins are nuclear-encoded. Transcripts for nuclear-encoded proteins are directed into the cytosol where translation occurs. Translation can occur on membrane-bound ribosomes, as is the case for many proteins which enter the secretory pathway, or on ribosomes which are free in the cytosol. Since proteins destined for mitochondria, peroxisomes, the nucleus, chloroplasts (in higher plant cells), and some secretory pathway proteins, are synthesised on free cytosolic ribosomes, there must be some system for targeting each type of protein posttranslationally to the correct compartment (Figure 1). Moreover, each compartment consists of several subcompartments, hence there must be a mechanism for 'sorting' proteins so that they reach the appropriate subcompartment, be it a particular soluble or membrane phase.

In recent years there has been considerable interest in the

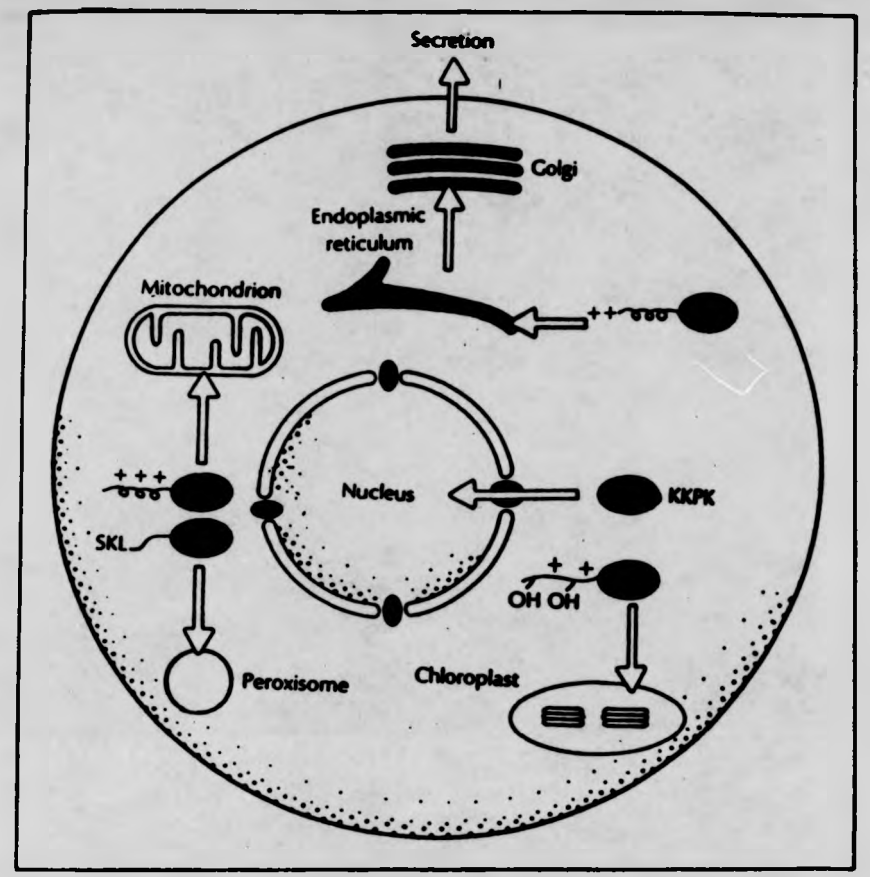


Figure 1. Schematic Illustration of Primary Protein Targeting Signals in Higher Plant Cells.

Nuclear-encoded proteins (large black ovals) are targeted to chloroplasts, mitochondria, peroxisomes, the nucleus and the secretory pathway. Secretory pathway proteins include those destined for the endoplasmic reticulum, Golgi apparatus, secretion and lysosomes; all these proteins are initially targeted to the endoplasmic reticulum. The main features of the primary targeting domain for each type of protein is shown. Proteins may also contain additional targeting information for intra-organelle sorting to a particular subcompartment.

S=Serine K=Lysine L=Leucine P=Proline

(Adapted from von Heijne, 1990).

mechanisms by which proteins are 'targeted' in eukaryotes and prokaryotes. Much progress has been made in deciphering the codes which label proteins for a particular destination. Many proteins must cross one or more membranes in order to reach their site of function; the mechanisms by which proteins are translocated across biological membranes, which are normally impermeable to macromolecules, have proved to be complex in the systems which have been studied in detail so far (see sections 1.3 to 1.4.4).

The aim of this project was to study the mechanism by which nuclear-encoded proteins are translocated across the thylakoid membrane of higher plant chloroplasts. In comparison to protein targeting to mitochondria and protein export from bacteria, the targeting of proteins to chloroplasts is poorly understood. Furthermore, when this project commenced, in 1989, the main body of work concerning protein import into chloroplasts had focussed on the import of stromal proteins and very little was known about protein translocation across the thylakoid membrane.

1.2 CHLOROPLAST STRUCTURE AND FUNCTION.

In terms of structure, higher plant chloroplasts are particularly complex organelles. As shown in Figure 2, the chloroplast consists of three distinct membrane types (the outer envelope membrane, the inner envelope membrane and the thylakoid membrane network) and three distinct soluble phases (the inter-envelope space, the stroma and the

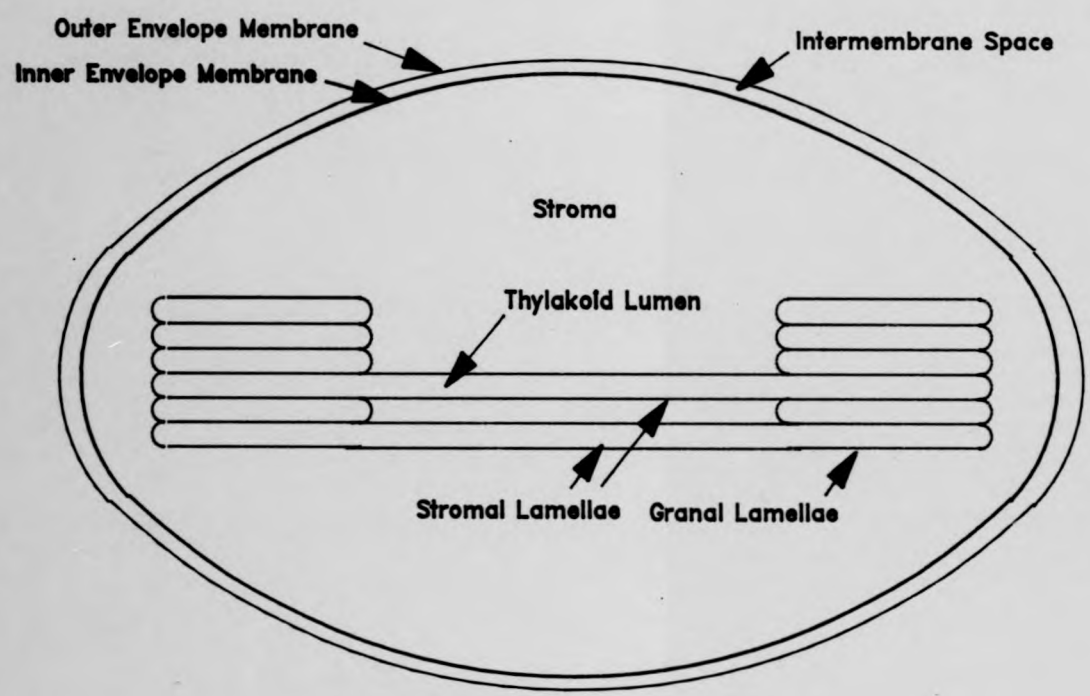


Figure 2. Diagram of Chloroplast Structure.

thylakoid lumen). The outer envelope membrane is relatively permeable to small molecules and is mainly a site of biosynthetic reactions such as lipid biosynthesis, while the inner envelope membrane is a selective permeability barrier (Heber and Heldt, 1981). Little is known about the function of the soluble phase between the envelope membranes, whereas the stroma is known to contain many soluble enzymes and to be the site of the dark reactions of photosynthesis. The thylakoid membrane constitutes about 99% of total chloroplast membrane and is involved in light capture, electron transport and photophosphorylation; it is arranged into stacked 'granal' lamellae which are connected by single 'stromal' lamellae. The three chloroplast membranes not only differ in protein composition but also in lipid content (reviewed by Douce and Joyard, 1990). All three have relatively low phospholipid content and relatively high galactolipid content. The outer envelope membrane contains the phospholipid phosphatidylcholine, unlike the inner envelope and thylakoid membranes. The thylakoid lumen is thought to be a single, continuous soluble phase. The protein and pigment composition of the thylakoid network is well characterised due to its major role in photosynthesis.

1.2.1 THE LIGHT REACTIONS OF PHOTOSYNTHESIS.

Photosynthesis is the light-driven fixation of CO_2 to form carbohydrates and other biological molecules. This process occurs in two stages:-

i) The light reactions in which light energy is captured and harnessed to generate ATP and NADPH. These reactions occur in the thylakoid membrane and lumen.

ii) The dark reactions are driven by the products of the light reactions and involve synthesis of carbohydrates from CO_2 and H_2O. The dark reactions begin in the stroma and continue in the cytosol.

Figure 3 shows the pathway of electron flow during the light reactions of photosynthesis. In higher plants and cyanobacteria, the light reactions involve the interplay of two photosystems. Photosystem I (PSI) is excited by light of wavelengths shorter than 700nm while photosystem II (PSII) is excited by light of wavelengths shorter than 680nm. Light absorbed by the chlorophyll molecules and accessory pigments of the light harvesting complex of PSII is funnelled to the reaction centre, P680. An electron is ejected, yielding photooxidised P680, ($P680^+$). P680 is regenerated by an electron extracted from H_2O by the PSII oxygen-evolving complex (OEC). This complex is associated with a cluster of four manganese ions at its catalytic centre. It binds two water molecules and cycles through a series of oxidation steps abstracting protons and electrons from the water molecules, finally releasing O_2 into the thylakoid lumen.

The electron transport chain consists of three protein complexes, PSII, the cytochrome b_6-f complex and PSI, which are electrically 'connected' by the diffusion of the electron carriers plastoquinone and plastocyanin. Electron

Figure 3. Pathways of Electron Flow During the Light Reactions of Photosynthesis.

Light excites the reaction centre of PSII (P680) causing it to lose an electron. The photooxidised reaction centre ($P680^+$) is reduced by an electron produced by water-splitting by the PSII oxygen-evolving complex (OEC). The ejected electron is passed down a series of electron carriers (black squares or named) to the photooxidised reaction centre of PSI ($P700^+$) which is then reduced (P700). P700 is excited by light, ejects an electron, and is thereby photooxidised. The electron is passed down a series of electron carriers to $NADP^+$ which is reduced to NADPH, or the electron passes from Fd to the cytochrome b_6-f complex (blue arrow).

Protons (H^+) accumulate in the thylakoid lumen, by the action of water splitting and the plastoquinone pump, or are removed from the stroma for the reduction of $NADP^+$; these reactions result in the acidification of the thylakoid lumen relative to the stroma.

QH_2=Reduced Plastoquinone PC=Plastocyanin Fd= Ferredoxin

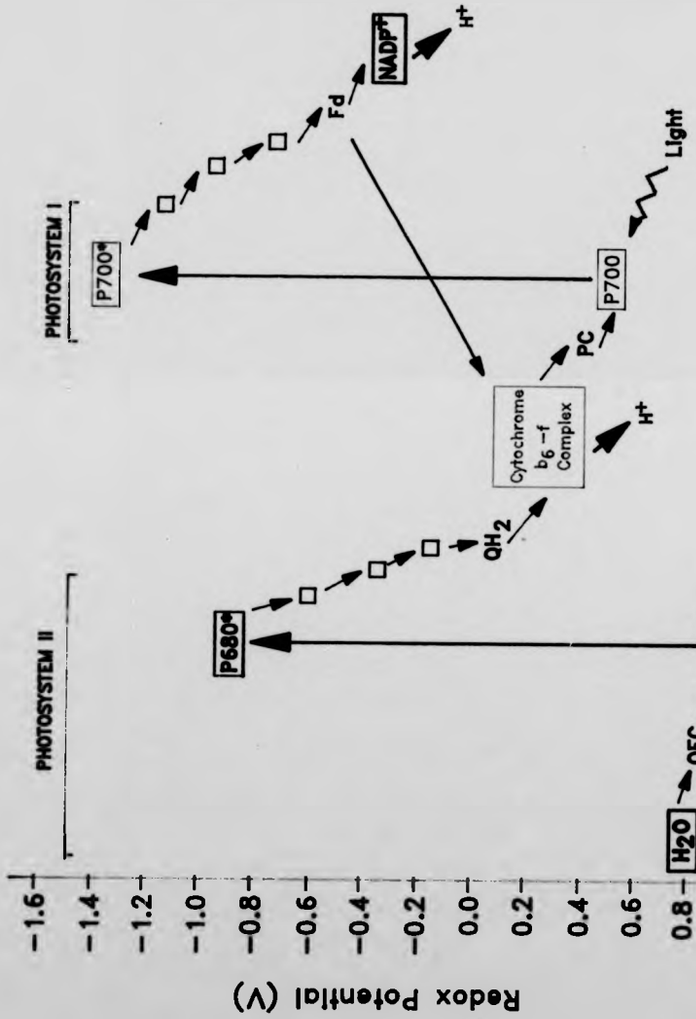


Figure 3. Pathway of Electron Flow During the Light Reactions of Photosynthesis

flow occurs from excited P680* down a series of electron carriers, sequential carriers being stronger oxidising agents than the previous one, to plastquinone. Reduced plastoquinone passes the electron on to the cytochrome b_6-f complex and in doing so is oxidised. During this process, protons are translocated from the stroma into the thylakoid lumen. Electrons are transferred from the cytochrome b_6-f complex, by plastocyanin, to the photooxidised reaction centre of PSI, P700*, thereby regenerating P700. Photon capture by PSI and the ejection of an electron from P700 leads to the generation of P700*. The ejected electron passes down a series of electron carriers to ferredoxin (Fd). Reduced Fd may pass electrons on to reduce $NADP^+$, generating NADPH in a process mediated by ferredoxin-$NADP^+$ reductase, or the electron may be returned cyclically, via the cytochrome b_6-f complex to the plastoquinone pool. Both cyclic and non-cyclic pathways involve proton-pumping into the thylakoid lumen by the cytochrome b_6-f complex. Water splitting by the PSII OEC also results in proton accumulation in the thylakoid lumen; a transmembrane pH gradient of about 3.5 pH units is generated, with the lumenal pH approaching 4. ATP synthesis is driven by the proton motive force (pmf) generated by the accumulation of protons in the lumen and the electrical charge difference generated, the lumen being more positively charged than the stroma. Flow of protons through the CF_oCF_1 ATP synthase drives ATP synthesis (Figure 4). Hence both cyclic and non-

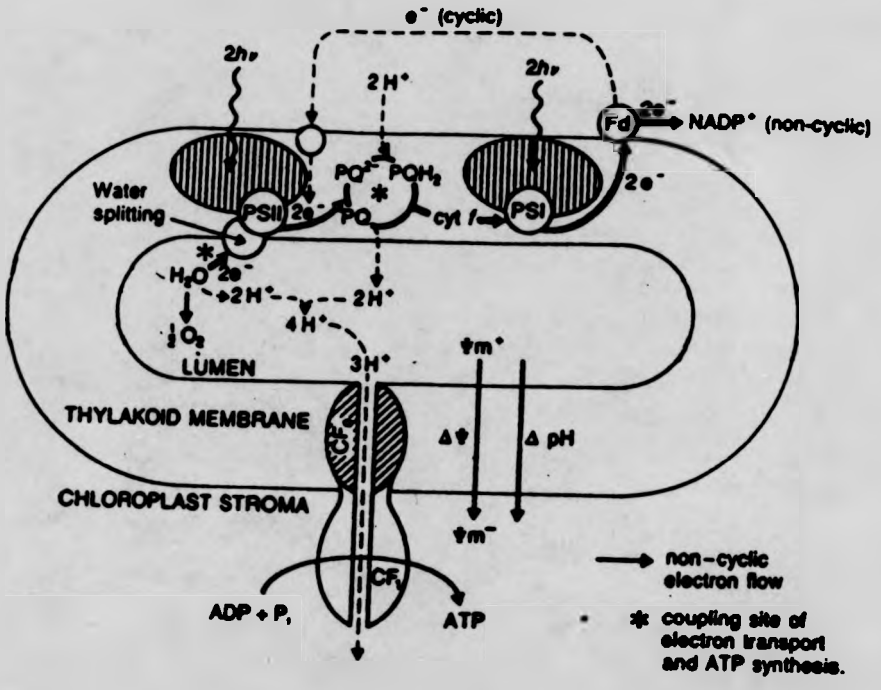


Figure 4. Schematic Relationship Between Electron Transport Driven by Light and ATP Synthesis.

Water-splitting and proton pumping by plastoquinone (PQ) result in the accumulation of protons (H^+) in the thylakoid lumen (Reduction of $NADP^+$ to NADPH also results in acidification of the lumen relative to the stroma). The difference in proton concentration across the thylakoid membrane constitutes a proton gradient, ΔpH. Electron transport and proton pumping also creates an electrical potential difference, $\Delta\psi$, across the membrane. Under the influence of ΔpH and $\Delta\psi$, protons move from the lumen to the stroma via ATP synthase, thereby generating ATP.

CF_0 and CF_1 are subunits of ATP synthase.

hv=Light cyt=Cytochrome Fd=Ferredoxin PQ=Plastoquinone PS=photosystem (Adapted from Lawlor, 1987).

cyclic pathways drive ATP synthesis but NADPH is only generated by non-cyclic electron flow. The cyclic pathway is favoured when levels of $NADP^+$ are low. The ATP and NADPH generated by the light reactions provide energy and reducing power respectively, to drive the dark reactions of photosynthesis.

The non-cyclic pathway of electron transfer involves water splitting and leads to oxygen evolution by the PSII OEC. The water splitting/ oxygen-evolving complex is situated on the lumenal face of the thylakoid membrane (Figure 5). Three major polypeptides form this complex, a 33kDa polypeptide (33K), a 23kDa polypeptide (23K) and a 17kDa polypeptide (17K). In some instances, a 10kDa polypeptide may also be associated with this complex (Webber et al., 1989). PSII contains a cluster of four manganese ions which is required for the oxidation of two water molecules at a time. The precise location of this cluster is uncertain; 33K can be isolated with some manganese ions associated, but the D1 and D2 components of PSII are also possible locations. Although the 17K, 23K and 33K polypeptides are required for oxygen evolution, their roles in this process are uncertain. Each of these polypeptides can be replaced by high concentrations of chloride and/or calcium ions. Hence, these polypeptides may increase the reaction centre's capacity to bind Ca^{2+} and Cl^- ions. It it also thought that together, these polypeptides form a protective environment for the manganese cluster from interaction with external reducing agents

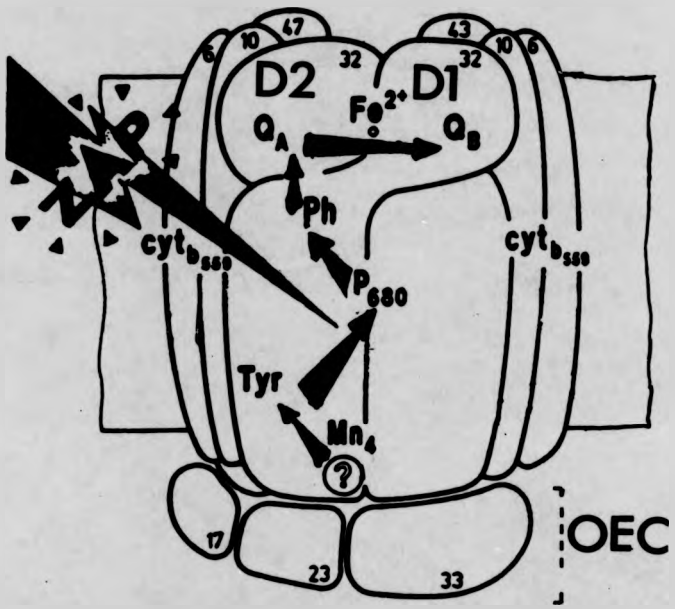


Figure 5. A Structural Model for PSII.
The PSII OEC consists of a 17kDa (17), a 23kDa (23) and a 33kDa (33) polypeptide on the lumenal face of the complex. Arrows indicate direction of electron flow. 'Zap' indicates absorption of an electron P680. Molecular masses are marked on each polypeptide. Electrons pass from the manganese centre (Mn_4), via a tyrosine residue (Tyr), to P680. Electrons ejected from this reaction centre pass to pheophytin (Ph), then to Q_A (a plastoquinone molecule associated with the D2 polypeptide) and on to Q_B (a plastoquinone molecule associated with the D1 polypeptide).
cyt=Cytochrome
(Adapted from Rutherford, 1989).

(reviewed in Rutherford, 1989).

The OEC proteins are nuclear-encoded and targeted to the thylakoid lumen posttranslationally. The 33K and 23K polypeptides were used during the course of this project to study protein transport across the thylakoid membrane.

1.3 TRANSPORT OF PROTEINS INTO, AND WITHIN, CHLOROPLASTS.

Chloroplast biogenesis is a complex process involving the activities of two genetic systems as shown in Figure 6. A small proportion of chloroplast proteins are encoded by chloroplast DNA and synthesised in the stroma, whereas the majority (about 80%) are nuclear-encoded, synthesised on cytosolic ribosomes and imported posttranslationally. Nuclear-encoded proteins are known to be targeted to all six chloroplast phases. The chloroplast is known to encode stromal and thylakoid proteins, and perhaps proteins which are destined for other chloroplast phases.

1.3.1 IMPORT OF NUCLEAR-ENCODED STROMAL PROTEINS.

The first studies on protein targeting to chloroplasts centred on an extremely abundant polypeptide, the small subunit of the stromal enzyme ribulose bisphosphate carboxylase oxygenase (RUBISCO). This enzyme constitutes up to 50% of soluble leaf protein and consists of 8 small subunits which are nuclear-encoded, and 8 large subunits, which are encoded by chloroplast DNA and synthesised in the stroma.

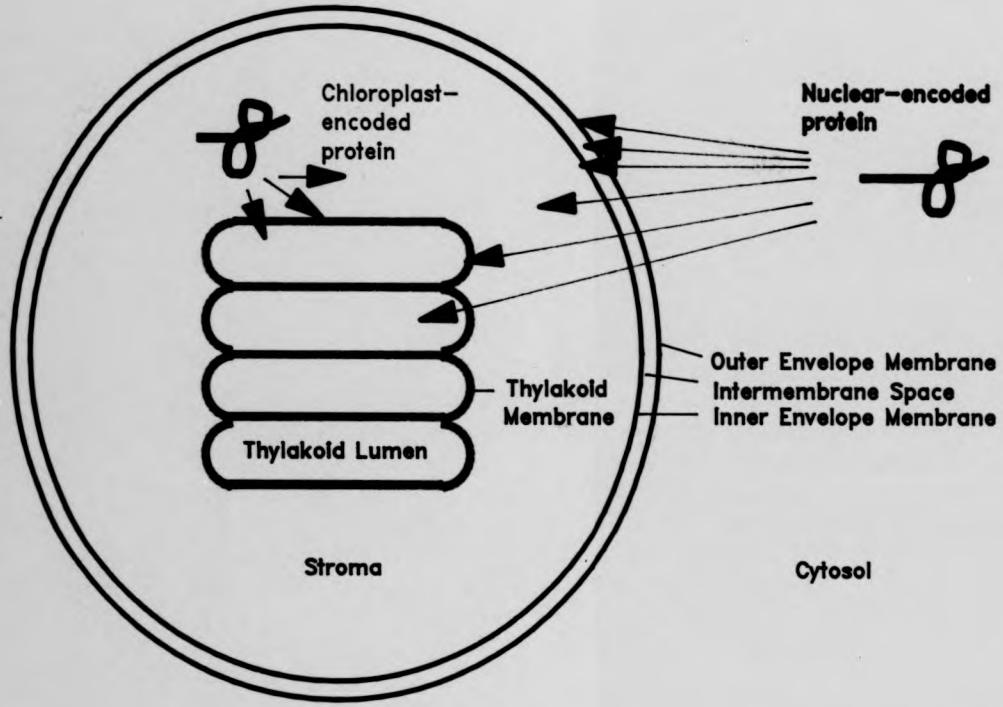


Figure 6. Diagram of Protein Transport During Chloroplast Biogenesis

Dobberstein *et al*. (1977), showed that the small subunit (SSU) is synthesised in a larger, precursor form (pSSU), while Highfield and Ellis (1978) and Chua and Schmidt (1978) demonstrated that pSSU could be imported into isolated chloroplasts posttranslationally. The use of isolated chloroplasts for the analysis of protein import (Figure 7) has proved very useful and has enabled the import of many other nuclear-encoded proteins to be studied *in vitro*.

1.3.1a STROMAL-TARGETING SIGNALS.

To reach their site of function, nuclear-encoded stromal and thylakoid proteins must traverse the chloroplast envelope. Such polypeptides are synthesised as larger, precursor forms which have N-terminal extensions known as presequences. The presequences of different stromal proteins show very little primary sequence similarity but tend to be rich in hydroxylated amino acyl residues, have few acidic residues and tend to share three simple features:-

i) an uncharged amino-terminal domain

ii) a central domain lacking acidic residues.

iii) a carboxy-terminal domain for which computer analysis of the amino acid sequence often predicts a high potential for forming an amphiphilic beta-strand. (von Heijne *et al*., 1989).

The targeting function of presequences can be demonstrated by constructing chimaeric genes which encode fusion proteins consisting of a particular presequence linked to a

Figure 7. The Use of Isolated Chloroplasts to Study Protein Import.

Isolated chloroplasts are incubated with <u>in vitro</u>-synthesised precursor proteins. Incubation with a stromal protein precursor will result in generation of the mature form in the stroma while some precursor remains unimported (A). After the import incubation, samples are treated with protease. A limited digestion results in degradation of proteins outside the chloroplast, whereas imported proteins are protected by the envelope from digestion (B). After import incubation, disruption of chloroplast membranes with detergent (or by sonication) and protease treatment is used to demonstrate that the imported protein is not inherently protease resistant (C). Incubation with a thylakoid lumen protein often results in the generation of an intermediate and a mature form within the chloroplast. Precursors are usually radioactively labelled and therefore samples can be analysed by SDS-PAGE followed by fluorography which enables resolution of different size forms.

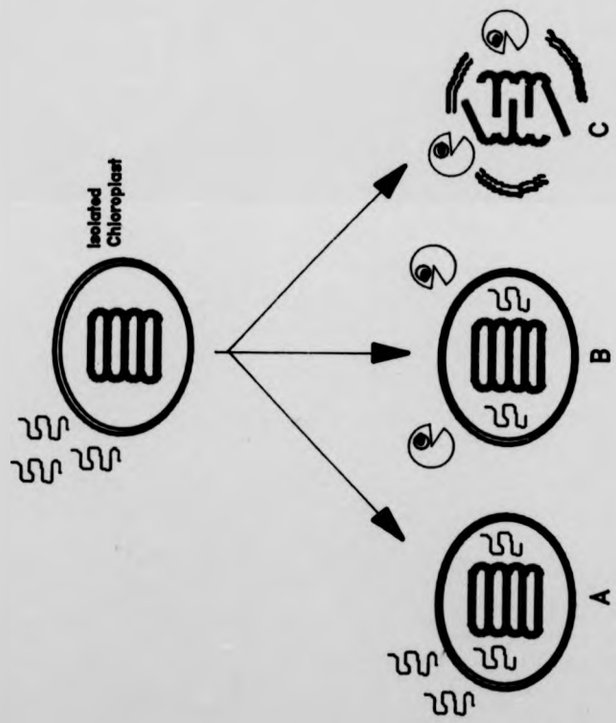


Figure 7. The Use of Isolated Chloroplasts to Study Protein Import.

'passenger protein'. The ability of a presequence to direct a foreign protein to a specific destination can then be determined. In such a manner, it was found that the presequences of several stromal proteins contain all the information required to direct import into the stroma. Schreier et al. (1985) constructed a chimaeric gene encoding a fusion protein consisting of the presequence and the first 22 amino acid residues from the N-terminus of SSU linked to the amino terminus of bacterial neomycin phosphotransferase II (NPT-II). An Agrobacterium vector was used to transfer and express this construct in tobacco plants and expression was monitored by assaying NPT-II activity. It was found that the fusion protein was targeted to the chloroplasts of transformed plants and the presequence proteolytically removed. All the information required for the targeting and processing of pSSU resides in the presequence since a fusion protein consisting of the presequence alone fused to NPT-II was found to be targeted to the chloroplasts of transformed tobacco and imported into isolated pea chloroplasts. In both cases the presequence was removed after, or during, import (Van den Broeck et al., 1985).

Joining a particular presequence to a passenger protein is not always sufficient for efficient targeting of a foreign protein. Several studies have found that fusing the presequence plus a stretch of residues from the N-terminus of the mature protein greatly improves efficiency of targeting of a foreign protein over that achieved without

the mature protein residues. While Van den Broeck et al. (1985) demonstrated that the presequence of SSU is sufficient to direct NPT-II import, Wasmann et al. (1986) found that including a stretch of 23 amino acid residues from the N-terminus of mature SSU before this passenger protein increased efficiency of import into isolated chloroplasts to levels close to those obtained for authentic pSSU. Kavanagh et al. (1988) constructed chimaeric genes encoding the presequence, and different numbers of residues from the mature form of the chlorophyll a/b binding protein (LHCP) fused to Escherichia coli beta-glucuronidase (GUS). The presequence together with N-terminal portions of the precursor form of LHCP (pLHCP), a nuclear-encoded thylakoid membrane protein, were tested for their ability to direct import of the bacterial protein into chloroplasts by introducing the chimaeric genes into tobacco then assaying the levels of GUS in leaf tissue and chloroplasts isolated from transformed plants. While fusion proteins containing 4 or 16 residues from the mature portion of LHCP were not imported into chloroplasts to any significant degree, those containing 24 or 53 such residues were efficiently imported. Increasing this stretch of linking residues to 126 drastically reduced import efficiency. Similar results were obtained when Lubben et al. (1989a) constructed a series of chimaeric genes encoding the SSU presequence linked to brome mosaic virus coat protein by 0, 14 or 177 amino acid residues from the N-terminus of mature SSU. Import of these

fusion proteins (which were radiolabelled) into intact chloroplasts was assayed. The chimaeric protein consisting of just the transit peptide linked to the coat protein was imported as efficiently as that including 14 residues of mature SSU. However, the fusion protein including most of the SSU polypeptide was not imported. All the chimaeric proteins were imported much less efficiently than authentic pSSU. Although the results of Wasmann et al. (1986) and Kavanagh et al. (1988) showed that including a certain number of residues from mature SSU in their chimaeric proteins was required for the efficient import of their passenger proteins, it is unlikely that mature SSU contains targeting information. More likely is that secondary or tertiary structural features of the chimaeric proteins affect their import. Wasmann et al. (1986) suggested that including a portion of mature SSU in their fusion proteins provided a spacer which separated the transit peptide from the passenger protein thereby allowing the presequence to assume its native conformation. Lubben et al. (1989a) suggested that chimaeric proteins may fold in such a way that the presequence is prevented from interacting with the chloroplast import apparatus or that segments of the passenger protein may inhibit conformational changes, such as unfolding, which may be necessary for import. Hence, although a particular transit peptide may contain all the information required for targeting, its function is

influenced by the passenger protein in question and the spacing between the presequence and passenger protein.

1.3.1b PROTEOLYTIC MATURATION OF STROMAL AND THYLAKOID MEMBRANE PROTEINS.

Under normal circumstances, precursor forms of imported chloroplast proteins cannot be detected in leaf tissue, indicating that the precursor is rapidly processed to the mature form.

Precursors of imported stromal and thylakoid proteins are processed by a stromal processing peptidase (SPP), either during, or shortly after, transport across the envelope (Figure 8). Robinson and Ellis (1984a) carried out size fractionation and inhibitor studies and found that SPP is a metal-dependent (i.e. chelator-sensitive) endopeptidase of about 180kDa. Processing by SPP is highly specific for imported precursors; partially purified SPP from pea chloroplasts has been found to process a number of precursors of stromal proteins to the mature size but does not cleave a variety of proteins normally targeted to other compartments within the cell (Robinson and Ellis, 1984a; Abad *et al.*, 1991).

Since the primary structures of many imported precursors are known, the nature of the signal which specifies cleavage by SPP has been investigated. It has been found that there is little primary sequence homology around the SPP site, although Gavel and von Heijne (1990) suggest that the

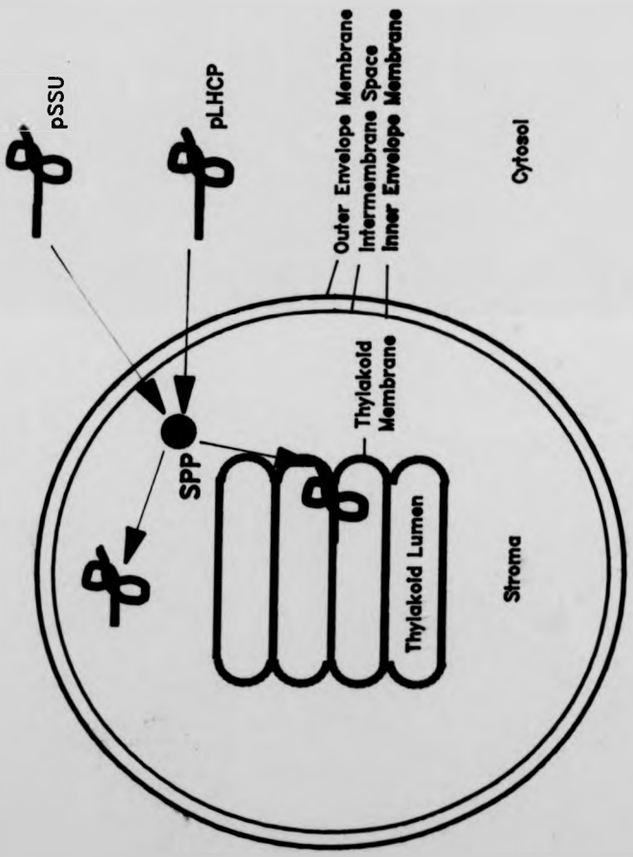


Figure 8. Diagram Showing the Import of pSSU and pLHCP.
The stromal protein SSU is synthesised in the cytosol as a higher molecular weight precursor (pSSU), and imported posttranslationally. Stromal processing peptidase (SPP) cleaves off the presequence to yield the mature size protein.
The thylakoid membrane protein LHCP is synthesised in the cytosol as a higher molecular weight precursor (pLHCP) which is imported posttranslationally. SPP cleaves off the presequence yielding mature size LHCP before, or possibly after integration into the thylakoid membrane.

majority of higher plant chloroplast presequences have "reasonable matches" to the conserved motif (Val/Ile)-X-(Ala/Cys)-Ala, cleavage taking place between the last two residues. It is generally believed that some structural features (as yet unknown), around the cleavage site, aid the specificity of this enzyme for the cleavage of imported chloroplast proteins.

While the removal of presequences is probably necessary for the correct functioning of the protein in question, it is not essential for translocation across the envelope, at least for the precursor forms of acyl carrier protein (pACP) and LHCP (pLHCP). Modification of pACP by carboxymethylation, blocks cleavage of this protein by SPP, but does not prevent import into the stroma of isolated chloroplasts (Dr. L. Barnett, University of Warwick, personal communication). The most abundant thylakoid membrane protein, LHCP, is nuclear-encoded and synthesised in a precursor form. Neither transport of pLHCP across the chloroplast envelope, nor its insertion into the thylakoid membrane is obligatorily coupled with proteolytic processing. pLHCP is processed by a stromal enzyme which is very similar to, or the same as, that identified by Robinson and Ellis (1984a). pLHCP is translocated across the chloroplast envelope and inserted into thylakoids of barley etioplasts in which the processing enzyme is inactive (Chitnis *et al.*, 1988). Clark *et al.* (1989) altered the amino acid sequence around the processing site of pLHCP so

that processing efficiency was greatly reduced and found that the mutant precursor was imported into isolated chloroplasts and inserted into the thylakoid membrane. Chitnis et al. (1988) suggested that, in vivo, pLHCP may be imported and inserted into the thylakoid membrane before processing to the mature form takes place.

In vitro studies have shown that SPP cleaves some precursors at more than one site. Robinson and Ellis (1984b) found that pSSU is cleaved to the the mature size in two steps. Since SPP has not been purified to homogeneity, it is not known whether SPP is a single enzyme or more than one enzyme of similar size and characteristics, hence whether the same enzyme is responsible for cleavage at both sites has yet to be determined. Whether this phenomenon occurs in vivo is not known; it is possible that the first cleavage is an artifact of the in vitro system, or perhaps precursor/enzyme interactions do not allow processing to the mature size in one step.

It is possible that in some instances cleavage of some precursors at more than one site has some functional significance. For example, in vivo, the LHCP associated with PSII is heterogeneous, being composed mainly of 26kDa and 25kDa species. It has been proposed that these species have different configurations in the thylakoid membrane and/or interact differently with other LHCP molecules. Part of this diversity is due to LHCP being encoded by a multigene family with two major classes, type I and type II. However, when

pLHCP encoded by a single type I gene is imported into isolated chloroplasts, both the 26kDa and 25kDa species are generated (Lamppa and Abad, 1988). Each species is produced by proteolytic processing at the N-terminus of the precursor, cleavage at the primary site yielding the 26kDa species and cleavage at the secondary site, 6 amino acyl residues closer to the C-terminus generating the 25kDa species. It has been proposed that the enzyme which processes pLHCP at the secondary site is the same as that described by Robinson and Ellis (1984a), since the enzyme, which has been enriched 7,000-fold from stroma in purification procedures, shows an identical pattern of cleavage to this enzyme for pSSU, pACP and pre-RUBISCO activase. However, the primary site of pLHCP is not recognised by the SPP in organelle-free reactions. This could be due to a different enzyme being responsible for cleavage at the primary site, which is not present or not active in this assay. Alternatively, both sites may be recognised by the same enzyme but the reaction conditions used greatly favour processing at the secondary site, or a component required for the recognition of the primary site is lacking or inactive in the organelle-free assay (Abad et al., 1991).

1.3.2 IMPORT OF NUCLEAR-ENCODED THYLAKOID LUMEN PROTEINS.

Approximately half of the known thylakoid proteins are encoded within the chloroplast and the remainder are

imported after synthesis in the cytosol. Nuclear-encoded thylakoid membrane proteins have similar presequences to imported stromal proteins, which target these proteins across the envelope membranes (section 1.3.1a); thylakoid lumen (TL) proteins must also carry information for translocation across the thylakoid membrane. The first thylakoid lumen protein to be cloned was plastocyanin (Smeekens et al., 1985), which is a small soluble electron carrier. Proteolytic processing and import assays using this protein and then others, resulted in three key observations which led to the proposal of a model for the import of thylakoid lumen proteins involving two membrane translocation steps Figure 9). These observation were:-

i) The presequences of TL proteins consist of two functionally distinct domains; the envelope transfer, and thylakoid transfer domains.

ii) The two-step proteolytic maturation sequence of TL proteins can be reconstructed in vitro. Partially purified SPP and thylakoidal processing peptidase (TPP) can process TL protein precursors to intermediate and mature sizes respectively (Hageman et al., 1986; James et al., 1989).

iii) Intermediate forms of several TL proteins have been observed with assays for import into isolated chloroplasts. Although the use of isolated chloroplasts to study protein translocation across the chloroplast envelope has proved very useful, this system is inadequate in some respects, for the analysis of the import of nuclear-encoded thylakoid

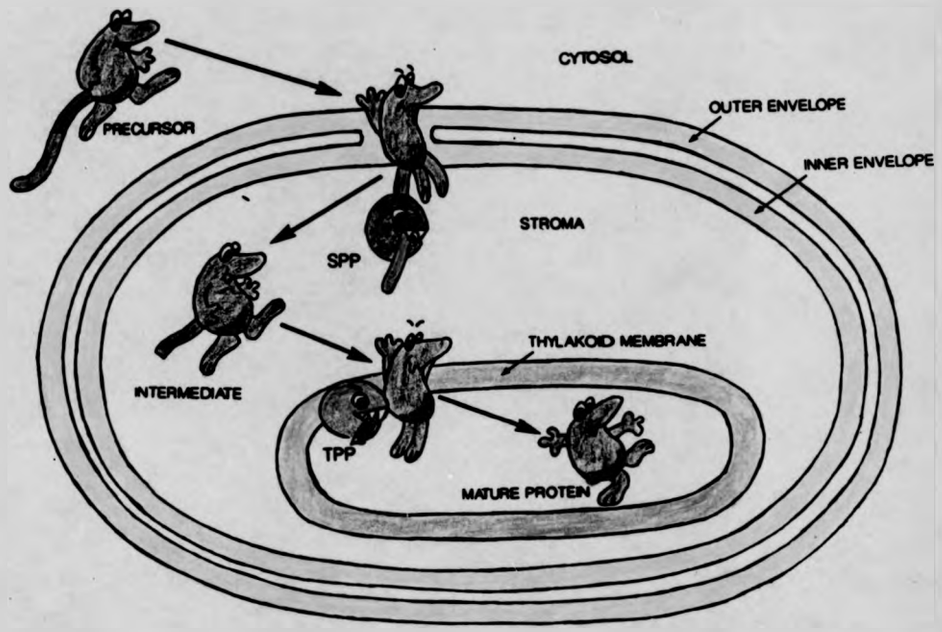


Figure 9. Model for the Import Pathway of Nuclear-Encoded Thylakoid Lumen Proteins.

The import pathway of nuclear-encoded thylakoid lumen proteins is believed to involve two translocation steps:-

1) The precursor protein is translocated across the envelope membranes into the stroma where the envelope transfer domain is removed by stromal processing peptidase (SPP), yielding an intermediate size protein.

2) The stromal intermediate is translocated across the thylakoid membrane and the thylakoid transfer domain removed by thylakoidal processing peptidase (TPP), yielding the mature size protein in the thylakoid lumen.

lumen proteins since protein translocation across the thylakoid membrane cannot be studied in isolation from translocation across the envelope. In view of this problem, Kirwin et al. (1989) developed an assay for the analysis of protein import into isolated thylakoids. Kirwin et al. (1989) found that p33K could be imported into isolated pea thylakoids in the presence of stromal extract and 10mM ATP/$MgCl_2$. The basic protocol for studying protein import into isolated thylakoids is illustrated in Figure 10. The first aim of this Ph.D. project was to further optimise the assay for import of proteins into isolated thylakoids. Protein transport across the thylakoid membrane is discussed further in Chapters 3, 4, 5 and 6.

1.3.2a THYLAKOID LUMEN TARGETING SIGNALS.

Nuclear-encoded thylakoid lumen proteins are targeted to the correct destination by composite presequences consisting of an envelope transfer domain, at the N-terminus, next to a thylakoid transfer domain (see Figure 11). The envelope transfer domain is structurally similar to the presequences of imported stromal proteins (section 1.3.1a).

Thylakoid transfer domains tend to be more hydrophobic than envelope transfer domains and are characterised by the following features:-

i) A hydrophobic core region.

ii) Small, uncharged, amino acid residues, often alanine, at

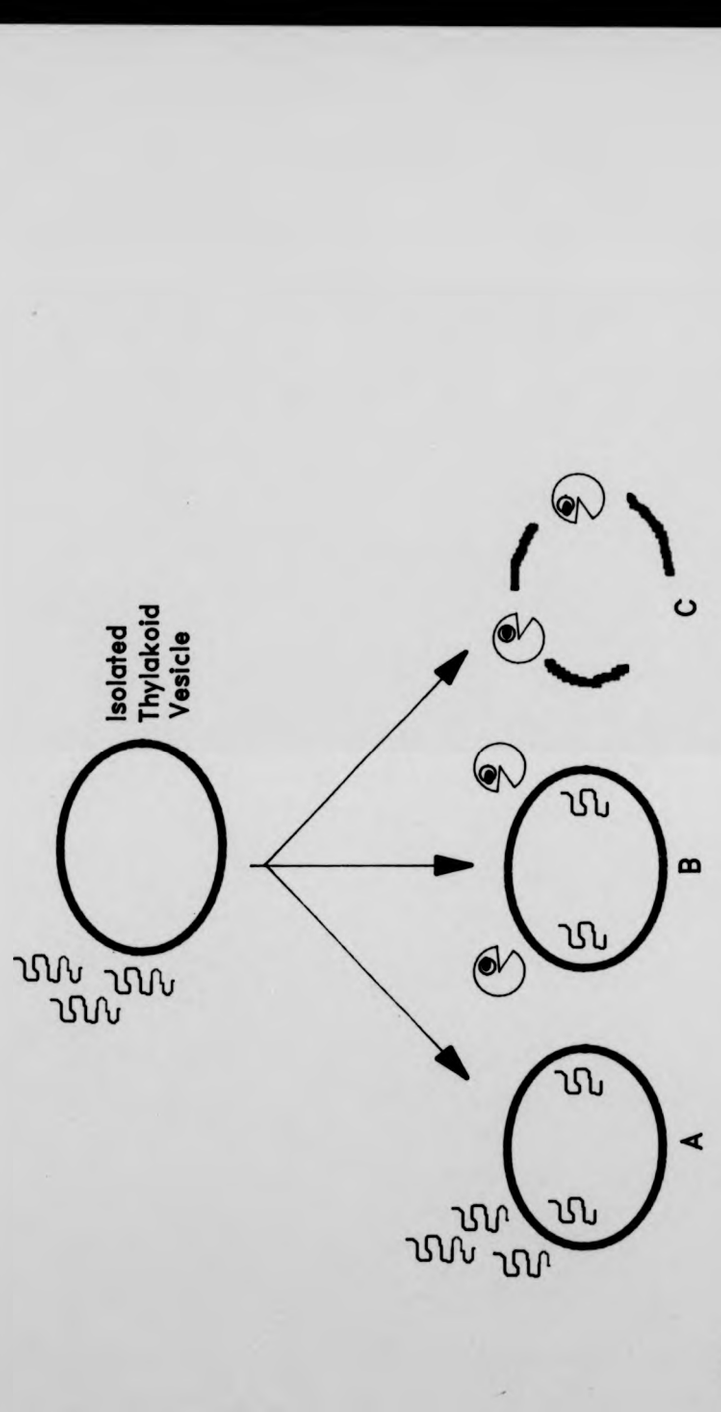


Figure 10. The Use of Isolated Thylakoids to Study the Import of Nuclear-Encoded Thylakoid Lumen Proteins.

Isolated thylakoids are incubated with precursors of nuclear-encoded thylakoid lumen proteins. Incubation in the presence of stromal extract results in the generation of the intermediate form. If import takes place, the mature form is located in the thylakoid lumen, while some precursor and intermediate form usually remain unimported (A). After the import incubation, samples are treated with protease. A limited digestion results in degradation of proteins outside the thylakoid, whereas imported proteins are protected by the thylakoid membrane from digestion (B). After import incubation, disruption of the thylakoid membrane with detergent (or by sonication) together with protease treatment is used to demonstrate that the imported protein is not inherently protease resistant (C). The precursors used in import incubations are radioactively labelled and therefore samples can be analysed by SDS-PAGE followed by fluorography which enables resolution of different size forms.

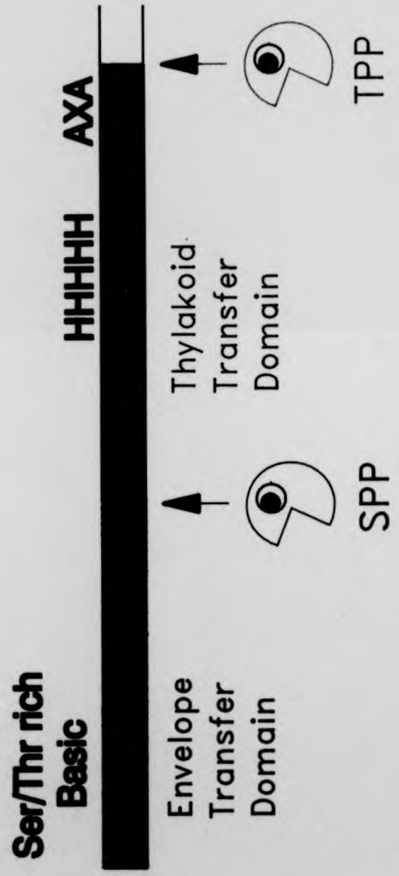


Figure 11. General Features of a Nuclear-Encoded Thylakoid Lumen Protein Presequence.

Figure 11. The General Features of Nuclear-Encoded Thylakoid Lumen Protein Presequences.

The envelope transfer domain has the same general features as the presequence of a nuclear-encoded stromal protein; the N-terminus being uncharged, the central region being rich in serine (Ser) and threonine (Thr) residues but lacking acidic residues, the end section having the potential to form an amphiphilic beta-strand.

The envelope transfer domain is cleaved off by stromal processing peptidase (SPP).

The thylakoid transfer domain has a hydrophobic, apolar core (HHHHH) and small, uncharged amino acid residues, usually alanine (A), at positions -3 and -1 relative to the thylakoidal processing peptidase (TPP) cleavage site.

positions -3 and -1, counting from the N-terminus of the mature protein, (also a feature of bacterial leader sequences and signal sequences which direct proteins across the endoplasmic reticulum membrane).
(von Heijne et al., 1989; Bassham et al., 1991).
The functions of thylakoid lumen presequence domains have been demonstrated in several ways. The envelope transfer domain of Silene pratensis plastocyanin can direct the import of the foreign protein, mouse dihydrofolate reductase (DHFR), into the stroma of isolated chloroplasts, indicating that it is functionally equivalent to the presequence of a nuclear-encoded stromal protein. Deletion of the proposed thylakoid transfer domain results in the accumulation of the protein in the stroma in import assays, but the complete presequence also fails to target mouse DHFR into the lumen (Hageman et al., 1990). The presequence of Silene pratensis plastocyanin has also failed to direct the import of a stromal protein, ferredoxin, and a foreign protein, yeast superoxide dismutase, to the thylakoid lumen (Smeekens et al., 1989; Smeekens et al., 1987). However, the presequence of wheat 33K with the first 22 amino acid residues from the N-terminus of the mature protein, can direct mouse DHFR into the thylakoid lumen of isolated chloroplasts (Meadows et al., 1989) and the presequence from Arabidopsis thaliana can direct SSU and DHFR, but not glycollate oxidase, to the thylakoid lumen in vitro (Ko and Cashmore, 1989). The failure of the presequence of Silene pratensis plastocyanin

to direct import of some proteins to the lumen may be due to some passenger proteins being more difficult to transport across membranes (such as glycollate oxidase), however, it also seems that some presequences are more efficient at targeting passenger proteins, since DHFR can be targeted to the thylakoid lumen by the presequence of 33K but not by the presequence of Silene plastocyanin. The thylakoid transfer domain of plastocyanin is shorter than that of 33K and hence it is possible that some targeting information resides in the mature part of preplastocyanin. Alternatively, import may be affected by interactions between the presequence and the passenger protein as outlined in section 1.3.1a. Interestingly, Roberts et al. (1991) found that a chimaeric protein, consisting of wheat 33K presequence plus 22 amino acid residues of the mature 33K linked to mature ricin A chain, was imported into isolated chloroplasts, but only a small fraction was located in the thylakoid lumen. Although all the imported protein was processed by TPP, the majority was located in the stroma. It is possible that the chimaeric protein was imported into the lumen and the presequence cleaved off before ricin A chain mediated its own translocation back across the thylakoid membrane. Alternatively, the majority of the protein was partially translocated across the thylakoid membrane, enabling removal of the presequence to take place, but then the protein was released back into the stroma.

1.3.2b PROTEOLYTIC PROCESSING OF IMPORTED THYLAKOID LUMEN PROTEINS.

As previously mentioned, processing of several thylakoid lumen proteins by thylakoidal processing peptidase has been observed. This enzyme has been partially purified and, unlike SPP, is a highly hydrophobic protein located in the thylakoid membrane. TPP activity is stimulated by metal-chelating agents such as EDTA and EGTA, has a pH optimum of 6.7 to 7.0, and activity of the isolated enzyme requires the presence of detergent. TPP is exclusively located in the non-appressed lamellae of the thylakoid network and is not intimately associated with any of the major thylakoid membrane protein complexes. The active site of TPP is on the lumenal face of the thylakoid membrane (Kirwin *et al.*, 1987; Kirwin *et al.*, 1988). In keeping with the structural similarities of thylakoid transfer domains with bacterial export signals and eukaryotic signal sequences, it has been found that the enzymes which cleave these presequences are essentially identical in terms of reaction specificity, although different in terms of structure. Muller *et al.* (1982) were the first to demonstrate that *E.coli* leader peptidase (LEP), which processes the leader sequences of bacterial proteins, and signal peptidase, which processes the leader signal sequences of proteins translocated across the ER membrane, have identical reaction specificities, while Halpin *et al.* (1989) demonstrated that LEP and TPP also have identical reaction specificities. Leader peptidase

purified from E.coli processes the wheat thylakoid lumen precursor proteins p23K and p33K to the correct mature size, while TPP partially purified from pea chloroplasts accurately cleaves the eukaryotic signal sequence from yeast prepro-alpha factor and the prokaryotic presequence from a chimaeric protein consisting of the leader peptide of Erwinia carotovora pectate lyase fused to an IgG light chain domain (Halpin et al., 1989). All three peptidases are integral membrane proteins with active sites on the trans side of the protein-translocating membrane, indicating that processing occurs during, or shortly after, translocation (Jackson and Blobel, 1977; Wolfe et al., 1983; Zimmermann and Mollay, 1986; Zimmermann et al., 1987). Although LEP and signal peptidase have identical reaction specificities, they differ markedly in structure, the former being a single polypeptide of 39kDa (Zwizinski and Wickner, 1980) and the latter consisting of at least two subunits (Evans et al., 1986). TPP has only been partially purified and hence its structure is not yet known (Kirwin et al., 1988). These data suggest that the processing sites of such proteins, rather than the processing enzymes, may have been subject to some conservation during evolution.

1.3.3 EARLY STAGES OF PROTEIN IMPORT INTO CHLOROPLASTS.

The early events in the import of proteins into chloroplasts have mainly been determined from studies on the import of

stromal proteins. Thylakoid proteins are believed to be transported across the envelope by the same mechanism as stromal proteins, mainly because of the close resemblance of envelope transfer domains to the presequences of stromal proteins.

1.3.3a BINDING TO THE CHLOROPLAST ENVELOPE.

The first step in the transport of proteins into chloroplasts is the specific binding of precursors to the envelope. Precursor binding is generally believed to involve interaction of the protein with the envelope membrane proteins and possibly membrane lipids.

Several lines of indirect evidence from early studies support the role of envelope proteins in precursor binding:-

i) A receptor or receptors will be exposed on the cytosolic face of the outer envelope membrane so that it is accessible for interaction with precursors, or digestion by added protease. Pretreatment of intact chloroplasts with thermolysin, to degrade outer envelope proteins, before incubation with radiolabelled precursors under conditions which enable binding but not translocation, resulted in significant (but not total) inhibition of pSSU binding and pLHCP binding. This inhibition of high affinity binding suggests the involvement of an outer envelope protein component or components in precursor binding (Cline *et al.* 1985).

ii) Since the number of binding sites on the chloroplast

surface is finite, they should be saturable. Pfisterer et al. (1982) carried out binding competition studies with radiolabelled translation products of poly(A) RNA from greening plants. The radiolabelled translation products were subject to competition for binding with the same products which had not been radiolabelled. It was also noted that SSU binds poorly to the envelope compared with pSSU.

There is little evidence for precursor/lipid interactions. The amphiphilic structures in mitochondrial protein precursors are thought to interact with membrane lipid and have been demonstrated to be involved in import into mitochondria (section 1.4.3). The presequences of chloroplast protein precursors (envelope transfer domains in the case of TL proteins) are also thought to form amphiphilic secondary structures.

Recently, several envelope proteins thought to be involved in protein translocation across the chloroplast envelope have been identified.

i) Cornwell and Keegstra (1987) modified pSSU with a photoactivatable cross-linking agent which they then incubated with isolated chloroplasts. They presumed that on activation, the modified precursor would bind to the pSSU receptor and that on activation of the cross-linker a conjugate of precursor and receptor would be formed. In this way a 66kDa protein was identified.

ii) Pain et al. (1988) made a synthetic peptide corresponding to a portion of pSSU presequence and raised

antibodies against this. By raising antibodies against the anti-peptide antibodies, anti-idiotypic antibodies were produced which were presumed to mimic the peptide and therefore interact with the same receptor as pSSU. The anti-idiotypic antibodies blocked import of pSSU, indicating that they did interact with the same receptor. The anti-idiotypic antibodies were actually found to bind to two different proteins; the large subunit of RUBISCO (for as yet unknown reasons) and a major 30kDa protein of the chloroplast envelope. Pain et al. (1988) concluded that a major 30kDa envelope protein was probably part of the pSSU receptor. However, this conclusion proved to be controversial; the only major 30kDa protein of the chloroplast envelope is the phosphate translocator which is an inner envelope protein constituting 10 to 20% of envelope protein. The phosphate translocator enables fixed carbon, in the form of triose phosphate, to be transported out of the chloroplast and the entry of phosphate into the chloroplast for ATP synthesis (Flugge et al., 1984). Joyard and Douce (1988) pointed out that the receptor protein labelled by Pain et al. (1988) may have been a minor 30kDa envelope protein, electrophoretically indistinguishable from the phosphate translocator and that the import receptor need not be an abundant protein to function efficiently. Kaderbhai et al. (1988) proposed that the phosphate translocator could have a dual function, being involved not only in phosphate/ triose phosphate exchange, but also in precursor binding. More

recently, Schnell *et al.* (1990) sequenced a cDNA of the putative receptor from pea and found that it had 84% homology with the phosphate translocator cDNA sequence from spinach (Flugge *et al.*, 1989). Schnell suggested that the sequences encoded essentially the same protein and that it was not the phosphate translocator but an outer envelope protein, actually of 36kDa, which has a role in pSSU binding.

iii) Kaderbhai *et al.* (1988) carried out cross-linking studies using an azido derivatised analogue of pSSU presequence. The modified presequence was found to bind to a 52kDa outer envelope protein and also a 30kDa inner envelope protein which they proposed to be the same protein as that identified by Pain *et al.* (1988), the phosphate translocator. However, Waegemann and Soll (1991) have found that pSSU binds tightly to isolated outer envelope membranes which were shown to be devoid of the phosphate translocator when western blots were carried out using antibodies raised to this protein. Inner envelope membrane which does contain the phosphate translocator was found not to bind pSSU significantly, suggesting that the phosphate translocator and pSSU receptor are not the same polypeptides.

Competition studies using different precursors may reveal which proteins have a receptor or receptors in common. Hinz and Flugge (1988) also identified a 51kDa outer envelope membrane protein (OEP 51) possibly the same as that identified by Kaderbhai *et al.* (1988). Hinz and Flugge

(1988) used a chimaeric protein consisting of pSSU presequence fused to mouse DHFR for their studies and found that binding of this precursor to the chloroplast induced a kinase activity which resulted in a specific increase in the phosphorylation state of OEP 51. They suggested that OEP 51 is either the receptor for pSSU import or a regulatory component intimately associated with the import apparatus.

iv) Waegemann and Soll (1991) incubated pSSU with isolated outer membrane and found that it bound tightly and was partially inserted into the membrane. The partially inserted pSSU could not be efficiently extracted by 1M NaCl or 0.1M NaOH washes. A complex containing pSSU was isolated after mild solubilisation of the membrane with detergent. Major components of this complex were a 86kDa outer envelope protein and a constitutively expressed homologue of heat shock protein of molecular mass around 70 (hsp70). Marshall et al. (1990) had previously noted that members of the heat shock protein (hsp) family are located in the outer chloroplast envelope and in the chloroplast stroma.

1.3.3b PROTEIN CONFORMATION DURING TRANSLOCATION ACROSS THE CHLOROPLAST ENVELOPE.

A number of studies have demonstrated that proteins unfold in order to be translocated across mitochondrial membranes (section 1.4.3). Della-Cioppa and Kishore (1988) studied whether chloroplast protein precursors must also unfold in order to be translocated across the envelope by analysing

the import of a stromal enzyme, 5-enolpyruvylshikimate-3-phosphsphate synthase (EPSP synthase), into isolated chloroplasts. This enzyme is the site of action of the herbicide glyphosate which binds tightly to the enzyme rendering it inactive. EPSP synthase is nuclear-encoded, synthesised with a presequence and imported posttranslationally into the chloroplast stroma. It was found that low concentrations of glyphosate (10uM) almost completely inhibited import of pEPSP synthase *in vitro* but not that of several other precursors of chloroplast proteins. It was concluded that inhibition of import was due to the herbicide tightly binding to pEPSP synthase, thereby preventing it from unfolding to the extent required for translocation.

Von Heijne and Nishikawa (1991) proposed that the presequences of nuclear-encoded chloroplast proteins are 'designed' to interact with different molecular chaperones in succession during their import pathway. Such chaperones prevent the protein being imported from folding in a manner, or interacting with other molecules, that would render it unable to interact correctly with the import apparatus or other essential proteins in its import/ assembly pathway. Molecular chaperones are involved in the import/ assembly of many proteins but are not present in the mature/ assembled structures. Von Heijne and Nishikawa (1991) have proposed that the envelope transfer domains of chloroplast proteins would have high affinity for a particular group of

chaperones, the hsp70 family. Recent work has suggested that hsp70s are involved in the import of proteins into mitochondria and the endoplasmic reticulum lumen. The activity of hsp70s requires ATP hydrolysis. The presence of hsp70 homologues in pea cytosol, chloroplast envelopes and stroma (Marshall et al., 1990) led von Heijne and Nishikawa (1991) to propose that import of chloroplast precursor proteins may first involve binding of a cytosolic hsp70, transfer to a envelope-bound hsp70 and on to stromal hsp 70s. Findings by Waegemann et al. (1990) lend some support to this hypothesis. Waegemann and co-workers found that import of pLHCP into isolated chloroplasts is dependent on the presence of two soluble proteins which are present in leaf extract but not in isolated chloroplasts. The effects of one of these proteins on pLHCP import can be mimicked by purified hsp70 while the effects of the other cannot.

1.3.3c PROTEIN TRANSLOCATION MAY TAKE PLACE AT CONTACT SITES BETWEEN THE CHLOROPLAST ENVELOPE MEMBRANES.

Although the chloroplast envelope is a major site of protein translocation in the plant cell, the site of the protein translocation apparatus is uncertain. Translocation is generally believed to take place at sites of contact between the outer and inner envelopes so that both membranes and the soluble phase between them are crossed in essentially one step. There is, as yet, little biochemical evidence for translocation at such 'contact sites' although the existence

of such sites and the localisation of a putative import receptor to the sites have been reported from electron microscopy and immuno-labelling studies (Pain *et al.*, 1988; Schnell *et al.*, 1990).

Protein translocation at contact sites between the outer and inner mitochondrial membranes has been well documented (see section 1.4.3); more research is required to confirm protein translocation at such sites in the chloroplast envelope.

1.3.4 ENERGY REQUIREMENTS FOR PROTEIN IMPORT INTO CHLOROPLASTS.

The translocation of proteins across biological membranes requires some form of energy input; the energy required depends on the system in question (sections 1.4.1 to 1.4.4). The energy requirements for translocation across the chloroplast envelope have been studied in some detail using a variety of approaches. All studies so far published agree that ATP is required for transport across the envelope although there has been some contradiction about the site at which ATP is required and what it is required for.

Early experiments demonstrated that light-stimulated import of pea poly(A) RNA translation products into isolated intact chloroplasts is due to ATP synthesis resulting from photosynthetic photophosphorylation. The addition of ATP in the dark mimics the stimulatory effect of light. Uncouplers of photophosphorylation, which prevent the generation of a proton motive force (pmf) across the thylakoid membrane

inhibit light-stimulated import while import was restored by the addition of ATP. Light and ATP did not act synergistically. It was concluded that ATP stimulated import and that this stimulation was not directly dependent on the generation of a proton motive force (Grossman et al., 1980). Cline et al. (1985) studied import of pSSU and pLHCP into intact chloroplasts. It was found that if chloroplasts were pretreated with the uncoupler nigericin (see section 4.2), when the precursors were added binding took place but translocation did not occur. Addition of ATP to such incubations resulted in the translocation of bound precursors across the chloroplast envelope.

Flugge and Hinz (1986), investigated import of pSSU into isolated spinach chloroplasts and demonstrated that MgATP and not an electrochemical gradient across the envelope, promotes import of this protein. It was also found that addition of a nonhydrolysable analogue of ATP did not stimulate import; the precursor was bound to, but not translocated across the envelope. Flugge and Hinz (1986) also addressed the question of which side(s) of the envelope the ATP-dependent steps of import occur. Alkaline phosphatase was used to hydrolyse external ATP without affecting stromal levels of ATP and was found to inhibit pSSU import. Stromal ATP was depleted in the dark by the addition of glycerate which is transported into chloroplasts via the glycerate translocator and subsequently converted to 3-phosphoglycerate at the expense of stromal ATP. Addition

of glycerate did not inhibit pSSU import and it was concluded that import of this protein was stimulated by external ATP, either cytosolic or within the intermembrane space.

Contrary to these findings, Pain and Blobel (1987) concluded from studies on the import of the same protein (pSSU) into isolated chloroplasts, that pSSU import is dependent on ATP hydrolysis within the chloroplast. These workers proposed the involvement of a previously uncharacterised ATPase located in the stroma or facing the stroma, in protein import into chloroplasts. In the dark, levels of stromal ATP were elevated by the addition of Calvin cycle intermediates, dihydroxyacetone phosphate (DHAP), oxaloacetate and inorganic phosphate which allow the Calvin cycle to run backwards past the step at which ATP is normally used in the conversion of 3-phosphoglycerate to 1,3-bisphosphoglycerate, thereby generating ATP. These additions stimulated pSSU import while the addition of non-penetrating ATP-consuming systems such as apyrase or glucose plus hexokinase, to deplete external ATP, did not significantly inhibit pSSU import. It was therefore concluded that internal ATP, stromal or possibly intermembrane space, is required for pSSU import. Pain and Blobel (1987) also demonstrated by the use of specific ionophores, that pSSU import is not dependent on gradients of H^+, Na^+, K^+ or divalent cations such as Ca^{2+} or Mg^{2+}, across the chloroplasts membranes.

Olsen *et al.* (1989) and Theg *et al.* (1989) set out to

resolve the question of where ATP is required for protein import into chloroplasts. Binding of precursors to the chloroplast envelope was generally believed not to require energy input. However, Olsen and co-workers demonstrated that binding, but not translocation, of pSSU, preferredoxin and preplastocyanin was stimulated by addition of low levels of ATP (50-100μM), whereas higher levels (1mM or more) were required for translocation of these proteins. Nonhydrolysable analogues of ATP could not substitute for ATP while other hydrolysable NTPs could, although they were less active than ATP, demonstrating that precursor binding requires NTP hydrolysis. The use of ionophores to collapse electrical potentials or pH gradients across membranes did not affect binding in the presence of ATP. To determine where ATP is required for precursor binding, internal ATP was generated in the dark as in Pain and Blobel (1987) or external ATP was reduced by adding glucose plus hexokinase. When internal ATP concentrations were high and little or no external ATP was present, pSSU binding occurred. When internal ATP was depleted due to the addition of glycerate, but external ATP was present, pSSU binding was inhibited. It was therefore concluded that stromal or intermembrane space ATP is required for pSSU binding.

Theg *et al*. (1989) manipulated internal and external ATP levels as in Olsen *et al*. (1989) and found that translocation of pSSU, preferredoxin and preplastocyanin required internal ATP. They also presented kinetic evidence

for stimulation of translocation by internal ATP. Import of these three proteins is found to occur at higher initial rates in the light compared to ATP-stimulated import in the dark. It was proposed that ATP is required internally and since ATP generated in the light is internal, it could be utilised immediately. ATP added to import incubations in the dark would need to be transported into the chloroplast by the adenylate translocator, before it could support protein import and there would therefore be a lag period before import could occur. In support of this proposal, it was found that preincubation of chloroplasts with ATP before addition of precursor abolished this 'lag' period and import occurred at initial rates very similar to those which occur in the light. Theg *et al*. (1989) found that glycerate varies in its efficiency as an internal ATP trap and proposed this as a reason why Flugge and Hinz (1986) drew different conclusions when using it. Flugge and Hinz themselves pointed out that using alkaline phosphatase as an external ATP trap probably affected import by some means other than removal of external ATP since two other external ATP traps did not significantly affect pSSU import.

Waegemann and Soll (1991) confirmed a requirement for ATP hydrolysis early in the import of pSSU when using isolated envelope membranes. They suggested that ATP might be required for the release of pSSU from cytosolic factors and simultaneous transfer to a receptor complex in the chloroplast envelope. Whether ATP is hydrolysed by a

cytosolic or envelope protein has yet to be determined. Hinz and Flugge (1988) have shown that OEP 51 is phosphorylated in an ATP-dependent manner, induced by precursor binding although it is not known at present which protein actually hydrolyses the ATP required.

Apart from the envelope transfer step of import into chloroplasts, integration of the proteins into the thylakoid membrane is an energy-requiring process. Cline et al. (1989) found that integration of pLHCP was inhibited by up to 70% when the thylakoidal proton motive force was dissipated by the addition of ionophores. Integration was inhibited when either the electrical component or the pH gradient component of the pmf was collapsed although maximum inhibition was achieved when the total pmf was dissipated. Cline and co-workers found that the proton gradient had a more major role than the electrical component of the pmf in stimulation of the pLHCP and mature LHCP integration.

In the dark, integration of pLHCP can be driven by ATP although uncouplers partially inhibit this process, suggesting that, although a pmf is not essential for pLHCP integration, it facilitates efficient integration.

The energy requirements for protein translocation across the thylakoid membrane were addressed as part of this Ph.D. project and are discussed in Chapters 4 and 6.

1.3.5 TARGETING OF PROTEINS TO THE CHLOROPLAST ENVELOPE.

In comparison with import of proteins to the stroma, very

little is known about targeting of proteins to the chloroplast envelope. Since the envelope constitutes only about 1% of total chloroplast membrane and is difficult to isolate, the cloning of envelope proteins has proved difficult. However, a few envelope proteins have recently been identified, some of which are believed to be involved in the protein translocation apparatus as mentioned above.

The most abundant chloroplast envelope protein, the phosphate translocator (also mentioned in section 1.3.3a) was cloned and sequenced by Flugge *et al*. (1989). This protein is nuclear-encoded and has a presequence which targets it to the inner envelope membrane in an ATP-dependent manner. The ATP requirement is on the chloroplast side of the outer envelope, possibly in the intermembrane space. Flugge *et al*. (1989) speculated that the location of the ATP required excluded the involvement of an ATP-dependent cytosolic factor but not the involvement of an ATP-dependent unfolding activity in the outer envelope energised by internal ATP.

Pretreatment of chloroplasts with protease prevents import of both proteins suggesting that they must interact with a proteinaceous receptor on the outer face of the outer envelope membrane to be imported.

Dreses-Weringloer *et al*. (1991) also cloned and sequenced a nuclear-encoded inner envelope protein of 37kDa. This protein is also synthesised with a presequence and imported into chloroplasts in an ATP-dependent manner.

The presequences of these inner envelope proteins contain an amphiphilic alpha-helix unlike the envelope-transfer domains of other imported chloroplast proteins which tend to have a C-terminal beta-strand (Willey et al., 1991; von Heijne et al., 1989). These proteins also lack the cleavage motif (Val/Ile)-X-(Ala/Cys)-*-Ala (the cleavage site denoted by *), which is found in most nuclear-encoded chloroplast presequences (Gavel and von Heijne, 1990). Dreses-Werringloer et al. (1991) have suggested that other structural elements may be involved in cleavage-site recognition for these proteins. The difference between the presequences of inner envelope proteins and other imported chloroplast proteins suggests that they may not share a common import pathway.

Genes encoding a spinach 6.7kDa outer envelope protein and a pea 14kDa outer envelope protein have been cloned by Salomon et al. (1990) and Li et al. (1991) respectively. Analysis of these proteins and their targeting to chloroplasts suggests that they are targeted in a very different manner from proteins destined for other chloroplast subcompartments. These proteins are not synthesised as higher molecular weight precursors, i.e. they are synthesised without cleavable presequences. Insertion into the outer envelope membrane of both these proteins does not require ATP, nor is it inhibited by pretreatment of the chloroplasts with thermolysin. Li et al. (1991) also reported that insertion of the 14kDa protein was not inhibited by synthetic peptide

analogues of SSU presequence which had been previously demonstrated to inhibit import of several precursors targeted to other chloroplast subcompartments (Perry *et al.* 1991). When this protein was synthesised *in vitro*, it was found to insert into the outer envelope membrane of chloroplasts but not the outer membrane of mitochondria, demonstrating specificity of insertion. From these results it was concluded that this protein either uses a different import receptor from the majority of imported proteins, or may interact directly with lipid components in the outer envelope membrane. Since pretreatment of chloroplasts with thermolysin did not affect integration of the 14kDa protein, it is possible that the protein does not interact with any protein component in this membrane; alternatively, its receptor may not be sensitive to thermolysin. If a protein receptor is not involved in 14kDa integration, specificity of insertion is ensured by some other interaction, perhaps involving the unique lipid composition of the outer envelope membrane.

It therefore seems that outer envelope proteins, and possibly inner envelope proteins, have a different import pathway or import mechanism to other imported chloroplast proteins. When more outer envelope proteins have been cloned and studied, a general mechanism for the import of these proteins may be determined.

1.3.6 THE ENDOSYMBIONT HYPOTHESIS FOR ORGANELLE EVOLUTION.

According to the endosymbiont hypothesis, the progenitors of eukaryotic cells were primitive cells without chloroplasts or mitochondria. A relatively early event, in evolutionary terms, was the establishment of a stable endosymbiotic relationship with a bacterium whose oxidative phosphorylation system was subverted for the use of the host. From such an internalisation event mitochondria are believed to have arisen. Chloroplasts are thought to have arisen from a separate endocytotic event involving the internalisation of a photosynthetic cyanobacterium-like cell, thereby creating the first primitive plant cell. This hypothesis is mainly based on the striking resemblance chloroplasts and mitochondria have to prokaryotes and evolutionary trees based on gene sequences (Schwartz and Dayhoff, 1978). Also, symbiosis between photosynthetic bacteria and other cells can be observed today; a close relative of present-day cyanobacteria lives within a host cell in a permanent symbiotic relationship, the two organisms being collectively known as Cyanophora paradoxa. Since the majority of genes which encode present-day chloroplast and mitochondrial proteins are located in the nucleus, it is proposed that after the initial endocytotic events, extensive gene transfer occurred from organellar to nuclear DNA.

The transfer of genes to the nucleus must have involved the evolution of 'envelope transfer' domains to target the gene

products into chloroplasts (Smeekens et al., 1986). The lumenal proteins of free-living cyanobacteria, such as 33K and plastocyanin are synthesised with presequences that closely resemble the thylakoid transfer domains of their higher plant counterparts (Kuwabara et al., 1987; Wallace et al., 1989). Hence it seems that transfer of genes encoding stromal and thylakoid proteins involved the addition of an envelope transfer domain while thylakoid lumen proteins already had the information required for targeting across the thylakoid membrane, and once in the stroma, utilise this more ancient pathway. Such 'conservative sorting' or 'evolutionary economy' has been suggested to occur during the import of most nuclear-encoded mitochondrial intermembrane space proteins (section 1.4.3)

1.3.7 USE OF PROTEIN TARGETING IN PLANT GENETIC ENGINEERING. The use of presequences to target specific proteins into chloroplasts may prove to be of great importance to plant genetic engineering. Foreign or altered proteins could be efficiently and specifically targeted to the required chloroplast subcompartment. Furthermore, analysis of other protein targeting mechanisms may result in our ability to target proteins to any required destination within the cell. At present such retargeting technology has not been used to alter the properties of chloroplasts in transformed plants apart from the following research by Della-Cioppa et al. (1987).

Crop productivity can be increased if the competition by weeds with the crop, for light and nutrients, is reduced. This can be achieved if the crop is resistant to a particular herbicide which can be used to kill the weeds. The herbicide glyphosate inhibits chloroplast EPSP synthases thereby preventing plant growth by blocking aromatic amino acid synthesis. A gene encoding a mutant form of EPSP synthase was isolated from E.coli and fused to a 5' cDNA sequence encoding the EPSP synthase presequence from petunia. This construct was expressed in vivo and gave rise to stable, glyphosate resistant enzyme within the chloroplasts of transformed tobacco plants. In vitro assays of import using isolated chloroplasts and radiolabelled precursor proteins showed that the efficiency of import of the plant/bacterial EPSP synthase was almost identical to that of the authentic plant enzyme. Della-Cioppa et al. (1987) suggested that efficient import of the fusion protein was due to its conformation being very similar to that of the authentic precursor since both are catalytically active. Such retargeting technology may be limited in several ways:-

i) interactions between the presequence and passenger protein may affect interactions between the protein and the import machinery.

ii) passenger protein structure may prevent passage through the import machinery.

iii) the number of plant species which can be efficiently transformed.

1.4 OTHER PROTEIN TRANSLOCATION SYSTEMS.

Our knowledge of the mechanism by which proteins are translocated across chloroplast membranes has tended to lag behind that gained about other protein translocating systems such as mitochondria and the endoplasmic reticulum. Indeed, many of the findings, from studies in these systems, have inspired similar research on chloroplast protein translocation. By comparing different protein translocating systems it may be possible to determine:-

i) whether such systems evolved from a common ancestral translocation apparatus

ii) the differences in targeting systems which ensure that a particular protein is targeted to the correct destination within the cell.

1.4.1 PROTEIN TRANSLOCATION ACROSS THE ER MEMBRANE.

A large proportion of proteins are translocated across the endoplasmic reticulum (ER) membrane, including ER, Golgi, lysosome and secretory proteins. Like nuclear-encoded chloroplast proteins, proteins targeted across the ER membrane are synthesised with N-terminal extensions not present in the mature protein, which are known as signal sequences. Signal sequences, which direct translocation across the ER membrane, share little amino acid sequence homology although they have basic features in common with bacterial export signals (section 1.4.3).

Unlike the translocation of nuclear-encoded proteins across

chloroplast membranes, protein translocation across the ER membrane is usually cotranslational. The events during translocation across the ER membrane can be divided into two phases, a signal recognition particle (SRP) cycle and actual translocation across the ER membrane (Figure 12);

i) The SRP cycle initiates as follows:-

Synthesis of a polypeptide to be translocated across the ER membrane begins on a ribosome which is free in the cytosol. Once the signal sequence of the nascent polypeptide chain emerges from the ribosome, it is recognised and bound by SRP which has an affinity for the ribosome. SRP consists of a 300 nucleotide RNA plus six different polypeptides. The interactions of SRP with a ribosome and a signal sequence are cooperative; SRP does not bind completed polypeptide chains released from ribosomes (Weidmann et al., 1987a). This association slows down elongation of the polypeptide chain (Walter and Blobel, 1981). The ribosome-nascent chain-SRP complex then specifically interacts with SRP receptor (also known as docking protein) which is located in the ER membrane (Gilmore et al., 1982; Meyer et al., 1982). SRP receptor is a dimer of alpha (69 kDa) and beta (30kDa) subunits. The receptor then binds GTP which may replace previously bound GDP (Connolly and Gilmore, 1989). After docking, SRP is released from the ribosome and the signal sequence in a process that requires GTP hydrolysis (Connolly and Gilmore, 1989), thus recycling SRP into the cytosol.

ii) After release from the SRP, the ribosome is thought to

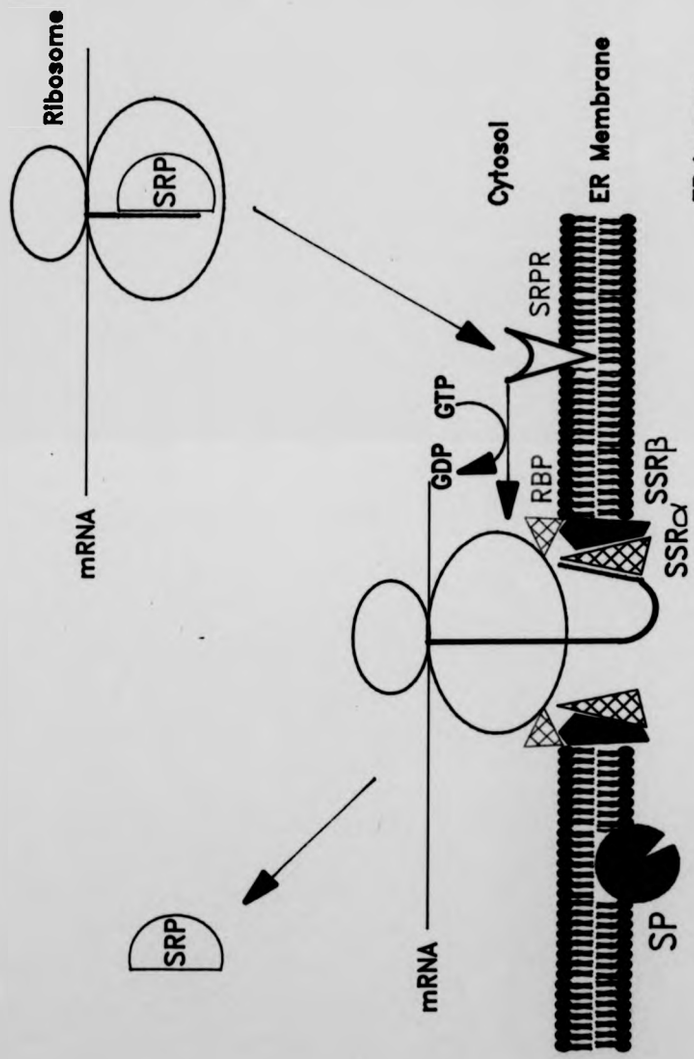


Figure 12. Cotranslational Protein Translocation Across the ER Membrane.

Figure 12. Cotranslational Protein Translocation Across the ER membrane.

Signal recognition particle (SRP) binds the presequence (shown in red) of the nascent precursor as it emerges from the ribosome, and to the ribosome itself. This complex interacts with SRP receptor (SRPR) which is located in the ER membrane, SRP is released in a process which requires GTP hydrolysis by SRP receptor. Some signal sequences have been found to interact with the alpha-subunit of an integral membrane protein, signal sequence receptor (SSR, shown in blue). The ribosome is thought to interact with ribosome binding protein (RBP). Translocation is believed to occur through an aqueous channel in the ER membrane and may involve heat shock protein 70 homologues in the ER lumen. During or after translocation the signal sequence is removed by the integral membrane protein, signal peptidase (SP).

interact with a membrane protein, ribosome binding protein (RBP). At least some signal sequences interact with an integral membrane protein complex, consisting of an alpha and beta subunit, termed signal sequence receptor (SSR) (Weidmann et al., 1987b).

How the subsequent protein translocation takes place is largely unknown, but the signal sequence probably dissociates from SSR in order for the protein to translocate across the ER membrane, which occurs as the nascent chain is elongated. Whether the translocation process requires ATP hydrolysis has proved difficult to determine since ATP is required for translation. The nature of the translocation machinery in the ER membrane is not fully understood. It is currently believed that translocation occurs through large aqueous tunnels in the ER membrane (Simon et al., 1989). Nicchitta and Blobel (1990) found that ER microsomes which were depleted in glycoproteins could bind preprolactin but their capacity to translocate this protein was greatly diminished, implicating an integral membrane glycoprotein in the translocation process. SSR, which is an integral membrane glycoprotein, has been found in close proximity to nascent chains during translocation and therefore may be a constituent of the putative tunnel or 'translocation pore' (Weidmann et al., 1987b; Kreig et al., 1989).

It has been found that translocation of certain small proteins (less than 100 residues) occurs posttranslationally in an SRP-independent manner (Muller and Zimmermann, 1987;

Rothblatt et al., 1987). This process in yeast is ATP dependent but does not require an electrical potential or proton gradient across the ER membrane. The involvement of constitutivley expressed hsp70 family proteins has been implicated in the posttranslational translocation of proteins across the ER membrane. In yeast the absence of hsp70-SSA1 results in the accumulation in the cytosol of certain proteins normally translocated across the ER membrane (Deshaies et al., 1988; Zimmermann et al., 1988). The addition of hsps in vitro can dramatically increase translocation of certain proteins into microsomes; however, this requirement for hsp70s can be bypassed if the protein to be translocated is denatured using urea (Chirico et al., 1988).

The requirement for hsp70s in posttranslational protein translocation across the ER membrane is thought to involve interaction of hsp70s with the protein to maintain it in a conformation which can be accepted by the translocation machinery, as may be the case for protein transport across the chloroplast envelope (section 1.3.3b). The involvement of hsp70s may reflect part or all of the requirement for ATP hydrolysis.

Signal sequences are removed by the integral membrane protein signal peptidase which, as previously mentioned in section 1.3.2b, has a similar or identical reaction specificity to TPP and bacterial leader peptidase.

1.4.2 PROTEIN TRANSLOCATION ACROSS THE BACTERIAL PLASMA MEMBRANE.

Studies on protein transport in prokaryotes have mainly focused on translocation of proteins across the plasma membrane of the gram-negative bacterium Escherichia coli (recently reviewed by Bassford et al., 1991). Protein synthesis occurs in the cytosol and proteins destined for the extracellular medium, the outer membrane and the periplasmic space, must cross the plasma membrane. As previously mentioned, the N-terminal leader sequences of 'exported' bacterial proteins, contain targeting information and are structurally similar to eukaryotic signal sequences. Most bacterial leader sequences are cleaved by leader peptidase (LEP), a 36kDa integral membrane protein which has a similar or identical reaction specificity to signal peptidase and TPP although these proteins have very different structures (Section 1.3.2b) (Date and Wickner 1981, Wolfe et al., 1983, Halpin et al., 1989). At least one other prokaryotic signal peptidase exists; Tokunaga et al. (1982) found that antibodies raised against LEP prevented processing of the normally exported polypeptide procoat protein but did not affect cleavage of prolipoprotein. It was concluded that another protease must be responsible for prolipoprotein processing. It is unclear whether processing of leader sequences occurs cotranslationlly or posttranslationally. Josefsson and Randall (1981) reported that some precursors are processed during translocation and

others after translocation, whereas some exhibit both modes of processing. Translocation across the E.coli plasma membrane can occur cotranslationally or posttranslationally. The relative simplicity of the E.coli genome has enabled a number of genes encoding components of the export/secretory machinery to be identified; such proteins are usually denoted 'Sec' for secretory.

The current model for protein translocation across the plasma membrane of E.coli is illustrated in Figure 13.

The precursor protein interacts with a cytosolic molecular chaperone and the precursor-chaperone complex interacts with the peripheral membrane protein SecA. ATP hydrolysis by SecA is required for protein translocation. SecA is bound to the membrane by interactions with acidic phospholipids and the SecE and SecY components of the translocation pore complex (Lill et al., 1989; Hartl et al., 1990). The translocation pore complex consists of integral membrane proteins, SecE and SecY, and is thought to form an aqueous pore in the plasma membrane. The pore complex may also contain the proteins SecD and SecF. It is not known whether processing of signal sequences occurs during or after translocation. Several proteins (SecB, trigger factor and GroEL) have been found to interact with E.coli precursor proteins in vitro and do not interact with globular cytosolic proteins. It is believed that these proteins are chaperones, like SRP and hsps, which recognise proteins to be translocated and maintain them in a conformation which can be translocated.

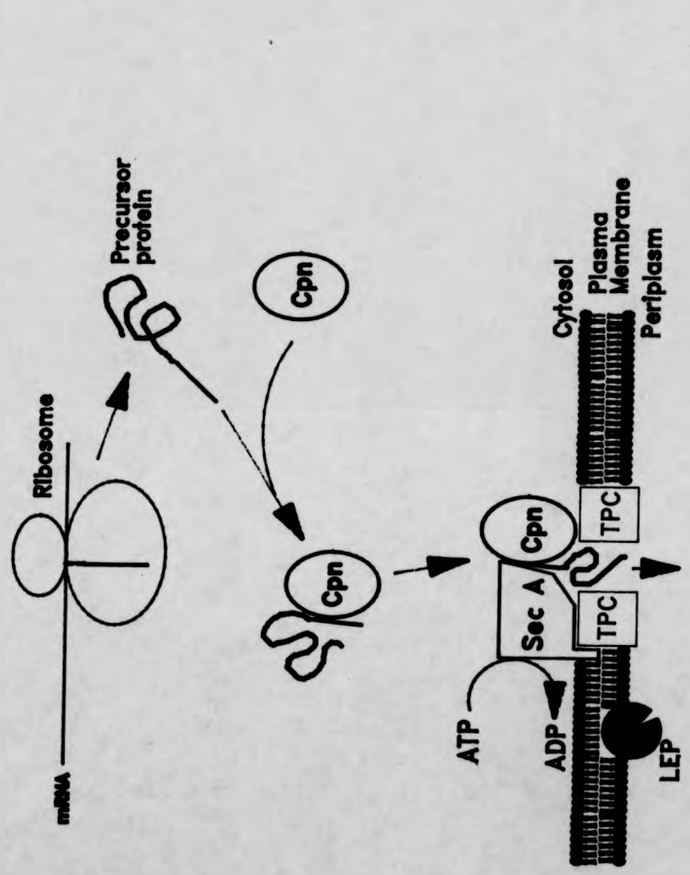
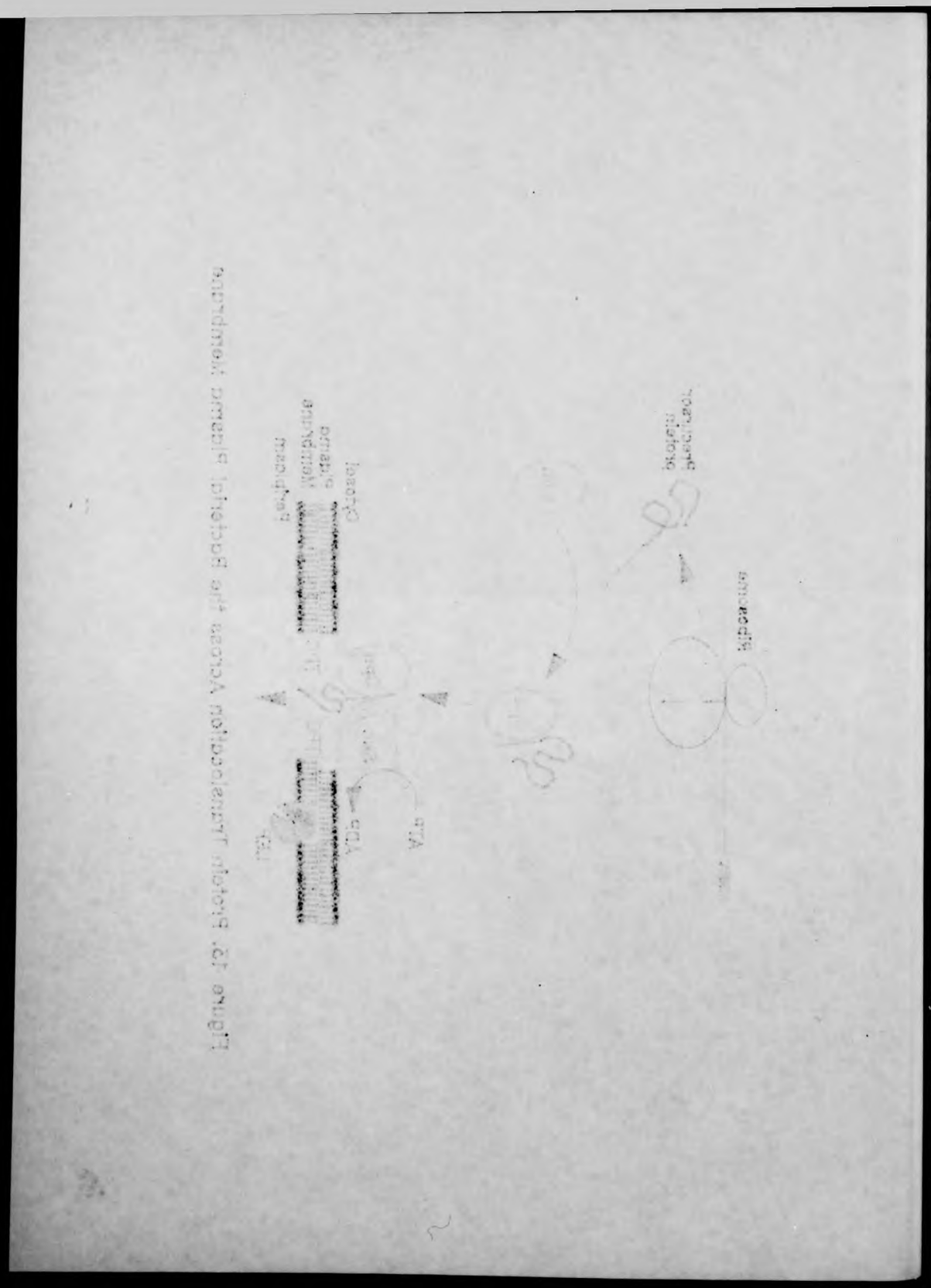


Figure 13. Protein Translocation Across the Bacterial Plasma Membrane.

The precursor protein interacts with a cytosolic molecular chaperone (Cpn) which is SecB, GroEL or trigger factor. This interaction maintains the precursor in a 'translocation-competent' conformation. The precursor-chaperone complex interacts with the peripheral membrane protein SecA. ATP hydrolysis by SecA is required for the translocation of the precursor. The translocation pore complex (TPC), is believed to form an aqueous channel through which translocation occurs. Leader sequences are removed during or after translocation, by the integral membrane protein leader peptidase (LEP).

Figure 13. Protein Translocation Across the Bacterial Plasma Membrane



The groups of precursors recognised by the chaperones are thought to overlap (Lecker et al., 1989). Recently, E.coli proteins Ffh and FtsY and a 4.5s RNA (Ffs) have been found to exhibit sequence similarities with subunits of mammalian SRP and SRP receptor (Reviewed in Bassford et al., 1991). These similarities led to the suggestion that cotranslational export from E.coli may involve an SRP/SRP receptor-type pathway. However, as yet the roles of Ffh, FtsY and Ffs in protein translocation across the bacterial plasma membrane have not been clearly demonstrated, unlike the involvement of the Sec proteins (Bassford et al., 1991). Although the ER membrane and bacterial plasma membrane translocation systems seem to consist of different components, they are, in some cases, capable of translocating the same proteins. For example, bacteriophage M13 procoat is a protein normally translocated across the bacterial plasma membrane in an ATP and Sec independent manner (Wolfe et al., 1985), but can also be translocated across ER membranes in vitro (Zimmermann et al., 1988).

Translocation of proteins across the plasma membrane of E.coli involves two energy requiring steps. No energy input is required until the point when the precursor is bound to the membrane via SecA. The energy of ATP binding to SecA allows the translocation of approximately 20 amino acid residues. Further translocation requires dissociation of the precursor from SecA which requires ATP hydrolysis by SecA. However, ATP hydrolysis does not drive translocation, a

proton motive force across the plasma membrane is required for this process (Schiebel et al, 1991). Bakker and Randall (1984) found that varying the contributions of the pmf, the electrical potential and the proton gradient, had no effect on translocation efficiency so long as the total pmf value was unchanged; that is, the proton gradient could substitute for the electrical potential component and vice versa. The precise role of the pmf in bacterial protein translocation is unclear. While an electrophoretic effect has been proposed for the role of membrane electrical potential in translocation across mitochondrial membranes (see section 1.4.3) this does not seem to be the case in bacteria, at least for translocation of M13 procoat protein. The presequence of bacteriophage M13 procoat protein normally has a net charge of -3 and it has been suggested that negatively charged presequences are attracted towards the periplasm, which is positively charged relative to the cytosol, by an electrophoretic effect. However when Kuhn et al. (1990) altered the net charge on this presequence to +3, they found little difference in the kinetics of its pmf-dependent translocation. Kuhn and co-workers concluded that the effect of the pmf may have more to do with interactions between the Sec proteins and the precursor rather than a direct electrophoretic effect.

It is possible that the pmf may cause changes in membrane proteins and/or phospholipid conformation thereby increasing translocation efficiency. Indeed the existence of voltage-

dependent channels across lipid bilayers and pmf-induced oligomerization of membrane proteins (reviewed by Honig et al., 1986; Kaback, 1986) adds support to this hypothesis.

Shiozuka et al. (1990) have suggested that a pmf may increase the affinity of the translocation apparatus for ATP, by facilitating the removal of ADP, possibly from SecA, thus enabling SecA to interact with another precursor. This hypothesis is consistent with the observation that a large excess of SecA, when precursor levels are non-saturating, in the absence of a pmf, can increase levels of translocation to those normally observed in the presence of a pmf. An increase in the affinity for ATP by the translocation machinery may be a result of acidification of the periplasm rather than a pmf per se.

Sasaki et al. (1990) found that the rate of translocation was almost constant when pH was varied over the range 6.5 to 8.0. They proposed that the effect of the proton gradient may be due to the difference in the proton concentration on each side of the membrane rather than absolute pH values. It is possible that translocation involves the antiport of protons (or the symport of hydroxyl ions) with the precursor; such coupled movements are known to occur with ions and solutes. A possible proton antiport mechanism could involve protonation of a residue of the translocation machinery on the periplasmic side of the membrane. Such an event could cause a conformational change rotating the protonated domain towards the cytosolic side of the

membrane. A possible candidate for such a residue is histidine. It is possible that several such events are required to complete translocation involving the proton(s) being passed from one component of the machinery to the next.

Reversal of translocation could be prevented by processes such as leader sequence cleavage, or folding of the translocated protein.

Driessen and Wickner (1991) reconstituted the E.coli translocation machinery using liposomes composed of E.coli phospholipids and purified SecA, SecE and SecY. By selectively collapsing the pH gradient and electrical components of the pmf, they demonstrated that binding of the precursor ProOmpA is independent of a pmf but that the ATPase activity of SecA and translocation itself were dependent on the cytosolic pH.

Driessen and Wickner also supplied evidence that the rate-limiting step of translocation involves proton-transfer, possibly involving the precursor, Sec proteins or both.

Although the electrical potential can substitute for the proton gradient component of the pmf and vice versa, both components do not necessarily promote import by the same mechanism. Decreasing one component may result in a compensating increase in the stimulatory effect of the other component, when the total pmf is maintained.

The possible multiple effects of the pmf may explain the variation in the stimulation of translocation by a pmf for

different proteins.

Although translocation across the bacterial membrane differs from that across the ER membrane in terms of a requirement for pmf, it shows similarities with protein integration into, and translocation across, the thylakoid membrane (see Chapter 4).

1.4.3 PROTEIN TRANSLOCATION ACROSS MITOCHONDRIAL MEMBRANES.

Mitochondria and chloroplasts are the only organelles which encode and synthesise some of their constituent proteins. The biogenesis of mitochondria, like that of chloroplasts, involves the posttranslational import of most of the organelle's proteins. The mitochondrion consists of four distinct phases; the inner and outer membranes which enclose a soluble phase known as the intermembrane space, and the matrix which is a soluble phase bound by the inner membrane. Protein targeting to mitochondria has mainly been studied in yeast and Neurospora crassa.

Nuclear-encoded proteins, synthesised in the cytosol, are targeted to each phase. Targeting information usually resides in an N-terminal presequence, which is cleaved off within the organelle. A minority of imported proteins do not have cleavable presequences, including cytochrome c (an intermembrane space protein), the ADP/ATP carrier and the matrix protein, 2-isopropyl malate synthase. The outer membrane protein porin also lacks a cleavable presequence and in this respect is similar to nuclear-encoded

chloroplast outer envelope proteins (Hartl et al., 1989). Like chloroplast protein presequences, mitochondrial protein presequences show low levels of sequence similarity. However, they do tend to have basic residues periodically arranged along their length and, unlike chloroplast protein presequences, analysis has revealed that mitochondrial protein presequences can form amphiphilic alpha-helices (von Heijne, 1986). Such a structure could have a high affinity for the surfaces of negatively charged phospholipids and hence mitochondrial presequences could facilitate interactions with membranes, perhaps required for their translocation. The high affinity of some mitochondrial presequences for membranes has been demonstrated by several workers including Roise et al. (1986) and Tam (1986), although a direct role for the alpha helix in targeting has yet to be found.

Import of nuclear-encoded mitochondrial proteins is believed to follow the sequence of events outlined below and shown in Figure 14. Some precursor proteins interact with cytosolic factors (chaperones), such as the hsp70s and presequence binding factor, which maintain them in a conformation that can be accepted by the import apparatus. ATP is probably required for release of precursors from such factors (Sheffield et al., 1990; Pfanner et al., 1990; Murakami and Mori, 1990). The precursor interacts with a receptor on the outer membrane. The receptor for most imported proteins, including all those with a cleavable presequence, is a 19kDa

Figure 14. Import of a Nuclear-Encoded Mitochondrial Matrix Protein.

The precursor protein is synthesised in the cytosol and interacts with a cytosolic factor such as hsp70 which maintains it in a 'translocation competent' conformation. The precursor then binds to a receptor on the organelle surface which is a mitochondrial outer membrane protein of 19kDa or 72kDa (MOM19 and MOM72, respectively). The precursor-receptor complex then interacts with an outer membrane protein, general insertion protein (GIP). Translocation of proteins into the mitochondrial matrix is believed to take place at contact sites between the outer membrane (OM) and the inner membrane (IM), through aqueous channels. X indicates the possible involvement of other membrane proteins in the translocation process. The presequences of matrix proteins are removed by the soluble matrix protein, mitochondrial processing peptidase (MPP) in conjunction with the processing enhancing protein (PEP). Completion of translocation may involve hsp70s in the matrix.

Matrix proteins are believed to interact with heat shock protein 60 homologues (hsp60) before folding. Translocation requires ATP hydrolysis and an electric potential across the inner mitochondrial membrane.

IMS=Intermembrane Space

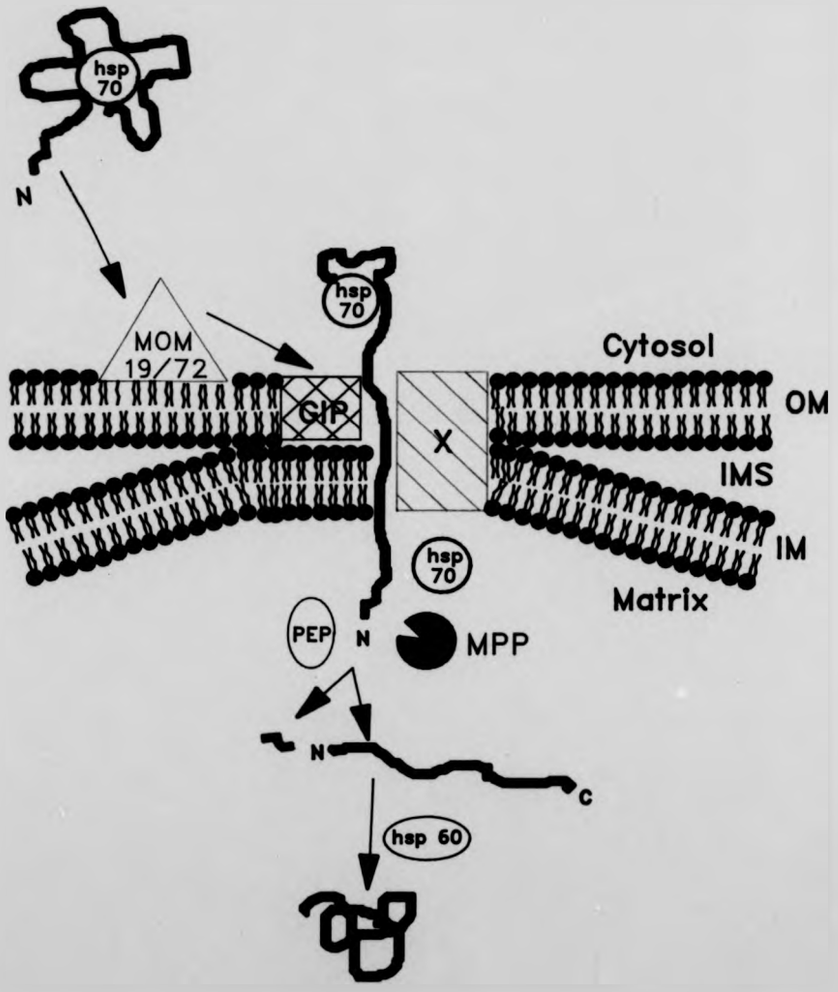


Figure 14. Import of a Nuclear-Encoded Mitochondrial Matrix Protein

outer membrane protein (MOM19) while the ADP/ATP carrier has been found to predominantly interact with a 72kDa outer membrane protein (MOM72) (Sollner et al., 1989; Sollner et al., 1990). The precursor-receptor complex then interacts with an outer membrane protein known as general insertion protein (GIP), which accepts the precursor and inserts it into the outer membrane. All translocation of proteins into and across mitochondrial membranes is believed to take place at sites of close contact between the outer and inner membranes. A 32kDa protein is thought to be involved in protein translocation through contact sites. Interestingly, the primary sequence of this protein was found to be identical to the inner mitochondrial membrane protein, the phosphate carrier (Meyer, 1990; Murakami et al., 1990). Like the postulated involvement of the phosphate translocator in protein translocation across the chloroplast envelope (see section 1.3.3a), this finding has caused some controversy and it may again be due to the approach used to identify the protein. Antibodies were raised against the presequence of a mitochondrial precursor and anti-idiotypic antibodies were raised against these. Although Pain et al. (1988) assumed that the anti-idiotypic antibodies would recognise the membrane components which normally recognise the precursor presequence, it is possible that the result obtained was due to cross-reactivity of the anti-idiotypic antibodies between the phosphate carrier and import components.

Translocation across the inner mitochondrial membrane is

believed to occur through proteinaceous channels. A mitochondrial hsp70 binds precursors emerging on the matrix side of the inner membrane thereby supporting the completion of translocation (Kang et al., 1990).

Presequences are processed by matrix processing peptidase (MPP), which like SPP is a metal dependent soluble enzyme, in conjunction with processing enhancing protein (PEP) (Attardi and Schatz, 1988; Hartl et al., 1989; Horwich, 1990).

Matrix proteins are folded in association with a hsp60, while intermembrane proteins interact with hsp60 before being targeted across the inner membrane by a different translocation system (Hartl and Schatz, 1989; Pfanner and Neupert, 1990; Horwich, 1990).

The biogenesis of mitochondrial intermembrane proteins is particularly interesting since, with the exception of cytochrome c which is translocated across the outer membrane only (Zimmermann et al., 1981), they are believed to be first translocated across both membranes into the matrix as described above and then targeted outwards, across the inner membrane to the intermembrane space. Like thylakoid lumen proteins, they are synthesised with bipartite presequences, the first domain directing translocation into the matrix and being processed by MPP in association with PEP. The second domain directs translocation from the matrix to the intermembrane space where it is cleaved by a second processing peptidase (see Figure 15). 'Inner membrane

Figure 15. Import of a Mitochondrial Intermembrane Space Protein.

Precursors of most intermembrane space proteins are synthesised with bipartite presequences. The first domain targets the precursor into the matrix via contact sites between the outer and inner membranes (OM and IM respectively) in the fashion outlined in Figure 14. The first domain is removed by mitochondrial processing peptidase (MPP) in conjunction with processing enhancing protein (PEP) producing an intermediate size protein.

The second domain targets the protein across the inner membrane, into the intermembrane space where it is removed by an integral membrane protein, inner membrane protease 1 (IMP1), yielding the mature size protein.

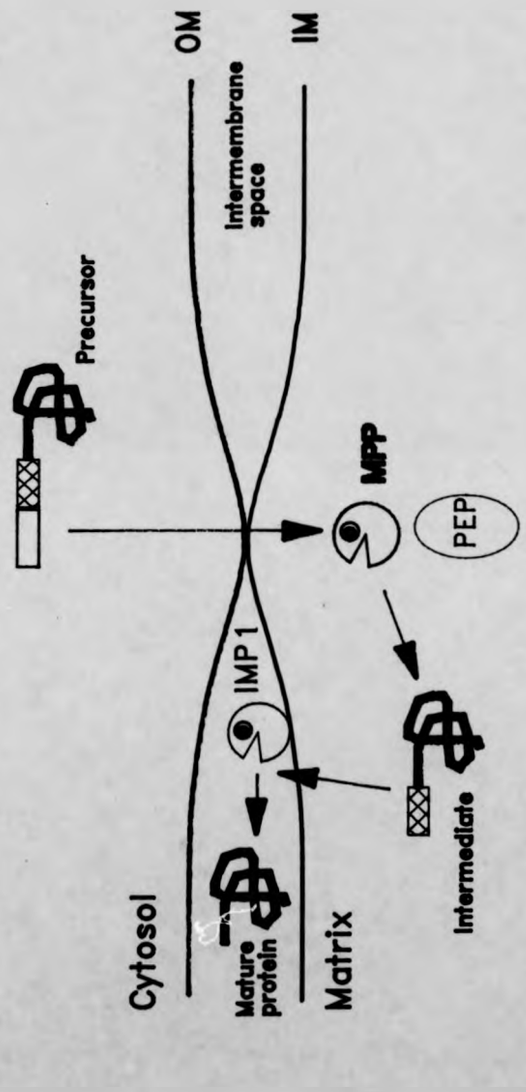


Figure 15. Import of a Mitochondrial Intermembrane Space Protein.

protease 1' (IMP1) is bound firmly to the outer face of the inner membrane; it contains a 21.4kDa subunit and may be a hetero-oligmer (Schneider et al., 1991)

This two step import mechanism may, as has been proposed for the import of thylakoid lumen proteins, utilise an ancestral transport system for the second translocation step. Mitochondria are thought to be descended from free-living prokaryotes (see section 1.3.6). Such ancestors would encode and synthesize proteins in what is now the matrix and such proteins would be exported across the inner membrane perhaps in a similar fashion to the export of proteins across the bacterial plasma membrane (see section 1.4.2). Hence before posttranslational import into the mitochondrial ancestor occurred, a system for translocation across the inner membrane already existed. Indeed, IMP1 and bacterial LEP have several characteristics in common, including a requirement for interactions with acidic phospholipids for activity (de Vrije et al., 1988).

Import of proteins into mitochondria requires ATP (or GTP) hydrolysis. Several reports have provided evidence for an NTP-dependent unfolding component early in the import pathways of many mitochondrial proteins (reviewed in Pfanner et al., 1988). As previously mentioned, release of cytosolic factors from precursors accounts, at least partially, for this requirement. Insertion of precursors into the inner mitochondrial membrane (at contact sites), requires an electrical potential across that membrane (reviewed in Hartl

et al., 1989). It has been proposed that the role of the membrane potential includes an electrophoretic effect; presequences or precursor domains with net positive charges being attracted towards the matrix side of the inner membrane which has a relative negative charge. A membrane potential is not required for completion of translocation; this may be achieved by hsp70s in the matrix binding to the precursor as it starts to emerge from the translocation machinery and effectively 'pulling' the precursor into the matrix (Kang et al., 1990). Protein folding in the matrix requires hsp60 binding and ATP hydrolysis (Ostermann et al., 1989). The energy requirements for translocation of intermembrane space proteins back across the inner membrane have yet to be determined.

1.4.4 PROTEIN IMPORT INTO PEROXISOMES.

Although peroxisomes (also called glyoxysomes and microbodies) are almost ubiquitous in eukaryotic cells, their biogenesis is much less well characterised than that of chloroplasts and mitochondria. With a few exceptions, peroxisomes are bound by a single membrane. Peroxisomes do not contain DNA and therefore all of their proteins are nuclear-encoded. Although it was once believed that peroxisomes form by budding from the rough ER, it is now believed that they form by division of existing peroxisomes and proteins are imported posttranslationally.

Peroxisomal proteins are not synthesised with presequences,

but have internal targeting information which tends to reside at the C-terminus of the protein. The nature of peroxisomal targeting domain is not well understood. Gould et al. (1987) identified two regions of peroxisomal luciferase which are required for import. One of these domains was C-terminal and found to be unusually rich in lysine, neutral and hydrophobic residues and may have a sharp turn around two lysine residues. However some other peroxisome proteins do not have domains with such features. Several workers including Gould et al. (1989) and Miyazawa et al. (1989) have suggested that the tripeptide alanine-lysine-leucine at the C-terminus functions as a peroxisomal targeting signal. Binding is thought to involve some kind of proteinaceous receptor since treatment of peroxisomes with protease abolishes their ability to import proteins (Santos et al., 1988b)

Peroxisomal proteins have been shown to bind specifically to peroxisomes and not mitochondria in vitro (Imanaka et al., 1987; Small et al., 1987)

Imanaka et al. (1987) studied the energy requirements for the import of acyl-CoA oxidase into peroxisomes. They found that binding occurred in the absence of ATP, while ATP hydrolysis was necessary for translocation of this protein. Import was not affected by collapsing the electrical potential or proton gradients across the membrane. Peroxisome membrane proteins may be imported by a different mechanism to other peroxisome proteins, since humans

suffering from Zeeweger syndrome have peroxisomes with normal membranes but lacking soluble peroxisomal proteins (Santos et al., 1988a; Santos et al., 1988b)

Perhaps in such cases the import pathway of soluble proteins is blocked. The import of chloroplast outer-envelope proteins seems to occur by a different pathway to other imported chloroplast proteins (section 1.3.5), and hence peroxisomal protein import may be similar to chloroplast protein import, there being a separate route/mechanism for import into the membrane.

1.5 AIMS OF PROJECT.

As previously mentioned in section 1.3.2, the use of isolated intact chloroplasts to study the import of thylakoid lumen proteins is not totally satisfactory, since it does not enable analysis of translocation across the thylakoid membrane to be carried out in isolation from translocation across the envelope membranes. However, the demonstration by Kirwin et al. (1989) that isolated thylakoids are capable of importing the thylakoid lumen protein 33K, paved the way for further analysis of the mechanism by which proteins traverse the thylakoid membrane using this system.

The aims of this Ph.D. project were:-

i) To further optimise the assay for import of thylakoid lumen proteins into isolated thylakoids, first developed by P. Kirwin and C. Robinson (University of Warwick).

ii) To determine the energy requirements for the transport of proteins across the thylakoid membrane.

iii) To determine the requirements, other than energy sources, for efficient protein import into isolated thylakoids.

CHAPTER 2. MATERIALS AND METHODS.

Solutions and media were sterilised by autoclaving which was carried out at 15psi for 25 minutes or filter-sterilised by passing through a 2 micron filter. Sucrose solutions were autoclaved at 10psi for 10 minutes or filter-sterilised.

The microfuge used during the protocols outlined below was an MSE microcentaur.

Sambrook *et al*. (1989) and Ausubel *et al*. (1987) are laboratory manuals and not necessarily the originators of the protocols described below.

2.1 GROWTH AND MAINTENANCE OF *ESCHERICHIA COLI* STRAINS.

2.1.1 *ESCHERICHIA COLI* STRAINS.

TG1 (Sambrook *et al*., 1989)

TG2 (Sambrook *et al*., 1989)

2.1.2 SHORT-TERM STORAGE OF *E.COLI*.

Stock strains of *Escherichia coli* (*E.coli*) were maintained by streaking out individual colonies onto glucose/minimal medium plates followed by incubation at 37°C overnight and storage at 4°C. This process was repeated monthly.

Glucose/Minimal Medium.	10xM9 Salts.
15g Minimal Agar in 900ml SDW	6% (w/v) Na_2HPO_4
100ml 10XM9 Salts	3% (w/v) KH_2PO_4
1ml 1M $MgSO_4$	1% (w/v) NH_4Cl
1ml 0.1M $CaCl_2$	0.5% (w/v) NaCl
1ml 1M Thiamine HCl	
10ml 20% (w/v) Glucose	

All components of glucose/minimal medium plates were made up with SDW, autoclaved or filter sterilised separately and cooled before mixing aseptically.

2.1.3 LONG-TERM STORAGE E.COLI.

A single bacterial colony was grown overnight at 37°C in 10ml L-broth in an orbital shaker at 200rpm. 850ul of overnight culture were transferred to a vial containing 150ul of sterile glycerol and mixed. Glycerinated cultures were stored at -80°C.

L-Broth.

1% (w/v) NaCl

1% (w/v) Bacto-Tryptone

0.5% (w/v) Yeast Extract

(To make L-agar, 2% agar was added to the above components before autoclaving).

2.2 TRANSFORMATION OF E.COLI.

2.2.1 PREPARATION OF COMPETENT CELLS.

E.coli cells of the appropriate strain were picked from a single colony and used to inoculate 10ml of L-broth which was grown overnight, to stationary phase, at 37°C in an orbital shaker at 200rpm. 40ml of L-broth were inoculated with 2ml of this overnight culture and grown at 37°C in an orbital shaker at 200rpm for 1½-2 hours, to mid-log phase (until the OD_{550} was approximately 0.3). Cells were pelleted by centrifugation at 2000xg for 5 minutes at 4°C, then

resuspended in 20ml sterile 100mM $CaCl_2$ which had been pre-chilled on ice. The resuspended cells were left on ice for 20 minutes before repeating the centrifugation step and resuspending in 4ml cold 100mM $CaCl_2$. Cells were left on ice for at least an hour before use, or stored at 4°C and used within 48 hours. The efficiency of transformation obtained, using cells prepared in this way, increases with time of storage then declines after 24 hours.

2.2.2 TRANSFORMATION OF E.COLI WITH PLASMID DNA.

Approximately 1ng of DNA or an aliquot of ligation mix (see section 2.4.8) was gently mixed with 100ul competent cells in pre-chilled Eppendorfs and incubated on ice for 45 minutes with intermittent gentle mixing. Cells were heat-shocked by incubation at 42°C for 2 minutes then diluted with 1ml L-broth and incubated at 37°C for 40 minutes. The transformed cells were then pelleted in a microfuge by spinning for 1 minute at low speed, and then resuspended in 100ul L-broth prior to spreading on L-agar plates (see L-broth), containing ampicillin if required.

When insertional inactivation of the lacZ gene in the plasmid was to be used for selection of recombinant DNA molecules, iosopropyl-1-thio-B-D-galactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-B-D-galactoside (X-GAL) were added to the plates.

Ampicillin.

Ampicillin was prepared as a 50mg/ml stock solution in SDW and stored at -20°C. This stock solution was added to liquid media or cooled agar-containing media just before pouring, to give a final concentration of 50ug/ml.

IPTG.

100mM stock (23.8mg/ml in SDW)

X-GAL.

100mM stock (20mg/ml in dimethylformamide)

(IPTG and X-GAL stock solutions were prepared just before use and added to cooled L-agar just before the plates were poured to give final concentrations of 7mM each).

The following controls were always carried out:

-A transformation with no added DNA to check for contamination.

-A transformation containing 1ng ds DNA to monitor cell competence. If IPTG and X-GAL were used, the control DNA used also conferred beta-galactosidase activity on the cell so that the effectiveness of the blue/white selection could also be monitored.

2.2.3 TRANSFORMATION OF E.COLI WITH M13 DNA.

Transformations using plasmids derived from M13 bacteriophage DNA were carried out essentially as above, with double-stranded or single-stranded bacteriophage, except that:

-300ul of competent cells were used.

-After heat-shock, Eppendorfs were placed on ice for 5

minutes then 2.8 mls of lawn mix were added. Samples were then transferred into 3mls of H-top maintained at 45°C, mixed quickly and poured onto pre-warmed H-agar plates. When the H-top had set, the plates were inverted and incubated overnight at 37°C. Plaques were stored on the plates at 4°C and used within 48 hours for preparation of DNA.

H-Top.

1% (w/v) Bacto-Tryptone
0.8% (w/v) NaCl
0.7% (w/v) Agar

H-Agar.

1% (w/v) Bacto-Tryptone
0.8% (w/v) NaCl
1.5% (w/v) Agar

2xTY

2% Bacto-Tryptone
1% Yeast Extract
0.5% NaCl

Lawn Mix.

2ml *E.coli* culture
grown overnight in 2xTY
400ul 100mM IPTG
400ul 100mM XGAL

2.3 PREPARATION OF DNA.

2.3.1 SMALL SCALE ISOLATION OF PLASMID DNA FROM *E.COLI*.

(Based on Holmes and Quigley, 1981)

10ml aliquots of L-broth containing ampicillin at 50µg/ml were inoculated with *E.coli* cells from individual colonies selected from L-agar plates containing 50µg/ml ampicillin. Cells were grown overnight at 37°C in an orbital shaker at 200rpm and then harvested by centrifugation at 2000g for 10 minutes at room temperature. Each pellet was resuspended in 100µl of 25% (w/v) sucrose/50mM Tris-Cl pH8.0.(filter-sterilised) and transferred to an Eppendorf. 600ul of MSTET

and 7µl of freshly prepared lysozyme 50mg/ml in Tris-Cl pH8.0 were added to each sample just before boiling for 60 seconds. The lysed cells were placed on ice to cool and then pelleted by centrifugation for 1 hour at room temperature in a microfuge. The supernatant from each sample was removed and incubated at 37°C for 30 minutes after the addition of 2µl ribonucleaseA. Each sample was phenol/chloroform extracted. DNA was precipitated by the addition of 1/10 volume 3M sodium acetate pH6.0 (autoclaved),and an equal volume of isopropanol followed by freezing on dry-ice for 30 minutes. Samples were pelleted by centrifugation as above for 15 minutes and dried under vacuum before resuspending in 100µl SDW. The samples were ethanol precipitated then resuspended in 100µl SDW or TE.

MSTET.

5% (w/v) Sucrose
50mM Tris-HCl pH8.0
50mM EDTA
5% (v/v) Triton X-100
(All components were autoclaved. The sucrose solution was filter-sterilised.

TE.

10mM Tris-Cl pH8.0
10mM EDTA pH8.0
(autoclaved)

RibonucleaseA.

Made up in SDW to 10mg/ml then boiled for 10 minutes and cooled before use.

2.3.1a PHENOL/CHLOROFORM EXTRACTION OF DNA.

All the following manipulations were carried out at room temperature, unless otherwise stated. The sample was mixed

with an equal volume of phenol in an Eppendorf tube until an emulsion formed, then spun at high speed in an microfuge for 3 minutes. The upper, aqueous, phase was transferred to a clean Eppendorf, care being taken to avoid the interface, and an equal volume of a 1:1 mixture of phenol and chloroform added. The sample was mixed, spun, and then the upper, aqueous phase transferred, as above. This step was repeated if there was a large amount of protein at the interface. An equal volume of chloroform was added to the aqueous phase and the sample which was then mixed, spun and transferred as above. The DNA was recovered by ethanol precipitation of the aqueous phase.

Preparation of Phenol.

100g of chromatography grade phenol was dissolved in 100ml of 1.5M Tris-Cl pH8.0 and 0.1% (w/v) 8-hydroxyquinoline was added to aid identification of the organic phase and to act as an antioxidant. The top (aqueous) phase was replaced with fresh 1.5M Tris-Cl pH8.0 and mixed with the organic phase; this process was repeated until the aqueous phase was between pH7.5 and pH8.0. The aqueous layer was then replaced with 10mM Tris-Cl pH8.0. After mixing, the phenol was stored in aliquots at -20^{o}C. When required, an aliquot was thawed, used at room temperature and stored for the short-term at 4^{o}C. (Tris-Cl solutions had been autoclaved).

2.3.1b ETHANOL PRECIPITATION OF DNA.

2 volumes of ice-cold ethanol and 1/10 volume 3M sodium acetate pH 6.0 (autoclaved) were added to the sample which

was then placed on dry-ice for 20 minutes or at -20°C for several hours. The sample was then spun at high speed in a microfuge at 4°C for 15 minutes and the supernatant carefully removed. The pellet was then washed with 200µl 80% (v/v) ethanol, which had been pre-chilled to -20°C. The supernatant was removed and the pellet dried under vacuum before resuspension in SDW or TE.

2.3.2 LARGE SCALE PLASMID DNA ISOLATION FROM E.COLI

(Based on Promega Biotec. Technical Bulletin N°9).

Cells containing plasmid were grown overnight in 10ml of L-broth and 50µg/ml ampicillin at 37°C in an orbital shaker at 200rpm. 250ml of L-broth containing 50ug/ml ampicillin was then inoculated with 2.5ml of this overnight culture and grown overnight at 37°C as above. Cells were pelleted by centrifugation at 2000g for 10 minutes at 4°C, then resuspended in 4ml of lysis buffer and placed on ice for 15 minutes. 8ml of alkali detergent solution were then added and the suspension which was gently mixed until transparent. After 10 minutes on ice, 5ml of 3M sodium acetate pH4.6 (autoclaved) were added and the suspension mixed until it was converted into a dense white precipitate. After 15 minutes on ice, this precipitate was pelleted by centrifugation at 17000g for 15 minutes at 4°C. The clear supernatant was transferred to a sterile universal and 5µl of a 10mg/ml solution of ribonucleaseA added to it before incubation at 37°C for 30 minutes. 0.5 volumes of

phenol/chloroform were then mixed with the supernatant which was then left at room temperature for 15 minutes. The mixture was centrifuged at 3000g for 10 minutes at room temperature, the upper phase transferred to a clean universal and the phenol/chloroform treatment repeated until the top phase was transparent. DNA was precipitated from the top phase by the addition of 0.6 volumes of isopropanol and incubation for 15 minutes on ice. The sample was then spun at 17000g for 10 minutes at 4°C and the resulting pellet resuspended in 1.6ml of SDW, to which 0.4ml of 4M NaCl and 2ml of 13% polyethylene glycol (autoclaved) were then added. The sample was left on ice for an hour and then spun at room temperature for 10 minutes at high speed in a microfuge. The supernatant was removed with a drawn-out Pasteur pipette and the pellet washed twice with 80% (v/v) ethanol, dried under vacuum and resuspended in 50µl of SDW or TE. DNA concentration was estimated by running an aliquot of the sample alongside samples of known concentration.

Lysis Buffer.	Alkali Detergent Solution.
25 mM Tris-Cl pH 8.0	0.2 M NaOH
10 mM EDTA	1% SDS
15% Sucrose	
2mg/ml Lysozyme	

(Tris-Cl, EDTA, sucrose and SDS solutions were autoclaved).

2.3.3 ISOLATION OF M13 REPLICATIVE FORM DNA FROM E.COLI.

Replicative form M13 DNA (double-stranded) was isolated from

E.coli using the above method with the following modifications:

-Cultures were grown in 2xTY (see page 79) without the addition of ampicillin

-10ml of 2xTY was inoculated with 0.1ml of an overnight TG2 culture and cells from one plaque. The culture was then grown at 37°C for 4-5 hours in an orbital shaker at 300rpm. Cells were pelleted by centrifugation at 2000g for 10 minutes at room temperature and 1ml of the supernatant used to inoculate 100mls 2xTY containing 1ml of a TG2 overnight culture. Cells were grown for 5 hours at 37°C in an orbital shaker at 250-300rpm and then pelleted by centrifugation at 2000g for 10 minutes at 4°C. The pellet was then resuspended in lysis buffer and the above method continued from this step.

2.3.4 SINGLE-STRANDED DNA PREPARATION.

All steps were carried out at room temperature unless otherwise stated. Plaques were picked from H-top agar using sterile Pasteur pipettes and transferred to 3ml of 2xTY containing 30µl of an overnight E.coli culture. After incubation at 37°C in an orbital shaker at 300rpm for 5 hours, the cells were spun down at 2000g in a bench-top centrifuge leaving phage particles in the supernatant. 1.5ml of supernatant was transferred to an Eppendorf and spun at high speed in a microfuge. If a cell pellet was visible, the supernatant was transferred to a clean

Eppendorf and respun. 1ml of supernatant was transferred to a clean Eppendorf and 200µl of PEG/NaCl solution (20% w/v polyethylene glycol 600o/2.5M NaCl, autoclaved) were added. The tube was shaken and then left to stand for 15 minutes. Phage was precipitated by spinning in a microfuge at high speed for 5 minutes. The supernatant was removed and the pellets respun for 3 minutes. Care was taken to remove any remaining supernatant, then the phage pellet resuspended in 100ul TE. The sample was phenol/chloroform extracted, ethanol precipitated and then resuspended in 30µl of TE or SDW.

2.4 DNA MANIPULATION AND ANALYSIS.

2.4.1 DIGESTION OF DNA WITH RESTRICTION ENDONUCLEASES.

The following were pipetted into a clean Eppendorf tube:

DNA (0.1µg to 4µg in SDW or TE)

2µl 10x Restriction Buffer

SDW (to make the final volume 20µl)

Restriction endonuclease was added at 1 to 5 units per ug DNA and the reaction incubated at the temperature recommended by the enzyme manufacturers (usually 37°C but 30°C for SmaI) for 1 to 4 hours.

The volume of restriction enzyme(s) added was less than 1/10 the reaction volume. Appropriate restriction buffers were supplied with the enzyme by the manufacturers.

In principle, 1 unit of restriction endonuclease completely digests lug of purified DNA in 60 minutes, using the

recommended assay conditions. However, crude DNA preparations, such as the small-scale preparations outlined in section 2.3.1, often required more enzyme and/or more time for complete digestion.

If digestion by two restriction endonucleases was required, a restriction buffer was chosen in which both enzymes are relatively active. If the buffers of the enzymes were too dissimilar, the DNA was first digested by the enzyme active at the lower salt concentration, then sufficient 1M NaCl (1 to 3µl for a 20µl reaction) so that the final concentration was appropriate for digestion by the second enzyme which was then added to the incubation.

2.4.2 PREPARATION AND RUNNING OF AGAROSE GELS.

A 1% (w/v) agarose gel was prepared by adding 0.5g agarose to 5ml 10xTBE and 45ml SDW and heating in a microwave oven with intermittent mixing until the agarose was completely dissolved. The solution was allowed to cool to approximately 50°C and then ethidium bromide added (from a stock solution of 10mg/ml in SDW) to give a final concentration of 0.5µg/ml. Gels were cast in a BRL "Horizon 58" horizontal slab gel apparatus with the required well-former inserted. When the gel was completely set, the well-former and end-sealers were removed and the gel immersed in 1xTBE containing ethidium bromide at 0.5µg/ml, to cover the gel to a depth of 1mm. DNA samples and appropriate DNA molecular weight markers, were mixed with at least 1/6 volume of 6x

DNA sample buffer, loaded into the wells and the gel run at a constant voltage of 1-6 V/cm. Since DNA is negatively charged, it migrates towards the anode. DNA in the gel was visualised with an ultra-violet transilluminator, since ethidium bromide bound to DNA displays an increased fluorescent yield compared to that free in solution.

10xTBE.	6x DNA Sample Buffer.
0.89M Tris Base	30% (v/v) Glycerol
0.89M Boric Acid	0.25% (w/v) Bromophenol Blue
0.02M EDTA (pH8.0)	0.25% (w/v) Xylene Cyanol
(autoclaved)	

2.4.3 ISOLATION OF DNA FROM AGAROSE GELS.

(Based on Grivitz et al., 1980).

The required DNA band was visualised using a U.V. transilluminator. A slot was cut just in front of the band (on the anode side) and a strip of Whatman number 1 paper which had been pre-soaked in sterile 1xTBE placed in the slot. A strip of dialysis tubing which had been prepared by boiling in 1mM EDTA and rinsed thoroughly in SDW, was placed in the slot in front of the paper. The gel was returned to the tank but 1xTBE added to the tank so that it was level with the top of the gel on each side but not immersing it. The gel was run at 50V for 5-10 minutes then the current reversed for 10 seconds. The gel was then viewed using a U.V. transilluminator. If the band had not run into the paper, the gel was run for a further period. The paper and

dialysis tubing were transferred to a 1.5ml Eppendorf with a hole in the bottom made with a pin. The 1.5ml Eppendorf was placed inside a 2ml Eppendorf. DNA was removed from the paper by adding 100µl TE to the sample and spinning the Eppendorfs at high speed in a microfuge for 1 minute. This step was repeated twice then the upper Eppendorf containing the paper discarded. The sample was spun at high speed for 2 minutes, the liquid transferred to a clean Eppendorf, phenol/chloroform extracted and ethanol precipitated.

2.4.4 PREPARATION AND RUNNING OF POLYACRYLAMIDE/TBE GELS. (Ausubel *et al*., 1987)

A 10% (v/v) polyacrylamide gel (nondenaturing) was used for resolution of small DNA fragments, between 50 and 300bp.

10% Polyacrylamide/TBE Gel.

30% (w/v) Acrylamide/0.8% (w/v) Bisacrylamide 6.7ml

Glycerol 100% (v/v) 1ml

SDW 10.3ml

10xTBE 2ml

TEMED 9.6µl

10% (w/v) Ammonium Persulphate 130µl

All components except the ammonium persulphate were premixed; ammonium persulphate was added to start the polymerisation reaction just before the gel was poured between 16.5cmx14cmx0.5cm gel plates with 1mm depth spacers, and the appropriate size well-forming comb inserted. After the gel had polymerised, the comb and bottom spacers were

removed and the wells rinsed with distilled water before the gel was mounted in a vertical gel electrophoresis tank (Scie-Plas). Running buffer (1xTBE) and sample buffer were as for agarose gel electrophoresis. Samples and size markers were loaded into the wells using a Hamilton syringe. The gel was run at 5V/cm until the desired resolution had been obtained. Bromophenol blue can be used as a tracking dye since it runs at approximately 30bp on a 10% (w/v) polyacrylamide/TBE gel. The gel plates were removed from the tank and prised apart, care being taken to leave the gel attached to one plate. The gel, still attached to the plate, was stained by submerging in 1xTBE containing 0.5µg/ml ethidium bromide for 20 minutes and then destained, if required, by submerging in SDW for 10 minutes. DNA in the gel was then visualised using a U.V. transilluminator after removing from the gel plate.

2.4.5 ISOLATION OF DNA FROM POLYACRYLAMIDE GELS.

The required DNA band was visualised using a U.V. transilluminator and excised. The strip of gel containing the DNA was cut into small pieces, placed in an Eppendorf and covered with 200-500µl of 0.5M ammonium acetate/10mM magnesium acetate/0.1% (w/v) SDS/0.1M EDTA (autoclaved). The sample was then incubated at 37°C for 2 hours with intermittent mixing, or overnight. Liquid was then transferred to a clean Eppendorf and phenol/chloroform extracted. 1.5ul of carrier tRNA (stock solution 10µg/ml)

were added to the sample before ethanol precipitation without the addition of further sodium acetate.

2.4.6 DEPHOSPHORYLATION OF PLASMID DNA.

(Sambrook et al., 1989)

When a ligation was carried out using a plasmid which had been cut yielding compatible ends, calf intestinal phosphatase (CIP) was used to remove 5' phosphate groups from DNA to prevent recircularisation of the plasmid without insertion of the required fragment during the ligation reaction.

Plasmid DNA (approximately 1µg) was digested to completion with the appropriate enzyme(s), phenol/chloroform extracted and ethanol precipitated. The DNA was dissolved in a minimum volume of 10mM Tris-Cl pH8.0, then 5µl 10xCIP buffer and SDW added to give a final volume of 48µl.

DNA with 5' overhangs was dephosphorylated by the addition of 0.1 units of CIP and incubation at 37°C for 30 minutes followed by addition of another 0.1 units and the incubation continued for a further 30 minutes.

DNA with 5' recessed termini or blunt ends was dephosphorylated by the addition of 0.1 units of CIP and incubation at 37°C for 15 minutes followed by incubation at 56°C for 15 minutes. After addition of another 0.1 units of CIP, incubation at both temperatures was repeated.

40µl of SDW, 10µl of 10x STE and 5µl of 10% (w/v) SDS were added to the sample before heating to 68°C for 15 minutes.

The sample was then phenol/chloroform extracted, ethanol precipitated and resuspended in 10ul of SDW.

10xCIP Buffer.	10xSTE.
0.5M Tris-Cl pH9.0	100mM Tris-Cl pH8.0
10mM $MgCl_2$	1M NaCl
1mM $ZnCl_2$	10mM EDTA
10mM spermidine	(filter-sterilised)

2.4.7 BLUNT ENDING OF 5' OR 3' OVERHANGS.

(Sambrook et al., 1989)

2µg of DNA was digested to completion with the appropriate restriction enzyme(s) in a 20µl reaction volume. 1µl of a solution containing dATP, dCTP, dGTP and dTTP each at 2mM in SDW was then added.

To blunt-end 5' overhangs, the recessed 3' termini were filled using the Klenow fragment of E.coli DNA polymerase 1. 1 unit of Klenow fragment was added for each microgram of DNA in the reaction and the sample incubated at room temperature for 15 minutes.

To blunt-end 3' overhangs, the protruding 3' termini were removed by utilising the 3' to 5' exonuclease activity of bacteriophage T4 DNA polymerase. 2 units of this enzyme were added for each microgram of DNA in the reaction and the sample incubated at 12°C for 15 minutes.

After the blunt-ending reaction, enzymes in the sample were inactivated by heating to 75°C for 10 minutes.

2.4.8 LIGATION OF BLUNT OR COHESIVE ENDS.

(Ausubel *et al.*, 1987).

The enzyme T4 DNA ligase was used for both blunt-end ligations and ligation of cohesive ends. Between 30ng and 100ng of cut plasmid and fragment were used in a ligation reaction. Cut plasmid and fragment were generally used at ratios of 1:1 and 1:2 (plasmid:fragment) in terms of approximate numbers of molecules. The required amounts of cut plasmid and fragment were made up to a volume of 9ul using SDW, then 10µl 2x Ligase buffer, and 1 unit of T4 ligase for cohesive-end ligations and 100 units T4 ligase for blunt-end ligations were added. Blunt-end ligations were incubated at room temperature for 3 hours while cohesive-end ligations were incubated at 15°C overnight. Incubation at the lower temperature favours the annealing of cohesive ends but diminishes enzyme activity. Blunt-end ligations require 10 to 100 times more enzyme to achieve the same efficiency as cohesive-end ligations since the ends are not brought together by annealing.

After ligation, *E.coli* TG2 were transformed with 1µl and 10µl aliquots of the sample (see section 2.2.2).

2x Ligase Buffer.

100mM Tris-Cl pH 7.5

20mM $MgCl_2$

20mM DTT

0.5mM ATP

(filter-sterilised)

2.4.9 DIDEOXY SEQUENCING WITH MODIFIED T7 DNA POLYMERASE.

Sequencing was carried out by the dideoxy chain termination method (Sanger et al., 1977) using a Sequenase™ DNA sequencing kit from United States Biochemical Corporation.

The chain termination method for sequencing involves synthesis of a DNA strand by DNA polymerase in vitro using a single-stranded DNA template. A synthetic oligonucleotide, complementary to a portion of the DNA template, is annealed to the template and the DNA polymerase initiates synthesis from this of a complementary strand. The primer is extended using dCTP, dGTP, dTTP and radioactively labelled [alpha-^{35}S] dATP. The synthesis reaction is terminated by random incorporation of dideoxynucleotides triphosphates (ddNTPs) which are nucleotide analogues which lack the 3'-OH required for DNA chain elongation .

Sequenase™ is a modified form of bacteriophage T7 DNA polymerase (Tabor and Richardson, 1987) which has low 3' to 5' exonuclease activity and, unlike Klenow fragment, has high processivity therefore DNA strands elongating from the template terminate only when a dideoxynucleotide is incorporated.

2.4.9a SEQUENCING M13 SINGLE STRANDED DNA TEMPLATES.

Single-stranded M13 DNA was prepared as in section 2.3.4.

Annealing of the primer to the template was carried out by adding 1µl (0.5 pmoles/µl) of M13 universal primer and 2µl of 5x Sequenase™ buffer (200mM Tris-Cl pH7.5/100mM $MgCl_2$/ 250mM NaCl) to 2µg of DNA template in 7µl SDW, heating to

65°C for 2 minutes then allowing the sample to cool to room temperature. The labelling reaction was carried out according to the manufacturer's instructions by adding 1ul 0.1M DTT, 2µl diluted labelling mix , 0.5 µl [alpha ^{35}S]dATP (10µCi/µl Amersham International) and 2µl diluted sequenase™, followed by incubation at room temperature for 5 minutes. For each sequencing reaction, a set of four Eppendorfs, labelled A,C,G and T, were pre-warmed to 37°C each containing 2.5µl of ddATP, ddCTP ddGTP, ddTTP termination mixes respectively. When the labelling reaction was complete, 2.5 µl of the sample was transferred to each termination mix and the incubation continued at 37°C for a further 5 minutes. 4µl of stop solution (95% (v/v) formamide/20mM EDTA/0.05% (w/v) Bromophenol Blue/0.05% (w/v) Xylene Cyanol) were then added to each Eppendorf and the samples stored at -20°C. Half of each sample was run on a sequencing gel (Section 2.4.10). Just before loading onto the sequencing gel, samples were heated to 80°C for 2 minutes.

2.4.9b SEQUENCING DOUBLE-STRANDED PLASMID DNA TEMPLATES.

Dideoxy sequencing can be readily performed on a double-stranded, closed circular DNA template that has been denatured with alkali (Chen and Seeburg, 1985).

To 2µg of DNA in a total volume of 9µl (made up with SDW), 1ul of 2M NaOH/0.02M EDTA was added and the sample incubated at room temperature for 5 minutes. The sample was then neutralised by the addition of 5µl 1M sodium acetate pH4.5

and ethanol precipitated by the addition of 40µl cold 100% (v/v) ethanol and placing on dry ice for 15 minutes. The sample was then spun at high speed in a microfuge for 10 minutes at 4°C, the supernatant removed and the pellet dried under vacuum. The pellet was then resuspended in 7µl SDW; 1µl primer (0.5pmoles/µl) and 2µl 5x Sequenase™ buffer were added and sequencing carried out using the Sequenase™ kit as in section 2.4.9a.

2.4.10 PREPARATION AND RUNNING OF SEQUENCING GELS.

Sequencing gels are high-resolution polyacrylamide gels which are used to fractionate radiolabelled single-stranded oligonucleotides on the basis of size. To reduce the effects of DNA secondary structure on electrophoretic mobility, gels contain 7M urea and are run at sufficient voltage to heat them to approximately 65°C.

Sequencing gel plates (49.8cm x 19.8cm x 0.5cm) were cleaned thoroughly and wiped with ethanol. The cuspid plate had Repelcote™ applied according to the manufacturer's instructions, then plates were assembled using 0.4mm spacers. A 6% sequencing gel was prepared by mixing 42ml of 6% acrylamide mix with 252µl 10% (w/v) ammonium persulphate and 11µl TEMED. The gel was poured immediately by sucking the mixture into a 60ml syringe then slowly squirting it between the plates, avoiding trapping air bubbles. A shark-tooth comb 0.4mm thick, was inserted upside down 1cm into the top of the gel to create a well, and the gel left to

polymerise at room temperature for 1 hour. Once polymerised, the comb and bottom spacer were removed and the exposed surfaces rinsed with distilled water. The gel was secured into the sequencing gel tank (BRL). The bottom reservoir was filled with 1xTBE and the top reservoir with 0.5xTBE. The buffer was squirted between the plates at the bottom of the gel to remove air bubbles. The gel was pre-warmed by running at 45V/cm until hot to the touch. Just before loading the gel, the comb-well was rinsed by squirting buffer into it then the shark-tooth comb was inserted the correct way up with the points just sticking into the gel. After the samples were loaded, the gel was run at 45V/cm for 1½ hours (until the bromophenol blue dye in the sequencing stop solution was at the bottom of the gel). When a sequence of over 200 bases needed to be read, half the sample was loaded and the gel run for approximately 90 minutes then the other half loaded and the gel run for a further 90 minutes.

After running, the plate treated with RepelcoteTM was removed and the plate with the gel attached submerged in gel fix for 15-20 minutes. The gel, still on the plate, was carefully removed from the gel fix. A piece of Whatman 3MM paper, slightly bigger than the gel, was laid on top of the gel then gently peeled off with the gel attached. The gel was dried onto the paper, under vacuum for 45 minutes at 80^{o}C. When dry, the gel was exposed to X-ray film (Fuji 35.6cmx43.2cm Medical) overnight. The film was developed in a dark-room using LX24 developer (Kodak) and fixed with

Unifix™ (Kodak) according to the manufacturer's instructions.

<u>6% Acrylamide Mix</u>

43g Urea

5ml 10xTBE

15ml 40% Acrylamide mix

Made up to 100ml with SDW

<u>40% Acrylamide Mix.</u>

38% (w/v) Acrylamide

2% (w/v) Bis-acrylamide

(Made up with SDW)

<u>Gel Fix.</u>

10% (v/v) Acetic acid

12% (v/v) Methanol

2.4.11 OLIGONUCLEOTIDE-DIRECTED MUTAGENESIS OF CLONED DNA.

2.4.11a PREPARATION OF OLIGONUCLEOTIDES

Oligonucleotides were synthesised on an Applied Biosystems 380B DNA synthesiser and purified by high performance liquid chromatography.

Oligonucleotides were 5'- phosphorylated as follows:

The oligonucleotide was diluted to approximately 20nmole/ml and 2.5µl transferred to a clean Eppendorf. The sample was gently mixed with 25µl of SDW, 3µl of 10xkinase buffer (1M Tris pH8.0/100mM $MgCl_2$/70mM DTT/10mM ATP, filter-sterilised) and 2 units of bacteriophage T4 polynucleotide kinase then incubated at 37°C for 15 minutes. The reaction was stopped by heating to 70°C for 10 minutes and the sample stored at -20°C until required.

2.4.11b OLIGONUCLEOTIDE-DIRECTED MUTAGENESIS.

Oligonucleotide-directed <u>in vitro</u> mutagenesis (site-directed

mutagenesis) was carried out on single-stranded M13 DNA with the required DNA fragment inserted, using an oligonucleotide designed so that its sequence carried the mutation of interest.

An oligonucleotide-directed *in vitro* mutagenesis system (version 2) kit (Amersham International) was used according to the manufacturer's instructions.

The mutagenic oligonucleotide was annealed to the single-stranded template and extended using the Klenow fragment of *E.coli* DNA polymerase I in the presence of bacteriophage T4 DNA ligase to generate a mutant heteroduplex. A thionucleotide analogue of dCTP (dCTP-alpha-S) was used in place of dCTP for the extension reaction which allowed selective removal of the nonmutant strand. Single-stranded template which had not been converted to the heteroduplex form during the extension reaction was removed by passing the sample through a nitrocellulose filter. Heteroduplex DNA DNA passes through the filter while single-stranded DNA binds to the filter. The filtration step greatly increases mutagenic efficiency. The nonmutant strand was selectively removed by incubation with the restriction endonuclease *NciI* which cannot cut phosphorothioate DNA (Nakamaye and Eckstein, 1986). Incubation with this enzyme results in nicks being introduced into the non-mutant strand. The nicked strand was removed by incubation with exonuclease III from *E.coli*, which degrades double-stranded DNA in a 3' to 5' direction from free 3' ends. The mutant strand was then

used as a template to create a mutant homoduplex using DNA polymerase I and T4 DNA ligase. 20µl of sample was used to transform 300µl of competent TG1 (section 2.2.3) and the remaining sample stored at -20°C.

2.4.11c ANALYSIS OF MUTANT PROGENY.

Single-stranded DNA was prepared from plaques (section 2.3.4) and used for sequencing (sections 2.4.9 and 2.4.10)). Sequenced samples were run along side a non-mutant sequence for easy comparison. Approximately 80% of samples contained the correct mutation.

Double-stranded DNA was prepared using phage supernatant, which had been stored for 2 days at 4°C saved from the single-stranded preparations. 2.5ml of phage supernatant was added to 250ml 2xTY containing 2.5ml of an overnight culture of E.coli TG2 and the procedure outlined in section 2.3.1 followed. The double-stranded DNA was cut with the appropriate restriction endonuclease(s) and ligated into the Riboprobe Gemini™ vector pGEM-4Z produced by Promega (section 2.4) for transcription of the mutant DNA. Once subcloned into this vector, the whole gene DNA sequence was checked using the method described in section 2.4.9. Primers which anneal to the SP6 and T7 promoter sites were used to sequence each end of the gene, and primers which were designed to anneal to specific sequences within the gene used to verify the remaining coding sequence.

2.5 TRANSCRIPTION, TRANSLATION AND PROTEIN ANALYSIS.

2.5.1 *IN VITRO* TRANSCRIPTION OF DNA IN pGEM^{TM}-4Z.

Full length cDNA inserts containing p33K (Meadows *et al.*, 1991) and p23K (James and Robinson, 1991) were cloned into the *Eco*RI site of the Riboprobe Gemini^{TM} vector pGEM-4Z (Promega), in the orientation for transcription from the SP6 promoter (kindly provided by J. Meadows and H. James respectively, University of Warwick). DNA encoding an artificial i23K (Ai23K) was cloned into the vector, in the orientation for transcription from the T7 promoter. DNA encoding an artificial i33K (Ai33K) was cloned into the vector, in the orientation for transcription from the SP6 promoter (see section 3.3).

The DNA was linearised before transcription by restriction endonuclease digestion. After digestion the DNA was phenol/chloroform extracted, ethanol precipitated and resuspended in SDW to 1ug/ul. The p23K and p33K constructs were linearised with *Sma*I. The artificial i23k and artificial i33K were linearised with *Eco*RI and *Hind*III respectively.

To 2µl of linearised DNA (1µg/ul) the following were added;

15.5µl transcription premix (40mM Tris-Cl pH7.5/ 6mM $MgCl_2$/ 2mM Spermidine/ 10mM DTT/ 500µM rATP/ 500µM rCTP/ 500µM rUTP/ 50µM rGTP/ 100µg per ml BSA)

20units RNasin (Promega. 40 units/µl)

0.25 units monomethyl cap (m^7G(5')ppp(5')G) (Pharmacia)

1µl of the appropriate RNA polymerase.

SP6 RNA polymerase (BRL.15units/µl) or 1µl T7 RNA polymerase (BRL.50units/µl)

The sample was incubated for 30 minutes at 37°C, then 1µl of 10mM GTP was added and the incubation continued for a further 30 minutes. The transcription reaction product was stored at -80°C until required for translation.

2.5.2 IN VITRO TRANSLATION USING A WHEAT GERM LYSATE SYSTEM.

In vitro transcription was carried out in a wheat germ lysate system (Roberts and Paterson, 1973).

1ul of transcription reaction product was gently mixed with 3.75µl wheat germ lysate, 2.4µl translation energy mix and 1µl [^{35}S]methionine (approximately 10µCi/µl; 1300 Ci/mM) in a sterile Eppendorf and the sample incubated at 27°C for 60 minutes. The translation reaction product was stored at -80°C until required. Wheat germ translation products were separated by SDS-PAGE and visualised by fluorography followed by autoradiography as outlined below.

2.5.3 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE).

2.5.3a PREPARATION AND RUNNING OF SDS-POLYACRYLAMIDE GELS. (Laemmli, 1970)

Gel plates (15.5cm x 12cm x 0.5cm) were cleaned and assembled using 1mm spacers on the vertical edges. The bottom of the gel was sealed with an agarose plug by placing the assembled plates into a plastic trough containing melted 1% (w/v) agarose. Once the plug had set, resolving gel mix

was poured between the plates , leaving enough space at the top of the plates for insertion of the well-forming comb. The gel was immediately overlaid with water saturated butan-1-ol. Once the resolving gel had polymerised, the butan-1-ol was rinsed away with distilled water and the gel surface carefully dried by inserting a piece of Whatman paper between the plates. Stacking gel mix was then poured into the remaining space between the plates and the required well-forming comb inserted. Once the stacking gel had polymerised, the comb was gently removed and the wells rinsed with distilled water to remove any unpolymerised acrylamide. The gel was then removed from the trough, taking care not to dislodge the agarose plug, and assembled in the gel tank (Scie-Plas). The gel tank reservoirs were filled with 1x Laemmli buffer and the sample wells rinsed out with this buffer using a Hamilton syringe. Samples were heated to 100°C for 2 minutes with at least an equal volume of 2x protein sample buffer before loading with a Hamilton syringe. Electrophoresis was carried out at 10V/cm until the bromophenol blue, in the sample buffer, ran into the agarose plug.

<u>2x Protein Sample Buffer.</u>

125mM Tris-Cl pH6.8
0.4% (w/v) SDS
20% (v/v) Glycerol
10% (v/v) 2-mercaptoethanol
0.1% (w/v) Bromophenol Blue

<u>10x Laemmli Buffer.</u>

25mM Tris-Cl pH 8.3
192mM Glycine
0.1% (w/v) SDS

Resolving Gel Mix.

% Gel (w/v) Acrylamide	12.5%	15.0%	20.0%
30%/0.8% (w/v) Acrylamide/Bis-acrylamide	8.3ml	10.0ml	13.3ml
Distilled water	9.0ml	7.3ml	4.0ml
3M Tris-Cl pH8.8	2.5ml	2.5ml	2.5ml
10% (w/v) SDS	200µl	200µl	200µl
10% (w/v) Ammonium persulphate	83µl	83µl	83µl
TEMED	8.3µl	8.3µl	8.3µl

Stacking Gel Mix.

30%/0.8% (w/v) Acrylamide/Bis-acrylamide	2.0ml
0.5M Tris-Cl pH6.8	2.5ml
Distilled water	5.4ml
10% (w/v) SDS	100µl
10% (w/v) Ammonium persulphate	50µl
TEMED	13µl

For both both resolving and stacking gels, the ammonium persulphate solution was made fresh and mixed with the other components just before pouring.

2.5.3b STAINING, DRYING AND AUTORADIOGRAPHY OF SDS-POLYACRYLAMIDE GELS.

The gel was then removed from the tank and the plates prised apart. The gel was carefully removed from the plates and placed in protein gel fix for at least 30 minutes, with gentle shaking. The gel was then placed in Amplify™ (Amersham) for 20 to 30 minutes then placed on two pieces of Whatman 3MM paper before drying under vacuum at 80°C for 2

hours. The gel was then placed in a light-proof cassette and exposed to X-ray film (Fugi 18cmx24cm Medical). The film was developed in a dark-room using LX24 developer (Kodak) and fixed with Unifix (Kodak) according to the manufacturer's instructions.

Protein Gel Fix.

0.25% (w/v) Coomassie brilliant blue

50% (v/v) Methanol

10% (v/v) Acetic acid

2.6 PREPARATION OF ISOLATED CHLOROPLASTS, THYLAKOID VESICLES AND IMPORT ASSAYS.

2.6.1 GROWTH OF PEA PLANTS.

Pea seeds (Pisum sativum variety Feltam First obtained from Booker seeds Ltd., Nottingham) were sown in compost (Fisons Levington Multipurpose), and grown for 10 days (unless otherwise stated) at 20°C +/- 2°C, under a 12 hour photoperiod. Light intensity was approximately 50$\mu E/m^2$/second using fluorescent lamps (Phillips warmlight). Tap water was applied after sowing and then on alternate days.

2.6.2 ISOLATION OF CHLOROPLASTS AND THYLAKOID VESICLES.

(Modified from Blair and Ellis, 1973 and Cline, 1986).

The method outlined below was used to prepare Percoll-purified intact chloroplasts. To avoid mechanical lysis of the chloroplasts, samples were always resuspended gently and

when pipetting was necessary, it was carried out slowly using wide-bore pipettes or plastic Gilson tips with the ends cut off to widen the apertures. Intact chloroplasts for use in chloroplast import assays were prepared using Percoll gradients while those to be lysed to make isolated thylakoid samples were isolated using Percoll pads, which are more convenient to make, or Percoll gradients.

Approximately 100g of pea leaves were ground using a Polytron homogeniser (Northern Media Supplies Ltd, Hull) at 75% full speed with three 4 second bursts, in 400ml of sucrose isolation medium (SIM). The homogenate was strained through 8 layers of muslin to remove debris and the resulting filtrate centrifuged at 4000g for 1 minute at 4°C to pellet the chloroplasts. The supernatant was discarded and the pellet(s) resuspended gently in approximately 100ml ice-cold SIM using a surgical swab (cotton bud). The centrifugation step was repeated then the pellet gently resuspended in 4ml 1xSRM using a clean surgical swab. The sample was carefully loaded onto two 4ml Percoll pads or two 10ml Percoll gradients which had been pre-cooled to 4°C.

-Percoll pads (4ml 35% Percoll/1xSRM in a 15ml Corex tube), were centrifuged at 2500g for 7 minutes at 4°C, with the centrifuge brake off. The supernatant and the green band at the the top of the pad (which includes broken chloroplasts) were removed, leaving the intact chloroplast pellet at the bottom of the tube.

-Percoll gradients (prepared by layering successively 80%,

65%, 45%, 25% and 10% (v/v) Percoll in 1xSRM in 15ml Corex tubes) were centrifuged at 1500g for 15 minutes at 4°C with the brake off. The middle band on the gradient, at the 65%/45% Percoll interface, (containing intact chloroplasts which are not aggregated) was carefully removed and placed in a clean tube.

The pellet from the Percoll pad or sample obtained from the Percoll gradient was washed by gently resuspending in 5ml 1xSRM with a cotton bud followed by centrifugation at 3000g for 2 minutes at 4°C. The washing step was repeated then the pellet resuspended in 300µl 1xSRM. The chlorophyll concentration of the sample was estimated using the method outlined by Arnon (1949) but, for speed, an aliquot of sample was mixed with acetone and left at room temperature for 5 minutes only, before centrifugation. The chlorophyll concentration of the sample was adjusted to 1mg/ml by adding more 1xSRM.

SIM	5xSRM
0.35M Sucrose	1.65M Sorbitol
25mM HEPES-NaOH pH7.6	250mM HEPES-KOH pH8.0
2mM EDTA	

(Both media were autoclaved at 10psi for 15 minutes and stored at 4°C. SIM was frozen to a slurry before use).

2.6.3 ASSAY FOR PROTEIN IMPORT INTO INTACT CHLOROPLASTS.

Transport of *in vitro* translation products into isolated chloroplasts was assayed using a procedure modified from

Chua and Schmidt (1978) and Ellis and Robinson (1987) (also see Figure 7).

Import incubations contained the following (final concentrations being approximate):-

55µl of isolated intact chloroplasts (at 1mg/ml chlorophyll)

25mM methionine (10µl of a 200mM stock solution)

8.8mM ATP (7µl of a 100mM stock solution)

8.8mM $MgCl_2$ (3.5µl of a 200mM stock solution)

3 to 8µl of *in vitro* translation product

(stock solutions were made up in 1xSRM).

The above components were gently mixed and incubated in glass tubes at 25°C in an illuminated water bath (300µE/m^2/second) for 20 to 40 minutes, with occasional shaking.

If inhibitors or ionophores were to be included in import incubations, samples were preincubated for 10 minutes at 25°C without the translation product to enable ATP to be taken up by the chloroplasts. ATP within chloroplasts is required for protein transport across the chloroplast envelope (see section 1.3.4), hence the addition of chemicals which uncouple ATP synthesis, such as the inhibitors and ionophores used in experiments described in Chapter 4, would inhibit transport across the envelope unless the chloroplasts contain sufficient ATP to support import. After preincubation, the inhibitors/ionophores were added and then the translation product and the samples incubated at 25°C in an illuminated water bath

(300μE/m^2/second) for 30 minutes, with occasional shaking. After this import incubation, each sample was split into two equal portions. Half of each sample was incubated on ice with 0.5mg/ml thermolysin (2mg/ml stock made up in 1xSRM) for 30 minutes and the other half incubated on ice with no additions for 30minutes. Samples were then washed in 5ml 1xSRM/50mM EDTA and the pellet resuspended in 60μl of 10mM Tris pH 7.6 before adding an equal volume of protein sample buffer and heating to 100°C for 2 minutes. Samples were analysed using SDS-PAGE followed by fluorography.

2.6.4 LOCALISATION OF PROTEIN IMPORTED INTO ISOLATED CHLOROPLASTS.

In order to determine the location of imported <u>in vitro</u> translation product within isolated chloroplasts, i.e. in the stroma or within the thylakoid lumen, import incubations were carried out as outlined above but with 4 times the quantities. After the import incubation, each sample was divided into four equal portions. The first portion (total import) was kept on ice until the rest of the samples were ready for addition of sample buffer, then it was washed with 5ml 1xSRM and resuspended in 60μl 10mM Tris pH7.6. The remaining three portions were treated with thermolysin as outlined in section 2.6.3. After washing with 1xSRM/50mM EDTA, each sample was centrifuged as above and the pellet resuspended in 60μl 10mM Tris pH7.6. The second sample (thermolysin-treated chloroplasts) was placed on ice until

the other samples were ready for addition of sample buffer. The remaining samples were transferred to 1.5ml Eppendorfs and centrifuged at full speed in a microfuge at 4°C for 10 minutes. The supernatant (stromal fraction) was transferred to a clean Eppendorf and the pellet washed twice with 1ml 10mM Tris pH7.6 and then resuspended in 60µl of this buffer. The fractions arising from the third sample were kept on ice until the other fractions were ready for addition of sample buffer. Fractions from the fourth sample were incubated on ice with proteinase K (200mg/ml) for 40 minutes. 20µl of PMSF (7mg/ml stock solution made up in 100% ethanol) was then added to each fraction. The thylakoid fraction was washed in 1ml 10mM Tris pH7.6 before resuspending in 60µl 10mM Tris pH7.6. An equal volume of protein sample buffer was then added to each sample or fraction before heating to 100°C for 2 minutes.

2.6.5 POSTTRANSLATIONAL PROTEIN IMPORT INTO ISOLATED THYLAKOID VESICLES.

Intact chloroplasts isolated on Percoll pads or on Percoll gradients were washed twice in 1xSRM then centrifuged at 3000g for 2 minutes at 4°C. The resulting pellet was resuspended in 10mM HEPES-KOH pH8.0 (autoclaved), to 1mg/ml chlorophyll, lysing the chloroplasts. The sample was transferred to Eppendorfs and left on ice for 10 minutes. This crude lysate can be used for import studies when washed thylakoids are not required. To separate thylakoid and

stromal fractions, the sample was spun in a microfuge at top speed for 5 minutes at 4°C. The supernatant (stromal extract) was transferred to a clean Eppendorf and the centrifugation step repeated to remove any remaining thylakoids in this fraction. The thylakoid pellet was washed twice in 10mM HEPES pH8.0 and then resuspended in 10mM HEPES pH8.0 (import buffer) to 1mg/ml chlorophyll. Unless otherwise stated, import incubations (50µl), contained isolated thylakoid vesicles (approximately 30µg chlorophyll), 10mM $MgCl_2$ (6.5µl of a 75 mM stock made up in 10mM HEPES pH8.0) and 1 to 5µl of *in vitro* synthesised precursor. If the inclusion of stromal extract was required for a particular incubation, 40ul of thylakoids (1mg/ml chlorophyll) were pelleted and resuspended in stromal extract or stromal extract diluted with import buffer to a volume of 40µl. The above quantities are convenient to use when several imports are to be carried out at the same time. For greater accuracy, larger import incubations can be done by scaling-up the above quantities. If import incubations are to contain the same amounts of thylakoids, stromal extract, $MgCl_2$ and *in vitro* translation product, these components are mixed together before aliquotting out to achieve more uniform incubations. Import mixtures were incubated in an illuminated water bath (300µE/m^2/second), at 25°C for 30 minutes. After import incubation, each sample was divided into two equal portions one of which was treated with protease (thermolysin at 0.5mg/ml for 30 minutes on ice

or proteinase K at 200µg/ml on ice for 40 minutes) while the other portion was kept on ice. Thermolysin-treated samples were washed in 1ml 10mM HEPES pH8.0/50mM EDTA before resuspending in 40µl of this buffer. Samples that had been treated with proteinase K were then washed in 1ml 10mM HEPES pH8.0 containing 50µl of PMSF (7mg/ml stock solution made up in 100% ethanol). The pellet was resuspended in 40µl of this buffer. Portions which had not been treated with protease were made up to 40µl with import buffer. Samples were heated to 100°C for 2 minutes after addition of 40µl of protein sample buffer, and then analysed using SDS-PAGE followed by fluorography.

2.6.6 DETERMINATION OF PROTEIN CONCENTRATION IN STROMAL EXTRACT.

Total protein concentration in stromal extracts was determined using the Bio-Rad protein assay (standard procedure) with the Bio-Rad protein assay kit I according to the manufacturer's instructions. This assay is based on the Bradford dye-binding procedure (Bradford, 1976).

2.6.7 SPUN-COLUMN PROCEDURE.

Sephadex G-50 columns were prepared in 1ml syringes as outlined in Sambrook *et al*. (1989) except that 10mM HEPES pH8.0 was used as the buffer throughout the procedure and the columns were used at 4°C.

2.6.7 DETERMINATION OF IMPORT EFFICIENCY.

All import incubations were analysed by SDS-PAGE followed by fluorography. The resulting autoradiographs were analysed using a Molecular Dynamics computing densitometer with the volume integration package (with the aid of J. Shackleton) in order to determine the efficiency of import. The efficiency of import by isolated thylakoids was calculated as being the density of mature protein band divided by the total density of protein bands (precursor, intermediate and mature forms) in a particular lane on the autoradiograph.

2.7 SUPPLIERS.

All materials used were of analytical grade if available. The majority of reagents were obtained from Sigma Chemical Co. (Poole, Dorset, England). The sources of other, specific materials are listed below.

Amersham.

(Amersham International PLC., Amersham, Buckinghamshire, England).

Amplify^{TM}, restriction endonucleases and buffers, Oligo-directed *in vitro* mutagenesis system (version 2) and T4 polynucleotide kinase.

[alpha-^{35}S]dATP (10mCi/ml, >1200Ci/mmol).

L-[^{35}S]methionine (15mCi/ml, >1000Ci/mmol).

Boehringer Mannheim.

(Boehringer Mannheim (UK) Ltd., Lewes, England).

M13mp18 RF DNA. ATP, CTP, GTP, TTP and UTP disodium salts.

DIFCO.

(DIFCO Laboratories, Detroit, Michigan, USA).

Bacto-agar, Bacto-peptone and Bacto-tryptone.

FSA/Fisons.

(FSA Laboratory Supplies, Loughborough, Leicestershire, England).

Analytical reagents: Acetic acid (glacial), ammonium chloride, magnesium chloride, potassium dihydrogen orthophosphate, urea, sodium acetate, sodium chloride and sodium hydroxide (pellets).

GIBCO/BRL.

(Life Technologies Ltd, Paisley, Scotland).

Restriction endonucleases and buffers, DNA polymerase I Klenow fragment, T4 DNA ligase, T4 DNA Polymerase, SP6 RNA Polymerase, T7 RNA Polymerase.

Kodak.

(Kodak Ltd., Hemel Hempstead, England).

Bis-acrylamide (N,N'-Methylene bisacrylamide, electrophoresis grade) and UNIFIX™.

MERK/BDH.

(BDH Chemicals Ltd, Poole, Dorset, England).

AnalaR™ Reagents: Ammonium acetate, ammonium persulphate, calcium chloride, chloroform, D-glucose, glycine, 8-hydroxy quinoline, magnesium acetate, magnesium sulphate, propan-2-ol, di-sodium hydrogen orthophosphate, sucrose, zinc chloride and Repelcote™ silicone treatment.

Acrylamide (Electran^{TM}), bromophenol blue (Electran^{TM}), butan-1-ol (general purpose), dimethylformamide, SDS (biochemical), phenol (chromatography), polyethylene glycol 6000 (biochemical), Tris(hydroxymethyl)methylamine (for culture work) and Triton X-100.

Northumbria Biologicals.

(Northumbria Biologicals Ltd., Cramlington, Northumberland, England).

Alkaline Phosphatase.

Oxoid.

(Unipath Ltd., Basingstoke, Hampshire, England).

Yeast extract (for in vitro diagnostic use).

Pharmacia.

(Pharmacia (United Kingdom) Ltd., Milton Keynes, Buckinghamshire, England).

m7G(5')ppp(5')G , Percoll.

Prolabo.

(Prolabo, Paris, France).

Normapur^{TM} analytical reagents: Boric acid (crystallised), EDTA (disodium salt). Glycerol (redistilled).

Promega.

(Promega Biotec., P & S Biochemical Ltd., Liverpool, England).

Riboprobe Gemini System pGEM-4Z, RNasin, SP6 promoter primer and T7 promoter primer.

United States Bioscience.

(USB, Cambridge Bioscience, Cambridge, England).

Sequenase^{TM} DNA sequencing kit, M13 "universal" sequencing primer.

Whatman.

(Whatman International Ltd., Maidstone, Kent, England).

3MM chromatography paper, filter paper (qualitative N^{o} 1).

RESULTS AND DISCUSSION

CHAPTER 3. DEVELOPMENT OF AN *IN VITRO* ASSAY FOR THE IMPORT OF PROTEINS BY ISOLATED THYLAKOIDS.

3.1 INTRODUCTION

During recent years, a great deal of information about the transport of nuclear-encoded proteins within cells has been obtained using *in vitro* systems to study protein translocation across membranes, the processing of translocated proteins and factors required for translocation. Such *in vitro* assays often offer a quick and practical means of studying protein transport. However, it should be noted that results obtained using *in vitro* systems do not necessarily correspond to the situation *in vivo*, which is much more complex. For example, when using isolated organelles for such assays, it should be noted that *in vivo* events taking place within the organelle and at its surface are influenced by events within the cell which, in turn, are influenced by surrounding cells, and so on in a chain of influence which starts with effects of the environment on the whole organism. Although *in vitro* assays have their limitations, they often yield useful information. The use of isolated chloroplasts to study the transport of nuclear-encoded proteins into chloroplasts has proved very useful for the analysis of protein translocation across the chloroplast envelope (section 1.3), but does not enable protein translocation across the thylakoid membrane to be analysed in isolation from translocation across the

envelope. In order to analyse the thylakoid protein transport system in detail, Kirwin *et al.* (1989) developed an assay for protein import into isolated thylakoids. Kirwin *et al.* (1989) found that in the presence of isolated thylakoids, stromal extract and ATP, p33K is converted to i33K (by SPP in the stromal extract) and also to the mature size. The mature size 33K was resistant to added protease, indicating that it was located in the thylakoid lumen (see Figure 10). However, the efficiency of import achieved with this assay was only 20%, or less, of the available precursor. The first aim of this Ph.D. project was to further refine the assay conditions to improve the efficiency of import. In the following discussion, the word efficient is used when levels of import are greater than those previously achieved, or are greater than those observed under different conditions in the same experiment; it is not meant as a comparison to the levels which occur *in vivo*.

3.2. OPTIMAL CONCENTRATIONS OF ATP/$MgCl_2$ REQUIRED FOR THE IMPORT OF p33K INTO ISOLATED THYLAKOIDS.

In order to increase the efficiency of p33K import into isolated thylakoids, the import protocol of P. Kirwin (University of Warwick) was followed as a control, and specific parameters of the protocol were altered in other incubations and the results compared with those from the control. Experiments were carried out in which the ratio of

ATP to $MgCl_2$ in the incubation was varied and it was found that equimolar amounts of ATP and $MgCl_2$, as in P. Kirwin's assays, resulted in the most efficient import (results not shown). Optimal concentrations of ATP/$MgCl_2$ were determined as shown in Figure 16. Thylakoids were prepared by lysing isolated chloroplasts in 10mM Tricine-KOH pH8.0, pelleting the thylakoids and removing the supernatant (stromal extract). Thylakoids were washed twice in 10mM Tricine-KOH pH8.0. Import incubations were carried out as in the figure legend. Maximum import, as demonstrated by protection of the mature form by the thylakoid membrane, from degradation by added protease, was achieved at 10mM ATP/$MgCl_2$; higher concentrations inhibit both processing of p33K to the intermediate form and import into the isolated thylakoids. Concentrations above 18mM usually cause clumping of the thylakoids. The effects of NTPs other than ATP on import were investigated and it was found that 10mM GTP, CTP or UTP could drive import but at levels lower than those achieved with ATP. When ATP was replaced with a non-hydrolysable analogue of ATP, adenosine 5'-O-(3-thiotriphosphate), no import was observed (results not shown).

3.3 EFFICIENT IMPORT OF 33K INTO ISOLATED THYLAKOIDS REQUIRES LIGHT AND THE ADDITION OF STROMAL EXTRACT.

In an attempt to further optimise the import of 33K, the effects of stromal extract concentration and illumination were investigated (Figure 17). Isolated thylakoids were

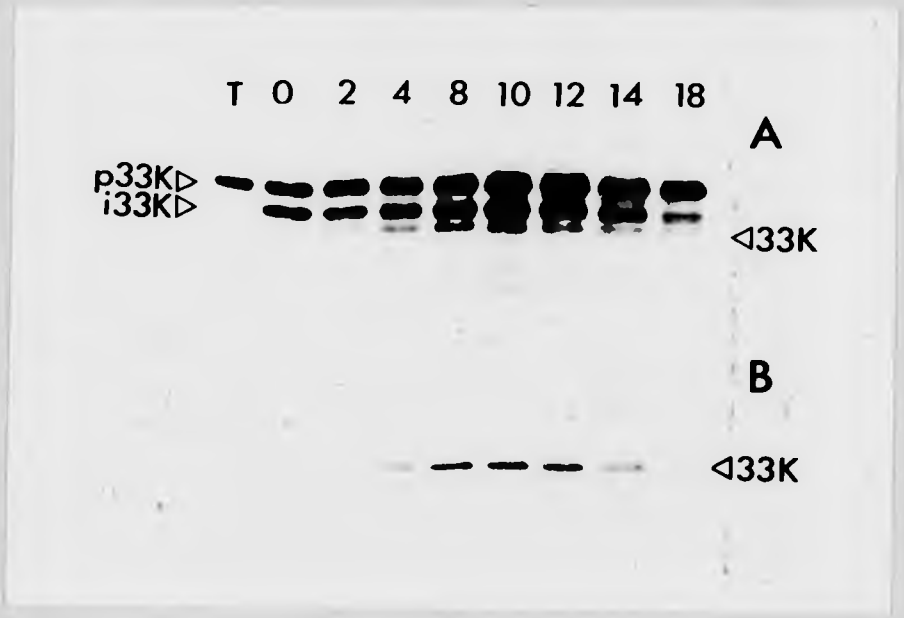


Figure 16. Optimal Concentrations of ATP/$MgCl_2$ Required for the Import of 33K into Isolated Thylakoids.

In vitro synthesised wheat p33K (lanes T) was incubated with isolated pea thylakoids (25µg chlorophyll) and stromal extract (30µg protein), in a total volume of 40µl for 60 minutes at 27°C with ATP/$MgCl_2$ concentrations as denoted by lane headings (mM). Samples were analysed directly (lanes denoted A), or after incubation with thermolysin at 400µg/ml on ice for 30 minutes followed by washing with 1ml 10mM Tricine-KOH pH8.0 before analysis (lanes denoted B). Samples were analysed by boiling with protein sample buffer before running on 15% SDS-polyacrylamide gels followed by fluorography. p33K, i33K and 33K are the precursor, intermediate and mature size forms of the 33kDa component of the PSII oxygen-evolving complex respectively.

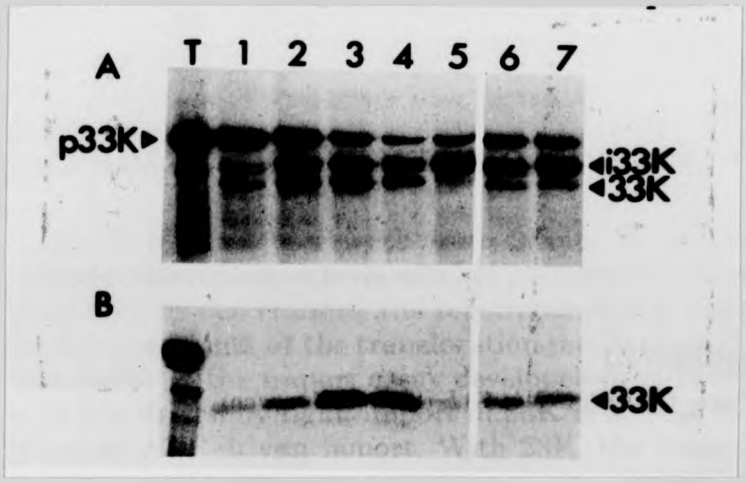


Figure 17. Efficient Import of 33K into Isolated Thylakoids Requires Light and Added Stromal Extract.

In vitro synthesised wheat p33K (lanes T) was incubated with isolated pea thylakoids in the absence of stromal extract (lanes 1) or in the presence of stromal extract at protein concentrations of 0.05, 0.2 and 1.0 mg/ml (lanes 2, 3, and 4 respectively). Import incubations shown in lanes 1 to 4 and lanes 7, were carried out under illumination at 300$\mu E/m^2$/second. Lanes 5, import incubation as lanes 4 but in the dark. Lanes 6, import incubation as lanes 5 but with 10mM ATP. Lanes 7, incubation as lanes 6 except that samples were illuminated. All import incubations were carried out at 27°C for 30 minutes. Half of each sample was analysed directly (lanes denoted A) or after incubation with thermolysin at 400μg/ml for 30 minutes on ice (lanes denoted B).

prepared by lysing isolated intact chloroplasts in 10mM HEPES-KOH pH8.0 followed by two washes in this buffer. Import incubations (50µl) contained isolated thylakoids (30µg chlorophyll) resuspended in 10mM HEPES-KOH pH8.0 and/or stromal extract, 10mM $MgCl_2$ and *in vitro* synthesised precursor. Imports were illuminated at 300µE/m^2/s or kept in the dark by carefully wrapping the tubes in aluminium foil. As shown in Figure 17, increasing the concentration of stromal extract (lanes 1 to 4) increases the efficiency of processing of p33K to i33K (by SPP in the stromal extract) and in the light is accompanied by an increase in levels imported as demonstrated by protection of the mature form from degradation by added protease. In the presence of stromal extract, but without illumination, p33K is efficiently processed to i33K but essentially no import takes place (lanes 5). In the presence of stromal extract but when driven by 10mM ATP, the import efficiency achieved is only approximately 40% of that which can be achieved in the light (lanes 6). Illuminating import incubations containing stromal extract and 10mM ATP, does not further increase the level of import achieved (lanes 7). These results indicate that efficient import of 33K requires stromal extract and light but addition of ATP is not required when import incubations are illuminated. However, a requirement for ATP cannot be ruled out since the wheat germ translation product contains 1.2mM ATP which is diluted to

between 40μM and 100μM (approximately) in import incubations.

3.4 EFFICIENT IMPORT OF 23K INTO ISOLATED THYLAKOIDS REQUIRES LIGHT BUT NOT THE ADDITION OF STROMAL EXTRACT.

In order to determine the mechanism(s) by which proteins are transported across the thylakoid membrane, it was essential that efficient import of more than one protein be achieved using the isolated thylakoid assay. Although ATP-driven import of 23K into isolated thylakoids was achieved, only approximately 5% of the precursor added was imported (Mould *et al.*, 1991). The effects of light and added stromal extract on the import of 23K into isolated thylakoids are shown in Figure 18. In the light, efficient import of p23K takes place both in the presence or absence of added stromal extract (lanes 1 to 3), as demonstrated by protection of the mature form from protease digestion (lanes 1B to 3B). Control experiments in which thylakoids are solubilised with non-ionic detergent (Triton X-100), after import incubation, demonstrate that when protease is added, the mature size 23K is digested and is not therefore inherently resistant to degradation (results not shown). In the presence of added stromal extract, when samples are kept in the dark, efficient processing to i23K takes place but no import is observed (lanes 4). Unlike the import of 33K into isolated thylakoids, efficient import of 23K occurs in the absence of added stroma (lanes 1) and in fact, a slight inhibition of

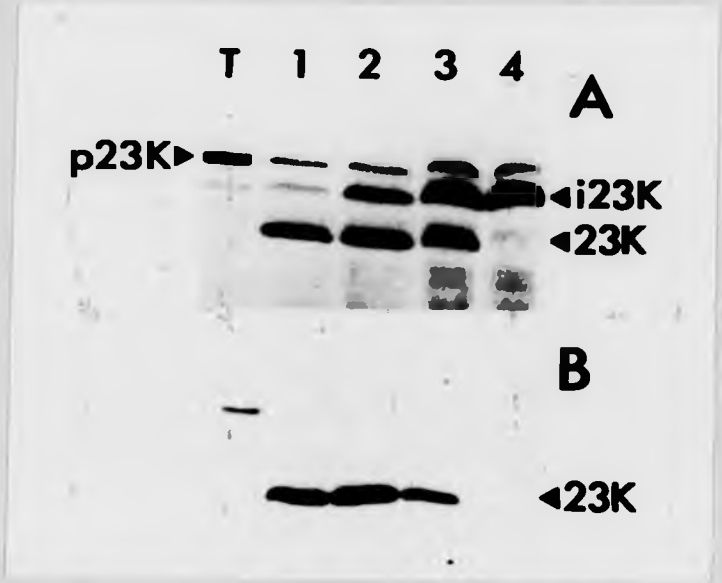


Figure 18. Efficient Import of 23K into Isolated Thylakoids Requires Light But No Addition of Stromal Extract.
In vitro synthesised wheat p23K (lanes T) was incubated with isolated pea thylakoids in the absence of stromal extract (lanes 1) or in the presence of stromal extract at protein concentrations of 0.2mg/ml (lanes 2) or 1.0 mg/ml (lanes 3) for 30 minutes at 25°C under illumination. Lanes 4 as lanes 2 except samples were kept in the dark. Samples were analysed directly (lanes denoted A) or after incubation with proteinase K at 200µg/ml on ice for 30 minutes (lanes denoted B). p23K, i23K and 23K are the precursor, intermediate and mature size forms of the 23kDa component of the PSII oxygen-evolving complex respectively.

import by the addition of stromal extract is often observed. These results suggest that processing to the intermediate-size is not essential for the import of 23K into isolated thylakoids since efficient import occurs in the absence of stromal extract. However, a low level of stromal processing peptidase activity occurs in the wheat germ lysate used for translation, as demonstrated by the presence of i23K in the wheat germ translation product and in the import incubation in which no stromal extract has been added (Figure 18 lanes T and 1 respectively). Under import conditions, the activity of these low levels of SPP may be stimulated, providing i23K for import by the isolated thylakoids. The ability of the thylakoid import apparatus to import p23K was confirmed by J. Shackleton (University of Warwick). p23K can be carboxymethylated so that cleavage by SPP is blocked; p23K modified in this way can still be imported into intact chloroplasts and isolated thylakoids (J. Shackleton in Mould *et al*., 1991). The intermediate form of 23K is also a substrate for import by isolated thylakoids. When p23K is processed to i23K by stromal extract in the presence of isolated thylakoids in the dark, subsequent illumination can 'chase' a proportion of this i23K into the thylakoid lumen. (results not shown but presented in Mould *et al*., 1991).

3.5 LIGHT IS REQUIRED DURING IMPORT OF 33K INTO ISOLATED THYLAKOIDS.

The stimulatory effect of light on the import of 33K was

further investigated by incubating various combinations of the components of import assays in the light before import incubation in the dark (i.e.'pre-illuminating' certain components). Illumination of the stromal extract could, for example, cause some essential modification of a stromal factor required for efficient import, or illumination of the thylakoids could result in modification of a 33K receptor (such as phosphorylation/dephosphorylation) required for successful 33K import. If such modifications were not too transient, the pre-illuminated component would stimulate subsequent import in the dark.

As shown in Figure 19, thylakoids and stroma pre-illuminated together with 10mM $MgCl_2$ are still capable of importing added p33K when further incubated in the light (lanes 1), but not in the dark (lanes 2). Hence the stimulation of 33K import by light only occurs if illumination is concurrent with the import incubation, or any modifications which make components of the import machinery active, are so transient that they are nullified before incubation is recommenced in the presence of p33K. As expected by the results shown in lanes 1 and 2, pre-illuminating stromal extract or thylakoids separately did not stimulate import in the dark. The effects of pre-illuminating p33K in stromal extract were not tested, hence modification of the precursor to render it 'import competent' cannot be ruled out.

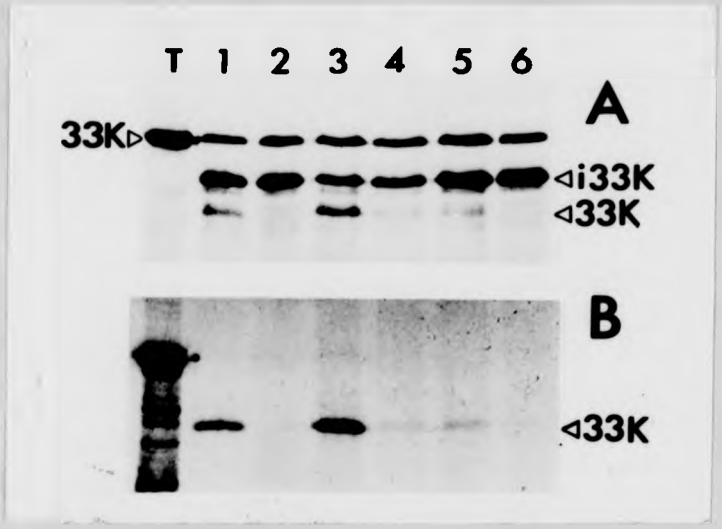


Figure 19. Light is Required During Incubation to Stimulate Import of 33K into Isolated Thylakoids.

In vitro synthesised wheat p33K (lanes T). Isolated pea thylakoids, stromal extract and 10mM $MgCl_2$ were pre-illuminated together before addition of p33K and further incubation under illumination for 30 minutes (lanes 1). Lanes 2 as lanes 1 except that after addition of p33K, incubation was carried out in the dark. Lanes 3 as lanes 1 except that after pre-illumination, thylakoids were replaced with thylakoids which had not been pre-illuminated. Lanes 4 as lanes 3 except that after addition of p33K, incubation was carried out in the dark. Lanes 5, thylakoids were pre-illuminated in the presence of stroma and $MgCl_2$ then pelleted and resuspended in in stroma/$MgCl_2$ which had not been pre-illuminated before addition of p33K and incubation in the dark. Lanes 6, stroma was pre-illuminated before the addition of thylakoids, $MgCl_2$ and p33K followed by incubation in the dark. All pre-illumination incubations were carried out at 25°C for 10 minutes and all import incubations were carried out at 25°C for 20 minutes. Samples were analysed directly (lanes denoted A) or after treatment with thermolysin (lanes denoted B).

3.6 SUMMARY OF CHAPTER 3.

It was found, using the assay for protein import by isolated thylakoids devised by Kirwin *et al.* (1989), that levels of 33K import were highest when 10mM ATP/$MgCl_2$ were included in import incubations. The levels of 33K import do not exceed 20% of the available precursor in such assays. The efficiency of 33K import has been improved by illuminating import incubations. In the light up to 40% of the available p33k can be imported. The import by isolated thylakoids of the lumenal protein 23K was achieved; typically about 50% (sometimes 70%) of the available precursor was imported in the light driven assay. Light is required during import incubations in order to drive efficient 33K and 23K import. Import of 33K into isolated thylakoids is dependent on the addition of stromal extract. This requirement may be due to processing to the intermediate form (by SPP in the stromal extract) being essential for 33K import and/or other factors in the stromal extract being needed for efficient import. Surprisingly, 23K import into isolated thylakoids differs from that of 33K in that it does not require the addition of stromal extract. These results suggested that p23K can be imported by isolated thylakoids without first being processed to the intermediate form; this possibility was confirmed by J. Shackleton (University of Warwick).

CHAPTER 4. ENERGY REQUIREMENTS FOR THE IMPORT OF PROTEINS INTO ISOLATED THYLAKOIDS.

4.1 INTRODUCTION.

In the previous chapter results were presented demonstrating light-stimulated import of 23K and 33K into isolated thylakoids. These results raised the possibility that translocation across the thylakoid membrane may be driven by the thylakoidal proton motive force (see Figure 4).

When the light reactions of photosynthesis take place, protons accumulate in the thylakoid lumen from water splitting and transport in from outside the thylakoids via plastoquinone. Electron transport and proton pumping also cause the generation of an electrical potential difference across the thylakoid membrane which is more positive within the thylakoid lumen than outside. Since the thylakoid membrane has only low permeability to protons, a proton gradient is generated. The proton gradient and the electrical potential difference both contribute to the total proton motive force (pmf). This force drives ATP synthesis by the thylakoid ATPase. To test the possibility that translocation across the thylakoid membrane is driven by a thylakoidal pmf, inhibitors and ionophores which block either the formation of the total pmf, or collapse the electrical potential or proton gradient components of the pmf selectively, were included in import incubations.

4.2 EFFECTS OF ELECTRON TRANSPORT INHIBITORS AND IONOPHORES ON 23K IMPORT BY ISOLATED THYLAKOIDS.

The effects on 23K import of collapsing the thylakoidal proton motive force or each of its components were investigated as shown in Figure 20. A combination of DCMU and methyl viologen was used to inhibit thylakoidal electron transport and therefore prevent the formation of a pmf. The electrical potential was selectively dissipated by the addition of valinomycin/KCl, while the proton gradient was selectively collapsed by the addition of nigericin/KCl. DCMU inhibits electron transfer from Q_A to Q_B, by binding to the D1 protein of the PSII complex and thereby blocking the site of Q_B attachment (see Figure 5). Methyl viologen inhibits cyclic electron transfer from PSI to PSII by transferring electrons to molecular oxygen, thereby creating free-radicals. Valinomycin increases membrane permeability to potassium ions thereby dissipating the electrical component of the pmf; this dissipation is accompanied by an increase in the proton gradient component, maintaining a constant pmf. Nigericin catalyses an electroneutral exchange of protons for other monovalent cations, thereby collapsing the proton gradient across the thylakoid membrane; a constant pmf is maintained by an increase in electrical potential across the membrane (Mills, 1986; Lawlor, 1987).

As shown in Figure 20 (lanes 1), incubation of p23K with isolated thylakoids, 10mM $MgCl_2$ and stromal extract in the light, leads to the generation of i23K by the activity of

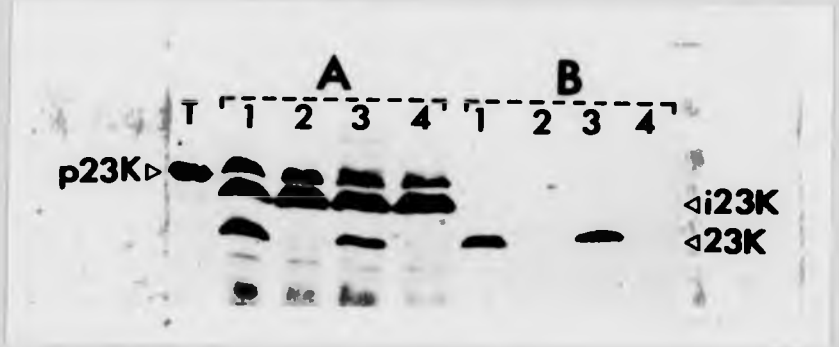


Figure 20. Effects of Electron Transport Inhibitors and Ionophores on p23K Import into Isolated Thylakoids.

In vitro synthesised wheat p23K (lanes T) was incubated with isolated pea thylakoids, stromal extract and 10mM $MgCl_2$ at 25°C for 20 minutes under illumination (lanes 1), in the presence of 15μM DCMU and 50μM methyl viologen (lanes 2), or 1uM valinomycin and 10mM KCl (lanes 3) or in the presence of 1uM nigericin and 10mM KCl (lanes 4). Samples were analysed directly (lanes denoted A) or after treatment with proteinase K (lanes denoted B).

SPP in the stromal extract, and the mature size form which has been imported as demonstrated by its protection from degradation by protease added to the sample (lane 1B). However, collapsing the total pmf (lanes 2), or the proton gradient component of the pmf (lanes 4) completely inhibits import of 23K. Dissipating the electrical component of the pmf leads to only a slight inhibition of import (lanes 3). These results demonstrate that an energised thylakoid membrane is required for efficient import of 23K by isolated thylakoids and that the proton gradient component of the pmf is essential whereas the electrical component is less significant. Similar results were obtained when using p33K as the import substrate under the same import conditions (results not shown).

4.3 EFFECTS OF INHIBITORS AND IONOPHORES ON p33K IMPORT INTO INTACT CHLOROPLASTS.

Experiments were carried out using intact chloroplasts instead of isolated thylakoids to determine whether the inhibitors and ionophores had similar effects in standard chloroplast import assays.

Figure 21 shows the effects of inhibitors and ionophores on the import of p33K by isolated chloroplasts. The results in lanes 1 show that p33K is efficiently imported into intact chloroplasts in the absence of inhibitors, as demonstrated by the generation of the intermediate and mature size forms within the chloroplasts. In the presence of DCMU and methyl

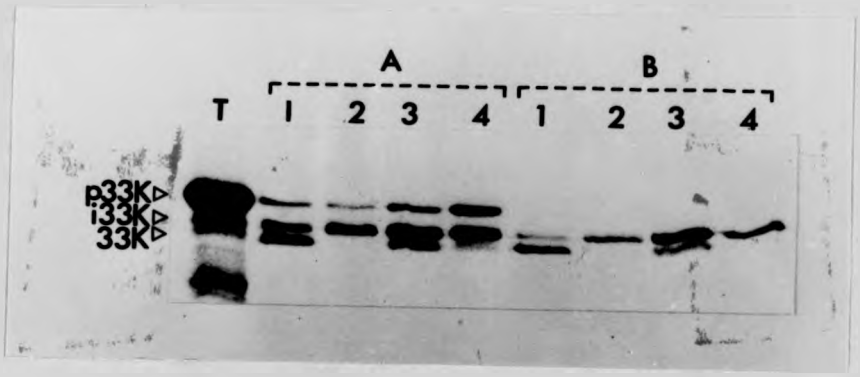


Figure 21. Effects of Electron Transport Inhibitors and Ionophores on p33K Import into Intact Chloroplasts.
Lanes T, in vitro synthesised wheat p33K. Isolated intact pea chloroplasts were preincubated with 5mM ATP/$MgCl_2$ for 20 minutes at 25°C before addition of p33K and incubation under illumination for 20 minutes at 25°C in the absence of inhibitors (lanes 1), or in the presence of DCMU/methyl viologen (lanes 2), or valinomycin/KCl (lanes 3) or nigericin/KCl (lanes 4). Samples in lanes denoted 'B' were treated with thermolysin after import incubation. Inhibitor and ionophore concentrations as Figure 20.

viologen (lanes 2) or nigericin/KCl (lanes 4), the i33K accumulates within the chloroplast but no mature size form is generated. In the presence of valinomycin/KCl (lanes 3), both intermediate and mature forms are produced, although the proportion of mature form generated of total protein imported is slightly lower than that in the control (lanes 1).

4.4 LOCALISATION OF 33K FORMS IN THE ABSENCE AND PRESENCE OF INHIBITORS.

While simple import experiments using intact chloroplasts such as those shown in Figure 21 demonstrate whether or not import has taken place and in which forms the protein are generated, they do not demonstrate the location of these forms within the chloroplast. For example, intermediate form within the chloroplast could be free in the stroma or bound to the thylakoid membrane, while mature form could be in the thylakoid lumen or perhaps spanning the thylakoid membrane with the presequence in the lumen so that processing to the mature size by TPP has taken place. Localisation experiments in which import incubations are carried out using intact chloroplasts, followed by fractionation of thylakoid and stromal compartments, give more information on the whereabouts of imported species within the organelle. Figure 22 shows such a localisation experiment using p33K as the import substrate. In the control incubation (lanes denoted A), p33K is imported efficiently and converted to the mature

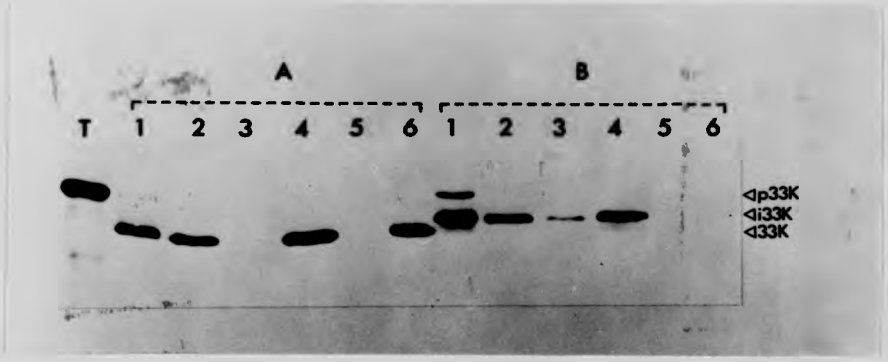


Figure 22. Localisation of 33K Forms Imported into Intact Chloroplasts in the Absence or Presence of Inhibitors.
Lanes T, <u>in vitro</u> synthesised wheat p33K. Isolated intact pea chloroplasts were preincubated with 5mM ATP/$MgCl_2$ for 20 minutes at 25°C before addition of p33K and incubation under illumination for 20 minutes at 25°C in the absence of inhibitors (lanes denoted A), or in the presence of DCMU/methyl viologen (lanes denoted B). After import incubations, samples were analysed after washing with 1xSRM (lanes 1) or after treatment with thermolysin (lanes 2). Thermolysin treated chloroplasts were washed with 1xSRM/50mM EDTA then lysed in 10mM HEPES pH8.0/50mM EDTA. Each lysate was separated into a stromal fraction (lanes 3) and a thylakoidal fraction which was washed once in 10mM HEPES pH8.0 (lanes 4). Lanes 5 and 6, as lanes 3 and 4 respectively, except that after fractionation samples were treated with proteinase K. Inhibitor concentrations as Figure 20.

size. No i33K is detected in this assay. The mature form is associated only with the thylakoid fraction (lane 4A) and is resistant to degradation when this fraction is treated with protease (lane 6A), showing that it is located within the thylakoid lumen. In the presence of DCMU and methyl viologen (lanes denoted B), the intermediate form accumulates within the chloroplast (lane 2B), some being associated with the stromal fraction (lane 3B) but the majority being associated with the thylakoid fraction (lane 4B). The i33K in both the stromal and thylakoid fractions is susceptible to degradation by protease treatment (lanes 5B and 6B respectively). These results show that in the presence of DCMU and methyl viologen, p33K is imported into intact chloroplasts and is processed to the intermediate form which is mainly located on the stromal face of the thylakoid membrane, but is not imported into the thylakoid lumen.

4.4 EFFECTS OF INHIBITORS AND IONOPHORES ON p23K IMPORT INTO INTACT CHLOROPLASTS .

Figure 23 shows the effects of the inhibitors and ionophores described above on p23K import into intact chloroplasts. Efficient import of 23K takes place in the absence of inhibitors (lanes 1). Degradation of the intermediate form by protease treatment (lane 1B), indicates that it is not located within the chloroplast, and probably reflects the presence of this species in the wheat germ translation

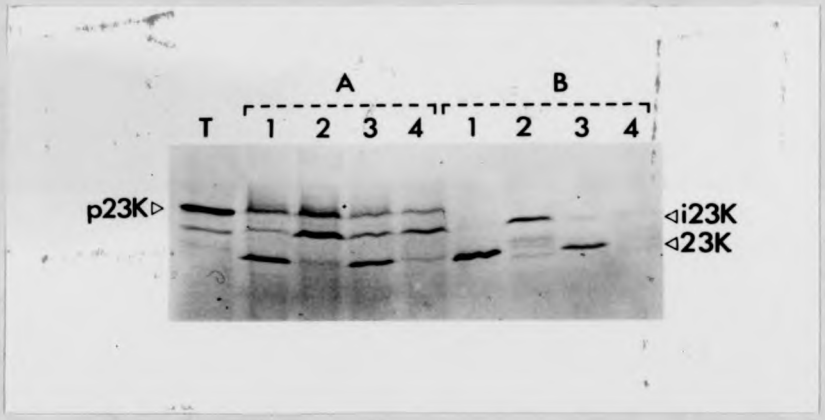


Figure 23. Effects of Electron Transport Inhibitors and Ionophores on p23K Import into Intact Chloroplasts.
Lanes T, in vitro synthesised wheat p23K. Intact chloroplasts were preincubated with 5mM ATP/$MgCl_2$ for 20 minutes at 25°C before addition of p23K and incubation under illumination for 20 minutes at 25°C in the absence of inhibitors (lanes 1), or in the presence of DCMU/methyl viologen (lanes 2), or valinomycin/KCl (lanes 3) or nigericin/KCl (lanes 4). Samples in lanes denoted 'B' were treated with thermolysin after import incubation. Inhibitor and ionophore concentrations as Figure 20.

(lanes T). No i23K is detected within the chloroplast; this could be due to generation of i23K in the stroma followed by rapid transport across the thylakoid membrane, or transport of the precursor across the chloroplast envelope membranes followed by rapid transport across the thylakoid membrane, without generation of the intermediate form, followed by processing to the mature form. In the presence of DCMU and methyl viologen (lanes 2), import into the chloroplast occurs, i23K accumulates but no mature size 23K is generated within the chloroplast. The effects of valinomycin/KCl on import are less pronounced; the mature form is generated within the chloroplast but low levels of i23K are also present, perhaps reflecting a slight inhibition or slowing down of transport across the thylakoid membrane. The effects of nigericin/KCl are more difficult to interpret. Although the generation of the mature form is almost completely inhibited, which is consistent with the data from the imports using isolated thylakoids, there is no corresponding increase in the levels of i23K within the chloroplast, unlike incubations carried out with DCMU and methyl viologen. The low levels of 23K forms within the chloroplast may be a consequence of nigericin/KCl-induced degradation of the 23K species or inhibition by nigericin/KCl of p23K import. This effect is reproducible when p23K is the import substrate but not when p33K (Figure 21, lanes 4) or pre-plastocyanin (Figure 25, lanes 4) are the import substrates. Although the nigericin/KCl data are difficult

to explain, the results obtained with DCMU and methyl viologen show that the mature size 23K is not generated within the chloroplast in the absence of a pmf, and the valinomycin/KCl data show that an electrical potential across the thylakoid membrane is not essential for generation of the mature form. It therefore appears extremely likely that a proton gradient is required for the generation of the mature size 23K within the chloroplast.

4.5 LOCALISATION OF 23K FORMS IN THE ABSENCE AND PRESENCE OF INHIBITORS.

Figure 24 shows the location of 23K forms within the chloroplast when import incubations are carried out in the presence or absence of DCMU/methyl viologen. In the absence of inhibitor (lanes denoted A), p23K is efficiently imported and the mature form is associated with the thylakoid fraction (lane 4A). The mature form is located within the thylakoid lumen as demonstrated by its resistance to degradation by protease added to the sample (lane 6A). The very low level of mature form in the stromal fraction (lane 3A) may be due to leakage from damaged thylakoid vesicles before fractionation, and is probably not due to contamination of the stromal fraction by intact thylakoids, since it is susceptible to digestion by proteinase K (lane 5A). When p23K import is carried out in the presence of DCMU and methyl viologen, i23K accumulates within the chloroplast (lane 2B) and is mainly associated with the thylakoid

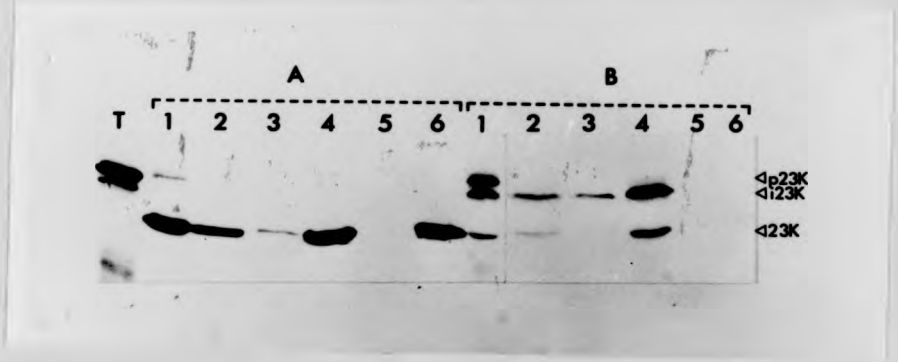


Figure 24. Localisation of 23K Forms Imported into Intact Chloroplasts in the Absence or Presence of Inhibitors.
Lanes T, in vitro synthesised wheat p23K. Isolated intact pea chloroplasts were preincubated with 5mM ATP/$MgCl_2$ for 20 minutes at 25°C before addition of p23K and incubation under illumination for 20 minutes at 25°C in the absence of inhibitors (lanes denoted A), or in the presence of DCMU/methyl viologen (lanes denoted B). After import incubations, samples were analysed after washing with 1xSRM (lanes 1) or after treatment with thermolysin (lanes 2). Thermolysin treated chloroplasts were washed with 1xSRM/50mM EDTA then lysed in 10mM HEPES pH8.0/50mM EDTA. Each lysate was separated into a stromal fraction (lanes 3) and a thylakoidal fraction which was washed once in 10mM HEPES pH8.0 (lanes 4). Lanes 5 and 6, as lanes 3 and 4 respectively, except that after fractionation samples were treated with proteinase K. Inhibitor concentrations as Figure 20.

fraction (lane 4B). i23K in the thylakoid and stromal fractions is susceptible to degradation by added protease (lanes 5B and 6B respectively). In this particular incubation, a low level of the mature species is generated within the chloroplast; although this mature form is associated with the thylakoid fraction (lane 4B), it is susceptible to digestion by proteinase K added to this fraction and therefore is not located within the thylakoid lumen. It is possible that these molecules are transmembrane translocation intermediates which have been partially transported across the thylakoid membrane so that the presequence is accessible to TPP. Alternatively, the active site of TPP was exposed to the external medium by damaged thylakoids, thus enabling processing to the mature form without import into the thylakoid lumen. However, it should be noted that the mature size 23K is not always generated in the presence of DCMU/ methyl viologen, and hence the significance of this observation is unclear.

Localisation experiments for both p33K and p23K import demonstrate that a proton motive force is essential for the import of these proteins into the thylakoid lumen but not for import across the envelope membranes into the stroma. In both cases, when a pmf is prevented from being generated by addition of DCMU and methyl viologen the majority of imported protein is located on the stromal face of the thylakoid membranes. The nature of this association, whether

specific, such as binding to a receptor, or non-specific, has yet to be determined.

4.6 EFFECTS OF ELECTRON TRANSPORT INHIBITORS AND IONOPHORES ON IMPORT OF PRE-PLASTOCYANIN INTO INTACT CHLOROPLASTS.

The results presented above clearly demonstrate that a pmf is essential for the translocation of 33K and 23K across the thylakoid membrane. In contrast, Theg *et al*. (1989) reported that the import of plastocyanin into the thylakoid lumen of intact chloroplasts was not affected by electron transport inhibitors and ionophores. To rule out the possibility that these differences were not simply due to minor differences in the import protocols or concentrations of inhibitors/ionophores used by Theg *et al*. (1989) compared to those described in this chapter, pre-plastocyanin was used as the import substrate in import assays using intact chloroplasts under the same conditions as those outlined in Figure 21. The results obtained (Figure 25), closely resemble those of Theg *et al*. (1989). In the presence or absence of a thylakoidal pmf, import occurs and the mature form is generated within the chloroplast. However, in the absence of inhibitors or ionophores (lanes 1), the ratio of mature form to intermediate form within the chloroplast is greater than this ratio for imports carried out in the presence of inhibitors or ionophores (lanes 2 to 4). These results suggest that although a pmf is not essential for import of plastocyanin and generation of the mature form

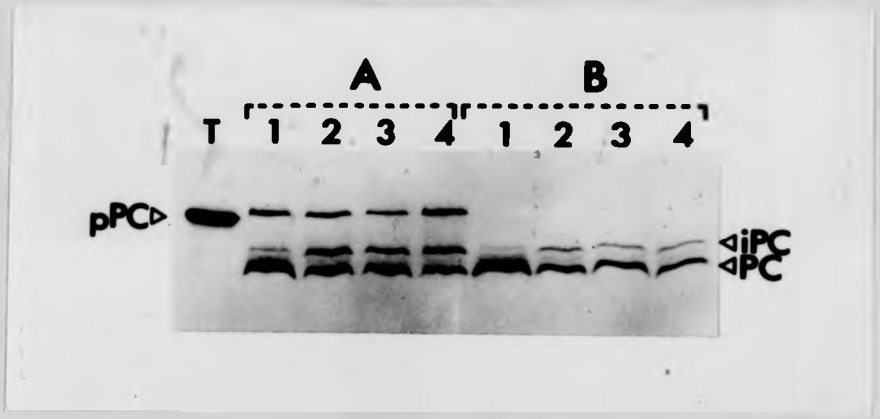


Figure 25. Effects of Electron Transport Inhibitors and Ionophores on pre-Plastocyanin Import into Intact Chloroplasts.

Lanes T, in vitro synthesised Silene pratensis pre-plastocyanin. Intact pea chloroplasts were preincubated with 5mM ATP/$MgCl_2$ for 20 minutes at 25°C before addition of pre-plastocyanin and incubation under illumination for 20 minutes at 25°C in the absence of inhibitors (lanes 1), or in the presence of DCMU/methyl viologen (lanes 2), or valinomycin/KCl (lanes 3) or nigericin/KCl (lanes 4). Samples were analysed directly (lanes denoted 'A') or after treatment with thermolysin (lanes denoted 'B'). pPC, iPC and PC are the precursor, intermediate and mature size forms of plastocyanin respectively. Inhibitor and ionophore concentrations as Figure 20.

within the chloroplast, a pmf across the thylakoid membrane may slightly increase efficiency of plastocyanin transport across this membrane.

4.6 SUMMARY OF CHAPTER 4.

The results presented in this chapter clearly demonstrate that import of 33K and 23K into the thylakoid lumen requires a thylakoidal proton motive force. The proton gradient component of the pmf is essential for the translocation of these proteins across the thylakoid membrane whereas the electrical component is much less important. In agreement with the results obtained by Theg *et al*. (1989), a thylakoidal pmf is not essential for the generation of mature size plastocyanin within intact chloroplasts. However, the efficiency of plastocyanin translocation across the thylakoid membrane appears to be slightly higher in the presence of a thylakoidal pmf.

It is possible that the import of plastocyanin is not fundamentally different from that of 33K and 23K, but differs in the level of pmf across the thylakoid membrane required to promote efficient import. Import experiments could be carried out using these proteins to determine the relationship between level of pmf across the thylakoid membrane and efficiency of import. For example, there may be a threshold level of pmf required to promote transport of each protein across the thylakoid membrane. As yet, the mechanism by which a pH gradient stimulates the transport of

some proteins across the thylakoid membrane has not been elucidated. Since the progenitors of chloroplasts are believed to be free-living prokaryotes, it is possible that many features of thylakoid import apparatus may have evolved from a prokaryotic transport system. It is possible that the pH gradient across the thylakoid membrane may stimulate protein translocation in a similar fashion to that in the prokaryotic system, perhaps by protonation of the translocation machinery (see section 1.4.2).

CHAPTER 5. GENERATION AND USE OF ARTIFICIAL INTERMEDIATE FORMS OF 33K AND 23K.

5.1 INTRODUCTION.

As demonstrated in Chapter 3, the import of 23K into isolated thylakoids does not require the addition of stromal extract. These results suggested that p23K can be imported by isolated thylakoids without first being processed to the intermediate form; this possibility was confirmed by J. Shackleton (University of Warwick).

The import of 33K into isolated thylakoids differs from that of 23K since it requires the addition of stromal extract. This requirement may reflect a difference in the substrates accepted by the thylakoid import apparatus; p23K and i23K may be substrates for translocation while 33K may only be a substrate as the intermediate form. Alternatively, stromal factors other than SPP (or in addition to SPP) may be required for 33K import. In order to determine whether i33K is a substrate for the thylakoid import apparatus and whether stromal factors, apart from SPP, are required for 33K import, an artificial i33K was generated which lacks the SPP cleavage site. Generation of an artificial 33K (Ai33K) enables any requirement for processing by SPP during the import pathway of 33K to be by-passed. An artificial i23K (Ai23K) was also generated so that its import could be compared with that of Ai33K, and possibly with p23K. The artificial intermediates were generated by altering cDNA encoding each protein; the authentic translation initiation

codon was removed and a new initiation codon introduced at a site equivalent to the SPP cleavage site by site-directed mutagenesis.

Bassham *et al*. (1991) determined the SPP cleavage sites of wheat p33K, wheat p23Kand *Silene pratensis* pre-plastocyanin; in each case cleavage occurred between a positively charged residue and an alanine residue. Since all three proteins have an alanine residue at the N-terminus of the intermediate species, it was considered that this residue may be of some significance, in terms of transport across the thylakoid membrane (perhaps being required for interaction with the import machinery) and therefore this residue was retained in the artificial intermediates. The artificial intermediates synthesised were therefore one amino acid residue larger than the authentic intermediates.

5.2 GENERATION OF AN ARTIFICIAL INTERMEDIATE FORM OF 33K.

A full-length cDNA encoding p33K cloned from a wheat (*Triticum aestivum* cv. Avalon) library by Dr. J. Meadows, (Kirwin *et al*., 1989, Meadows *et al*., 1991) was used to produce a cDNA encoding an artificial intermediate form of the 33K (Ai33K) using site-directed mutagenesis as outlined below.

An *Eco*RI/*Kpn*I fragment encoding the presequence of the wheat p33K was subcloned into M13mp19 and an *Nco*I site introduced at the initiation codon by Dr. J. Meadows (University of Warwick). Site-directed mutagenesis was performed on this

DNA construct using the Amersham International site-directed mutagenesis kit and the manufacturer's protocol to introduce a translation initiation codon at the site which would correspond to the SPP cleavage site in the precursor protein (Bassham et al., 1991). The required alteration was a change in codon 31 from CGG (arginine) to ATG (methionine) which results in the creation of an NcoI restriction site in this DNA sequence as shown in Figure 26. This alteration was detected by preparing single-stranded DNA from the plaques produced at the end of the mutagenesis protocol and sequencing this ssDNA using the USB DNA sequencing kit and the M13 universal primer. Figure 27 shows part of the sequence of the DNA used as template for the mutagenesis reaction (lanes denoted 1) alongside the sequence of DNA in which the required alteration has been produced (lanes denoted 2).

The strategy for subcloning the Ai33K coding region into pGEM-4Z is outlined in Figure 28. Double-stranded DNA was prepared from a clone with the correct alteration and digested with NcoI and KpnI. The 210bp NcoI/KpnI fragment was isolated using polyacrylamide gel electrophoresis. pKK233-2 containing a full-length cDNA clone of wheat p33K with an NcoI site introduced at the initiation codon (kindly provided by J. Meadows, University of Warwick) was digested to completion with NcoI then partially digested with KpnI. Vector with the 300bp NcoI/KpnI fragment cut out was isolated using agarose gel electrophoresis. A sticky-end

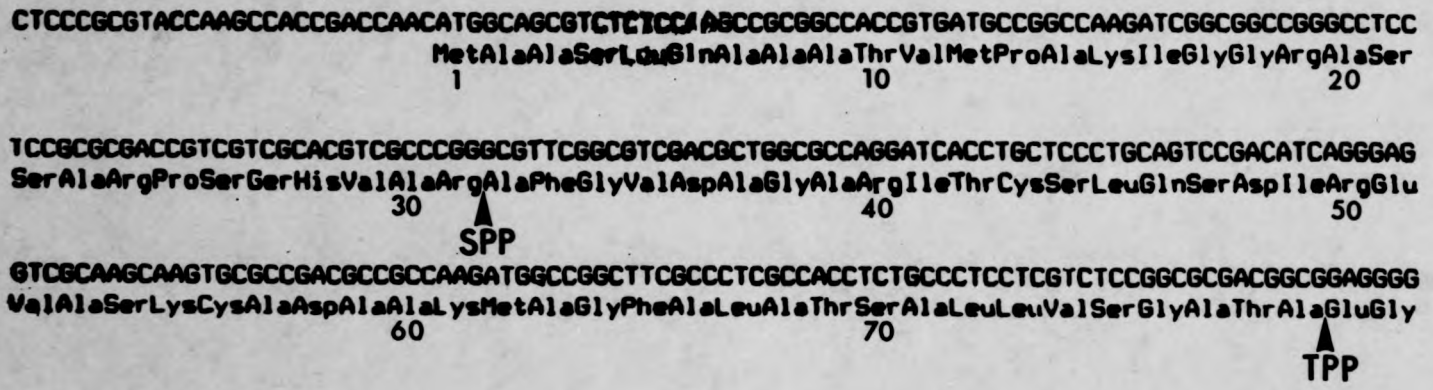


Figure 26. DNA and Amino Acid Sequences of the Wheat p33K Presequence.

The DNA sequence is shown above the corresponding amino acid sequence. The amino acid sequence is numbered from the N-terminal amino acid residue of the precursor protein. Stromal processing peptidase (SPP) cleaves between amino acid residues 31 and 32. Thylakoidal processing peptidase (TPP) cleaves between residues 79 and 80.

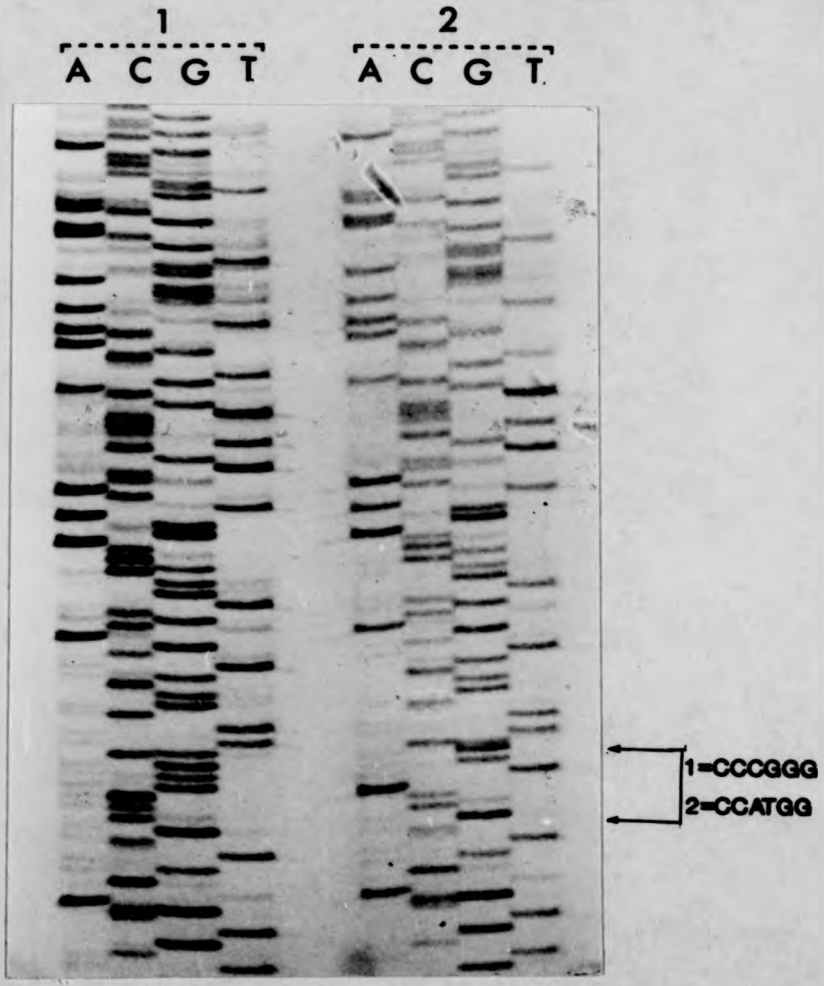
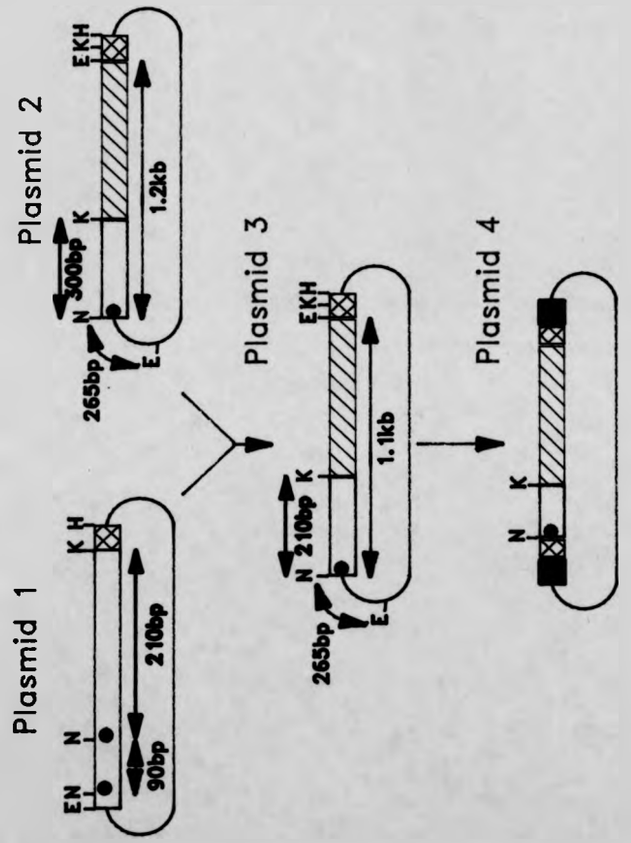


Figure 27. Introduction of a Translation Start Codon into DNA Encoding p33K.

The Figure shows the DNA sequence of a portion of template (spanning the region encoding the SPP cleavage site) which was used for site-directed mutagenesis in lanes denoted 1. The corresponding region after the mutagenesis protocol is shown in lanes denoted 2. The sequence should be read from bottom to top. Arrows indicate the region in which the sequence has been altered from CCCGGG (lanes 1) to CCATGG (lanes 2).

Figure 28. Strategy for Cloning Ai33K DNA into pGEM-4Z.
Plasmid 1 is ds M13mp19 containing a fragment encoding the presequence of wheat p33K. The authentic translation start codon is shown as a black disc while that introduced by site-directed mutagenesis is shown as a red disc.
Plasmid 2 is pKK233-2 containing the complete coding sequence for p33K which has had an NcoI site introduced at the authentic start codon (provided by Dr. J. Meadows).
The 210bp NcoI/KpnI fragment was isolated from Plasmid 1 and ligated into Plasmid 2 which had been prepared by removing the 300bp NcoI/KpnI fragment, thereby creating Plasmid 3.
Plasmid 3 was digested with NcoI and EcoRI followed by blunt-ending with DNA polymerase I Klenow fragment. The 1.1kb fragment was isolated and ligated into pGEM-4Z which had been prepared by digestion with SmaI followed by CIP treatment, thereby creating Plasmid 4.
Plasmid 4 is the coding region for an artificial i33K in pGEM-4Z.
(E=EcoRI, N=NcoI, K=KpnI, H=HindIII, blue box=SP6 promoter, green box=T7 promoter, purple hatch=polylinker).



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Plasmid 4 is the coding region for an artificial i33K in pGEM-4Z.
(E=EcoRI, N=NcoI, K=KpnI, H=HindIII, blue box=SP6 promoter, green box=T7 promoter, purple hatch=polylinker).

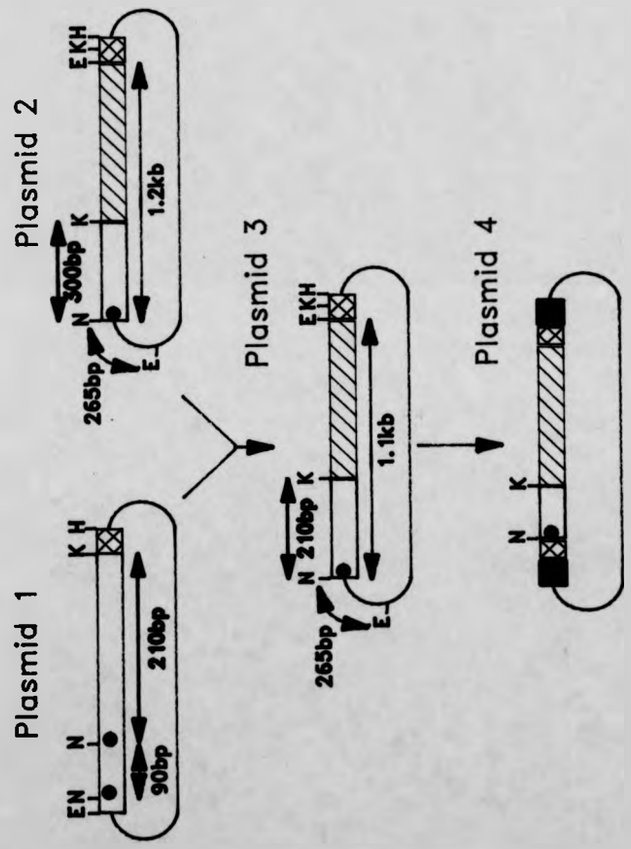

Figure 29. Strategy for Cloning Ai33K DNA into pGEM-4Z.

ligation reaction was carried out using this vector and the 210bp NcoI/KpnI fragment and the ligation mix was used to transform E.coli. Colonies were screened by preparing dsDNA from them followed by digestion of this DNA with NcoI and KpnI, or EcoRI. Plasmid giving the expected size fragments on digestion should contain a coding region for the artificial i33K and this construct could be used for expression of this protein in E.coli. The Ai33K coding region was transferred to pGEM-4Z for in vitro transcription and translation as outlined below.

Ai33K in pKK233-2 was digested to completion with NcoI and EcoRI, then the resulting fragments blunt-ended using DNA polymerase I Klenow fragment. The 1.1kb NcoI/EcoRI fragment was isolated by agarose gel electrophoresis. This fragment was ligated into pGEM-4Z which had been digested to completion with SmaI followed by treatment with CIP to prevent self-ligation. Aliquots of ligation were used to transform E.coli. Transformed cells were plated out onto ampicillin/IPTG/X-GAL plates. Double-stranded DNA was prepared from white colonies using the small scale isolation method and digested with EcoRI and HindIII to screen for the expected size insert. Plasmids containing the expected size fragment were further analysed by digestion using restriction endonucleases.

Figure 29 illustrates the construct obtained and Figure 30 (lanes 1 to 5) shows analysis of the construct by restriction digestion followed by agarose gel

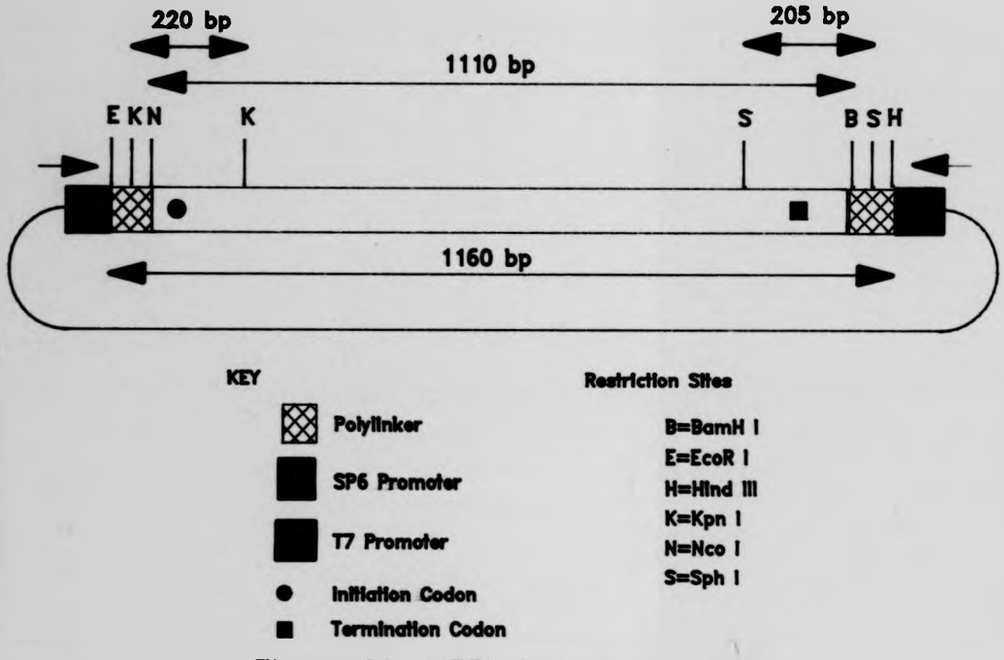

Figure 29. Ai33K DNA in pGEM-4Z.

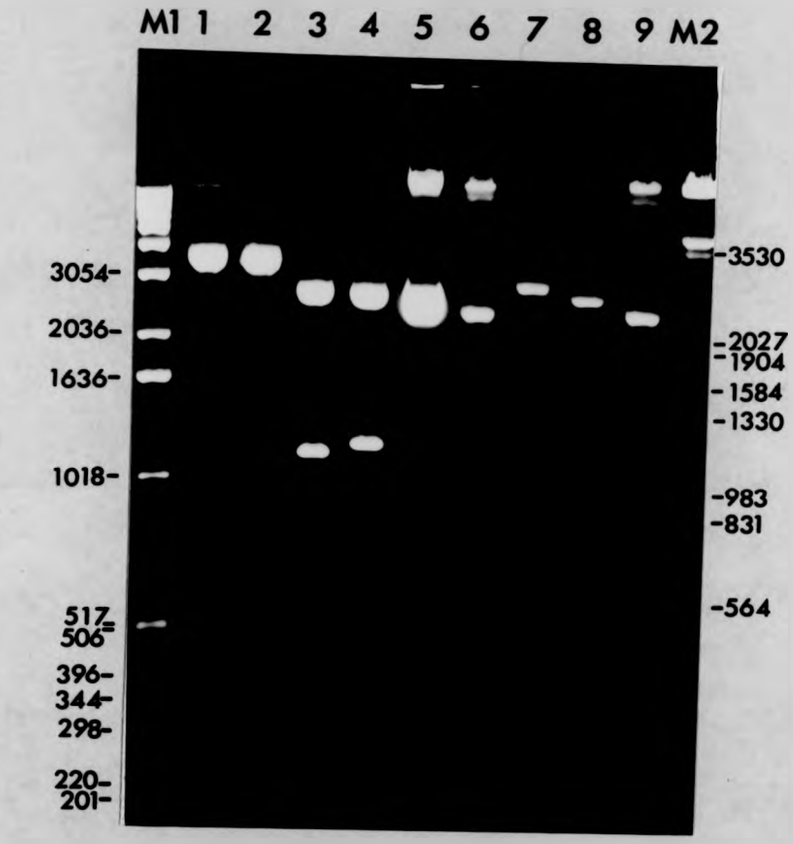

Figure 30. Analysis of DNA Constructs by Restriction Digests.

Figure 30. Analysis of DNA Constructs by Restriction Digests.

Restriction digests of pGEM-4Z containing the coding regions for Ai33K (lanes 1 to 5) or Ai23K (lanes 6 to 9), analysed by agarose gel electrophoresis.

M1= 1kb ladder DNA fragment size markers. M2= Phage lambda DNA digested with EcoRI and HindIII to produce DNA fragment size markers. DNA fragment sizes are in base pairs (bp).

For lanes 1 to 5, refer to the plasmid shown in Figure 29.

Lane 1= SphI digest yielding a fragment of between 201bp and 220bp.

Lane 2= KpnI digest yielding a fragment of approximately 220bp.

Lane 3= NcoI/BamHI digest yielding a fragment of between 1018bp and 1330bp.

Lane 4= EcoRI/HindIII digest yielding a fragment of between 1018bp and 1330bp.

Lane 5= uncut plasmid.

For lanes 6 to 9, refer to the plasmid shown in Figure 34.

Lane 6= BglI digest yielding no fragments.

Lane 7= BamHI digest yielding a fragment of between 564bp and 831bp.

Lane 8= EcoRI/HindIII digest yielding a fragment of approximately 980bp.

Lane 9= uncut plasmid.

electrophoresis. The results shown in lanes 4 and 5 demonstrate that a fragment of approximately the correct size has been inserted into pGEM-4Z, while those in lanes 1 and 2 demonstrate that the insert is in the orientation for transcription from the SP6 promoter. Plasmid yielding the correct size fragments was plasmid sequenced to confirm that no undesirable sequence changes had occurred in the inserted DNA.

5.3 GENERATION OF AN ARTIFICIAL INTERMEDIATE FORM OF 23K.

A full-length cDNA encoding p23K was cloned from a wheat (Triticum aestivum cv. Avalon) library by H. James (James and Robinson, 1991). Single-stranded DNA of this clone in M13mp19 (kindly provided by H. James) was used to generate an artificial intermediate form of 23K (Ai23K) using site-directed mutagenesis as outlined below. The Amersham International site-directed mutagenesis was used according to the manufacturer's protocol to introduce a translation initiation codon at the site which would correspond to the SPP cleavage site in the precursor protein (Bassham et al., 1991). The required alteration was a change in codon 34 from AAG (lysine) to ATG (methionine) as shown in Figure 31. This alteration was detected by preparing single-stranded DNA from the plaques produced at the end of the mutagenesis protocol and sequencing this ssDNA using the USB DNA sequencing kit and the M13 universal primer. Figure 32 shows part of the sequence of the DNA used as template for the

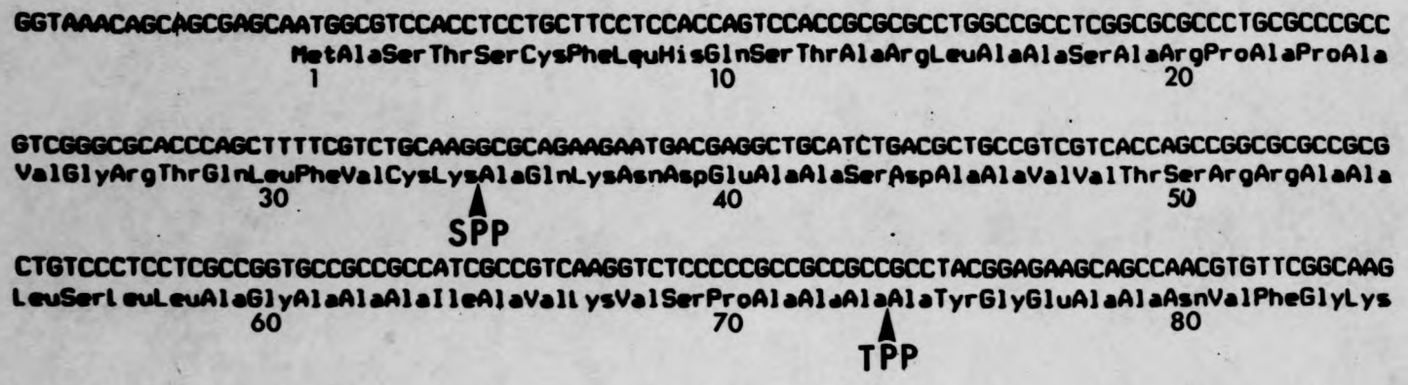

Figure 31. DNA and Amino Acid Sequences of the Wheat p23K Presequence.

The DNA sequence is shown above the corresponding amino acid sequence. The amino acid sequence is numbered from the N-terminal amino acid residue of the precursor protein. Stromal processing peptidase (SPP) cleaves between amino acid residues 34 and 35. Thylakoidal processing peptidase (TPP) cleaves between residues 73 and 74.

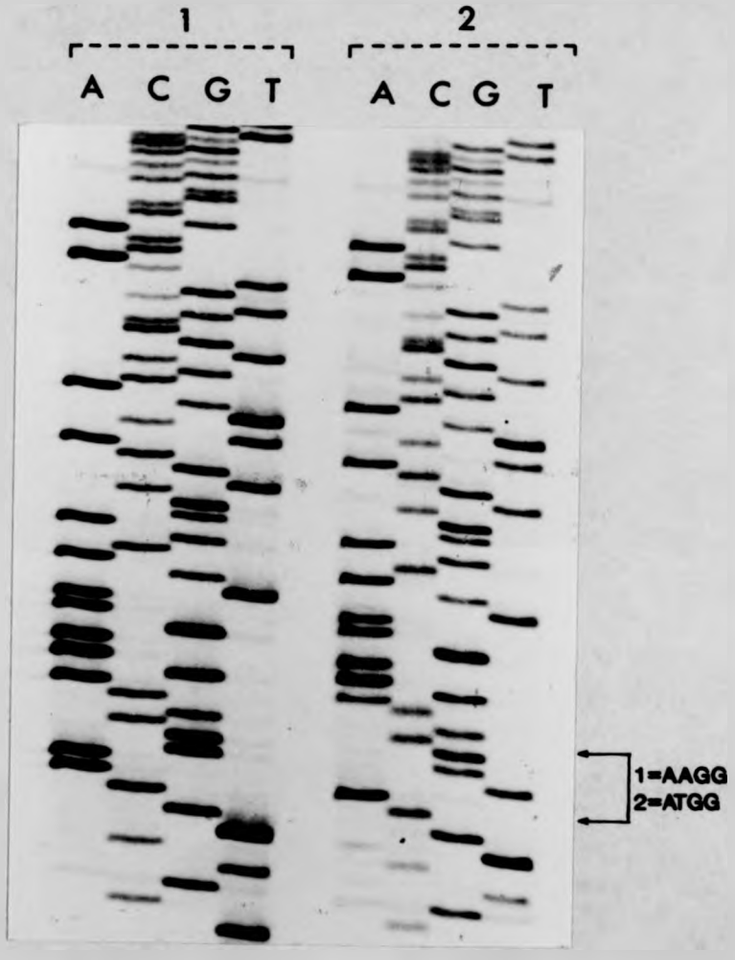

Figure 32. Introduction of a Translation Start Codon into DNA Encoding p23K.

The Figure shows the DNA sequence of a portion of template (spanning the region encoding the SPP cleavage site) which was used for site-directed mutagenesis in lanes denoted 1. The corresponding region after the mutagenesis protocol is shown in lanes denoted 2. The sequence should be read from bottom to top. Arrows indicate the region in which the sequence has been altered from AAGG (lanes 1) to ATGG (lanes 2).

mutagenesis reaction (lanes denoted 1) alongside the sequence of DNA which has the required alteration has been produced (lanes denoted 2).

The strategy for subcloning the Ai23K coding region into pGEM-4Z is outlined in Figure 33. Double-stranded DNA was prepared from a clone with the correct alteration and digested to completion with BglI to cleave between the introduced initiation codon and the authentic initiation codon. The cut plasmid was then blunt-ended using T4 DNA polymerase and then digested to completion with EcoRI. The 910bp EcoRI/blunt-ended fragment was isolated using agarose gel electrophoresis. The isolated fragment was ligated into pGEM-4Z which had been prepared by digestion to completion with SmaI and EcoRI followed by isolation by agarose gel electrophoresis. The BglI site is destroyed by this procedure. Ligations were transformed into E.coli and blue/white selection used to screen for clones containing pGEM-4Z with an inserted fragment. Double-stranded DNA was prepared, using the small scale isolation method, from white colonies and the plasmid DNA screened for the correct size insert by digestion with BamHI and EcoRI. Further digestions using restriction endonucleases were carried out on a DNA sample yielding the correct size fragment to confirm that the insert was that required.

Figure 34 illustrates the construct obtained, while the results of restriction digests of this DNA are shown in Figure 30 (lanes 6-9). The results shown in Figure 30 lanes

Figure 33. Strategy for Cloning Ai23K DNA into pGEM-4Z.
Plasmid 1 is ds M13mp19 containing the coding region for wheat p23K. The authentic translation start codon is shown as a black disc while that introduced by site-directed mutagenesis is shown as a red disc.
Plasmid 1 was digested with BglI, followed by blunt-ending with T4 DNA polymerase then digestion with EcoRI. The resulting 900bp fragment was isolated.
Plasmid 2 (pGEM-4Z) was prepared by digestion with SmaI and EcoRI, then the 900bp fragment ligated into it, thereby creating Plasmid 3.
Plasmid 3 is the coding region for an artificial i23K in pGEM-4Z.
(B=BglI, E=EcoRI, S=SmaI, H=HindIII, blue box=SP6 promoter, green box=T7 promoter, purple hatch=polylinker).

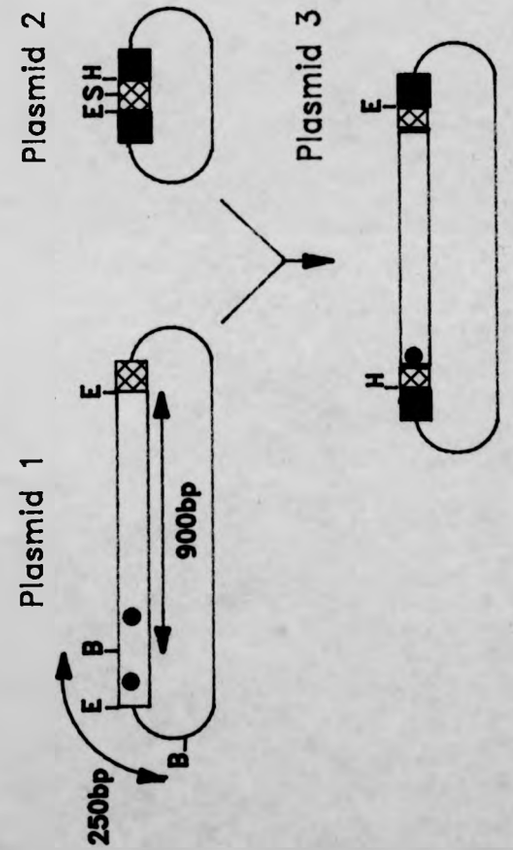

Figure 33. Strategy for Cloning Ai23K DNA into pGEM-4Z.

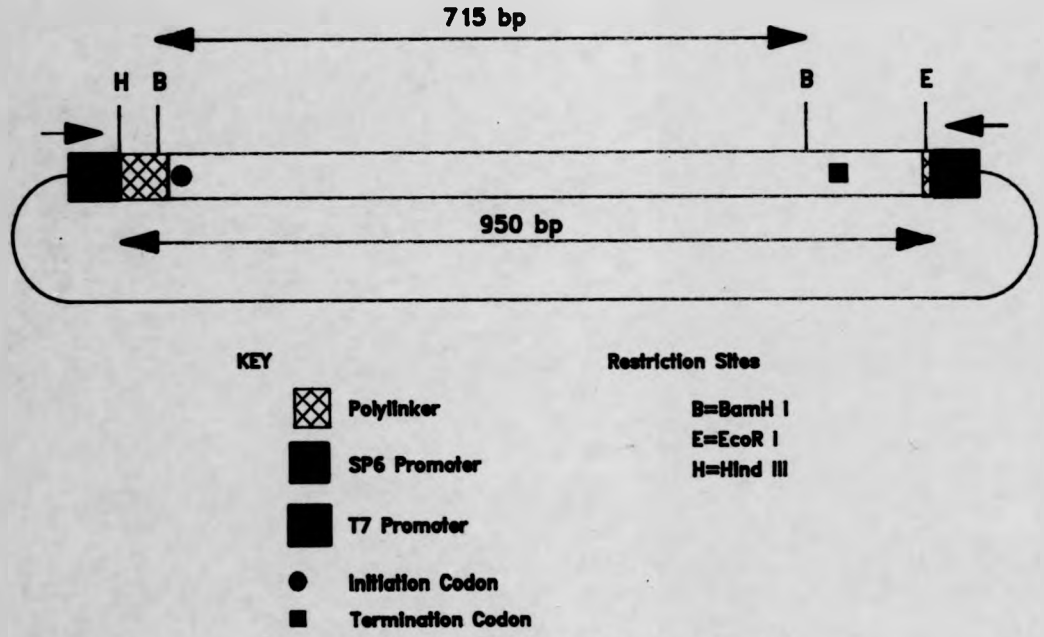

Figure 34. Ai23K DNA in pGEM-4Z

7 and 8 demonstrate that a fragment of approximately the correct size has been inserted into pGEM-4Z, while the result shown in lane 6 demonstrates that the BglI site has been destroyed, as expected. Plasmid DNA yielding the correct size fragments after restriction digests was plasmid sequenced to confirm that no undesirable sequence changes in the inserted DNA had occurred.

5.4 IN VITRO TRANSCRIPTION AND TRANSLATION OF Ai33K AND Ai23K.

Double-stranded DNA encoding Ai33K and Ai23K in pGEM-4Z was prepared using the large-scale isolation method. Ai33K in pGEM-4Z was linearised using EcoRI, phenol/chloroform extracted then ethanol precipitated. The linear DNA was then transcribed using SP6 RNA polymerase. Ai23K in pGEM-4Z was linearised by digestion with HindIII, phenol/chloroform extracted then ethanol precipitated. The linear DNA was then transcribed using T7 RNA polymerase. Both types of transcript were translated in a wheat germ system in the presence of [^{35}S]-methionine. As shown in Figure 35, the artificial intermediates are very similar, in terms of gel mobility, to their true intermediate counterparts produced by processing of the appropriate precursor form with partially purified SPP.

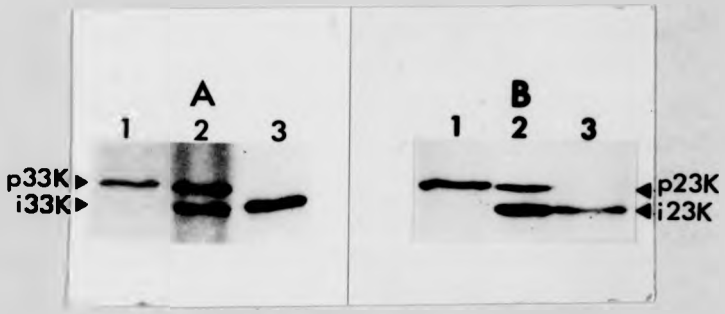

Figure 35. Comparison of Gel Mobilities of the Artificial Intermediates with Intermediates Produced by Precursor Processing by SPP.

Figure shows in vitro synthesised radiolabelled translation products separated by SDS-PAGE. Lane 1A shows p33K. Lane 2A shows p33K which has been incubated with partially purified stromal processing peptidase for 90 minutes at 27°C, and hence contains the 'authentic' intermediate form of the protein below the precursor. Lane 3A shows the artificial intermediate 33K.

Lane 1B shows p23K. Lane 2B shows p23K which has been incubated with partially purified stromal processing peptidase for 90 minutes at 27°C, and hence contains the 'authentic' intermediate form of the protein below the precursor. Lane 3B shows the artificial intermediate 23K (Ai23K).

Partially purified stromal processing peptidase was kindly provided by D. Bassham (University of Warwick).

5.5 A STROMAL FACTOR IS REQUIRED FOR THE IMPORT OF Ai33K, BUT NOT Ai23K, INTO ISOLATED THYLAKOIDS.

Transport of the artificial intermediates into isolated thylakoids was analysed (Figure 36) using the light-driven assay described in Chapter 3. Figure 36A shows that, in the presence of stromal extract and 10mM $MgCl_2$ Ai33K is imported into isolated thylakoids under illumination and is processed to the mature size (lanes 1A). In the absence of stromal extract, however, no import is observed (lanes 2A). These results demonstrate that Ai33K is a substrate for the thylakoid import machinery and that the stromal extract contains one or more factors required for transport of Ai33K into isolated thylakoids. Ai23K is also a substrate for the thylakoid import machinery as shown in Figure 36B; import is observed in both the presence and absence of added stromal extract (lanes 1B and 2B respectively). This result is consistent with the observation that added stromal extract is not required for import of p23K into isolated thylakoids. As would be expected, neither Ai33K nor Ai23K are imported into intact chloroplasts (results not shown).

These results show that Ai33K and Ai23K are substrates for the thylakoid import apparatus, while p23K is also such a substrate (see section 3.3). Bauerle and Keegstra (1991) have shown that preplastocyanin can be imported into isolated thylakoids whereas the intermediate form of this protein cannot.

The observation that full precursor forms can be

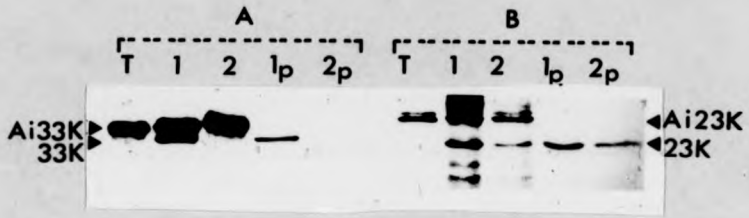

Figure 36. Import of Ai33K and Ai23K into Isolated Thylakoids.

Lanes denoted A:-

In vitro synthesised Ai33K (lane T) was incubated with washed, isolated thylakoids (approximately 30µg chlorophyll) resuspended in stromal extract at 5mg/ml protein in 10mM HEPES pH8.0 (lanes 1) or 10mM HEPES pH8.0 (lanes 2). Import incubations also contained 10mM $MgCl_2$ and were carried out at 25°C for 30 minutes under illumination. Samples were analysed directly, or after treatment with thermolysin (lanes denoted P).

Lanes denoted B, as above except that Ai23K was used as the import substrate.

translocated across the thylakoid membrane brings into question the validity of some aspects of the two step model for the import of nuclear-encoded thylakoid lumen proteins (see Figure 9). This model originally proposed that the generation of a stromal intermediate is required prior to translocation across the thylakoid membrane.

These results raise the interesting possibility that, *in vivo*. SPP may not be involved in the import pathways of plastocyanin or 23K. Perhaps processing by SPP was required earlier in chloroplast evolution, when the removal of envelope transfer domains was essential for recognition of the thylakoid transfer domain of imported TL proteins by the thylakoid import machinery, but is now unnecessary.

As yet, the import of p33K into isolated thylakoids, without the generation of the intermediate form, has not been demonstrated. Determining whether precursor and/or intermediate forms of TL proteins are translocated across the thylakoid membrane in isolated chloroplasts may give clues to the role of SPP in TL protein import *in vivo*. Although kinetic studies by Bauerle *et al*. (1991) using isolated chloroplasts showed that i33K behaved like a substrate for the thylakoid import machinery (levels increasing within the organelle during incubation then decreasing as the mature form accumulates), the possibility that the precursor is translocated across the thylakoid membrane and that the intermediate form is degraded in the stroma, could not be discounted. Pulse-chase experiments

using radiolabelled precursor quickly followed by the addition of high levels of unlabelled precursor to chloroplast import assays, may reveal whether or not stromal intermediates are translocated across the thylakoid membrane in intact chloroplasts.

5.6 PARTIAL CHARACTERISATION OF A STROMAL FACTOR REQUIRED FOR Ai33K IMPORT.

The results of further analysis of Ai33K import into isolated thylakoids are shown in Figure 37. The concentration of stromal extract added to the import incubations ranged from zero to 5mg/ml protein (lanes 1 to 4); over this range, the level of substrate imported increases, and it is probable that import efficiency could be further increased above the level shown in lanes 4 to 6 if more concentrated stromal extract were added. In practice, use of more concentrated stromal extract causes overloading of the gel system used for these studies. The activity of the stromal factor(s) is completely destroyed by heating to 65°C for 10 minutes (lanes 5), indicating that it may be a protein. The requirement for stromal extract was further analysed by investigating whether relatively large molecules such as proteins, or small molecules, such as NTPs, in the stromal extract are required for Ai33K import. Relatively small molecules (less than 10kDa) were removed from the stromal extract using the spun-column procedure

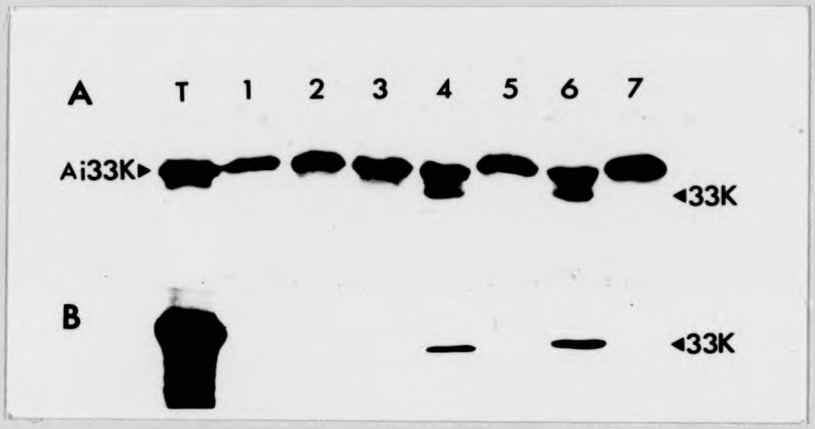

Figure 37. Partial Characterisation of Stromal Factor(s) Required for Ai33K Import into Isolated Thylakoids.
Import incubations were carried out as in Figure 36, with the following exceptions:-
Thylakoids were resuspended in stromal extract at 0, 0.2, 1, and 5mg/ml protein (lanes 1, 2, 3 and 4, respectively). Stromal extract was heated to 65°C for 10 minutes then cooled on ice before use (lanes 5). Stromal extract was filtered through G-50 Sephadex using the spun column procedure before use (lanes 6). Stromal extract was omitted and purified pea stromal chaperonin 60 (1mg/ml protein) used to resuspend the thylakoids (lanes 7). Samples were analysed directly (lanes denoted A), or after treatment with thermolysin (lanes denoted B).

with G-50 Sephadex. This procedure did not significantly affect import efficiency, indicating that a large molecule is required to stimulate Ai33K import (lanes 6).

An abundant hsp60-type stromal protein, chaperonin 60 (Hemmingson et al., 1988) is believed to mediate the refolding and assembly of several imported stromal proteins (Gatenby et al., 1988; Lubben et al., 1989; Gatenby and Ellis, 1990). Since it seemed possible that chaperonin 60 may also be involved in the 33K import pathway, possibly maintaining the stromal intermediate in an 'import competent' conformation, the chaperonin was added to an import incubation. Partially purified chaperonin 60 (kindly provided by Dr. L. Barnett, University of Warwick) was used in place of stromal extract in a thylakoid import incubation but did not significantly stimulate import of Ai33K (lanes 7). This result does not rule out the possibility that this chaperonin is involved in the import pathway; it is possible that an additional stromal factor is required or that the activity of the chaperonin was much reduced due to freezing prior to use.

Another possibility considered, was that the stromal factor required for integration of LHCP into the thylakoid membrane (Cline, 1986; Chitnis et al., 1987) may be involved in the import pathway of 33K. This factor is proteinaceous and is believed to maintain LHCP in a soluble form in the stroma, thus preventing LHCP aggregation. The factor alone is not sufficient for LHCP integration, suggesting the involvement

of an additional stromal factor (Payan and Cline, 1991). Fulson and Cline (1988) have shown that the integration factor's activity is highly sensitive to N-ethylmaleimide (NEM), which covalently modifies free sulphydryl groups. As shown in Figure 38, preincubation of stromal extract with NEM, followed by removal of the unreacted compound before addition of the extract to an import incubation, does not significantly inhibit Ai33K import into isolated thylakoids. This preliminary result suggests that the NEM-sensitive stromal factor involved in LHCP integration is not involved in the 33K import pathway.

Although filtering stromal extract with a G-50 Sephadex spun column does not affect the efficiency of Ai33K import (Figure 37, lanes 6), preliminary results show that when the stromal extract and wheat germ translation products are filtered together using the spun-column procedure, no import is observed (results not shown). This result indicates that small molecules in the wheat germ translation are required for Ai33K import. Import was restored to the levels of unfiltered samples by the addition of 1mM ATP to the import incubations (results not shown). A wheat germ translation normally contains 1.2mM ATP which is diluted to between 40μM and 100μM in an import incubation. These results suggest that import of Ai33K into thylakoids requires ATP, which is normally provided by the wheat germ translation in import assays using isolated thylakoids, and relatively large molecules present in the stromal extract.

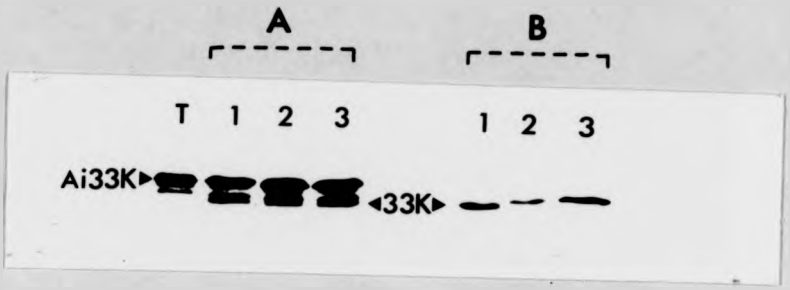

Figure 38. Effect of NEM on Stromal Factor Activity.
Import incubations were carried out essentially as in Figure 36, stromal extract being used at 5mg/ml protein and being treated as follows:-
Stromal extract was pre-incubated on ice for 30 minutes before use (lanes 1). Stromal extract was pre-incubated as in lanes 1 but with 2% (v/v) ethanol (lanes 2) or 2% (v/v) ethanol plus 2mM N-ethylmaleimide (NEM) (lanes 3). All stromal samples were then filtered through Sephadex G-50 using the spun-column procedure. Samples were analysed directly (lanes denoted A) or after treatment with thermolysin (lanes denoted B).

The activity of the stromal factor(s) required for Ai33K import does not seem to be affected by repeated freezing and thawing and hence stromal extracts may be stored frozen without significantly reducing the required activity (results not shown).

The results presented above demonstrate that a stromal factor or factors (and probably ATP) are required for the import of Ai33K into isolated thylakoids. The nature of the stromal factor(s) could be further determined by protease and RNase treatment of stroma before import assays to determine whether the factor is proteinaceous in nature and/or has RNA components. Given that molecular chaperones are known to be involved in protein translocation across the bacterial plasma membrane, the ER membrane, across the chloroplast envelope and for the import of some mitochondrial proteins (see Chapter 1) it is likely that the stromal factor(s) required for translocation of Ai33K across the thylakoid membrane functions as a molecular chaperone.

Interestingly, throughout the course of this project, the import competence of p33K and Ai33K has varied according to the wheat germ fraction used for translation (results not shown). It is possible that molecular chaperones in certain wheat germ fractions bind to p33K and Ai33K preventing their misfolding/ aggregation (see Chapter 6).

5.7 SUMMARY OF CHAPTER 5.

Artificial intermediates of 33K and 23K were generated using site-directed mutagenesis.

The ability of isolated thylakoids to import p23K demonstrated that processing by SPP to generate i23K is not essential for 23K import and raised the possibility that i23K may not be a substrate for the thylakoid import apparatus. However, the import of Ai23K by isolated thylakoids demonstrates that the intermediate form, as well as the full precursor, can be imported by thylakoids. Whether both p23K and i23K are substrates for the thylakoid import apparatus *in vivo* is unknown.

Since the import of p33K into isolated thylakoids requires the addition of stromal extract, i33K is always generated and it is therefore difficult to determine whether p33K is a substrate for the thylakoid import apparatus. The import of Ai33K, however, demonstrates that the intermediate form is a substrate for the thylakoid import apparatus.

The import of Ai33K and Ai23K into isolated thylakoids differs substantially in that stromal factors, other than SPP, are required for Ai33K import but not for Ai23K import. The import of p23K by isolated thylakoids, like that of Ai23K, does not require the addition of stromal extract suggesting that, at least *in vitro*, no soluble stromal factors are required for 23K translocation across the thylakoid membrane. It should, however, be noted that soluble proteins in the wheat germ lysate used for

translation may be involved in 23K import into isolated thylakoids.

The import of Ai33K requires soluble stromal factors other than SPP. This stromal factor (or factors) is larger than 10kDa (approximately), likely to be a protein, and preliminary results suggest that its activity is not sensitive to treatment with NEM. Further purification and characterisation of this factor is required in order to determine what role it has in the import pathway of 33K.

The transport of 23K and 33K into the thylakoid lumen also differ in terms of ATP dependence. Cline *et al.* (in press) have found that import of p23K into isolated thylakoids in the light is not dependent on the presence of ATP whereas preliminary data, mentioned above suggest that 33K import by isolated thylakoids also requires ATP. Repetition of the filtration experiment outlined above is required in order to confirm that import of Ai33K is dependent on ATP. Such a requirement may correlate with the dependence of Ai33K import on stromal extract; ATP may be required for the activity of stromal factors involved in 33K import.

CHAPTER 6. FINAL DISCUSSION.

6.1 THE USE OF AN *IN VITRO* ASSAY FOR IMPORT OF PROTEINS BY ISOLATED THYLAKOIDS.

During the course of this project the efficiency of 33K import by isolated thylakoids has been improved from approximately 20% of the available precursor (Kirwin *et al.*, 1989) to approximately 40%. The import of another TL protein, 23K, has also been achieved with up to 70% of the available precursor being imported. Further refinement of import assay conditions may result in higher efficiencies of import for these proteins being achieved.

The import of several other thylakoid proteins has recently been achieved using the assay system described in Chapter 3. Import of wheat 17K, spinach plastocyanin and the integration of a thylakoid membrane protein, CF_oII (the CF_o subunit II of the thylakoid ATP synthase), have been demonstrated (Klosgen *et al.*, in press; Klosgen, Herrmann and Robinson, unpublished data). Cline *et al.* (in press) have independently demonstrated the import of pea 17K and pea 23K into isolated pea thylakoids but were unable to achieve efficient import of pea 33K or pea plastocyanin. It is likely that the import of many more thylakoid proteins by isolated thylakoids will be achieved in the near future and that this system will prove of great use in the analysis of protein transport across, or integration into, the thylakoid membrane.

Although the field of thylakoidal protein transport is still in its infancy, the studies outlined in this thesis and those of Cline *et al*. (in press) and Klosgen *et al*. (in press) have made significant progress on some points, in particular, the energy requirements for protein transport across the thylakoid membrane. Even though the import of only a handful of thylakoid proteins has been studied, these studies together reveal a surprisingly wide variety of import requirements (see sections 6.2 and 6.3).

6.2 THE MECHANISM OF PROTEIN IMPORT INTO, AND ACROSS, THE THYLAKOID MEMBRANE.

Recently, the energy requirements for the import of several thylakoid proteins have been determined:-

i) A transthylakoidal proton gradient is essential for the transport of 33K, 23K and 17K across the thylakoid membrane (Mould and Robinson, 1991; Klosgen *et al*., in press).

ii) A transthylakoidal proton gradient is not required for the transport of plastocyanin across the thylakoid membrane or for the integration of CF_0II (Klosgen *et al*., in press; Klosgen, Herrmann and Robinson, unpublished data).

iii) A transthylakoidal proton gradient is not essential for, but increases the efficiency of, LHCP integration (Cline *et al*., 1989).

iv) The import of 23K and 17K into isolated thylakoids does not require ATP (Klosgen *et al*., in press: Cline *et al*., in press).

v) Preliminary results suggest that the import of 33K into isolated thylakoids requires ATP (see section 5.6).

The requirement for stromal factors in import assays also depends on the protein in question;-

i) The import of 33K and plastocyanin into isolated thylakoids requires the addition of stromal extract (Mould *et al*., 1991; Klosgen, Herrmann and Robinson, unpublished data).

ii) The import of 23K and 17K into isolated thylakoids does not require the addition of stromal extract (Mould *et al*., 1991; Klosgen *et al*., in press).

At present, it is difficult to rationalise this seemingly bewildering array of requirements. These observations raise the possibility that several different thylakoid protein transport systems may operate in parallel, with different groups of proteins utilising different systems. If this were the case, perhaps one system is ATP-dependent and another dependent on a transthylakoidal proton gradient; certain proteins such as LHCP may be able to utilise both systems.

Alternatively, there may be a single transport system. Most of the proteins mentioned above have similar presequences which would tend to suggest that they are recognised by a single import system. If this were the case, the single system would be particularly adaptable, its mode of action depending on the characteristics of the protein to be imported.

In order to determine whether these proteins utilise the

same transport system, competition studies could be carried out. For example, saturating quantities of p23K could be added to import incubations with relatively low levels of p33K. If the two proteins are imported via the same pathway, the p23K would outcompete the import of 33K, whereas if they utilised different pathways, the efficiency of 33K import would not be affected by the presence of large quantities of p23K.

The mechanism by which a transthylakoidal proton gradient drives the import of some thylakoid proteins is at present obscure. The thylakoidal proton gradient can be monitored using concentration quenching of 9-aminoacridine fluorescence (protocol reviewed in Mills, 1986). The use of this technique would enable the relationship between the proton gradient and the import of different proteins to be determined. It is not known whether proton flux across the thylakoid membrane drives the import of certain proteins or whether a pH difference (i.e. an acidic lumen) is the essential factor.

If protein transport is coupled with the movement of protons out of the thylakoid lumen, high rates of protein import may produce a decrease in the pH gradient which can be detected. Whether an acidic thylakoid lumen is required for the import of certain proteins could be determined by manipulating the lumenal pH, as described by Hind and Jagendorf (1965), and monitoring its affect on protein import. The export of some proteins across the bacterial plasma membrane also requires

a transmembrane proton gradient (see sections 1.4.2). Since the progenitors of chloroplasts are believed to be free-living prokaryotes, it is possible that features of thylakoid import apparatus may have evolved from a prokaryotic system, perhaps including a means of harnessing a proton gradient to drive protein translocation. The mechanism by which this a proton gradient drives protein export in bacteria has also yet to be determined.

6.3 THE ROLE OF STROMAL FACTORS IN THE IMPORT OF THYLAKOID PROTEINS.

Recent work on the substrate specificity of the thylakoid import apparatus has brought into question the involvement of SPP in the import pathways of thylakoid lumen proteins. Results outlined in this thesis and obtained by J. Shackleton (in Mould *et al.*, 1991) clearly demonstrate that the precursor form of 23K can be imported by isolated thylakoids and therefore processing to the intermediate form by SPP is not essential for 23K import.

Bauerle and Keegstra (1991) have reported that preplastocyanin but not the intermediate form of this protein can be imported by isolated thylakoids. However, the validity of these results is questionable since only 1 to 6% of the available precursor was imported. In addition, Bauerle and Keegstra (1991) reported that the import of plastocyanin does not require stromal factors; however, Klosgen, Herrmann and Robinson (unpublished data) have found

that the addition of stromal extract is required for the efficient import of plastocyanin into isolated thylakoids. Although part of the two step model for the import of nuclear-encoded thylakoid lumen proteins proposes that processing by SPP to generate a stromal intermediate is required in order for transport across the thylakoid membrane to take place (see Figure 9), SPP has not yet been demonstrated to have an essential role during the import of any thylakoid lumen protein. Indeed, the results presented in Bauerle and Keegstra (1991) and Mould *et al.* (1991) raised the possibility that the precursor forms of imported TL proteins are the true substrates for the thylakoid import apparatus and that the intermediates observed during *in vitro* import assays are simply artefacts produced in these assays. Bauerle *et al*. (1991) have suggested that the substrates of the thylakoid import apparatus (precursor and/or intermediate forms) could be be determined by incubating isolated chloroplasts with radiolabelled precursors for a short period of time then flooding the import apparatus with unlabelled precursor. The fate of imported protein could then be followed over a period of time; the appearance of radiolabelled intermediate in the stroma followed by the appearance of the mature form would suggest that the intermediate form is transported across the thylakoid membrane. This approach, however, does not provide direct evidence for the transport of intermediate forms across the thylakoid membrane. Results presented in this

thesis (Chapter 5) do provide direct evidence that intermediate forms of nuclear-encoded TL proteins are substrates for the thylakoid import apparatus. Whether precursor and/or intermediate forms are translocated across the thylakoid membrane *in vivo* may prove difficult to determine. Perhaps, during the early evolution of the import pathway for nuclear-encoded thylakoid lumen proteins, removal of the envelope transfer domain was essential for the recognition of such proteins by the ancestral thylakoid import apparatus. If this was the case, the system may have evolved to become more efficient such that processing by SPP is now unnecessary.

The results outlined in sections 5.5 and 5.6 demonstrate the involvement of at least one stromal factor, other than SPP, in the import pathway of 33K. Stromal factors have also been found to be involved in the import/integration of the thylakoid membrane protein, LHCP. These factors are believed to prevent misfolding of LHCP in the stroma (Cline *et al.*, 1986; Payan and Cline, 1991). It is possible that the factor(s) involved in 33K import also prevent the misfolding of 33K in the stroma and thus maintain it in a loose, import competent conformation. Since many molecular chaperones require ATP for their activity, it is possible that the ATP requirement for 33K import by isolated thylakoids is linked to a molecular chaperone activity in the stroma. The protease sensitivity of a protein can be used as an indication of how tightly it is folded; a limited digestion

carried out over a given period of time will produce smaller fragments if the protein is loosely folded than would be produced if it is tightly folded. This technique could be used to determine if the addition of stromal extract results in 33K becoming more loosely folded or more tightly folded and if the addition of ATP causes such an alteration in protein conformation. If the stromal extract contains factors which mediate the unfolding of 33K required for its transport across the thylakoid membrane, this requirement could be by-passed by unfolding p33K or Ai33K in 8M urea. The unfolded protein would be diluted into an import incubation with isolated thylakoids but without stromal extract. If ATP is required for successful import of the unfolded protein this would indicate that ATP is required at some other step in the import pathway apart from, or in addition to, interaction with stromal molecular chaperones. The import of p33K *in vitro* seems to be affected by factors in the wheat germ fraction used for translation in addition to stromal factors. These 'wheat germ factors' may replace cytosolic chaperones, which *in vivo* maintain p33K in a conformation competent for translocation across the envelope membranes whereas the stromal factors prevent misfolding in the stroma. Import assays using isolated thylakoids and Ai33K as the import substrate, demonstrate that while factors in the wheat germ may prevent misfolding of the protein, they are not sufficient for translocation across the thylakoid membrane, since the addition of stromal

extract is required for this to occur. Perhaps part or all of the ATP requirement for Ai33K translocation across the thylakoid membrane can be explained by ATP hydrolysis being essential for the removal of hsp70-type chaperones from Ai33K so that the stromal factor(s) can then bind to the polypeptide. Precedents for this model, involving at least two chaperone-type molecules in the import pathway of p33K, are supplied by similar models for the import of mitochondrial matrix proteins, entry of proteins into the secretory pathway and the observation that different chaperones do not necessarily have interchangeable roles (reviewed in Gething and Sambrook, 1992). Further analysis of the stromal factor(s) and possible involvement of cytosolic factors is required to confirm or refute this model for the import of p33K.

Import of p23K, p17K and Ai23K into isolated thylakoids, does not require the addition of stromal extract (Mould *et al.*, 1991: Klosgen *et al.*, in press). During the course of this project it has been noted that import of p23K and Ai23K import into isolated thylakoids does not show a marked variation in efficiency depending on the wheat germ fraction used for translation, unlike the import of p33K and Ai33K. It is possible that the p23K and Ai23K adopt conformations which are acceptable to the thylakoid import machinery without the participation of any other molecules. Alternatively, p23K and Ai23K may interact with a factor in the wheat germ, found in most fractions, which maintains

p23K in a conformation suitable for the import machinery. Although *in vivo* p23K may be 'handed on' from a cytosolic factor to a stromal factor, such a step may not be essential for import *in vitro* using isolated thylakoids.

The import pathway of p23K probably does not involve a hsp70-type protein in the stroma, since its translocation across the thylakoid membrane does not require ATP (Cline *et al.*, in press).

In summary, studying the import of p23K, Ai23K, p33K and Ai33K has yielded some interesting results. Since 23K and 33K are both nuclear-encoded and assembled into the same complex, it seemed likely that they would share a common import pathway. Indeed, the import of both p23K and p33K, unlike that of plastocyanin, requires light which stimulates import by generating a pH gradient across the thylakoid membrane (Theg *et al.* 1989; Mould and Robinson 1991). However, the import of 23K and 33K, differ markedly in some respects. The import of p23K requires neither ATP nor added stromal extract (Mould *et al.*, 1991; Cline *et al.*, in press), while that of p33K and/or i33K requires both ATP and stromal factors. Perhaps the differing requirements for import of these proteins are a direct result of the manner in which they fold.

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