PROTEOLYTIC PROCESSING OF

IMPORTED CHLOROPLAST PROTEINS

BY

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SUMMARY

Three proteins located in the thylakoid lumen, plastocyanin and the 23KDa and 33KDa oxygen evolving polypeptides of photosystem II, are synthesised in the cytoplasm as higher molecular weight precursors, with N-terminal transit peptides. Import of these proteins involves removal of the first part of the transit peptide by a stromal processing peptidase to yield an intermediate. Maturation, by removal of the remaining transit peptide is performed by a thylakoidal processing peptidase (TPP). TPP has been partially purified and characterised from pea thylakoids and found to be an integral thylakoid membrane protein with the active site on the lumenal (trans) side of the membrane. TPP has a molecular weight of less than 250 000 and is not associated with any supra-molecular complex. Partial purification has yielded ten bands on a Coomassie stained SDS-PAGE gel; however TPP has not been attributed to any of these bands.

TPP displays specificity for chloroplast protein precursors with transit peptides containing a thylakoid transfer domain; however, no species specificity is displayed. TPP exhibits similarities in reaction specificity to Escherichia coli leader peptidase (LEP) in that both peptidases cleave the same eukaryotic and bacterial precursor as well as cleaving higher plant lumenal precursors at the predicted cleavage site.

No standard protease inhibitor has been found to abolish TPP activity; however, a synthetic signal sequence polypeptide will inhibit TPP and LEP.

A thylakoidal endopeptidase (EP5) has been discovered which cleaves lumenal precursors to a size slightly larger than the mature size. EP5 displays different inhibitor sensitivities to TPP and is either a high molecular weight protein or associated with a supramolecular complex. EP5 is assumed to be involved in the turnover of thylakoid proteins.

Work presented in this thesis has been included in the content of the following publications.

Halpin, C., Elderfield, P.D., James, H.E., Zimmerman, R., Dunbar, B. and Robinson, C., (1989). EMBO. J.,8, 3917-3921.

Kirwin, P.M., Elderfield, P.D. and Robinson, C., (1987). J. Biol. Chem., 262, 16386-16390.

Kirwin, P.M., Elderfield, P.D., Williams, R.S. and Robinson, C., (1988). J. Biol. Chem, 263, 18128-18132.

Kirwin, P.M., Meadows, J.W., Shackleton, J.B., Musgrove, J.E., Elderfield, P.D., Mould, R., Hay, N.A. and Robinson, C., (1989). EMBO. J., 8, 2251-2255.

Musgrove, J.E., Elderfield, P.D. and Robinson, C., (1989). Plant Physiol., 90, 1616-1621.

During the course of this project the following collaboration and assistance was provided.

cDNA clones encoding the various precursors used were supplied by the following people; wheat pre-33KDa OEC polypeptide (J. Meadows, Warwick), wheat pre-23KDa OEC polypeptide (H. James, Warwick), spinach pre-33KDa OEC polypeptide (R. Herrmann, Munich), pre-PC and PCi (P. Weisbeek, Utrecht), prepro-alphafactor (D Meyer, UCLA), Erwinia pre-pectate lyase (R. Spooner, Warwick). Purified LEP was provided by W. Wickner (UCLA). The synthetic signal sequence inhibitor was synthesised by R. Merck (Genezentrum, Munchen). Antisera to cyt b6f was supplied by J. Gray (Cambridge). Microsequencing of cleaved proteins was carried out in collaboration with B. Dunbar (Aberdeen).

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LIST OF ABBREVIATIONS

Ala	alanine
ATP	adenosinetriphosphate
BSA	bovine serum albumin
cpDNA	chloroplast DNA
cpm	counts per minute
CTP	cytidine triphosphate
DEAE	diethylaminoethyl
DHFR	dihydrofolate reductase
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	[ethylenebis (oxyethylenenitrilo)] tetraacetic acid
EPSPS	5-enolpyruvylshikimate-3-phosphate synthetase
EP1..EP5	endo peptidase 1 .. endo peptidase 5
ER	endoplasmic reticulum
FPLC	fast protein liquid chromatography
GIP	general insertion protein
GTP	guanosine triphosphate
HEPES	4-(-2-hydroxyethyl)-1-piperazine ethane sulphonic acid
Kbp	kilo base pairs
LEP	leader peptidase
Leu	leucine
LHCP	light harvesting chlorophyll protein
Lys	lysine
MBP	maltose binding protein
MPP	matrix processing peptidase
mRNA	messenger RNA

NADP	nicotinamide adenine dinucleotide phosphate
OEC	oxygen evolving complex
PBS	phosphate buffered saline
PEP	protease-enhancing protein
PC	plastocyanin
PCi	plastocyanin intermediate
PMSF	phenylmethylsulphonylfluoride
PQ	plastoquinone
pre-PC	pre-plastocyanin
pre-23KDa	pre-23KDa OEC polypeptide
pre-33KDa	pre-33KDa OEC polypeptide
PSI	photosystem I
PSII	photosystem II
PTH	phenylthiohydantoin
PVDF	polyvinylidene difluoride
RNA	ribonucleic acid
RNAase	ribonuclease
Rubisco	Ribulose bisphosphate carboxylase oxygenase
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SIM	sucrose isolation medium
SPP	stromal processing peptidase
SRP	signal recognition particle
SSU	small subunit of Rubisco
TEMED	N,N,N, tetramethylenediamine
Tris	2-amino-2-hydroxymethylpropan-1,3-diol
Triton X-100	polyoxethylene p-t-octyl phenol
TPP	thylakoid processing peptidase
UTP	uridine triphosphate

v/v	volume/volume
w/v	weight/volume
^{35}[S] met	^{35} sulphur labelled methionine
^{3}[H] lys	tritium labelled lysine
^{3}[H] leu	tritium labelled leucine

SINGLE LETTER AMINO ACID CODE

A	-	Alanine
R	-	Arginine
N	-	Asparagine
D	-	Aspartic acid
C	-	Cysteine
Q	-	Glutamine
E	-	Glutamic acid
G	-	Glycine
H	-	Histidine
I	-	Isoleucine
K	-	Lysine
M	-	Methionine
F	-	Phenylalanine
P	-	Proline
S	-	Serine
T	-	Threonine
W	-	Tryptophan
Y	-	Tyrosine
V	-	Valine

I : LITERATURE REVIEW

INTRODUCTION

I.1. CHLOROPLAST STRUCTURE

The chloroplast is the organelle within plant cells responsible for photosynthetic assimilation of carbon dioxide into metabolisable compounds. Chloroplasts belong to a family of organelles common to eukaryotic photosynthetic organisms termed plastids. The presence of the green pigment chlorophyll distinguishes chloroplasts from other plastids (see Schnepf, 1980 for review). The dimensions of chloroplasts vary but all are typically lens shaped with a length of approximately 10μm and diameter of 5μm. In addition to their photosynthetic capacity chloroplasts perform a variety of other metabolic processes which include synthesis of lipids, amino acids as well as a variety of sugars. The presence of DNA and protein synthesising machinery means that chloroplasts, unlike other organelles (with the exception of mitochondria) encode and synthesise their own proteins (see Section I.2.B). Chloroplasts divide during cell division with accompanying chloroplastic DNA replication (Rose et al., 1975; Boffey et al., 1979).

Chloroplasts consist of three major structural units. A double membrane ENVELOPE forms the boundary between the rest of the plant cell and soluble STROMA in which the THYLAKOID membrane is located. A schematic representation is presented in Figure 1. A more detailed description of each unit is discussed below.

I.1.A. THE ENVELOPE

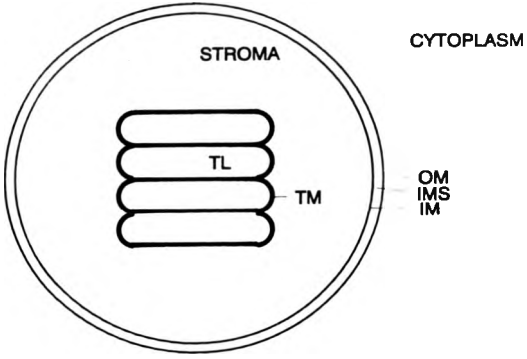
A double membrane, termed the envelope membrane, surrounds the chloroplast. The inner and outer membranes of the envelope are separated by an inter membrane space. Permeability between the plant cell and chloroplast is regulated by the inner membrane which displays selective permeability properties (Herber and Held, 1981). Outer and inner membranes differ in their composition of lipid and protein. (Block et al., 1983 a, b). Enzyme activities of the chloroplast envelope also show segregation between inner and outer membranes. A Mg^{2+} ATPase, galactosyl transferase and phosphatidic acid phosphatase are associated with the inner envelope membrane whilst acylCoA synthetase resides in the outer envelope membrane (Block et al., 1983 b).

I.1.B. THE STROMA

The soluble phase which is enclosed by the envelope membrane is known as the stroma. This compartment houses the enzymes responsible for the metabolic steps described in Section I.1

FIGURE 1 - Schematic representation of a chloroplast showing; double membrane envelope, soluble stroma phase surrounding the chlorophyll containing thylakoid membranes

The predominant enzyme present in the stroma is that responsible for the conversion of CO_2 and ribulose-bisphosphate to 3 phosphoglycerate, thereby performing fixation of CO_2. This enzyme is known as ribulose-bisphosphate carboxylase oxygenase (Rubisco) and accounts for over 50% of stromal protein (Foyer, 1984).



TL - thylakoid lumen
TM - thylakoid membrane
OM - outer membrane
IM - inner membrane
IMS - inter membrane space

along with DNA and protein synthesis machinery (Boulter et al., 1972).

I.1.C. THE THYLAKOID MEMBRANE

The thylakoid membrane is a set of disc like membranes which enclose an intra thylakoid space known as the thylakoid lumen (Whitmarsh, 1986). These membranes house the electron transport systems of photosynthesis, along with the chlorophyll containing protein complexes of photosystems I and II and their associated antenna complexes. Higher plant thylakoid membranes show a distinct structural arrangement in which regions of stacked or appressed membranes (grana) are interconnected by unstacked non-appressed membranes (stromal lamellae) (Barber, 1987 and Staehelin, 1986). The interface between opposing membranes in stacked regions provide a separation of 4nm (Ryrie et al., 1980). The purpose of this structural organisation is the matter of some debate and includes possible regulatory mechanisms in order to produce a balance of excitation energy between the two photosystems (Miller and Lyon, 1985 and Staehelin, 1986).

The thylakoid membrane consists of approximately 50% protein, 40% lipid, and 10% pigment by weight (Gounaris et al., 1986). A large number of thylakoid membrane proteins have been identified, and four major complexes are known; these are photosystem I and II, cytochrome b6f and the chloroplastic ATPase (Gounaris et al., 1986). The two

photosystems exist as individual complexes each with their own chlorophyll and proteins associated with harvesting light energy for the synthesis of ATP and $NADPH_2$ in the following manner (see Anderson and Anderson, 1985; Ort, 1986).

Light energy is harvested by 'antenna' chlorophyll molecules and passed on to a small number of 'reaction centre' chlorophyll molecules. An excited electron from photosystem II is then transferred to photosystem I involving a number of small molecules as well as plastocyanin and cytochrome b6f complex. The electron received by photosystem II is then excited and transferred to NADP involving the soluble stromal protein ferredoxin. The inter-connection for the two photosystems and respective electron transport chains forms the 'Z scheme' as proposed by Hill and Bendall (1960).

A proton gradient is generated across the thylakoid membrane. ATP is synthesised as protons flow through a chloroplastic ATPase down the proton gradient into the stroma.

The arrangement of proteins in the thylakoid membrane shows a high degree of lateral and transmembrane asymmetry (Masojidek et al., 1987). (For reviews see Anderson, 1982; Staehelin, 1986; Barber, 1987.) The ATPase and ferredoxin NADP oxidoreductase are located exclusively on the outer surface of thylakoid membranes in contact with the stroma. The two photosystems are distributed such that photosystem

II predominates in appressed membranes whilst photosystem I predominates in non-appressed regions (Anderson and Haehnel, 1982). The mobile electron carrier plastocyanin may also show lateral heterogeneity depending on light intensity (Haehnel et al., 1989). However cytochrome b6f complex appears to be uniformly distributed (Anderson, 1982). A description of the two photosystems and plastocyanin is presented below.

(i) Photosystem I

Photosystem I complex consists of at least 7 polypeptides, P700 chlorophyll and Fe-S centres. P700 receives electrons from plastocyanin in the lumen and after excitation passes the electron onto ferredoxin on the stromal face.

(ii) Photosystem II

The first step in electron transport of photosynthesis is mediated by photosystem II. The source of electrons for this step comes from photolysis of water

$$2H_2O \longrightarrow 4H^+ + 4e^- + O_2$$

therefore photosystem II can be regarded as a water splitting enzyme. Photosystem II is a multimeric complex, composed of a group of at least three proteins involved in the water splitting step, and six reaction centre core proteins (Gounaris et al..

1986; Ort, 1986). A schematic representation is presented in Figure 2.

(a) The Oxygen Evolving Complex (OEC)

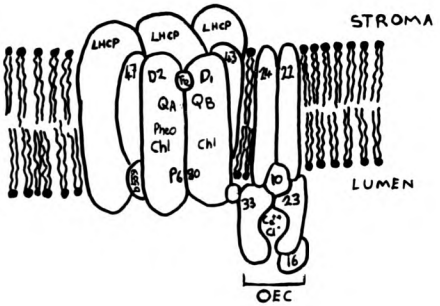
This complex is responsible for the photolysis of water and comprises three nuclear encoded polypeptides of 16KDa, 23KDa and 33KDa. The 33KDa and 23KDa polypeptides are loosely attached to three intrinsic polypeptides of 22KDa, 24KDa and 10KDa as shown by immuno-precipitation nearest neighbour analysis (Ljunberg et al., 1984). Sequential rebinding of OEC polypeptides to inside-out membranes (which have had the OEC complex removed by salt washing) suggest that the 16KDa polypeptide binds to the 23KDa polypeptide as shown in Figure 2 (Andersson et al, 1984). The release of the 33KDa polypeptide from inside-out vesicles is usually accompanied by the loss of manganese ions and oxygen evolving capability (Andersson, 1985; Barber, 1987 and Rutherford, 1989). However, under conditions of 1M CaCl, the 33KDa polypeptide can be removed without the loss of manganese ions and oxygen evolution (Barber, 1987). It is therefore currently agreed that the role of the 33KDa polypeptide is for the protection and stabilisation of photosystem II bound

FIGURE 2 - Structure and orientation of photosystem II in the thylakoid membrane

Photosystem II consists of:

- a reaction centre core containing D1, D2, 47KDa and 43KDa chlorophyll binding proteins and cytochrome b559
- light harvesting complex which surrounds the reaction centre core. These two components are membrane bound
- 16KDa, 23KDa and 33KDa polypeptides of the oxygen evolving complex bound to the intrinsic 10KDa, 24KDa and 22KDa proteins

Numbers indicate the size of the proteins in KDa.


STROMA
LHCP
LHCP
LHCP
43
D2
D1
24
22
QA
QB
Pheo
Chl
Chl
Pe
30
10
33
23
Mn
16
LUMEN
OEC

manganese and hence performs a structural as opposed to a catalytic function. The 16KDa and 23KDa polypeptides can be functionally substituted by calcium and chloride ions and it is believed that these polypeptides enhance the affinity of the oxygen evolving site for chloride and calcium ion cofactors (Barber, 1987; Andersson, 1982). However, Wales *et al*. (1989) using secondary structure predictions propose that the 33KDa polypeptide possesses a putative calcium binding site and therefore may also be involved in OEC calcium binding.

(b) Reaction Centre Core

This is composed of several proteins whose function is the transfer of electrons from water to plastoquinone. The proteins of the reaction centre core are synthesised within the chloroplast and are of 47KDa, 43KDa, 10KDa, 4KDa and two in the region of 31-34KDa (Barber, 1987).

- 47KDa and 43KDa proteins; bind chlorophyll and function as a light harvesting antenna system for the D1 and D2 proteins.

- D1; is one of the 31-34KDa proteins and the probable location of the manganese binding site associated with oxygen evolution (Rutherford, 1989). D1 also binds several other agents of photosynthesis including pheophytin, chlorophyll, non-heme iron and the secondary electron acceptor quinone Q_B, which is interchangeable with unbound plastoquinone in the thylakoid. The D1 protein is also the target of a large number of herbicides such as atrazine and diuron which block the transfer of electrons to Q_B.

- D2; this protein is the other 31-34KDa protein of photosystem II and binds chlorophyll, non-heme iron, pheophytin and Q_A the primary electron acceptor.

- 10KDa and 4KDa; are the polypeptides of cytochrome b559 which performs a function which is not fully understood.

(iii) Plastocyanin

Plastocyanin is an 11KDa nuclear encoded protein which is freely diffusable in the lumen, its function being electron transfer from cyt b6f to photosystem I (Haehnel et al., 1989). Its redox activity employs changes in the oxidation state of a

bound copper ion. The timing of the copper addition i.e. before or after import into the chloroplast or thylakoid is unknown but due to conformational constraints of imported proteins bound to ligands during membrane transport (discussed in Section I.2.E.) the copper is likely to be added after import into the lumen.

The relative locations of the photosystems and plastocyanin in the thylakoid is illustrated in Figure 3.

FIGURE 3 - The Z scheme of photosynthesis

Diagrammatic representation showing the relative locations of proteins and electron carriers which facilitate the transfer of electrons from water to NADP during photosynthesis, as proposed by the Z scheme of Hill and Bendall (1960).

PSI - photosystem I

PSII - photosystem II

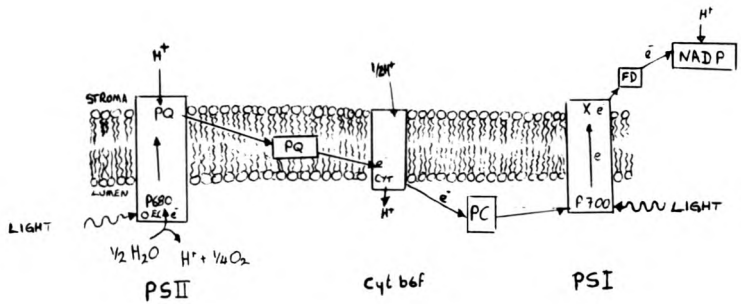
cytb6f - cytochrome b6f

PC - plastocyanin

PQ - plastoquinone

X - iron sulphur centre

FD - ferredoxin


H+
STROMA
PQ
PQ
LUMEN
P680
e
LIGHT
½ H2O
H+ + ¼ O2
1/8H+
Cyt
H+
e-
PC
X
e
e
F700
LIGHT
FD
e-
H+
NADP
PSII
Cyt b6f
PSI

I.2. CHLOROPLAST PROTEIN SYNTHESIS

The synthesis of chloroplast proteins involves two separate genomes, i.e., the nuclear and chloroplastic genome. Confirmation of the existence of chloroplastic DNA (cpDNA) was provided by electron microscopy with Chlamydomonas moewusii (Ris and Plaut, 1962). However, a non-Mendelian pattern of inheritance of chloroplast phenotype mutants had been obtained half a century earlier (see Groot, 1984).

I.2.A. cpDNA

Three reviews discuss the chloroplast genome (Bohnert et al., 1982; Groot, 1984 and Hoober, 1987) and describe the distinctive properties of cpDNA. It is a covalently closed circular piece of DNA which varies in size between species typically 120-160Kbp for higher plants with a coding capability of 55 proteins up to 2100 residues in length (Hoober, 1987). In many higher plants repeated 20-24Kbp segments of DNA occur arranged in an inverted fashion (termed inverted repeats), which are separated by a section of single copy DNA varying in size between species from 12 to 76Kbp. Some higher plants such as Pisum sativum lack the inverted repeat structure (Bohnert et al., 1982). In contrast to nuclear DNA, cpDNA lacks modified bases such as methylcytosine. Replication of cpDNA occurs in young dividing cells and is then distributed among dividing chloroplasts during primary leaf expansion, resulting in chloroplasts with multiple copies of cpDNA (Bohnert et al., 1982).

The locations of many genes on cpDNA have been determined as shown below (reviewed by Bohnert *et al*. (1982) and Groot (1984)). The entire sequence is known for liverwort (Ohyama *et al*., 1986) and tobacco (Shinozaki *et al*., 1986).

(i) rRNA: the 4 ribosomal RNA genes 23S, 16S, 5S and 4.5S rRNA of chloroplast ribosomes reside in the inverted repeat sequences.

(ii) tRNA: between 30 and 40 tRNA genes have been identified which show an even distribution throughout cpDNA.

(iii) Protein: up to 100 chloroplast encoded proteins have been identified (Hoober, 1987) including large sub unit of Rubisco (Hartley *et al*., 1975) cytochrome b6f and D1 protein.

I.2.B. THE SYNTHESIS OF CHLOROPLAST PROTEINS

Bottomley and Bohnert (1982) and Gray *et al*. (1984) review various *in vitro* and *in vivo* techniques which have revealed that most chloroplast proteins are encoded by the nuclear genome.

(i) Selective inhibition of chloroplast ribosomes with antibiotics such as chloramphenicol.

(ii) *In vitro* synthesis of chloroplast proteins in cell free systems programmed with mRNA of nuclear origin.

Using a wheat-germ lysate system, Highfield and Ellis (1978) translated isolated poly (A) mRNA from pea and showed that Rubisco SSU was one of the translation products. Since nuclear mRNA is poly adenylated but chloroplast mRNA is not (Westhoff, 1981; Herrmann *et al.*, 1982) translation of SSU from poly (A) mRNA demonstrates that this protein is encoded on the nuclear genome. Bottomley and Bohnert (1982) suggest that the majority of chloroplast proteins are nuclear encoded.

I.2.C. PROTEIN TRANSPORT INTO CHLOROPLASTS

Chloroplasts possess the capability of synthesising their own proteins, as discussed in Section I.2.B. However, not all proteins located in the chloroplast are encoded on the chloroplast genome. The remainder are encoded by the nuclear genome and are therefore synthesised in the cytoplasm, (see Section 1.2.B.). Consequently cytoplasmically synthesised chloroplast proteins have to be imported by the organelle. This process has received widespread attention recently and a greater understanding of the mechanism involved has been obtained.

The import of precursor proteins into chloroplasts involves the following processes.

(i) Binding of precursors to the chloroplast

(ii) Translocation of precursors across the envelope membranes

(iii) Proteolytic cleavage of precursors in the stroma

(iv) Intra-organellar sorting of cleaved precursors to one of four possible destinations

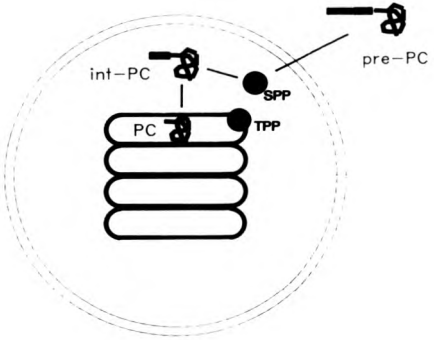
(a) The stroma
(b) The envelope
(c) The thylakoid membrane
(d) The thylakoid lumen

The latter includes a two step targeting process. Firstly, precursors are imported into the stroma and cleaved to an intermediate size by stroma processing peptidase (SPP). This intermediate is then targeted to and translocated across the thylakoid membrane. Finally, intermediates are cleaved to yield the mature sized protein in the lumen.

Figure 4 shows a schematic representation of transport and processing of the thylakoid lumen protein precursor preplastocyanin. Each of these steps is discussed in greater detail below.

FIGURE 4 - Schematic representation of protein transport into chloroplasts

The thylakoid lumen protein precursor preplastocyanin (pre PC) is synthesised in the cytoplasm with an N-terminal transit peptide. The precursor is imported into the chloroplast stroma and cleaved to an intermediate size (int-PC) by stromal processing peptidase (SPP). The intermediate is transported across the thylakoid membrane and cleaved to yield the mature sized protein (PC) by thylakoidal processing peptidase (TPP).


pre-PC
int-PC
SPP
PC
TPP

I.2.D. THE STRUCTURE AND INVOLVEMENT OF THE TRANSIT PEPTIDE IN TARGETING OF PRECURSOR PROTEINS TO CHLOROPLASTS

All chloroplast proteins synthesised in the cytoplasm are synthesised as higher molecular weight precursors with cleavable N-terminal transit peptides (von Heijne et al., 1989; see Keegstra 1989 for review). The involvement of a transit peptide in targeting precursors of Rubisco small subunit, a cytoplasmically synthesised stromal protein to the chloroplast was shown by Mishkind et al. (1985). These workers demonstrated that in in vitro experiments, Rubisco SSU possessing its transit peptide was imported into isolated chloroplasts. SSU which lacked a transit peptide was not imported into isolated chloroplasts. In most cases the information for targeting of precursors to the chloroplast resides within the transit peptide. In some cases foreign (non chloroplast) proteins can be targeted to the chloroplast (Van den Broeck et al., 1985). These workers achieved targeting, both in vivo and in vitro, of a bacterial protein into the chloroplast stroma. A chimeric gene protein product was used, in which the transit peptide from Rubisco SSU was fused to neomycin phosphotransferase. Schreier et al. (1985) also targeted a similar construct into the stroma in vivo.

The amino acid sequence of many chloroplast transit peptides has been determined (see von Heijne et al., 1989; Keegstra et al., 1989). No extensive primary structure homologies have been observed between the transit peptides of different

proteins from the same species. In numerous examples primary sequence similarities have been observed from different plant species for individual precursors (see von Heijne et al., 1989; Keegstra et al., 1989). Conserved structural features exist which are important for recognition of the transit peptide by the import machinery. In the case of ferredoxin a region of transit peptide in positions -2 to -7 (relative to the cleavage site) is important for binding to the chloroplast (Smeekens et al., 1989). These workers showed that precursors with deletions in this region failed to bind and import into isolated chloroplasts. A functionally similar sequence occurs relative to the stromal processing site in the transit peptide of the thylakoid lumen protein plastocyanin (Smeekens et al., 1989). In some cases deletion of only a few amino acids from the mature sequence will also reduce in vitro import and binding efficiency (Wassmann, et al., 1986; Reiss et al., 1987 and Smeekens, et al., 1989). This suggests that part of the mature protein is involved in import.

Secondary structure rather than primary structure may therefore be the essential feature recognised by the import apparatus. This would parallel the mitochondrial system (von Heijne et al., 1989) and eukaryotic signal sequences (von Heijne, 1985). The form of secondary structure essential for recognition by the import apparatus remains as yet unknown. However, the secondary structure of mitochondrial and chloroplast transit peptides must be

different. According to *in vivo* experiments with transgenic plants, N-terminal fusion of a mitochondrial or chloroplastic transit peptide to the same reporter gene resulted in targeting to the correct organelle (Boutry, 1987). Smeekens *et al.* (1987) showed that import into mitochondria could not be achieved in *in vitro* experiments with a chloroplastic transit peptide fused to a mitochondrial protein. Inefficient targeting of proteins into yeast mitochondria, using a chloroplastic transit peptide has been reported (Hurt *et al.*, 1986). This study employed transit peptides with large C terminal deletions, which possibly may have removed specific targeting information (Smeekens *et al.*, 1987).

The transit peptide of thylakoid lumen proteins is more complicated than that of stromal proteins and can be divided into two domains (Smeekens *et al.*, 1986; Hageman *et al.*, 1986). The envelope transfer domain occurs at the N-terminus and is responsible for targeting to the stroma. This domain is proteolytically removed by SPP. A more hydrophobic segment of the transit peptide termed the 'thylakoid transfer domain' is then responsible for targeting to the thylakoid. Structural similarities exist between the thylakoid transfer domain and signal sequences of precursors in eukaryotic and bacterial systems (Smeekens and Weisbeek, 1988). These similarities may reflect a common evolutionary origin, from bacteria to chloroplast. Thus, after transfer of genes for thylakoid lumen protein precursors from the chloroplast to nucleus the sequence

encoding the envelope transfer domain would have to be acquired (Smeekens et al., 1989). Cheung et al. (1988) have successfully relocated a chloroplast gene, psbA, fused in frame to a sequence encoding SSU transit peptide, to the nuclear genome and observed correct import and assembly of the chimeric protein in vivo in transformed plants. This indicates that relocation of a chloroplast gene to the nucleus can yield a product capable of correct localisation in chloroplasts. Studies in yeast have shown that genes for mitochondrial proteins devoid of transit peptides can acquire weakly active N-terminal targeting sequences, by DNA rearrangement (Verner and Schatz, 1988). A similar process may occur in chloroplasts, with selective 'fine tuning' of newly acquired targeting sequences. Figure 5 shows a diagrammatic representation of a thylakoid lumen protein precursor.

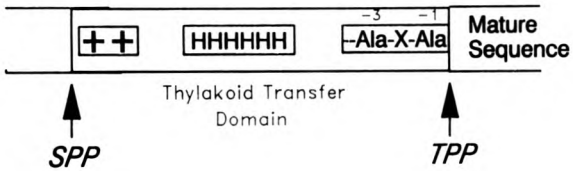
One exception to the system described above has been observed in Chlamydomonas (Grim et al., 1989). These workers desribe a 22KDa heat shock protein which apparently lacks a cleavable transit peptide. This protein may exploit an alternative import pathway in a similar manner to that of cytochrome C in mitochondria (see Pfanner et al., 1988). However, further work is required for conclusive proof of the transit peptide deficiency from this protein (Grim et al., 1989).

FIGURE 5 - Diagrammatic representation of a thylakoid lumen protein precursor

Thylakoid lumen protein precursors possess a two domain N-terminal transit peptide consisting of an envelope targeting and thylakoid transfer domain.

The envelope targeting domain is cleaved by stromal processing peptidase (SPP) at the point indicated by the arrow. The remaining thylakoid transfer domain is cleaved by thylakoidal processing peptidase (TPP) at the point indicated by the arrow. The two cleavage steps yield the mature sized protein.

The thylakoidal transfer domain possess a hydrophilic N-terminus (++) and hydrophobic core (H). The sequence immediately prior to the cleavage site is $Ala_{-3} - x - Ala_{-1}$


++
HHHHHH
−3 −1
-Ala-X-Ala
Mature Sequence
Thylakoid Transfer Domain
SPP
TPP

I.2.E. BINDING AND TRANSLOCATION OF CHLOROPLAST PROTEIN PRECURSORS REQUIRES ATP

Protein import into chloroplasts occurs post translationally (Grossman et al., 1980; Chua and Schmidt, 1978; Highfield and Ellis, 1978). Chloroplast protein precursors synthesised in the cytoplasm bind to the chloroplast surface (Pfistner et al., 1982). The binding mechanism has been shown to be protease sensitive and specific for chloroplast protein precursors. This suggests that involvement of a proteinaceous receptor (Cline et al., 1985). Binding of a variety of precursors including pre SSU, preferredoxin and preplastocyanin has a requirement for ATP hydrolysis (Olsen et al., 1989). A variety of techniques have been used to identify a putative receptor protein. Cornwell and Keegstra (1987) crosslinked the precursor of Rubisco small subunit with a 66KDa envelope protein using a photoactivatable crosslinker. Pain et al. (1987) raised anti-idiotypic antibodies to a 30 residue synthetic peptide of Rubisco small subunit transit peptide which bound to a 30KDa envelope protein. The identification of two putative receptor proteins may be due to the existence of a multimeric receptor protein. Alternatively one of the identified proteins may be involved elsewhere in the import complex (Keegstra, 1989).

The import of chloroplast protein precursors has an energy requirement which differs from that of mitochondria (see Section I.3.A.). Mitochondrial protein import has a

requirement for ATP hydrolysis as well as a membrane potential (Eilers and Schatz, 1988). However, chloroplast protein import has a requirement for ATP hydrolysis but not an energy potential (Cline et al., 1985; Grossman et al., 1980; Pain and Blobel, 1987 and Flugge and Hinz, 1986). ATP hydrolysis occurs during the binding of precursors to the putative receptor protein (Olsen et al., 1989). The hydrolysis of ATP may be required for phosphorylation of a 51KDa envelope protein (Hinz and Flugge, 1988). Included in the observations of these workers was the fact that the 51KDa protein was specifically phosphorylated during the binding of chloroplast precursor proteins in vitro. Phosphorylation did not occur in the presence of mature protein alone. The location of the ATP hydrolysis in chloroplast precursor binding and translocation remains to be confirmed (Keegstra, 1989; Theg et al., 1989).

The conformation of proteins translocated across the chloroplast envelope membrane has been studied. Incorporation of stop transfer sequences (a hydrophobic segment of protein which anchor translocated proteins in the membrane) in pre SSU did not prevent translocation across the envelope membrane (Lubben et al., 1988). This may be due to the concealment of the stop transfer region by the folded protein or a highly specific translocation apparatus, which does not recognise ER stop transfer sequences. Della Cioppa and Kirshore (1988) have shown that the precursor pre-5 enolpyrurylshikimate-3-phosphate synthetase (pEPSPS) exists in an active conformation. These workers showed that this

precursor displays some degree of unfolding during translocation (Della Cioppa and Kirshore, 1988) and that import of pEPSPS was inhibited when held in a rigid conformation by bound ligand (glyphosate). Unfolding of precursors during mitochondrial protein import has been demonstrated (Eilers and Schatz, 1986); a parallel mechanism may occur in chloroplasts. Unfolding may occur during the binding to a receptor and/or translocation complex and require ATP hydrolysis. An alternative hypothesis discussed by Keegstra (1989) is for temporary rearrangement of the envelope membrane bilayer. This hypothesis provides a possible explanation as to how precursors with inserted stop transfer sequences, and many foreign proteins, can import into chloroplasts with the appropriate transit peptide (see Keegstra, 1989) but fails to explain why a folded precursor cannot be imported (Della Cioppa and Kirshore, 1988). Further investigation is required for a full understanding of the conformation of chloroplast imported proteins.

I.2.F. INTRA CHLOROPLAST SORTING

The fate of precursors translocated across the envelope membrane depends on their final destination, which may be any of the following locations:

(i) The envelope

(ii) The stroma

(iii) The thylakoid membrane

(iv) The thylakoid lumen

Targeting to each location is discussed below.

(i) The Envelope

Very little information is available on the biogenesis of chloroplast envelope proteins (Smeekens et al., 1990; Keegstra, 1989). These two reviews indicate that this area is an emerging technology. Keegstra (1989) argues that parallels with mitochondrial membranes may occur, involving import and then retargeting to the envelope membrane. Flugge et al. (1989) have demonstrated in vitro import, processing and retargeting to the inner envelope membrane of the triose phosphate 3 phosphoglycerate phosphate translocator into isolated chloroplasts.

(ii) The Stroma

During or shortly after import of precursors across the envelope membrane a soluble processing peptidase (SPP) cleaves precursors to the mature size (Chua and Schmidt, 1978) (see Section I.2.D). In in vitro systems cleavage of pre SSU in the stroma was followed by assembly into the holoenzyme (Chua and Schmidt, 1978). The maturation of precursors by a stromal processing peptidase represents the final step in targeting to the stroma. Proteins are retained in the stromal compartment in the absence of any additional targeting information, which may

target the protein to the thylakoid membrane or thylakoid lumen as discussed below.

(iii) The Thylakoid Membrane

The light harvesting chlorophyll a/b binding protein (LHCP) of photosystem II is an integral thylakoid membrane protein encoded by a family of nuclear genes (Schmidt et al., 1982). Precursors to LHCP (pre LHCP) have an N-terminal targeting transit peptide which is proteolytically removed in the stroma by a stromal peptidase (Schmidt et al., 1984, Chitinis et al., 1986). Recent studies have uncovered the likely mechanism of pre LHCP targeting to thylakoid membranes. Lamppa (1988) replaced pre LHCP transit peptide with that of pre SSU and achieved correct targeting and assembly of LHCP in the thylakoid membrane. The information for thylakoid membrane targeting must therefore reside in the mature protein (Lamppa, 1988). Interaction between stromal factors and pre-LHCP is required for insertion into the thylakoid membrane (Cline, 1986). These factors are proteinaceous but differ from signal recognition particle of the ER (see Section I.4.B) since they are ribonuclease resistant and thus contain no RNA element (Fulson and Cline, 1988). In vitro studies by Cline (1986) suggest that insertion into the thylakoid membrane requires ATP hydrolysis. Smeekens et al. (1990) suggest that this may be for precursor unfolding.

Evidence suggests that cleavage of the transit peptide is performed after membrane insertion, since observation of intact precursors in the membrane has been made. (Chitinis et al., 1986, 1987, 1988; Cline, 1986). This appears to contradict the observation that precursors targeted to the chloroplast are processed soon after or during import (Robinson and Ellis, 1984a). This awaits further study (Chitinis et al., 1988).

The evidence discussed above indicates that targeting of thylakoid membrane proteins requires information residing in the mature sequence and assistance from stromal factors.

(iv) The Thylakoid Lumen

The transit peptides of thylakoid lumen proteins possess information which targets proteins to the stroma and thylakoid. This is reflected in the two domain structure of the transit peptide suggested by Smeekens et al. (1986). In in vitro chloroplast import systems attempts have been made to target foreign proteins to the thylakoid lumen (Smeekens et al., 1986, 1987; Meadows et al., 1989). Smeekens et al. (1986, 1987) fused the plastocyanin transit peptide onto the N terminus of a foreign protein. This resulted in accumulation of intermediates in the stroma. Lack of transport of these constructs

into the lumen can be attributed to either blockage of translocation, due to the adoption of an unfavourable conformation of the passenger protein, or the absence of the essential targeting information present within the mature sequence of plastocyanin (Smeekens et al. 1986, 1987). Smeekens et al. (1987) suggest that stromal intermediates of chimeric constructs using plastocyanin transit peptides and foreign proteins are found attached to the stromal side of the thylakoid membrane.

This would suggest that targeting information resides in the transit peptide and additional information, from the mature sequence, is required for translocation. Binding in these experiments is very weak and may be due to lack of mature sequence contributing the necessary information for tight binding to the membrane. Meadows et al. (1989) achieved in vitro import of a foreign protein, dihydrofolate reductase (DHFR) into the lumen, by fusing the transit peptide of pre-33KDa OEC to the N terminus of DHFR. This construct contained 22 residues of the mature protein and therefore this portion of mature protein may have contributed towards targeting to the lumen.

Kirwin *et al*. (1989) demonstrated an ATP requirement for *in vitro* import of thylakoid lumen protein precursors by isolated thylakoids. This requirement may be in the form of energy for precursor unfolding as observed in mitochondria (Eilers and Schatz, 1986; Pfanner *et al*., 1988). Regions within the mature sequence may be recognised by a putative 'unfoldase' enzyme. Stromal intermediates translocated across the thylakoid membrane are cleaved to the mature size by a specific thylakoid processing peptidase. (See Section I.2.C.) Further work is required to unravel the import mechanism and possible locations of targeting information within the mature sequence.

I.3. PROTEIN TRANSPORT INTO MITOCHONDRIA

The import of cytoplasmically synthesised proteins into mitochondria has been extensively investigated as reviewed by Nicholson and Neupert (1988) and Pfanner et al. (1988). An in-depth analysis is not presented in this thesis; however, to permit a comparison with the chloroplastic mechanism the important aspects are presented below.

The vast majority of mitochondrial proteins are synthesised in the cytoplasm and transported into the organelle (Hurt and Van Loon, 1986). Cytoplasmically synthesised mitochondrial proteins are usually made as precursors with N-terminal targeting sequences (Maccecchini et al., 1979; Lewin et al., 1980). However, certain cases exist in which mitochondrial proteins have no N-terminal pre-sequence but still contain targeting information (Hase et al., 1984). Both types of protein are imported into mitochondria post translationally (see Nicholson and Neupert, 1988). The following steps are involved in mitochondrial protein transport.

I.3.A. BINDING TO THE OUTER MEMBRANE

Targeting information for the majority of mitochondrial proteins synthesised in the cytoplasm resides in the transit peptide (see Schatz, 1987). These precursors bind loosely to one of three proteinaceous receptors on the mitochondrial surface and are then inserted into a common receptor in the

outer membrane termed the general insertion protein (GIP). Association with GIP conveys resistance to externally added proteases (see Pfanner et al., 1988). This binding and insertion step has a requirement for hydrolysis of ATP (Eilers and Schatz, 1986, 1988; Pfanner et al., 1988). Using a mouse mitochondrial pre-sequence fused to dihydrofolate reductase (DHFR), Eilers and Schatz (1986, 1988) demonstrated that unfolding of precursor was required for import. They showed that holding DHFR in a conformationally rigid state, by binding ligand (methotrexate) inhibited import into isolated mitochondria.

I.3.B. TRANSLOCATION ACROSS THE MEMBRANE

For protein translocation to occur, mitochondrial inner and outer membranes adopt a special conformation called contact sites (Schleyer and Neupert, 1985). Contact sites are formed by close association of the two membranes by a single polypeptide (Schleyer and Neupert, 1985; Pfanner et al., 1988). It has been proposed by Pfanner et al. (1988) that contact sites contain specific proteins involved in the translocation step. The involvement of contact sites was observed in various experiments in which translocation of bound protein was blocked (see Pfanner et al., 1988). Insertion of precursor proteins into contact sites, but not completion of precursor translocation across the membrane, requires a membrane potential but not ATP hydrolysis (Schleyer et al., 1982; Pfanner et al., 1988). The import of only the amino terminus was shown to require a membrane

potential (Schleyer et al., 1982). Pfanner et al. (1988) and Nicholson and Neupert (1988) propose that the membrane potential is required for electrophoresis of the positively charged N-terminal pre-sequence into the membrane receptor complex.

The mechanism for translocation of the remaining protein is as yet unknown. However, Verner and Schatz (1988) suggest that translocation may be powered by folding to a conformation more thermodynamically stable on the trans side of the membrane. They suggest that whilst the difference in free energy is small, proteins will be 'locked' within the matrix by lack of unfolding machinery.

I.3.C. PROTEOLYTIC PROCESSING IN THE MATRIX AND RETARGETING TO THE INTER MEMBRANE SPACE

Precursor proteins translocated into the matrix are proteolytically cleaved by a specific metal ion dependent protease (matrix processing peptidase MPP). MPP is assisted by an additional 'enhancing protein', Processing Enhancing Protein (PEP) (Bohni et al., 1980, 1983; Henning and Neupert, 1981; Haulitshetk et al., 1988). For proteins targeted to the inter membrane space e.g., cytochrome b2, cleavage by MPP removes the hydrophilic N-terminal region of the transit peptide. The hydrophobic region, which facilitates targeting back across the inner membrane, is then removed by a peptidase on the outer surface of the inner membrane (see Nicholson and Neupert, 1988). Pfanner

et al. (1988) suggest that mitochondrial proteins are sorted to their final destination via the matrix thereby following their evolutionary pathway. However, some inner membrane proteins may reach their destination by lateral diffusion from contact sites.

Ctyochrome c is imported by a different mechanism. The precursor binds to a putative protein receptor, then to cytochrome c lyase after translocation. Cytochrome c is released into the inner membrane space after addition of heme (see Pfanner et al., 1988).

I.4. PROTEIN TRANSPORT ACROSS THE ER MEMBRANE

Secretory proteins destined for export from eukaryotic cells are initially synthesised in the cytoplasm on membrane bound ribosomes of the rough endoplasmic reticulum (ER).

This section outlines the transport mechanism for secretory proteins which involves the following stages:

(i) Precursor protein synthesis on ER bound ribosomes
(ii) Translocation of protein into the ER lumen
(iii) Transport through the Golgi to secretory vesicles
(iv) Exocytosis and release of protein into extra cellular medium.

For reviews see Perara and Lingappa (1988) and Hanover and Dickson (1988).

The 'Signal sequence hypothesis' is the proposed mechanism for protein translocation across the ER (Blobel and Dobberstein, 1975 a,b). This hypothesis recognises the following key processes.

I.4.A. TARGETING INFORMATION RESIDES IN THE SIGNAL SEQUENCE

Milstein *et al*. (1972) demonstrated that immunoglobulin light chain was synthesised *in vitro* as a larger product than the authentic mature protein. Blobel and Dobberstein (1975 a,b) showed that secretory proteins were synthesised

with N-terminal 'signal sequences'. A cleavable signal sequence is found on nearly all eukaryotic secretory proteins (Perara and Lingappa, 1988). Signal sequences are typically 15-30 residues in length and show little sequence homology but extensive secondary structure homology (von Heijne, 1985). Common structural features include a hydrophobic core flanked by hydrophilic residues. Small side chain residues occur close to the cleavage site (von Heijne, 1984). The presence of a signal sequence has been shown to be sufficient for targeting and translocation of precursor proteins across the ER membrane (Lingappa et al., 1984). However, Perara and Lingappa (1988) review work which demonstrates that exceptions occur. In some cases secretory proteins may lack a signal sequence but contain a functional equivalent within the mature protein.

I.4.B. BINDING OF NASCENT PROTEIN TO RECEPTORS

The binding of the nascent polypeptide involved interaction with a multimeric protein - RNA complex. This complex, termed signal sequence recognition particle (SRP), migrates to and becomes associated with the ER membrane (Meyer et al., 1982). SRP temporarily halts translocation of the nascent protein (Walter and Blobel, 1981). Thus the function of SRP is to permit interaction of the nascent chain with an ER surface receptor (see below). Wickner (1989) proposes that SRP acts as a chaperonin (see Ellis and Hemingsen, 1989) to prevent aggregation prior to receptor binding. The SRP-ribosome complex has an affinity for a

receptor. Binding of the complex releases SRP from the ribosome permitting association of the transported protein with the receptor. Association between receptor and an aqueous pore may exist (see Perara and Lingappa, 1988).

Perara and Lingappa (1988) describe examples of proteins which do not recognise SRP and bypass the mechanism described above. In some instances signal sequences are capable of binding to a receptor without the involvement of SRP in a post-translational mechanism which may be abolished by treating the membranes with protease (Prehn et al., 1980, 1981; Gilmore and Blobel, 1985). Pre-promelittin is an example of a precursor which undergoes SRP independent, signal sequence specific translocation (Zimmerman and Mollay, 1986). Perara and Lingappa (1988) propose that the reason why some proteins undergo co-translational translocation and other post-translational translocation may be due to a necessity to maintain signal sequences in a conformation accessible by the receptor. For larger precursors, the duration when the signal sequence is accessible to the receptor is increased by association with the SRP.

I.4.C. TRANSLOCATION ACROSS THE MEMBRANE

Translocation of nascent protein across the ER membrane does not occur spontaneously or require ongoing protein synthesis. However, it does require energy in the form of NTP hydrolysis, which may be necessary for

- Maintenance of nascent chain in a transport-competent state

- Assembly or activation of a proteinaceous channel through the membrane or

- Direct motion of the protein through the membrane (see Perara and Lingappa, 1988). This hypothesis suggests that translocation occurs through an aqueous tunnel created by integral membrane proteins, for which Gilmore and Blobel (1985) have provided evidence.

(i) Maturation of Imported Proteins

An integral membrane processing protease (signal peptidase) cleaves the signal sequence from the nascent protein as it emerges into the ER lumen (Jackson and Blobel, 1977).

(ii) Secretion From the Cell

From the ER lumen mature proteins are exported by exocytosis involving the formation of secretory vesicles (Palade, 1975; Faquhar and Palade, 1981). This occurs in an orderly sequence of distinct isolatable compartments (see Hanover and Dickson, 1988 for a review).

I.5. BACTERIAL PROTEIN EXPORT

Extensive study has been performed on bacterial protein transport and a model for the mechanism has been proposed in a review by Randall *et al*. (1987). Some parallels and differences exist between this model and protein transport across the ER (see Section I.9.). The salient features are presented below.

I.5.A. BINDING OF PRECURSORS TO THE CYTOPLASMIC MEMBRANE

Precursors destined to be exported from the bacterial cytoplasm interact with soluble cytoplasmic factors prior to binding to the membrane. Two cytoplasmic factors have been identified.

(i) Trigger Factor

This binds a precursor named pro outer membrane protein A (pro ompA), destined for the outer membrane either during or after translocation (Crooke *et al*., 1988). The complex produced by pro ompA and trigger factor is targeted to the periplasmic space. Pro ompA then binds to a receptor protein *sec Y*, with the sub- sequent loss of trigger factor (see Meyer, 1988).

(ii) Sec B Protein

This protein performs a functionally analogous binding and targeting reaction to trigger factor (see Meyer, 1988) for a subset of proteins, including the periplasmic maltose binding protein (MBP) Weiss et al. (1988). The interaction between precursor of MBP (pre-MBP) and sec B protein transiently holds pre-MBP in an unfolded state to permit translocation (Weiss et al., 1988; Collier et al., 1988).

It is possible that trigger factor and sec B protein perform an analogous function to SRP in eukaryotes (See Section I.4.B.). Their role may therefore be to prevent the folding of the precursor and allow subsequent targeting to a membrane receptor (Lill et al., 1988; Collier et al., 1988; Randall et al., 1987). In this capacity trigger factor and sec B may therefore represent chaperonins (see Ellis and Hemingsen, 1989). Meyer (1988) reviews work which demonstrates that SRP and trigger factor can be functionally exchanged supporting the suggestion of analogous roles.

Randall et al. (1988) suggest that precursors bind to sec Y protein via the leader sequence. They suggest that part of the mature protein may also be associated with sec Y protein binding thereby maintaining the precursor in an unfolded state. Also proposed by these workers is the involvement of an ATP requirement for the dissociation of precursor from

sec B or trigger factor. The ATP hydrolysis may be performed by another soluble factor, sec A protein (Meyer, 1988).

I.5.B. TRANSLOCATION

The translocation function is probably performed by sec Y in a translationally-independant process which appears to require an energy potential. However, this demands further characterisation (see Randall et al., 1987).

I.5.C. CLEAVAGE

Exported precursors are proteolytically cleaved to yield mature protein. Cleavage is performed via a mechanism independent of translation by an integral membrane protease termed leader peptidase (LEP) (Zwinzinski and Wickner, 1980). LEP is discussed in more detail in Section I.6.D.

I.6. PROCESSING PEPTIDASES

Previous sections of this thesis have discussed the translation and targeting of precursors across chloroplast, mitochondrial, ER and bacterial membranes. All of these mechanisms exploit the use of an N-terminal targeting pre-sequence which is proteolytically removed during or shortly after transport. Removal of this targeting sequence appears to be essential since it is ubiquitous in protein transport across membranes. The cleavage step may be vital for the protein to adopt the correct conformation for assembly (Taylor et al. 1988). This section considers the properties of these proteases and compares characteristics and mechanisms.

I.6.A. STROMAL PROCESSING PEPTIDASE (SPP)

Cytoplasmically synthesised precursor proteins targeted to the stroma are cleaved by a soluble peptidase located in the stroma, during or shortly after import (Smith and Ellis, 1979; Robinson and Ellis 1984a). Precursors destined for the thylakoid lumen are cleaved to an intermediate size by the same peptidase (Robinson and Ellis, 1984a; Chia and Arntzen, 1986). The peptidase responsible for cleavage in the stroma is a specific metallo-endopeptidase with a molecular mass of around 180KDa (Robinson and Ellis 1984a). Processing of pre SSU targeted to the stroma is carried out as a two step process (Robinson and Ellis, 1984b; Marks et al., 1986) probably by the same enzyme (Robinson and Ellis,

1984b). SPP may recognise secondary rather than primary structure of the transit peptide (Robinson and Ellis, 1985). Wassman et al. (1986) propose that in some cases a specific sequence at the C terminus of the transit peptide (Ile-Thr-Ser) is required for processing, but do not suggest possible secondary structure influence for recognition.

A stromal peptidase is responsible for maturation of the precursor of the thylakoid membrane proteins LHCP (Abad et al., 1989). It is possible that the same protease cleaves stromal and thylakoid membrane precursors to the mature size. Verification of this will have to await purification to homogeneity.

I.6.B. MATRIX PROCESSING PEPTIDASE (MPP)

The majority of protein precursors entering the mitochondrial matrix are cleaved by a soluble protease (Bohni et al., 1980, 1983) to the mature size (Cerletti et al., 1983) in a two step mechanism (Schmidt et al., 1984) whilst most proteins of inner membranes' outer surface are cleaved only to an intermediate (Zimmerman and Neupert, 1980). MPP processing activity has been ascribed to a 57KDa protein and has a requirement for a 52KDa 'enhancing protein' cofactor (PEP) (Haulitshetk et al., 1988). PEP may act as a chaperonin (see Ellis and Hemingsen, 1989 for review) during presentation of precursor to MPP. The gene for MPP has been identified in yeast (mas I) and occurs as a precursor mediating its own cleavage (Varner and Schatz, 1988).

MPP and SPP display similar characteristics in requirement for metal ion cofactors and soluble proteins. However they differ in molecular weight. SPP has not been purified to homogeneity and contaminating bands in the purification may represent an 'enhancing' protein. The difference in molecular weight between MPP and SPP is quite large. It is therefore conceivable that this difference is due to association with another essential protein.

I.6.C. MITOCHONDRIAL INTER MEMBRANE SPACE PROTEASE

For proteins targeted to the mitochondrial inter membrane space, transport and cleavage in the matrix is followed by retargeting to the inner membrane. (Pfanner et al., 1988). A second cleavage may also occur in some cases involving an additional peptidase. This cleavage step involves removal of the hydrophobic terminal sequence by a peptidase on the outer surface of the inner membrane (Pfanner et al., 1988).

I.6.D. LEADER PEPTIDASE (LEP)

Exported bacterial precursors are cleaved to the mature size by a specific peptidase, Leader Peptidase (LEP) (Zwinzinski and Wickner, 1980). Wolfe et al. (1983) have shown this peptidase to be an integral membrane protein of the periplasmic membrane, with a molecular weight of 36KDa. The active site is on the trans side of the membrane, i.e., in the periplasm (Zimmerman et al., 1982). The cleavage step performed by LEP is essential for the release of exported

proteins from the membrane (Dalbey and Wickner, 1985). Release of exported proteins in this manner may be due to the fact that the leader peptide is capable of anchoring the precursors to the membrane, preventing release (Dalbey and Wickner, 1985). LEP is synthesised with a non-cleavable N-terminal leader peptide (Wolfe *et al.*, 1983) in common with other *E. coli* membrane proteins. Inhibition *in vitro* of LEP can be produced by the synthetic leader sequence to bacteriophage M13 procoat (Wickner *et al.*, 1987). This suggests that the leader sequence contains the information for recognition by LEP (Wickner *et al.*, 1987). In the absence of sequence homology a hydrophobic central core and short chain residues at -1 and -3 (relative to the processing site) may be key features recognised by LEP (von Heijne, 1983, 1985).

Additional processing peptidases are involved in the maturation of other translocated proteins in bacteria such as lipoproteins (see Randall *et al.*, 1987).

I.6.E. SIGNAL SEQUENCE PEPTIDASE

The signal sequence of proteins translocated into the ER lumen is proteolytically removed by sequence peptidase (Blobel and Dobberstein, 1975 a,b; Jackson and Blobel, 1977). This processing peptidase is an integral membrane protein (Lively and Walsh, 1983). Purification of signal peptidase shows it to exist as a multimeric complex (Evans *et al.*, 1986). Since LEP exists as a single protein, Evans

et al. (1986) suggest that some of the additional proteins are part of a translocation pore complex. The active site of the protease is on the lumenal face (Jackson and Blobel, 1977).

I.6.F THYLAKOIDAL PROCESSING PEPTIDASE (TPP)

Thylakoid lumen protein precursors are cleaved to the mature size by a protease in the thylakoid (Hageman et al., 1986).

This peptidase is the subject of work presented in this thesis and is therefore not discussed in detail here.

A summary of the requirements of protein transport across the membranes of chloroplasts, mitochondria, bacteria and ER is presented in Table 1.

TABLE 1 - Summary of requirements and properties of protein import into chloroplasts and mitochondrian and protein translocation across ER and bacterial plasma membrane

Organelle or Membrane System	Processing Peptidase involved	Soluble Factors Associated	Membrane Receptor involved	Translocation Energy Requirement
CHLOROPLASTS				
Destination of protein				
1. Stroma	SPP (soluble metallo peptidase)	Putative unfoldase	Putative in envelope	ATP - i - to cross envelope
2. Thylakoid Membrane	SPP (soluble metallo peptidase)	Putative unfoldase	Putative in envelope	ATP - i - to cross envelope - ii - incorporation in the thylakoid
3. Thylakoid Lumen	TWO STEP - SPP - TPP (membrane bound)	Putative unfoldase	Putative in envelope and thylakoid	ATP - i - to cross envelope - ii - to cross thylakoid membrane
MITOCHONDRIA				
Destination of protein				
1. Matrix	MPP (soluble metallo)	Yes	3 separate in outer membrane plus GIP	ATP + electrical potential
2. Inter	TWO STEP - MPP - Inter membrane space protease	Yes		
BACTERIAL PLASMA MEMBRANE	LEP (membrane bound)	Trigger factor sec B	sec Y	ATP + electrical potential
E.R.	Signal peptidase (membrane bound)	SRP	Docking protein	ATP

I.7 AIMS AND APPROACHES

The maturation of the thylakoid lumen protein precursor plastocyanin (pre-PC) involves successive cleavages by a stromal processing peptidase (SPP) and a thylakoidal processing peptidase (TPP) (Hageman *et al.*, 1986). The discovery of TPP came from work by Hageman *et al.* (1986) who showed that the stromal intermediate of plastocyanin could be converted to the mature size by Triton X-100 solubilised pea thylakoids. In the light of the discovery of this enzyme the aims of the work presented in this thesis were to characterise TPP in detail and thereby gain a greater understanding of the involvement of TPP in the import of thylakoid lumen protein precursors.

The major objectives of this thesis were as follows:

(i) To partially purify and characterise TPP

(ii) To analyse the organisation and orientation of TPP in the thylakoid network

(iii) To determine the reaction specificity of TPP; Hageman *et al.* (1986) showed that TPP processes pre-PC to the mature size but did not test whether the import of other thylakoid lumen proteins involves cleavage by TPP

(iv) To compare the TPP reaction mechanism with those of other processing peptidases.

II : MATERIALS AND METHODS

II.1. CHEMICALS

The majority of chemicals were purchased from Sigma (Poole, Dorset). Listed below are the sources of the other main chemicals used during this project.

Amersham International (Amersham, Bucks.);

[^{35}S] methionine >1000 Ci/mmol

[^{3}H] lysine approx 10 Ci/mmol

[^{3}H]leucine approx 10 Ci/mmol

Amplify

Biotin streptavidin Western Blot kit

SP6 RNA polymerase

BDH (Poole, Dorset);

EDTA

Acrylamide (Electran grade)

Bis-acrylamide

TEMED

HEPES

Acetone

Sucrose

Pharmacia (Uppsala, Sweden);

DEAE-Sephacel

RNase inhibitor (placental)

CAP ($m^7G(5')ppp(5')G$)

Biorad (Richmond, California);

Hydroxyl apatite

Fluka (Glossop, Derbyshire);

EGTA

Mercaptoethanol

II.2. GROWTH OF PLANTS

Pea seeds (*Pisum sativum* variety Feltham First) were obtained from Booker Seeds, Ltd., Sleaford. Seeds were sown in compost (Fisons Levington Multipurpose) watered and placed under 'warmlight' fluorescent lamps (Philips) with a 12h photo period. Light intensity was approximately 50μE/m^2/sec and the growth room temperature was held at 20°C. Water was applied on alternate days. The age of plants was measured from the time of sowing.

II.3. ISOLATION OF THYLAKOIDS

II.3.A. PREPARATION OF CRUDE CHLOROPLAST PELLETS

Chloroplasts were isolated using a slightly modified version of the method described by Blair and Ellis (1973). Approximately 100g of leaves from ten day old pea seedlings were homogenised, using a Polytron (Northern Media Supplies, Hull) at setting 7 for three 4 second bursts in 400cm^3 of sucrose isolation medium (SIM) which contained

0.35M sucrose
2mM EDTA
25mM HEPES - NaOH pH 7.6

The homogenate was filtered through eight layers of muslin and the filtrate was centrifuged at 4000 x g for 1min at 4^{o}C. The supernatant was discarded and the chloroplast pellets were gently resuspended in a minimal volume of ice cold SIM using a cotton bud. Resuspended pellets were pooled and centrifuged at 4000 x g for 2min at 4^{o}C to give a washed chloroplast pellet.

II.3.B. PREPARATION OF WASHED THYLAKOID PELLETS

Washed chloroplast pellets (Section II.3.A.) were vigorously resuspended in lysis buffer, which contained

10mM Tris-HCl pH 7.0
5mM $MgCl_2$

using a $5cm^3$ wide bore pipette and held on ice for 15min to lyse. Thylakoid membranes were separated from stroma by pelleting in a centrifuge at 5000 x g for 10min at $4^{o}C$. Thylakoid pellets were washed four times in four volumes of resuspension medium which contained

20mM Tris-HCl pH 7.0
5mM $MgCl_2$
15mM NaCl

to remove any contaminating stromal proteins, pelleting membranes as before between each wash.

II.4. CELL FREE TRANSCRIPTION AND TRANSLATION OF THYLAKOID LUMEN PROTEIN PRECURSORS

II.4.A. SP6 mRNA TRANSCRIPTION

Clones encoding various thylakoid lumen protein precursors were obtained. Full length cDNA inserts encoding pre-33KDa and pre-23KDa OEC polypeptides were cloned (by J. Meadows and H. James) into the EcoRI site of plasmid pGEM4Z, in the correct orientation for transcription by SP6 polymerase. An artificial intermediate to preplastocyanin (PC1) which possesses a few residues of the envelope targeting domain and the entire thylakoid transfer domain of the transit peptide (Hageman et al., 1986) was a gift from Professor Weisbeek (Utrecht). The transcription mix contained

20 units RNase inhibitor (0.5µl)
0.25 units CAP (m^7G(5')ppp(5')G) (1.0µl)
2 µl Non linearised DNA at 1µg/µl

A 15.5ul aliquot of pre mix from lab stocks was added to the transcription mix giving a final concentration of

0.1mg/ml BSA
10.5mM dithiothreitol
41.7mM Tris-HCl pH 7.5
6.0mM $MgCl_2$
2.0mM Spermine

520uM ATP

520μM CTP

520μM UTP

50μm GTP

1.0μl of SP6 RNA polymerase (0.25 units) was the last constituent added to the mix which was then incubated at 40°C for 30min. 0.5μl of GTP was then added to a concentration of 1mM and incubation continued for a further 30min. Transcriptions were stored in 5μl aliquots at -80°C.

II.4.B. WHEAT-GERM LYSATE TRANSLATION

Thylakoid lumen protein precursor mRNA was translated in a wheat-germ lysate system according to the method of Roberts and Patterson (1973). Translation was performed in sterile microcentrifuge tubes using lab stocks of wheat-germ lysate and energy mix to the following concentrations.

25% (v/v) wheat-germ extract

100mM K acetate

2mM Mg acetate

1mM Tris-ATP

100uM GTP

10mM creatine phosphate

4mM DTT

250uM Spermidine

50uM Spermine

50μM every protein amino acid except methionine

22.5mM HEPES-KOH pH 7.6

50μg/ml creatine phosphokinase

500μCi/ml [^{35}S] methionine

1μl mRNA *in vitro* transcription

The wheat-germ extract was the last constituent added and the mixture incubated for 60min at 27°C. Translations sizes were typically 12.5, 25 or 50μl.

II.4.C. MEASUREMENT OF [^{35}S] METHIONINE INCORPORATED INTO PROTEIN

After the translation incubation (Section II.4.B.), 2μl aliquots were transferred to 1cm x 1cm strips of Whatman No.1 paper and left to dry. The strips were boiled in 10% (w/v) trichloroacetic acid (25cm^3/strip). The strips were left in hot acid for 20min and then washed in an equal volume of 10% trichloroacetic acid at room temperature, followed by two washes in 100cm^3 ethanol. The strips were left to dry and counted in 4cm^3 scintillant (Optiphase) in an LKB Minibeta scintillation counter.

II.5. ANALYSIS OF PROTEINS BY POLYACRYLAMIDE-GEL ELECTROPHORESIS

II.5.A. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Slab gels (15.5cm x 12cm x 0.5cm) using the system of Laemmli (1970) were used. A resolving gel of approximately $20cm^3$ contained the following components.

15% (w/v) acrylamide
0.4% (w/v) bis acrylamide
0.1% (w/v) SDS
375mM Tris-HCl pH 8.8
.9.5% (w/v) ammonium persulphate

Immediately prior to pouring the resolving gel 8.3µl of TEMED were added. The resolving gel was poured, overlaid with water saturated butan-1-ol and allowed to polymerise. The butan-1-ol was washed off with water before pouring the stacking gel, which contained

6% (w/v) acrylamide
1.6% (w/v) bis acrylamide
1% (w/v) SDS
125mM Tris-HCl
1% (w/v) ammonium persulphate

Immediately prior to pouring the stacking gel 13µl of TEMED were added. The gel was poured, a well-former inserted and the gel allowed to polymerise. The well-former was then

removed, the well rinsed with H_2O and the gel assembled in an electrophoresis tank. Electrophoresis buffer contained

25mM Tris-base
192mM glycine
0.1% (w/v) SDS
pH 8.3

Samples were prepared for electrophoresis by addition of one volume of sample to one volume of 2 x sample buffer which contained

125mM Tris-HCl pH 6.8
2% (w/v) SDS
10% (v/v) glycerol
5% (v/v) 2-mercaptoethanol

The samples were boiled for 2min prior to loading into gel wells. Electrophoresis was carried out at 40mA for 1.5h and the gel removed for staining.

II.5.B. COOMASSIE BLUE SDS-PAGE GEL STAINING

Protein bands in SDS-PAGE gels (Section II.5.A.) were stained by soaking for 2h in a solution containing;

0.25% (w/v) Coomassie brilliant blue
50% (v/v) methanol
8% (v/v) acetic acid

Surplus stain was removed by washing in the following solution

40% (v/v) methanol
7% (v/v) acetic acid

Gels run to determine the protein profile were dried prior to storage. Gels used for assay, containing radiolabelled wheat-germ lysate translation products were fluorographed.

II.5.C. SILVER NITRATE SDS-PAGE GEL STAINING

Where required SDS-PAGE gels were silver nitrate stained according to Wray et al. (1981). SDS-PAGE gels were washed in 50% methanol for 16h and then stained for 15min with stain made up as follows; 4ml of 20% (w/v) silver nitrate was added dropwise to 21ml of 0.36% NaOH, 6.25% (w/v) NH_4OH and made up to 100ml with water. The gel was washed in copious amounts of distilled water and developed using a solution of 1mM citric acid, 10mM formaldehyde. After the gel had developed it was fixed in 40% (v/v) methanol, 7% acetic acid.

II.5.D. FLUOROGRAPHY

Gels stained with Coomassie blue (Section II.5.B.) were soaked for 15min in Amplify and the gel was dried. Orientation spots of radioactive ink were added to the edge of the gel prior to exposure to X-ray film.

II.6. PARTIAL PURIFICATION OF THYLAKOIDAL PROCESSING PEPTIDASE (TPP)

II.6.A. A STRATEGY

Purification of the TPP involved conventional, well characterised techniques. TPP activity was purified from isolated thylakoids of pea leaves (Section II.3.A, B) by Triton X-100 solubilisation, acetone precipitation, ion exchange and hydroxylapatite chromatography. Details of the purification are given below (Section II.6.B) and the assay protocol is described in Section III.1.B.

Solubilisation of thylakoid membranes with Triton X-100 was carried out by a modified method of Hageman et al. (1986). A range of concentrations were tested to optimise recovery of activity and limitation of contaminating proteins.

A concentration step was chosen prior to column chromatography to maximise the amount of activity loaded onto the column. A range of acetone concentrations and resolubilisation methods were explored. Alternative precipitation methods including ammonium sulphate and ethanol abolished processing activity.

Two column chromatography steps were employed, a hydroxylapatite column followed by DEAE-Sephacel ion exchange chromatography. Test runs were used to determine the best flow rates, column size and gradients.

Column eluates were assayed (Section II.7) to establish which fractions contained a peak of processing activity. Peak fractions were pooled for use in the next purification step. Difficulty was experienced in constructing reliable column profiles of absorbance at 280nm due to interference of absorbance by chlorophyll. The extent of each purification was observed by analysis of Coomassie and silver nitrate stained SDS-PAGE gels.

II.6.B. PURIFICATION PROTOCOL

All procedures were performed at $4^{o}C$.

(i) Preparation of 30 000 x g Supernatant

Washed thylakoid pellets from 1000g of pea leaves (Section II.3.A.) were resuspended in resuspension buffer (Section II.3.A.) to a chlorophyll concentration of $1mg/cm^3$, a typical yield being 350mg. Chlorophyll concentration was determined according to the method of Arnon (1949). Triton X-100 was added from a stock 25% v/v solution in 20mM Tris-HCl pH 7.0 to a final concentration of 0.15% (v/v). The mixture was stirred in the dark for 30min and centrifuged at 30 000 x g for 30min. The supernatant containing TPP (30 000 x g supernatant) was removed from the unsolubilised membrane pellet. Storage if required was at $-80^{o}C$.

(ii) Acetone Precipitation

30 000 x g supernatant (Section II.6.B. (i)) was mixed with an equal volume of ice cold acetone and left to precipitate for 30min. The sample was centrifuged at 3000 x g in 50cm^3 polypropylene tubes for 4min. The supernatant was discarded and the pellet thoroughly resuspended in 10cm^3 of 20mM Tris-HCl pH 7.0, 0.15% Triton X-100. Resuspension was performed by repeated drawing of sample into a 5cm^3 pipette, followed by repeated drawing into a 10cm^3 syringe with 25 gauge needle. The fine suspension produced was centrifuged at 3000 x g for 4min. The supernatant was removed and further centrifuged at 12 000 x g for 10min. The supernatant contained TPP activity and was therefore retained. The pellets were resuspended in a minimal volume of 20mM Tris-HCl pH 7.0, 0.15% Triton X-100 using a pipette and syringe as before. Low and high speed centrifugation and resuspension was repeated. The resulting active supernatants (acetone precipitate) were pooled and stored at -80^{o}C if required.

(iii) Hydroxyl Apatite and DEAE Chromatography

Acetone precipitate ((200ml) (Section II.6.B. (ii)) was loaded onto a 30ml hydroxyl apatite column. The column was pre-washed with 1M potassium phosphate

buffer pH 7.0, 0.15% Triton X-100 and pre-equilibrated with 10 column volumes of 20mM Tris-HCl pH 7.0, 0.15% Triton X-100. A 120ml linear gradient 0-100mM potassium phosphate pH 7.0, with 0.15% Triton X-100 was run at 20ml/h and 1ml fractions were collected using a Pharmacia Frac 100 collector. Activity of fractions was assayed (Section II.6.B. (i)) and the active fractions were loaded onto a 3ml DEAE-Sephacel column. The column was pre-washed in 20mM Tris-HCl pH 7.0, 0.15% Triton X-100, 1m NaCl and pre-equilibrated with 10 column volumes of 20mM Tris-HCl pH 7.0, 0.15% Triton X-100 and 0.5ml fractions were collected. The fractions were assayed for TPP activity.

II.7. ASSAY FOR TPP ACTIVITY

The presence of TPP was assayed by incubating samples with precursors to PCi pre-23KDa or pre-33KDa synthesised *in vitro* using a wheat-germ lysate (Section II.4.B.). Incubations contained 1µl of translation product, 1µl of 200mM EGTA and 18µl of sample. The sample was mixed by repeated pipetting and incubated for 90min at 27°C. The processing reaction was stopped by the addition of sample buffer (Section II.5.A.) and boiling for 2min. Samples were electrophoresed on a 15% SDS-PAGE gel and fluorographed (Section II.5.C.). The presence of TPP activity was demonstrated by the appearance of a radiolabelled band which co-migrated with the mature size of the precursor. Column fractions which produced the greatest quantity of labelled mature size protein indicated a peak of TPP activity.

II.8. N-TERMINAL AMINO ACID SEQUENCING BY EDMAN DEGRADATION

The N-terminal sequence of TPP cleaved thylakoid lumen protein precursors was determined by Edman degradation using an Applied Biosystems sequenator. Radiolabelled pre-23KDa and PCi proteins were produced by translation of the corresponding mRNAs in a 100µl wheat-germ lysate (Section II.4.B.), containing 2.1µCi/ml [^{3}H]lysine or 1.6µCi/ml [^{3}H]leucine, respectively. Translation products were cleaved by incubating with 600µl of acetone precipitate (Section II.6.B. (i)), at 27^{o}C for 90min and snap frozen with liquid nitrogen prior to freeze drying. The sample was resuspended in 100µl distilled water, and 100µl x 2 sample buffer (Section II.5.A.) was added before boiling for 2min.

Cleavage products were separated by SDS polyacrylamide gel electrophoresis. A 15% slab gel for Hoefer Scientific (California) Mighty Small II tank was prepared (Section II.5.A.) and pre run, with 50µM glutathione in the electrophoresis buffer, at 3mA for two hours. Samples were electrophoresed at 7mA for 1h with 0.1mM sodium thyiogylcolate in the upper reservoir electrophoresis buffer. The gel was blotted onto PVDF membrane and prepared for sequencing according to the method of Matsudaira (1987). Proteins were sequenced using an Applied Biosystems sequenator model 470A with on line PTH analysis using the 03RPTH program. PTH derivatives were analysed on a reverse phase Brownlee C18 column. Fractions (120µl) were collected from the sequenator and mixed with optiphase scintillant and counted as in Section II.4.C.

II.9. ARTIFICIAL INSERTION OF PCi INTO THE THYLAKOID LUMEN BY SONICATION

Washed isolated thylakoid pellets (Section II.3.B.) were resuspended in 20mM Tris-HCl pH 7.0 to a chlorophyll concentration of 1.5mg chlorophyll per ml. 10µl of wheat-germ translation containing PCi were added to a 400µl aliquot of thylakoids. The mixture was subjected to repeated two second bursts of sonication at 12µ followed by incubation at 27^{o}C for 2min. At various time points 10µl aliquots were removed and centrifuged at 100 000 x g. The supernatants were analysed by SDS-PAGE (Section II.5.A.). Control experiments were performed in which membranes were subjected to sonication prior to the addition of wheat-germ translation products.

II.10. SUCROSE GRADIENT AND SUCROSE PAD CENTRIFUGATION OF THYLAKOID EXTRACTS

II.10.A. SUCROSE GRADIENT CENTRIFUGATION

30 000 x g supernatant was prepared (Section II.6.B. (i)). A 4cm^3 aliquot was loaded onto a 6cm^3 linear sucrose gradient containing

0.1M - 0.7M sucrose
20mM Tris-HCl pH 7.0
2mM $MgCl_2$
0.1% Triton X-100

and centrifuged at 100 000 x g for 16h at 4^oC. The gradient was fractionated into 1cm^3 fractions.

II.10.B. SUCROSE PAD CENTRIFUGATION

30 000 x g supernatant was prepared (Section II.6.B. (i)). A 2.5cm^3 aliquot was loaded onto a 2.5cm^3 sucrose pad containing

0.2M sucrose
20mM Tris-HCl pH 7.0
2mM $MgCl_2$
0.1% Triton X-100

and centrifuged at 100 000 x g for 16h at 4°C. The sample was fractionated into 500µl fractions and the pellet resuspended in 200µl 20mM Tris pH 7.0, 0.5% Triton X-100.

II.10.C. ULTRACENTRIFUGATION OF 30 000 X G SUPERNATANT

30 000 x g supernatant was prepared as described in Section II.3.B. and subjected to ultracentrifugation at 200 000 x g, 4°C for 5h. The supernatant was removed and the pellet resuspended in an equal volume of 20mM Tris-HCl pH 7.0, 0.15% Triton X-100.

II.11 WESTERN BLOTTING

SDS-PAGE gels were blotted onto nitrocellulose membrane by electrophoresis for 90min at 60A in transfer buffer which contained

1% (w/v) glycine
25mM Tris
20% methanol

Nitrocellulose was then removed and blocked by shaking in phosphate buffer saline (PBS), 0.1% Triton X-100, 2% Marvel for 30min. The blot was sealed in polythene and antisera was added to the bag along with 5ml PBS, 2% Marvel and shaken for 4h at 20°C. The blot was removed from the bag and liberally soaked in PBS, 0.1% (v/v) Tween for 25min.

The blot was developed using the Biotin-Streptavidin kit from Amersham International as follows: biotinylated protein A was added to 0.33% (w/v) in PBS, 2% Marvel and shaken with the blot for 1h at 20°C. The blot was washed liberally in PBS, 0.1% Tween for 25min prior to incubation with biotinylated streptavidin at 0.33% (w/v) in PBS, 1% Tween for 30min followed by a rinse in PBS 1% Tween. The colour reaction was developed by shaking the blot in 10ml of 50mM Tris pH 7.3, 0.09% NaCl containing 6mg diaminobenzidine and 15µl H_2O_2. Developed blots were washed in water and photographed.

III : RESULTS AND DISCUSSION

III.1. PARTIAL PURIFICATION AND CHARACTERISATION OF TPP

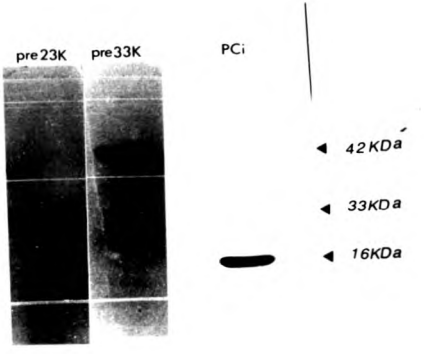
III.1.A. AN *IN VITRO* ASSAY FOR TPP ACTIVITY

During this work an *in vitro* assay based on that described by Hageman *et al*. (1986) was used to test for the presence of TPP in thylakoid extracts and column eluates. The assay employed ^{35}[S]met radiolabelled *in vitro* translation products of an artificial stromal intermediate of pre-plastocyanin. This clone was generated by P. Weisbeek (Utrecht) by isolation of a cDNA clone for preplastocyanin from *Silene pratensis*. The full length clone was subjected to exonuclease degradation to remove the initiation codon, translation therefore commences at the next initiation codon which fortunately produces a translation product just a few residues larger than the authentic stromal intermediate. Radiolabelled translation products generated using a wheat-germ lysate system were incubated with thylakoid extracts to test for the presence of TPP. Cleavage of PCi to the mature size, visualised by autoradiography of SDS-PAGE gels indicates the presence of TPP activity.

Figure 6 shows an autoradiograph of ^{35}[S]met labelled PCi *in vitro* translation products. This autoradiograph also shows *in vitro* translation products of precursors to two additional thylakoid lumen protein precursors analysed

FIGURE 6 - Wheat-germ lysate translation products for thylakoid lumen protein precursors pre-23KDa, pre-33KDa and PCi

mRNA transcripts were prepared *in vitro* by SP6 polymerase transcription of cDNA, as described in Section II.4.A. The transcripts were translated using an *in vitro* wheat-germ lysate system in the presence of ^{35}[S]met. 1µl aliquots were analysed by SDS-PAGE and visualised by autoradiography.


pre23K
pre33K
PCi
42KDa
33KDa
16KDa

during this project i.e. pre-23KDa and pre-33KDa of photosystem II oxygen evolving complex. Clones to pre-23KDa and pre-33KDa were isolated in this laboratory by H. James and J. Meadows from aλGT11 cDNA expression library of wheat. Studies on these precursors are described in Sections III.3. and III.4. Both PCi and pre-33KDa appear as discrete bands. However, pre-23KDa translation products occur as two bands, the upper band corresponds to the precursor of 23KDa. The additional band is likely to be due to translation from an internal initiation site within the transit peptide, or alternatively due to pre-23KDa cleavage to the stromal intermediate by SPP in the wheat-germ lysate mixture.

A typical <u>in vitro</u> processing assay is illustrated in the time course study presented in Figure 7. A crude extract containing TPP (30 000 x g supernatant, see Section II.6.B.) was prepared by Triton X-100 solubilisation of isolated pea thylakoids. <u>In vitro</u> translation products of PCi were incubated with 30 000 x g supernatant for various time intervals. Analysis of samples by SDS-PAGE and autoradiography reveals the presence of PCi in each track of Figure 7. The apparent difference in migration between the track 0 and the neighbouring track are due to distortion at the edge of the gel. However, samples incubated for at least 40min display an additional lower molecular weight band due to the presence of TPP in 30 000 x g supernatant. This can be inferred since the lower band co-migrates with that of mature plastocyanin prepared by <u>in vitro</u> import experiments, in which isolated chloroplasts import and

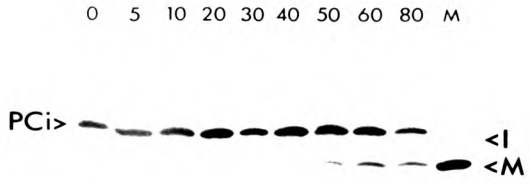
FIGURE 7 - Time course analysis of PCi maturation by TPP

A 100µl aliquot of 30 000 x g supernatant was mixed with 10µl of PCi and incubated at 27°C. At the time intervals indicated (minutes) a 10µl aliquot was removed, mixed with sample buffer and boiled for 2min. Samples were analysed by SDS-PAGE and autoradiography.

PCi - artificial intermediate translation product

I - authentic intermediate size

M - mature sized plastocyanin produced by the incubation of pre PC with isolated pea chloroplasts.


0 5 10 20 30 40 50 60 80 M
PCi>
<I
<M

process radiolabelled pre PC to the mature size. The slight variation of PCi migration in tracks 0 and 5 are due to distortion at the margin of the gel.

Processing activity is dependent upon the concentration of enzyme in the assay. Figure 8 shows processing can be observed with 15µl of 30 000 x g supernatant although activity in this preparation was weak.

FIGURE 8 - PCi processing; dependence on TPP concentration

2µl aliquots of PCi translations were mixed with 30 000 x g supernatant (amount in µl shown above each track). Each sample was made up to 20µl using 20mM Tris-HCl pH 7.0, 0.15% Triton X-100 and incubated at 27°C for 90 min. Samples were mixed with sample buffer, boiled for 2min and analysed by SDS-PAGE and visualised by autoradiography.

PCi - artificial plastocyanin intermediate

PC - mature sized plastocyanin


0
2
5
10
15
18
PCi
PC

III.1.B. PARTIAL PURIFICATION OF TPP ACTIVITY

Partial purification of TPP activity was required for two reasons. Firstly, a highly concentrated and active preparation of enzyme was deemed necessary for detailed characterisation. In addition purification was performed with the possibility of yielding a band on an SDS-PAGE gel to which TPP activity could be attributed. Antisera raised against the putative band could then be used for further investigation of TPP characteristics.

The purification protocol employed two chromatography techniques; hydroxyl apatite and DEAE-Sephacel chromatography. For an explanation of the purification strategy see Section II.6.A. PCi processing assays (Section II.1.A.) were used to monitor the eluates from chromatography columns for the presence of TPP activity. The protein profiles of each step were determined by analysis on silver nitrate stained SDS-PAGE gels, since absorbance at 280nm proved to be unreliable due to the presence of chlorophyll (Section II.6.A.).

(i) Hydroxyl Apatite Column Chromatography

Acetone precipitate (Section II.6.B.) was loaded onto a hydroxyl apatite column and eluted with a phosphate gradient as described in Section II.6.B. (ii). Figure 9 shows that in PCi processing assays TPP activity eluted in fractions 55 to 75. This

FIGURE 9 - Fractionation of PCi processing activity by hydroxyl apatite chromatography

30 000 x g supernatant was subjected to an acetone precipitation concentration step. Approximately 250ml of acetone precipitate was loaded onto a 30ml hydroxyl apatite column pre-equilibriated with 20mM Tris-HCl pH 7.0, 0.15% Triton X-100 and eluted with a linear 0-100mM phosphate gradient. 1ml fractions were collected and assayed for TPP activity. An 18µl aliquot was mixed with 1µl of PCi translation products, 1µl 300mM EGTA (see Section III.1.C.) and incubated for 90min at 27°C. Samples were then mixed with sample buffer, boiled for 2min and analysed by SDS-PAGE and autoradiography.

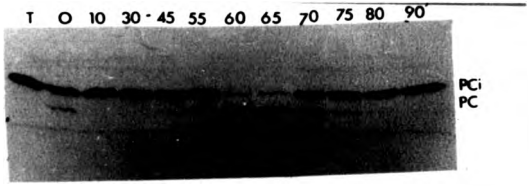
T - translation product

O - original sample loaded onto column

PCi - artificial plastocyanin intermediate

PC - mature size plastocyanin

Column fraction numbers are indicated above each track.


T O 10 30 45 55 60 65 70 75 80 90
PCi
PC

corresponds to the fractions containing the bulk of proteins visualised in the silver nitrate stained SDS-PAGE gel in Figure 10. However processing activity was separated from additional proteins, which were retained on the column and eluted in later fractions, thus partial purification was achieved.

(ii) DEAE-Sephacel Column Chromatography

Active fractions from hydroxyl apatite chromatography were subjected to further purification by DEAE-Sephacel chromatography. Some inconsistency as to the exact position of TPP elution from DEAE-Sephacel columns was experienced. Figure 11 shows PCi processing activity assays for TPP activity eluted with the wash volume and the protein profile (shown by the silver stained SDS-PAGE gel) in Figure 12 shows that a number of proteins were washed through the column along with TPP activity. Later column fractions show that additional proteins bound to the column before elution, thus again achieving partial purification of TPP.

Figure 13 shows a Coomassie stained gel for a typical purification of TPP from 30 000 x g supernatant using hydroxyl apatite and DEAE chromatography. This figure demonstrates the low recovery obtained, and illustrates the loss of major bands during the purification protocol.

FIGURE 10 - Fractionation of 30 000 x g proteins by hydroxyl apatite chromatography

Hydroxyl apatite column chromatography was performed on acetone precipitate (see Figure 9). A 30µl aliquot of each fraction was analysed by SDS-PAGE and stained by the silver nitrate method.

Fraction numbers are indicated above each track

O - original sample loaded onto column

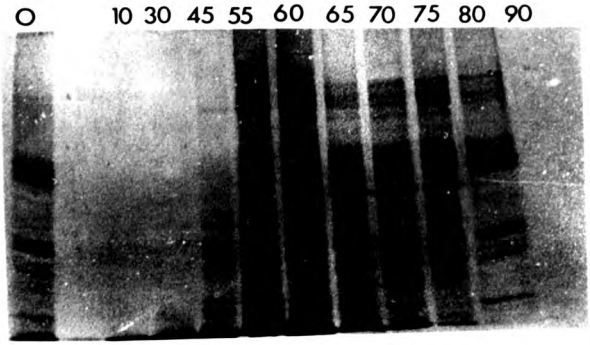

0
10 30 45 55 60 65 70 75 80 90

FIGURE 11 - Fractionation of PCi processing activity by DEAE-Sephacel chromatography

30 000 x g supernatant was partially purified by acetone precipitation and hydroxyl apatite column chromatography (see Section II.6.B.). Fractions from hydroxyl apatite chromatography which contained TPP activity were pooled and loaded onto a 3ml DEAE-Sephacel column, pre-equilibriated with 20mM Tris-HCl pH 7.0, 0.15% Triton X-100. The column was washed with approximately 25ml of 20mM Tris-HCl pH 7.0, 0.15% Triton X-100 and collected in 3 aliquots. A 0-100mM NaCl gradient was then used to elute the protein and 0.5ml fractions were collected. Column fractions were assayed for TPP activity, an 18µl aliquot was mixed with 1µl PCi translation product, 1µl 300mM EGTA and incubated for 90min at 27°C. Samples were mixed with sample buffer and boiled for 2min prior to analysis by SDS-PAGE and autoradiography.

Fraction numbers are indicated above each track.

T - translation product

O - original sample

W1 - column wash fraction 1

W3 - column wash fraction 3

PCi - artificial plastocyanin intermediate

PC - mature sized plastocyanin

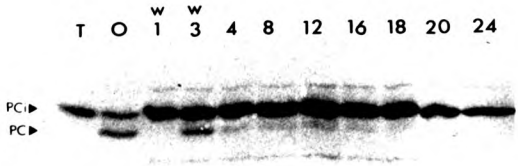

T
O
w 1
w 3
4
8
12
16
18
20
24
PCi
PC

FIGURE 12 - Fractionation of hydroxyl apatite eluate by DEAE-Sephacel chromatography

DEAE-Sephacel column chromatography was performed on hydroxyl apatite column fractions containing TPP activity (see Figure 11). A 30µl aliquot was analysed by SDS-PAGE and stained by the silver nitrate method.

Fraction numbers are indicated above each track.

Molecular weight marker sizes are indicated on the left.

O - original sample loaded onto column

1 - column wash fraction 1

3 - column wash fraction 3

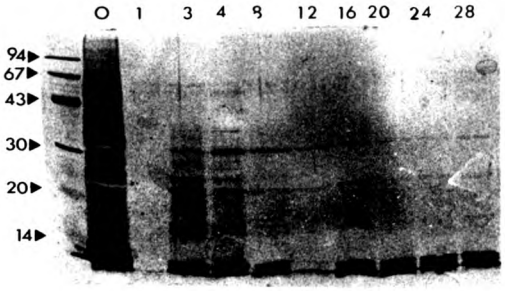

0 1 3 4 8 12 16 20 24 28
94
67
43
30
20
14

FIGURE 13 - Stages of TPP partial purification

30µl of sample containing peak PCi processing activity was loaded onto an SDS-PAGE gel and Coomassie stained, a duplicate sample of DEAE-Sephacel eluate was silver stained.

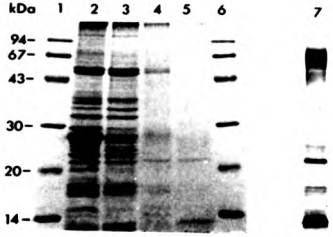
2 - total thylakoid protein

3 - 30 000 x g supernatant

4 - hydroxyl apatite eluate

5 - DEAE-Sephacel eluate

7 - as 5 but silver stained


kDa
1
2
3
4
5
6
7
94-
67-
43-
30-
20-
14-

Additional chromatography methods were attempted including; butyl agarose column chromatography (in which the protein bound to the column is eluted with a Triton X-100 gradient), FPLC using a column based on DEAE, chromatofocusing and gel filtration. All of these chromatography techniques failed to produce adequate resolution and recovery of TPP activity for further exploitation. A non-denaturing gel electrophoresis method was also attempted which failed to produce an adequate recovery of TPP activity.

The assay technique used during this thesis allowed only limited quantitation and it was estimated that the purification protocol produced between 5 and 10% recovery of activity. With the failure of additional purification techniques and a partially purified TPP sample containing low activity an alternative approach to purification was considered.

Analysis on Coomassie stained SDS-PAGE gels of the wash volume from DEAE chromatography containing TPP activity revealed ten bands (not all visible in the photograph). It was possible that one or more of these bands could represent TPP. In light of this possibility bands were excised, electro-eluted and antisera raised in mice to each band. The presence of antibodies to TPP in the resulting antisera was tested by immuno precipitation. In these experiments antisera were mixed with extracts containing TPP followed by in vitro processing assays. If antisera contained anti TPP antibodies, appearance of TPP activity in the precipitate

and a corresponding decrease in the supernatant would be expected.

Electro-eluted samples were analysed by SDS-PAGE and western blotting with various thylakoid protein antisera in order to eliminate putative bands representing these proteins. The silver nitrate stained SDS-PAGE gel in Figure 14A shows that tracks 5 to 10 contain distinct bands, however, tracks 1 to 4 are less distinct. The western blot with a mixture of antisera to cytochrome b6f, 23KDa and 33KDa in Figure 14B shows cross reactivity with band 9, suggesting that this band is not TPP. All bands were used to raise antisera which was then mixed with 30 000 x g supernatant and precipitated with protein A sepharose beads. Both precipitated and supernatant fractions were assayed for the presence of pre-23KDa processing activity. Some fractions cleaved pre-23KDa but none to the mature size (not shown). This system requires extensive optimisation before TPP can be assigned to any of these electro eluted bands.

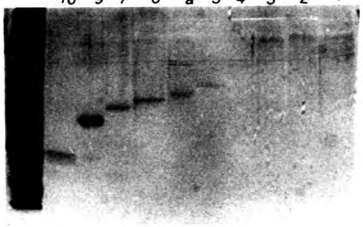
FIGURE 14A - Electro-eluted proteins from DEAE-Sephacel chromatography SDS-PAGE gel

The column wash (fraction 3) was run on a SDS-PAGE gel and Coomassie stained. The 10 bands revealed were individually excised and electro eluted into 1ml of Laemmeli electrophoresis buffer. A 30µl aliquot of each band was analysed by SDS-PAGE and silver stained. Track 0 contains 5µl of 30 000 x g supernatant.

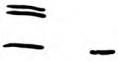
FIGURE 14B - Western Blot of electro eluted bands against cytochrome b6f, 23KDa OEC and 33KDa OEC antisera

A duplicate gel of (A) was Western Blotted in the presence of antisera to cytochrome b6f, 23KDa OEC and 33KDa OEC, and developed using the biotin streptavidin method.

A



B



III.1.C. THE EFFECT OF PROTEASE INHIBITORS OF TPP PROCESSING ACTIVITY

This section aimed to assign TPP to a class of protease by the use of diagnostic protease inhibitors. Most proteases fall into one of four classes depending on the mechanism used to cleave peptide bonds as follows;

(i) Thiol proteases; contain an essential thiol group in their active site.

(ii) Serine proteases; contain an essential serine residue in their active site.

(iii) Metallo proteases; are associated with a metal ion co-factor.

(iv) Acid proteases; contain an essential carboxylic acid group.

Incubation of proteases with diagnostic inhibitors for each class of reaction mechanism can be used in the classification of proteases. Thus, iodoacetate will covalently modify the thiol groups of cysteine residues in thiol proteases, whereas serine proteases can be inhibited by incubation with phenyl methyl sulphonyl fluoride (PMSF). Metal chelating agents such as EDTA will inhibit metallo proteases by chelation of essential metal ion cofactors.

In order to assign TPP to a particular class of protease, inhibitors from each class were tested in TPP activity assays. Figure 15 shows the autoradiogram for PCi processing assays in the presence of protease inhibitors. The extent of PCi processing in the presence of inhibitors, with the exception of EDTA and EGTA, is identical to that of the inhibitor-free control. The presence of metal chelating agents EDTA and EGTA produced a greater degree of PCi processing than the inhibitor-free control. These data suggest that no standard protease inhibitor abolishes the activity of TPP. The presence of the metal chelating agents EDTA and EGTA causes a marked stimulation of TPP activity.

A range of other protease inhibitors were tested, including iodoacetamide, N-ethylmaleimide (thiol protease inhibitors), benzamidine (serine protease inhibitor) and microbial carboxyl protease inhibitors pepstatin and bestatin. All of these inhibitors failed to reduce TPP activity (not shown).

FIGURE 15 - Effect of protease inhibitors on TPP activity

17µl aliquots of 30 000 x g supernatant were mixed with 1µl of PCi translation products and 2µl of inhibitor. Samples were incubated for 90min at 27°C and mixed with sample buffer. Analysis of samples was performed by SDS-PAGE and autoradiography.

M - in vitro chloroplast import marker (I = intermediate size PC, M = mature size PC)

1 - PCi translation products

2 - PCi translation products + 30 000 x g supernatant

3 - as 2 + 100µg/ml leupeptin

4 - as 2 + 2mM phenyl methyl sulphonyl fluoride (PMSF)

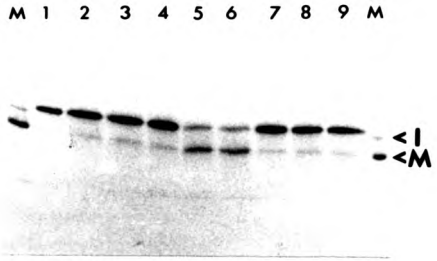
5 - as 2 + 10mM EDTA

6 - as 2 + 10mM EGTA

7 - 30 000 x g mixed with 1mM iodoacetate 4°C 60min prior to being mixed with PCi

8 - as 7 but using 10mM iodoacetate

9 - as 2


M 1 2 3 4 5 6 7 8 9 M
<I
<M

III.1.D. DISCUSSION

Partial purification of TPP activity has been achieved by a combination of techniques. However, further purification must be performed to identify a putative TPP band on SDS-PAGE gels. TPP is likely to be a low abundance protein; this coupled with its low stability and poor recovery encountered during this project suggest that it may be difficult to purify to homogeneity.

The ability of TPP to retain activity in the presence of diagnostic protease inhibitors suggests that it cannot be assigned to any of the standard protease mechanisms described in Section II.1.A. It is therefore possible that TPP exploits a novel proteolytic mechanism. However, a continued investigation into TPP inhibitor sensitivity using a more highly purified and active preparation is recommended.

The presence of EDTA and EGTA causes a marked stimulation of TPP activity. This initially suggests that inhibition by divalent metal ions occurs. However, this remains doubtful due to the stimulation provided by EDTA and EGTA in 20mM Tris-HCl pH 7.0, 0.15% Triton X-100 which is devoid of divalent metal ions (not shown). Rearrangement of the Triton X-100 micelle (containing TPP) by the presence of a divalent cation may produce an inhibitory effect, if the active site is made inaccessible to precursors. Such inhibition could be removed by chelation of metal ions by

EGTA and EDTA. Incubation of TPP extracts with a range of divalent metal ions failed to produce any marked inhibition of activity (not shown). The exact cause of the EGTA and EDTA stimulatory effect remains to be resolved. However, in the light of this discovery EGTA was routinely added to *in vitro* TPP activity assays to maximise processing activity.

III.2. LOCATION AND ORIENTATION OF TPP WITHIN THE THYLAKOID

III.2.A. INTRODUCTION

The aim of this section was to establish more precisely the location and orientation of TPP within the thylakoid network. A number of possibilities were considered:

(i) Loose association of TPP with the thylakoid membrane. Hageman et al. (1986) demonstrated that salt washing of thylakoid membranes did not remove TPP, suggesting that loose association would be most likely to involve the lumenal face of the thylakoid as opposed to the stromal face.

(ii) TPP may exist as a soluble protein in the lumen.

(iii) TPP could be an integral membrane protein tightly bound to the thylakoid membrane.

(iv) Lateral segregation of TPP within the thylakoid could occur. Many thylakoid membrane proteins display a high degree of lateral segregation between appressed and non-appressed membranes (see Section I.1.C.). Similar lateral distribution may occur.

A number of techniques were employed to establish the location of TPP.

III.2.B. ULTRACENTRIFUGATION OF 30 000 X G SUPERNATANT

Solubilisation of thylakoid membranes with Triton X-100 releases PCi processing activity along with additional thylakoid proteins. Centrifugation of extract at 30 000 x g separates unsolubilised thylakoids, predominately stacked grana containing photosystem II (Kirwin et al., 1987), from a supernatant containing processing activity. Ultracentrifugation of 30 000 x g supernatant at 200 000 x g (Section II.10.A.) generates a clear supernatant and a chlorophyll containing pellet. Under these conditions, 30 000 x g supernatant partitions into a supernatant predominantly containing hydrophilic proteins and a pellet containing hydrophobic proteins. Analysis of both phases on Coomassie stained SDS-PAGE gels support this.

Figure 16A shows a Coomassie stained SDS-PAGE gel of supernatant and pellet fractions from ultracentrifugation of 30 000 x g supernatant at 200 000 x g. The supernatant contains characteristic hydrophilic thylakoid lumen proteins; plastocyanin, which is soluble in the lumen and 16KDa, 23KDa and 33KDa OEC polypeptides of photosystem II, which are loosely associated with the lumenal face of the membrane. No major thylakoid membrane protein is apparent in the supernatant. The pellet contains hydrophobic thylakoid membrane proteins, such as ATPase and photosystem II complex subunits.

FIGURE 16A - Fractionation of 30 000 x g proteins by ultracentrifugation

30 000 x g supernatant was subjected to ultracentrifugation at 200 000 x g for 5h and separated into supernatant and pellet fractions. The pellet was resuspended in the original volume of 20mM Tris-HCl pH 7.0, 0.15% Triton X-100. Samples were mixed with sample buffer boiled for 2min and analysed on a Coomassie stained SDS-PAGE gel.

1 - 30 000 x g supernatant

2 - ultra-centrifugation supernatant

3 - ultra-centrifugation pellet

Molecular weights are indicated on the right. 33KDa, 23KDa and 16KDa on the left hand side indicate the positions of the oxygen evolving polypeptides.

FIGURE 16B - Fractionation of TPP activity by ultracentrifugation

The 30 000 x g supernatant, ultracentrifugation supernatant and pellet fractions were assayed for PCi processing activity by mixing 18µl of sample with 1µl PCi translation product, 1µl 300mM EGTA and incubating at 27^{o}C for 90min. Samples were boiled for 2min in sample buffer and analysed by SDS-PAGE and autoradiography.

1 - PCi translation products

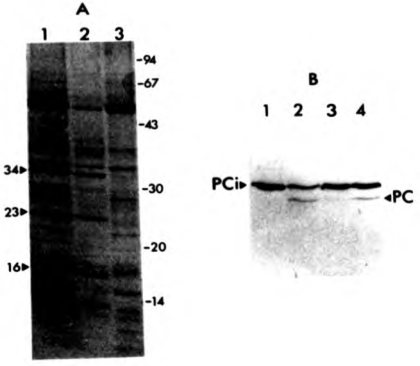
2 - PCi + 30 000 x g supernatant

3 - PCi + 200 000 x g supernatant

4 - PCi + 200 000 x g pellet

PCi - artificial plastocyanin intermediate

PC - mature size plastocyanin


A
1 2 3
94
67
43
34
30
23
20
16
14
B
1 2 3 4
PCi
PC

Supernatant and resuspended pellet fractions from ultracentrifugation preparations were assayed for TPP activity (Section III.1.A.). Figure 16B demonstrates that TPP activity migrated with the hydrophobic detergent micellar phase and is located exclusively in the pellet fraction. No processing activity is apparent in the soluble supernatant phase. These results suggest that TPP is an integral membrane protein, a suggestion which is supported by the observation that TPP activity has an absolute requirement for detergent (Kirwin et al. 1987). During the extraction process TPP presumably partitions into the detergent micelles along with additional thylakoid membrane proteins, whereas hydrophilic proteins remain in the soluble phase.

Ultracentrifugation of 30 000 x g supernatant results in a decrease of TPP activity, as shown by comparing track 2 (containing 30 000 x g supernatant) with track 4 (containing 200 000 x g pellet) of Figure 16B. Loss of activity may not entirely be due to the denaturation of TPP, which is stable at 4^{o}C over 16 hours in dialysis experiments, (not shown). An alternative possibility is that an essential stabilising protein or ion is separated from TPP under these conditions. A 'protease enhancing protein' is associated with matrix processing peptidase of mitochondria (see Section I.6.B.) and a similar protein associated with TPP may have been removed during ultracentrifugation. A reversible association with such a factor should permit restoration of TPP activity. However, remixing of pellet and supernatant fractions did not significantly increase the level of processing activity.

III.2.C. SUCROSE DENSITY GRADIENT CENTRIFUGATION OF 30 000 X G SUPERNATANT

Ultracentrifugation of 30 000 x g supernatant suggests that TPP is located in the thylakoid membrane (Section III.2.B.). Under these conditions TPP pellets along with thylakoid membrane complexes e.g. ATPase, raising the possibility that TPP may be associated with one of these complexes. In order to address this question, 30 000 x g supernatant was subjected to sucrose density gradient centrifugation. This method separates protein complexes according to size and density and hence resolution of TPP from supramolecular complexes may be achieved.

After centrifugation the gradient was separated into $1cm^3$ fractions (Section II.10.A) and subjected to analysis by SDS-PAGE, TPP activity assay using PCi, and western blotting against cytochrome b6f antisera. These analyses are presented in Figure 17A, B and C. In each figure the first four tracks represent the sample volume loaded, with the gradient commencing in track 5. The protein profile in Figure 16A shows that hydrophilic thylakoid lumen proteins were completely retained in the sample volume (tracks 1 to 4). This is clearly demonstrated by the location of 33KDa OEC, which is distributed evenly throughout the sample volume, but did not enter the gradient. Cytochrome b6f complex partially migrates into the gradient with a peak in fractions 4 to 5 as shown in the western blot in Figure 17B. The ATPase complex migrates mid way into the gradient,

FIGURE 17A - Fractionation of 30 000 x g proteins by sucrose gradient centrifugation

4ml of 30 000 x g supernatant was layered onto an 11ml linear gradient of 0-0.7M sucrose in 20mM Tris-HCl pH 7.0, 0.15% Triton X-100, 2mM $MgCl_2$ and centrifuged at 100 000 x g for 16h at $4^{o}C$. Approximately 1ml fractions were collected and analysed by Coomassie stained SDS-PAGE. Fractions, starting from the top of the tube are numbered at the head of each track (1-4 represent the sample volume). ATPase doublet and 33KDa OEC are indicated.

FIGURE 17B - Western Blot of 30 000 x g protein fractionated on sucrose gradient using cytochrome b6f antisera

Antisera to cytochrome b6f were used to immunoblot a duplicate SDS-PAGE gel as shown in Figure 17A.

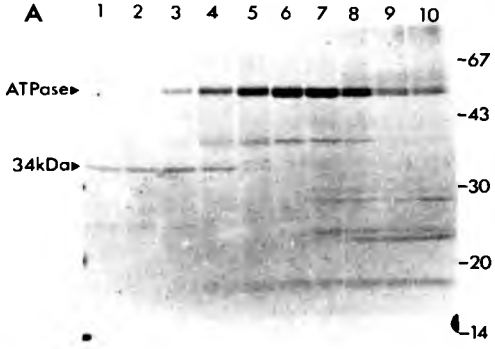
S - original sample loaded onto gradient.

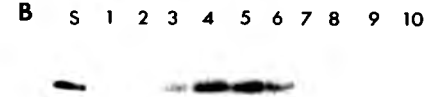
FIGURE 17C - Fractionation of TPP activity by sucrose gradient centrifugation

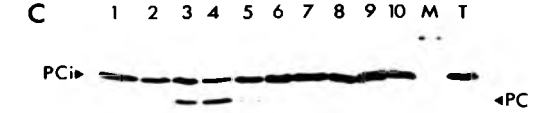
18µl aliquots of each sample were mixed with 1µl PCi translation products and incubated at $27^{o}C$ for 90min. Samples were boiled in sample buffer for 2min and analysed by SDS-PAGE and autoradiography. Numbers above track correspond to fraction numbers.

M - mature plastocyanin from in vitro chloroplast import of pre PC

T - PCi translation products


A
1 2 3 4 5 6 7 8 9 10
ATPase
34kDa
67
43
30
20
14


B
S 1 2 3 4 5 6 7 8 9 10


C
1 2 3 4 5 6 7 8 9 10 M T
PCi
PC

peaking at fraction 7. Photosystem I complex migrates towards the bottom of the gradient with a corresponding chlorophyll peak between fractions 8 to 10. TPP activity assays with PCi in Figure 17C show that the processing activity remains towards the top of the tube with a peak at fractions 3 to 4. Slight migration of TPP into the gradient occurs since activity can be detected in fractions 5 and 6. TPP and cytochrome b6f are not fully separated under these conditions. However, the separation of peaks indicates that co-migration of these two proteins did not occur. This result indicates that TPP is not associated with cytochrome b6f, photosystem I or ATPase complexes.

In this system TPP migrates closely behind cytochrome b6f which has a molecular weight of about 250 000. This indicates that the molecular weight of TPP is below 250 000, but this system does not permit a more accurate estimation.

III.2.D. LOCATION OF THE TPP ACTIVE SITE WITH RESPECT TO THE THYLAKOID MEMBRANE

This section considers the location of the active site of TPP in the thylakoid membrane. Evidence presented in Section III.2.C. suggests that TPP is an integral thylakoid membrane protein, therefore possible orientations for the active site include;

(a) the stromal face

(b) the lumenal face

(c) within the membrane in a pore assembly.

Hageman et al. (1986) failed to detect TPP activity in the absence of detergent using isolated thylakoids in vitro. An explanation for this observation may be that the active site of TPP is not present on the stromal face and hence inaccessible to precursors.

The possibility of TPP active site residing on the lumenal face was explored and two approaches were considered. Preparation of inside out thylakoid vesicles (by everting the membrane) would expose the lumenal face and precursors incubated with inside out thylakoid vesicles would then encounter the active site (if it resided on the lumenal face) and become processed to the mature size. This approach was considered, however, initial attempts (in this laboratory) to prepare inside out thylakoids were unsuccessful, and an alternative approach was therefore

adopted. Hauska *et al*. (1971) successfully introduced plastocyanin into the thylakoid lumen by sonication and this approach was employed, to introduce *in vitro* synthesised PCi into the lumen. Thylakoid vesicles were mixed with PCi and the membranes sheared by repeated sonication. PCi can therefore enter the lumen during sonication, before spontaneous resealing of vesicles occurs. Processing of PCi to the mature size should then occur if TPP is located in the lumen or the active site resides on the lumenal face of the membrane.

Using this technique thylakoids were sonicated briefly and incubated at 27°C for 2min before further rounds of sonication (Section II.9.), under these conditions PCi was cleaved to the mature size. A time course assay in which thylakoids in the presence of PCi were subjected to varying extents of sonication is presented in Figure 18. This indicates that the active site is located on the lumenal face of the thylakoid membrane. In control experiments thylakoids which were subjected to an equivalent degree of sonication prior to incubation with PCi failed to release TPP activity. Kirwin *et al*. (1988) also demonstrated that extreme sonication fails to release TPP activity into the soluble phase.

These data confirm that TPP is an integral membrane protein with its active site on the lumenal face of the thylakoid. In this location the TPP active site is accessible to artificially introduced precursors, which are cleaved to the mature size.

FIGURE 18 - Processing of PCi artificially inserted into the thylakoid lumen using sonication

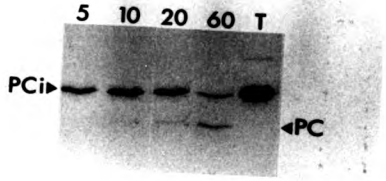
Washed thylakoids were resuspended in 400µl 20mM Tris-HCl pH 7.0 at a chlorophyll concentration of 1.5mg/cm^3 and mixed with 10µl of PCi translation products. The mixture was repeatedly sonicated for 2sec bursts and incubated for 2min at 27°C between sonications. At various time intervals a 10µl sample was removed and centrifuged at 100 000 x g for 10min to pellet the thylakoid membranes. The supernatants were mixed with sample buffer, boiled for 2 min and analysed by SDS-PAGE and autoradiography.

The times above each track indicate the period of sonication in seconds.

T - translation products

PCi - artificial plastocyanin intermediate

PC - mature size plastocyanin


5
10
20
60
T
PCi
PC

III.2.E. DISCUSSION

This section has provided evidence concerning the location of TPP and has shown it to be an integral membrane protein, independent of major thylakoid membrane complexes, with its active site on the lumenal face.

Ultracentrifugation of 30 000 x g supernatant separates hydrophilic thylakoid lumen proteins from hydrophobic membrane proteins. TPP activity is associated with the hydrophobic phase indicating that TPP is a membrane protein. This evidence explains why TPP processing activity cannot be released from isolated thylakoids by salt washing procedures (Hageman et al., 1986) or extensive sonication (Kirwin et al., 1988). Therefore, TPP is an integral membrane protein. After Triton X-100 extraction from isolated thylakoids the detergent provides the correct hydrophobic environment to retain an active conformation of TPP.

Separation of 30 000 x g supernatant by sucrose density gradient centrifugation partially resolves TPP activity from other thylakoidal proteins and complexes. Distinct separation of TPP from photosystem I and ATPase is achieved due to mobility of the larger complexes into the sucrose gradient. A peak of processing activity is also partially resolved from a peak of cytochrome b6f, demonstrating that no link exists between these complexes and TPP. This is not entirely unexpected since TPP is not involved in the

function of any of these complexes. However, Evans et al. (1986) have proposed the existence of a supramolecular import complex in the endoplasmic reticulum with which signal peptidase is associated. The limited migration of TPP into the top of the gradient suggests that TPP is not strongly linked to any such supramolecular complex. The association of TPP with such a structure is therefore unlikely unless it involves weak association which is disrupted under these conditions.

The migration of TPP activity behind cytochrome b6f indicates that the molecular weight of TPP is below 250 000. A more accurate estimation requires a more sensitive technique such as calibrated gel filtration.

Artificial introduction of PCi into the lumen by sonication results in cleavage to the mature size. This demonstrates that the active site is on the lumenal face of the thylakoid membrane, which is supported by evidence from Kirwin et al. (1988) who demonstrated that TPP activity is resistant to cleavage by exogenous protease added to isolated thylakoids.

It is not known whether TPP cleavage of stromal intermediates operates in a co-translocational or post-translocational manner. Co-translocational cleavage would be expected where translocation of stromal intermediate involved a translocation complex with which TPP was associated. Evans et al. (1986) have proposed association

of signal peptidase with a translocation complex in the E.R. Evidence described in Section I.3.B., I.4.C. and I.5.B. suggests that a translocation complex is involved in other membrane translocation systems. However, evidence presented in this section demonstrates that TPP is not associated with such a complex, unless the association is weak. Thus, co-translocational cleavage via such a complex appears unlikely. A post-translocational cleavage process can be envisaged where after entry into the lumen the stromal intermediate diffuses until it encounters TPP, whereupon it is cleaved to the mature size. Further investigation of the translocation event is required (see Section III.6.B.)

Kirwin *et al*. (1988) demonstrated lateral segregation of TPP within the thylakoid membrane. This shows that TPP activity predominates in the stromal lamellae and non-appressed regions of the thylakoid membrane. Thus, TPP is present only in regions of the membrane in contact with stroma and hence incoming stromal intermediate forms. Processed proteins such as the 16KDa, 23KDa and 33KDa OEC polypeptides must therefore reach their target site in the grana after cleavage to the mature size (see Section III.3).

III.3. TPP REACTION SPECIFICITY

III.3.A. INTRODUCTION

Evidence presented in this thesis demonstrates that a processing peptidase activity, involved in the biogenesis of plastocyanin, can be partially purified from pea thylakoid extracts. This section considers whether the same peptidase is involved in the biogenesis of other thylakoid lumen proteins.

In addition to plastocyanin other prominent proteins located in the thylakoid lumen include the 16KDa, 23KDa and 33KDa oxygen evolving complex (OEC) polypeptides of photosystem II (see Section I.C.1 and Barber (1987)). Like plastocyanin, these proteins are nuclear encoded and synthesised in the cytoplasm as higher molecular weight precursors (Barber, 1987; Tyagi et al., 1987; Jansen et al., 1987). It was considered possible that the import of these precursors follows a similar route to plastocyanin, thus, maturation may employ the same processing peptidase which cleaves PCi to yield the mature sized protein. Alternatively a unique peptidase may be involved in each case. To address this question thylakoid extract containing PCi processing activity (30 000 x g supernatant) was incubated with wheat-germ lysate translation products of pre-23KDa and pre-33KDa in in vitro processing assays. Additionally, column eluates from the partial purification of TPP were used in in vitro

processing assays with pre-23KDa and PCi in order to establish whether PCi processing activity co-eluted with that of pre-23KDa.

Further work in this section is concerned with possible reaction similarities between TPP and membrane bound processing peptidases from other protein transport systems. The targeting and membrane transport of proteins across the endoplasmic reticulum, bacterial periplasmic membrane, mitochondrial and chloroplast membranes involves proteolytic cleavage of transit peptides and signal sequences. (Verner and Schatz, 1988. See also Sections I.2.D., I.3., I.4. and I.5.) A notable example is the targeting of protein precursors to the periplasmic space in E. coli. In this system, leader peptidase (LEP) cleaves the leader peptide from protein precursors targeted to the periplasmic space (Zwizinski and Wickner, 1980). TPP and LEP share several features in common. Both are integral membrane proteins with a detergent requirement for in vitro activity (Kirwin et al., 1987, Zwinzinski and Wickner, 1980). The active sites of both peptidases reside on the trans sides of the membranes i.e. the lumen for TPP and periplasm for LEP (Section III.2.D., Kirwin et al., 1988; Wolfe et al., 1983). Furthermore, the leader sequence of E. coli periplasmic protein precursors, and thylakoid transfer domains of thylakoid lumen protein precursors display structural similarities: both have small unchanged amino acids at residues -3 and -1 upstream from the processing site (von Heijne, 1983, 1985). In thylakoid transfer domains this is

almost always $\bar{A}^{3}a$ - x - $\bar{A}^{1}a$. A hydrophobic central core is also common to both types of presequence (von Heijne, 1983, 1985; von Heijne et al., 1989). These similarities in presequence structure and properties of TPP and LEP raise the possibility of a similarity in the reaction specificity of these peptidases. Tests were therefore carried out to determine whether TPP is able to cleave E. coli periplasmic space protein precursors to the mature size, or whether LEP is capable of maturation thylakoid lumen protein precursors.

III.3.B. MATURATION OF PRE-23KDa AND PRE-33KDa EMPLOYS A SIMILAR TWO STEP MECHANISM TO PRE-PC INVOLVING A STROMAL PEPTIDASE AND PROBABLY A COMMON THYLAKOIDAL PEPTIDASE

During the course of this work clones encoding wheat pre-23KDa and pre-33KDa and spinach pre-33KDa were isolated by J. Meadows, H. James (in this laboratory) and R. Herrmann (Munich) respectively and became available for *in vitro* processing assays. *In vitro* translation products of each precursor were incubated with either SPP extracts (supplied by J. Musgrove) or 30 000 x g supernatant in order to test for a possible two step processing mechanism.

Figure 19A shows SPP and TPP processing activity assays using pre-23KDa and pre-33KDa. In each case the precursor was processed by 30 000 x g supernatant to the mature size and no further. The two bands apparent in pre-23KDa translations are both processed to the mature size. The lower band may be due to internal initiation of pre-23KDa translation of SPP activity in the wheat-germ lysate. Processing of pre-23KDa by 30 000 x g supernatant is more extensive than that of pre-33KDa suggesting that pre-23KDa is a better substrate for TPP. Incubations of precursors with SPP extracts produce a single intermediate sized cleavage product in each case. This evidence suggests that like pre-PC, pre-23KDa and pre-33KDa are processed in two steps, firstly to an intermediate size by a stromal peptidase and finally to the mature size by a thylakoidal peptidase, present in 30 000 x g supernatant. However,

FIGURE 19A - Processing of Spinach pre-33KDa by SPP and TPP

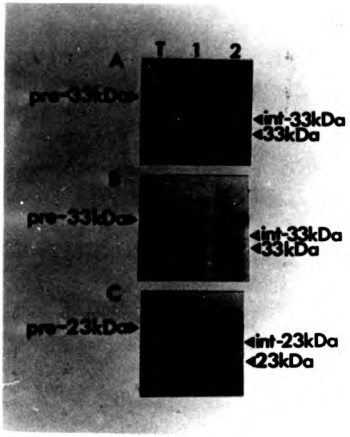
1 µl of spinach pre-33KDa translation products were incubated with (1) 19µl SPP sample (J. Musgrove, Warwick), (2) 18µl of 30 000 x g supernatant + 1µl of 300mM EGTA and incubated at 27°C for 90min; samples were mixed with sample buffer boiled for 2min and analysed by SDS-PAGE and autoradiography.

FIGURE 19B - Processing of Wheat pre-33KDa by SPP and TPP

A duplicate method to 19A was run in which spinach pre-33KDa was replaced by wheat pre-33KDa.

FIGURE 19C - Processing of Wheat pre-23KDa by SPP and TPP

A duplicate method to 19A was run in which spinach pre-33KDa was replaced by wheat pre-23KDa.


T 1 2
A
pre-33kDa
int-33kDa
33kDa
B
pre-33kDa
int-33kDa
33kDa
C
pre-23kDa
int-23kDa
23kDa

pre-23KDa and pre-33KDa can be cleaved directly to the mature size in vitro in a single step suggesting that the envelope targeting domain does not impede cleavage by the thylakoidal peptidase.

It is not possible to establish from these data whether a single thylakoidal peptidase is responsible for the processing of PCi, pre-23KDa and pre-33KDa. To address this question PCi and pre-23KDa were incubated with hydroxyl apatite column eluates from a partial purification of TPP (Section III.1.B.). Separation of processing activity peaks would be expected if different peptidases were involved in the final cleavage step of each lumenal protein precursor.

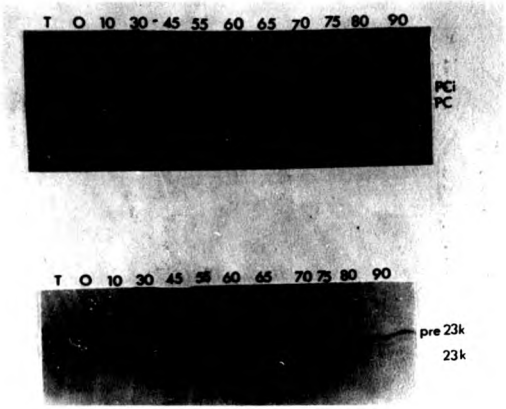
Figure 20 shows autoradiograms of PCi and pre-23KDa processing assays using hydroxyl apatite column eluates. Under the conditions of this purification the PCi processing activity elutes in fractions 55 to 75, pre-23KDa processing activity elutes in fractions 45 to 75. The peaks of processing activity for each precursor coincide; however, pre-23KDa processing activity displays a broader profile. This probably reflects the fact that pre-23KDa is a better substrate than PCi. These data (within the limitations of this purification technique) suggest that the same peptidase is responsible for the cleavage of the thylakoid transfer domain of both precursors. However, the possibility that two separate peptidases which co-purify under these conditions, resulting in coincident activity peaks, cannot be eliminated.

FIGURE 20 - Comparison of PCi and pre-23KDa processing profiles for partial purification of TPP by hydroxyl apatite chromatography

Partial purification of TPP was performed by hydroxyl apatite column chromatography (see Section II.6 and Figure 9). Duplicate assays were set up to test for processing activity using pre-23KDa and PCi. A 1µl aliquot of translation product was mixed with 1µl 300mM EGTA, 18µl of column eluate and incubated for 90min at 27°C. Samples were mixed with sample buffer boiled for 2min and analysed by SDS-PAGE and autoradiography.

Column fraction numbers are indicated above each lane.

T	-	translation product
O	-	original sample applied to column
PCi	-	artificial plastocyanin intermediate
PC	-	mature sized plastocyanin
pre-23KDa	-	precursor sized 23KDa OEC
23KDa	-	mature sized 23KDa OEC


T O 10 30 45 55 60 65 70 75 80 90
PCi
PC
T O 10 30 45 55 60 65 70 75 80 90
pre 23k
23k

III.3.C. REACTION SIMILARITIES BETWEEN TPP AND LEADER PEPTIDASE (LEP)

TPP and LEP display some similar structural characteristics as described in Section III.3.A. In order to test whether these properties extend to the cleavage mechanism, precursors to thylakoid lumen, periplasmic space, and eukaryotic secretory proteins were assayed with both TPP and LEP *in vitro*. Purified leader peptidase was provided by R Zimmerman (Munich) according to the method of Zwizinski and Wickner (1980).

Incubations of pre-23KDa or pre-33KDa with either TPP or LEP results in cleavage to the mature size and no further, as shown in Figure 21. Pre-23KDa is almost completely processed and represents a better substrate than pre-33KDa for both peptidases. The putative internal-initiation band in pre-23KDa (see Section III.3.B.) is also processed to the mature size by both peptidases. Time course analyses of LEP and TPP processing of pre-23KDa are presented in Figure 22. In both cases processing yields a band corresponding to the mature sized protein, and no other cleavage product was observed. This illustrates that the *in vitro* processing of pre-23KDa by both TPP and LEP occurs in a single step and yields no product except the mature sized protein. Therefore, as with partially purified TPP, LEP recognises and processes higher plant thylakoid lumen protein precursors in a single step.

FIGURE 21 - Comparison of processing activity between TPP and LEP

Duplicate processing assays were set up containing pre-23KDa or pre-33KDa translation products. Aliquots of wheat-germ lysate translations of pre-23KDa and pre-33KDa were incubated with 20µl of 20mM Tris-HCl pH 7.0 0.15% Triton X-100 (lane 1) or 20µl TPP (lane 2) or 20µl LEP (lane 3) in the same buffer. Samples were incubated for 60min at 27°C.

pre-33KDa - precursor size of 33KDa OEC

33KDa - mature size 33KDa OEC

pre-23KDa - precursor size of 23KDa OEC

23KDa - mature size 23KDa OEC

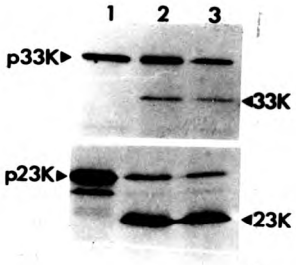
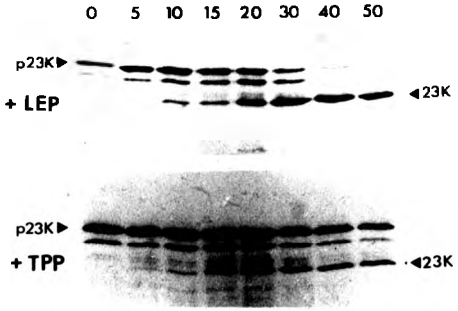

1
2
3
p33K
33K
p23K
23K

FIGURE 22 - Time course analysis of pre-23KDa processing by LEP and TPP

A 10µl aliquot of pre-23KDa was mixed with either 200µl LEP or 200µl TPP and incubated at 27°C. A 20µl aliquot was removed from both samples at the time (in minutes) indicated above each lane and the reaction terminated by mixing with sample buffer and boiling for 2min. Samples were analysed by SDS-PAGE and autoradiography.

The mobility of pre-23KDa and mature 23KDa was indicated.


0 5 10 15 20 30 40 50
p23K
+ LEP
23K
p23K
+ TPP
23K

TPP and LEP were assayed for their capability of cleaving eukaryotic signal sequence-bearing precursors (preproalpha factor) and a fusion construct which possesses the bacterial leader sequence from Erwinia pectate lyase (from R. Spooner (Warwick). It was observed that cleavage was markedly improved when the processing peptidases were present in the translation mixes. The autoradiograph of these processing assays is present in Figure 23. The appearance of a single cleavage product was observed with each peptidase. This band was presumed to be the mature size since mature sized marker proteins were not available. The greatly enhanced processing of pre-pro-alpha factor by TPP when present in translation mixes suggests that after translation it adopts a conformation in which the signal sequence becomes inaccessible to TPP.

Precursors with presequences which did not resemble the thylakoid transfer domain or leader peptides e.g. ferredoxin were not processed by TPP (data not shown).

LEP activity is inhibited by a consensus signal peptide (Wickner et al., 1987 and Austen et al., 1984). A marked reduction in LEP and TPP processing activity was observed when a synthetic 19 residue signal sequence (Austin et al., 1984) was present in in vitro pre-23KDa processing assays, as shown in Figure 24. Competitive inhibition of pre-23KDa processing activity due to the 19 residue consensus signal sequence being in vast excess of precursor is the likely cause of inhibition.

FIGURE 23 - Maturation of leader and signal sequence bearing precursors by TPP and LEP

A. 8µl wheat-germ lysate translations of preproalpha factor (ppaF) were performed in the presence of 2µl 20mM Tris-HCl pH 7.0, 0.15% Triton X-100 (lane 1) 2µl TPP (lane 2) or 2µl LEP (lane 3) in the same buffer. Samples were incubated at 27°C for 60min, mixed with sample buffer, boiled for 2min and analysed by SDS-PAGE and autoradiography.

B. Transcripts of Erwinia pectate lyase construct (pPL) were translated in a wheat-germ lysate system. A 2µl aliquot of translation product was incubated with 20µl 20mM Tris-HCl pH 7.0, 0.15% Triton X-100 (lane 1) or 20µl TPP (lane 2) or 20µl LEP (lane 3) in the same buffer, at 27°C for 60min. Samples were mixed with sample buffer, boiled for 2min and analysed by SDS-PAGE and autoradiography.

The arrows indicated migration of processed products.

Synthetic peptide sequence

K-K-S-A-L-L-A-L-M-Y-V-C-P-G-K-A-N-K-E

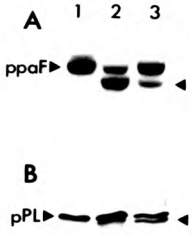
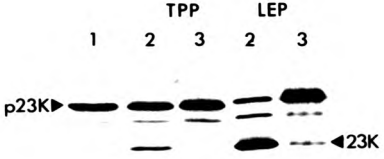

A
1
2
3
ppaF
B
pPL

FIGURE 24 - The effect of a synthetic signal sequence on TPP and LEP processing activity

TPP and LEP were incubated with pre-23KDa in the absence (lane 2) and presence (lane 3) of a synthetic signal sequence at a concentration of 0.5mM. Samples were incubated for 60min at 27°C, mixed with sample buffer, boiled for 2 min and analysed by SDS-PAGE and autoradiography.

1 - products from the translation of pre-23KDa transcripts


TPP
LEP
1
2
3
2
3
p23K
23K

III.3.D. DISCUSSION

This section has been concerned with the reaction specificity of TPP. Hageman et al. (1986) demonstrated TPP activity from Triton X-100 solubilised thylakoids against PCi, but TPP had not been assayed with any other precursors prior to the work presented in this thesis. Data presented in this section demonstrate that precursors which follow a similar import route to plastocyanin (pre-23KDa and pre-33KDa) are processed to an intermediate size by SPP preparations and finally to the mature size by TPP, suggesting a common import pathway for thylakoid lumen proteins.

30 000 x g supernatant is a crude preparation and consequently may contain more than one processing peptidase. Processing activity assays of hydroxyl apatite column eluates (used in the partial purification of TPP) with PCi and pre-23KDa demonstrate that these processing activity peaks coincide. It is therefore probable that the same peptidase is responsible for the maturation of PCi and pre-23KDa. This cannot be confirmed however, until TPP is purified to homogeneity. Evidence in this section also demonstrates that TPP from pea thylakoids is capable of cleaving precursors from a range of plant species, i.e. PCi from white campion and pre-23KDa and pre-33KDa from wheat, demonstrating that TPP displays no species specificity amongst higher plants. Data in this section also demonstrate that TPP is able to cleave leader sequences from

bacterial precursors and signal sequences from eukaryotic secretory proteins.

Work presented in this section has centred on exploring the processing of nuclear encoded thylakoid lumen protein precursors (PCi, pre-23KDa and pre-33KDa). However, the chloroplast possesses its own DNA and protein synthesising machinery (Section I.2 and I.2.A.). It is therefore interesting to speculate whether TPP is capable of cleaving chloroplast encoded precursor proteins. A number of chloroplast encoded proteins have been investigated by other workers including;

(a) the 32 KDa 'herbicide binding' protein of photosystem II (also called D1 protein) (Mattoo and Edelman, 1987; Taylor et al., 1988, see Section I.1.C. (i)). This protein is associated with binding manganese in the photosystem II complex and is synthesised as a precursor with a 1.5KDa C-terminal extension (Gounaris et al., 1986; Grebanier et al., 1978; Reisfeld et al., 1982). Cleavage of the C-terminal extension involves a processing peptidase bound to the thylakoid membrane (Inagaki et al., 1989). It is not possible to rule out whether D1 processing peptidase and TPP are the same enzyme until purification to homogeneity has been achieved. However, Taylor et al. (1988) suggest that D1 maturation is not performed by TPP since a specific (LF-1) mutant in Scenedesmus obliquis is

incapable of D1 maturation whilst thylakoid lumen protein precursors are present in the mature form. Further investigation is required including additional characterisation of TPP and D1 processing peptidases.

(b) Cytochrome f is a chloroplast encoded thylakoid membrane protein (Doherty and Gray, 1979) and synthesised as a higher molecular mass precursor on thylakoidal ribosomes (Gray et al., 1984). This represents a more likely candidate for maturation by TPP than D1 protein. However work performed in this laboratory in collaboration with the laboratory of J. Gray (Cambridge) (not shown) failed to process pre cytochrome f. It is possible that the cleavable N-terminal sequence is exposed and available for cleavage only during translation thus adopting a non-cleavable conformation after synthesis in vitro. The success of processing eukaryotic precursors with signal sequences in which TPP is present during the translation (Section III.3.C.) suggests that a similar mechanism is required for cytochrome f processing. A mechanism may therefore be envisaged whereby the N-terminus traverses the membrane and is cleaved from the nascent chain by TPP as it enters the thylakoid lumen. The mechanism of cytochrome f processing merits further investigation.

TPP and LEP display similar properties (as discussed in Section III.3.A.). Evidence in this section suggests that these similarities extend from basic characteristics e.g. both enzymes are extremely hydrophobic, to the level of reaction specificity, i.e. various precursors to

(a) thylakoid lumen proteins with transit peptides
(b) bacterial proteins with leader sequences
(c) eukaryotic proteins with signal sequences

are all recognised and processed by TPP and LEP, demonstrating that the reaction specificity of these processing peptidases is very similar if not identical. The origin of this reaction similarity may be based on the structural resemblance of signal, leader and transit peptides (von Heijne, 1983; von Heijne et al., 1989), with subsequent similarity in active site structures. The observed inhibition of LEP and TPP by a consensus signal sequence polypeptide supports this suggestion. Other structural parallels may also exist; however, the failure of LEP antisera to cross react with TPP, and the inactivation of TPP by protein purification techniques e.g. ethanol precipitation used for LEP (not shown) suggest that the two enzymes differ structurally.

The conserved reaction mechanism between TPP and LEP suggests a possible evolutionary link. Cyanobacteria, the probable predecessors of chloroplasts, possess internal thylakoid membranes in many cases. Proteins targeted to the

cyanobacterial thylakoid lumen are synthesised as higher molecular weight precursors with leader peptide type pre-sequences, cleavage of which involves a processing peptidase (Wallace et al., 1989) which may be related to TPP and LEP. Evidence from this project (not shown), has demonstrated that cyanobacterial thylakoid extracts are capable of cleaving wheat thylakoid lumen protein precursors to the mature size. Thus, during the evolutionary steps from bacteria to cyanobacteria to higher plants, a specific leader sequence or transit peptide cleavage mechanism has been conserved. However, a divergence in peptidase structure has probably taken place.

It is important to consider that cyanobacterial thylakoid lumen protein precursors and proteins destined to be exported from the cell are likely to possess transit peptides and leader sequences with similar features. However these presequences must contain a distinguishing characteristic which assures that correct targeting is achieved, alternatively correct targeting may be achieved after association with a common factor.

Evidence in this section demonstrates that PCi, pre-23KDa and pre-33KDa are probably cleaved to the mature size by TPP. Kirwin et al. (1988) demonstrated that TPP displays lateral segregation within the thylakoid membrane, predominantly occurring in the non-appressed regions. Thus, imported proteins must be translocated through regions exposed to the stroma, i.e. non-appressed regions where they

become cleaved to the mature size. This raises the question as to how these proteins reach their target in the appressed regions and become assembled with the photosystem II complex. Possible explanations include;

(a) after cleavage by TPP the mature protein diffuses through the thylakoid lumen to the appressed regions where it becomes assembled into photosystem II.

(b) the mature protein is assembled with photosystem II in the non-appressed regions and completed photosystem II complex then diffuses to the appressed region through the thylakoid membrane.

III.4. CLEAVAGE FIDELITY OF TPP AND LEADER PEPTIDASE (LEP)

III.4.A. INTRODUCTION

This section is concerned with establishing the position of the TPP cleavage site in thylakoid lumen protein precursors, in order to determine the accuracy of TPP cleavage *in vitro*. It was deemed particularly important to establish that TPP from one species was capable of recognising the correct cleavage site of a thylakoid lumen protein precursor from another species, since TPP and precursors used during *in vitro* assays were derived from different species. The accuracy of pre-23KDa cleavage by LEP was also investigated, to establish whether TPP and LEP display identical reaction specificities.

The work presented in Section III.3.B. shows that TPP processes PCi, pre-23KDa and pre-33KDa to the mature sizes. The cleavage of lumenal protein precursors to the mature size during *in vitro* assays was inferred when the cleavage products (visualised by fluorography) displayed identical mobility on SDS-PAGE gels to the Coomassie stained mature protein. Thus, pre-23KDa cleavage products co-migrated with Coomassie stained 23KDa OEC protein from thylakoid extracts (not shown). However, it cannot be guaranteed that co-migrating bands on SDS-PAGE gels have precisely the same molecular weight. This evidence therefore is insufficient to confirm that the cleavage products in such assays are the authentic mature sized protein.

TPP *in vitro* may not accurately recognise and cleave thylakoid lumen protein precursors at the correct position in some cases. This may occur in the TPP activity assays described in this thesis, since TPP, PCi and the OEC precursors are derived from different plant species. Therefore any species specificity could result in the production of cleavage products a few amino acids larger or smaller than the authentic mature sized protein. Similar cleavage products may also be produced in assays where LEP was used to cleave pre-23KDa (see Section III.3.).

A more accurate technique for establishing whether the cleavage products of thylakoid lumen protein precursors were authentic mature sized protein was required, and this problem was addressed by radiosequencing, in which the precise location of radiolabelled amino acids in the cleavage product can be established. Comparison of the N-terminal sequence for mature protein with the location of radiolabelled amino acids then reveals the cleavage site within a precursor.

III.4.B. RADIOSEQUENCING OF CLEAVAGE PRODUCTS GENERATED BY TPP AND LEP

Lumenal protein precursors were radiolabelled with ^{3}H lysine (pre-23KDa) or ^{3}H leucine (PCi) as detailed in Section II.8. Pre-23KDa was processed with TPP or LEP, whilst PCi was processed with TPP only. Radiosequencing was performed on the cleavage products using an Applied Biosystems sequenator, (in collaboration with B. Dunbar at SERC micro protein sequencing facility, Aberdeen) as described in Section II.8. Figure 25 shows CPM $^{3}[H]$ for each sequencing cycle of pre-23KDa cleavage products using TPP and LEP. Figure 26 shows CPM ^{3}H for each sequencing cycle of the cleavage product generated by incubation of PCi with TPP.

For pre-23KDa cleavage products produced by either TPP or LEP, peaks of radioactivity occur in cycles 11, 13 and 14 in both cases. The position of lysine residues in 23KDa was determined by microsequencing of a mature sized 23KDa sample isolated from wheat by H. James. A comparison of the position of the peaks of radioactivity was made with the position of lysine residues in the authentic mature sized protein. For PCi cleavage products generated by TPP, peaks of radiolabel occur in cycles 4, 5 and 12, as shown in Figure 26, which correspond to the positions of leucine residues in the mature sequence of plastocyanin (Smeekens et al., 1985). In each case the peak of radioactivity is at least three-fold greater than the background level and confirms the presence of radiolabelled amino acids at these

FIGURE 25A - Amino terminal sequencing of purified wheat-23KDa OEC polypeptide

Purified wheat-23KDa (supplied by H. James, Warwick) was resolved on an SDS-PAGE gel as specified in Applied Biosystems User Bulletin No. 25. The gel was blotted onto Immobilon membrane (Millipore UK) and the 23KDa band excised, inserted in the cartridge of an Applied Biosystems 470A protein sequenator with a 120A on line PTH analyser using the standard 03R PTH program, and the sequence established.

FIGURE 25B - Radiosequencing of [^{3}H]lysine labelled pre-23KDa OEC polypeptide after TPP cleavage

A 60µl aliquot of TPP processing assay was sequenced as described in (A). Fractions from each cycle of the sequence were counted using an LKB Mini Beta scintillation counter to establish the location of radiolabel.

FIGURE 25C - Radiosequencing of [^{3}H]lysine labelled pre-23KDa OEC polypeptide after LEP cleavage

An identical procedure as described in (B) was performed on a pre-23KDa LEP processing assay.

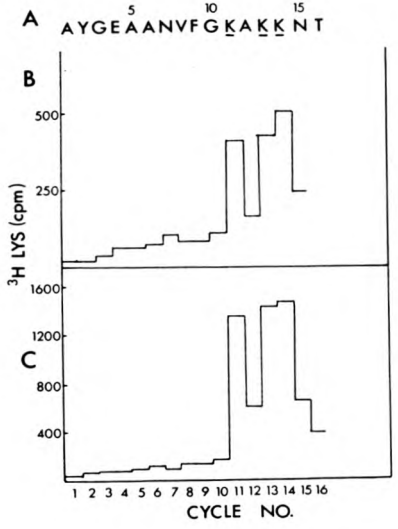
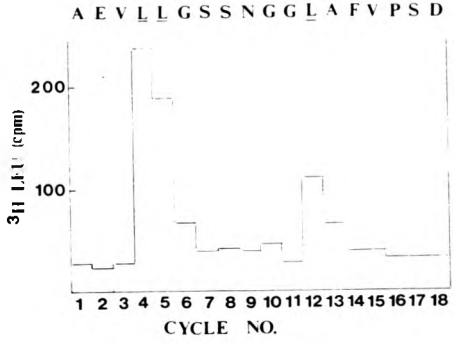

A
AYGEAANVFGKAKKNT
5
10
15
B
500
250
C
1600
1200
800
400
^{3}H LYS (cpm)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
CYCLE NO.

FIGURE 26 - Radiosequencing of [^{3}H]leucine labelled PCi after TPP cleavage

A 60µl aliquot of PCi processing assay using TPP was subjected to sequencing as described in Figure 25(A) and determination of radiolabel position as described in Figure 25(B). PC mature sequence indicated above the graph (Smeekens et al., 1985).


A E V L L G S S N G G L A F V P S D
200
100
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
CYCLE NO.

positions according to Cerletti et al. (1983). These data clearly indicate that precise cleavage at the predicted cleavage site of two lumenal precursors is performed by both TPP and LEP.

III.4.C. DISCUSSION

The data presented in this section demonstrate that processing of pre-23KDa by TPP or LEP, and of PCi by TPP results in cleavage at the predicted site. This confirms that the mature size bands on SDS-PAGE gels due to cleavage of precursors by TPP and LEP were genuine mature proteins. Thus, both of these processing peptidases cleave pre-23KDa and PCi to the mature size.

TPP is capable of recognising and accurately processing precursors of thylakoid lumen proteins from diverse species to the mature size, suggesting that the TPP cleavage mechanism is not species specific. Additionally, these data show that LEP precisely cleaves pre-23KDa at the predicted cleavage site. This result reinforces evidence from Section III.3. demonstrating that TPP and LEP have reaction similarities. Cleavage of precursors to the mature size in a single step by both peptidases suggest that the reaction mechanisms for TPP and LEP must be similar if not identical.

The vast majority of lumenal protein precursors have a sequence immediately prior to the cleavage site of $\bar{A}la^{-3}$ - x - $\bar{A}la^{-1}$ as shown in Figure 27. Sequence data from this laboratory (by H. James) demonstrate that the sequence around the cleavage site for wheat pre-23KDa is $\bar{A}la^{-3}$ - Ala - $\bar{A}la^{-1}$ - Ala. If the sequence $\bar{A}la^{-3}$ - x - $\bar{A}la^{-1}$ alone is sufficient for cleavage site recognition, wheat pre-23KDa would offer two possible cleavage sites i.e. $\bar{A}la^{-3}$ - x - $\bar{A}la^{-1}$

FIGURE 27 - Comparison of leader peptides and thylakoid transfer peptides

The cleavage site is indicated by *, apolar regions are underlined. Only the C terminal portion of transit peptides is shown.

SPINACH 33KDa protein (Tyagi *et al.*, 1987)

... K C V D A T K L A G L A L A T S A L I A S G A N A*

Silene plastocyanin (Smeekens *et al.*, 1985)

... I K A S L K D V G V V V A A T A A A G I L A G N A M A*

SPINACH 23KDa protein (Jansen *et al.*, 1987)

... G V S R R L A L T V L I G A A A V G S K V S P A D A*

Erwinia pre-pectate lyase (Lei *et al.*, 1987)

M K Y L L P T A A A G L L L L A A N P A M A*

YEAST pre-pro-alpha factor (Kurjan and Herskowitz 1982)

M R F P S I F T A V L F A A S S A L A*

MUSTARD 23KDa (Wennig *et al.* 1989)

... B S R R L A L T L L V G A A A V G S K V S P A D A*

and $^{-2}$Ala - x - Ala^{+1}. A reaction mechanism which will permit recognition of both sequences as cleavage sites would yield a spread of radiolabel in cycles 10, 11, 12, 13 and 14 during radiosequencing using ^{3}[H] lysine. No such trend was observed. This demonstrates that a degree of specificity within the transit peptide for cleavage site recognition is displayed by both TPP and LEP in order to ignore a second putative cleavage site, and cleave only at $\bar{A}la^{3}$ - x - $\bar{A}la^{1}$. Recognition of the cleavage site is therefore not solely sequence specific directly around the -3 to +1 region. Other constraints, probably in conjunction with the $\bar{A}la^{3}$ - x - $\bar{A}la^{1}$ sequence must operate to specify the cleavage site.

It has been proposed that the features of leader sequences required for cleavage by LEP include the -1 and -3 small neutral residues prior to the cleavage site (Dierstein and Wickner, 1986; Kuhn and Wickner, 1985). The accurate cleavage of pre-23KDa by LEP described in this section confirms that in lumenal precursors LEP must recognise more than the $\bar{A}la^{3}$ - x - $\bar{A}la^{1}$ sequence. Similarly cleavage of lumenal transit peptides by TPP requires recognition of constraints in addition to the $\bar{A}la^{3}$ - x - $\bar{A}la^{1}$ sequence. Dierstein and Wickner (1986) proposed that the hydrophobic cores of leader sequences are not involved in cleavage site recognition which may suggest that the same feature in transit peptides of lumenal precursors is not recognised by TPP. However, the features required by TPP for cleavage site recognition await further investigation.

TPP will cleave the entire transit peptide from pre-23KDa in one step (Section III.3.B.). This *in vitro* assay therefore presents TPP with a transit peptide larger than it would encounter *in vivo*, since pre-23KDa possesses both the envelope targeting and thylakoid transfer domains. Thus, although *in vivo* the stromal intermediate is probably the substrate for TPP, the evidence in this section confirms that TPP cleavage fidelity is not affected by the presence of the envelope transfer domain. The conformation of the transit peptide in these processing assays is therefore such that the envelope transfer domain does not impede cleavage by TPP at the terminal cleavage site. This evidence also suggests that the additional amino acids present at the N-terminus of the artificial stromal intermediate PCI have an insignificant effect on TPP activity.

The presence of the envelope targeting domain is essential since it is responsible for targeting of the precursor to the chloroplast (Section I.2.D.). Evidence in this section demonstrates that cleavage by SPP is not a prerequisite for TPP activity *in vitro*. It is therefore interesting to consider the requirement of the stromal cleavage step *in vivo*. SPP cleavage must perform an essential function which could include the following.

(a) Recognition by a thylakoid receptor; it is possible that translocation of thylakoid lumen protein precursors across the thylakoid membrane involves interaction with a translocation complex, as is

observed in mitochondria and eukaryotic systems (see Sections I.3.B, I.4.C. and I.5.A.). Such a complex may recognise a specific sequence or conformation which is accessible in the intermediate form but recognition is prevented by the presence of the envelope targeting domain.

(b) Release from import complex; the release of precursor from a putative receptor-translocation complex may be performed by SPP and thus may be essential to the import mechanism.

(c) Locking of proteins within the chloroplast; cleavage by SPP may be essential to 'lock' the protein within the chloroplast, preventing reassociation with a putative import complex.

The evidence presented in this section therefore shows that the single step cleavage of thylakoid lumen protein precursors by TPP and LEP yields the authentic mature sized protein and that these peptidases share an identical reaction specificity.

III.5. DISCOVERY OF AN ENDOPEPTIDASE (EP5) IN PEA THYLAKOIDS

III.5.A. INTRODUCTION

This section is involved in the study of a thylakoidal endopeptidase not directly associated with the maturation of thylakoid lumen protein precursors.

Proteases carry out two important functions within cells

(a) Biogenesis; previous sections of this thesis have described the involvement of TPP and SPP in the maturation of imported chloroplast precursors.

(b) Degradation; stromal and thylakoidal proteins are degraded (Schmidt and Mishkind, 1983; Bennett, 1981) possibly as a recycling or regulatory mechanism.

The characteristics of four stromal endopeptidases, termed EP1 - EP4 , have been described by Lui and Jagendorf (1986) and Musgrove et al. (1989). The activity of thylakoidal proteases have been observed (Bennett, 1981; Kyle et al., 1985 and Mattoo et al., 1984); however, prior to this thesis none had been characterised.

III.5.B. ENDOPEPTIDASE (EP5) ACTIVITY IN THYLAKOID EXTRACTS

Two peptidase activities are resolved by centrifugation (at 100 000 x g) of 30 000 x g supernatant layered onto a 0.2M sucrose pad. (Section III.10.B.) These peptidases are separated by their differential mobility through the pad. Figure 28 shows a PCi processing assay for fractions of a sucrose pad preparation, demonstrating the separation of the two proteolytic activities. TPP, under these conditions accumulates towards the interface of the sample applied and sucrose pad (fractions 1 to 2). An additional peptidase migrates into the pellet fraction (fraction 4). TPP processes PCi to the mature size and no further whilst the endopeptidase in the pellet (here after termed EP5) cleaves PCi to a size slightly larger than the mature size.

In Section III.1.C. it has been demonstrated that TPP is not inhibited by the presence of diagnostic protease inhibitors. PCi processing assays were set up using sucrose pad (EP5) preparations in the presence of diagnostic protease inhibitors, to establish whether EP5 and TPP display different inhibitor sensitivities. Inhibitors were used at the following concentrations:

5mM 1,10 phenanthroline	metal chelator
1mM phenylmethylsulphonylfluoride	serine protease inhibitor
10mM iodoacetate	thiol protease inhibitor
5mM EDTA	metal chelator

FIGURE 28 - Fractionation of TPP and EP5 processing activity

(A) 30 000 x g supernatant was layered onto a $3cm^3$, 0.2M sucrose pad and centrifuged at 100 000 x g for 16h at $4^{o}C$. Fractions from the top of the tube (lane 1) to the bottom (lane 3) and the pellet, which was resuspended in a minimal volume of 20mM Tris-HCl pH 7.0, 0.15% Triton X-100, were assayed for peptidase activity. A 1µl aliquot of PC1 translation product was incubated with 18µl of sample and 1µl 300mM EGTA for 90min at $27^{o}C$. Samples were analysed by SDS-PAGE and autoradiography.

(B) A duplicate experiment was performed on wheat thylakoid extracts.

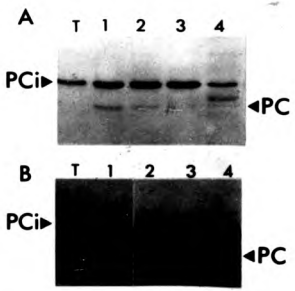

A
T 1 2 3 4
PCi
PC
B
T 1 2 3 4
PCi
PC

Figure 29 shows that some inhibition of EP5 activity occurs with PMSF and 1,10-phenanthroline. Whilst activity is abolished by the presence of 10mM iodoacetate, EP5 sensitivity to protease inhibitors therefore differs from that of TPP, which is not inhibited by any of these agents (see Section III.I.C.). These data therefore indicate that EP5 is a novel peptidase found in the thylakoid network which exploits a different cleavage mechanism to that of TPP.

FIGURE 29 - The effects of protease inhibitiors on EP5 activity

Sucrose pad preparation samples containing EP5 activity (see Figure 27) were mixed with protease inhibitors as follows.

Lane 1 - PCi translation products

2 - PCi translation products + EP5 extract

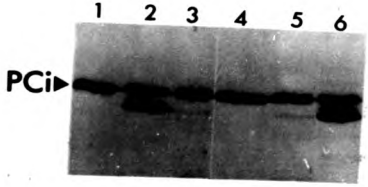
3 - as 2 + 5mM 1,10-phenanthroline

4 - as 2 + 1mM phenylmethylsulphonylfluoride

5 - EP5 extract was incubated at 4°C for 1h with 10mM iodoacetate prior to mixing with PCi

6 - as 2 + 5mM EGTA

All samples were incubated at 27°C for 90min. Samples were then mixed with sample buffer, boiled for 2min and analysed by SDS-PAGE and autoradiography.


1
2
3
4
5
6
PCi

III.5.C. DISCUSSION

The existence of proteases in the thylakoid responsible for protein turnover has been suggested by Bennett (1981). EP5 may be such an activity, and this assay may prove useful for further studies on this enzyme. EP5 activity becomes apparent after centrifugation of 30 000 x g supernatant through a sucrose pad; under these conditions, EP5 activity is found in the pellet fraction, as are large complexes such as photosystam II and the ATPase (not shown). This suggests either that EP5 is of high molecular weight, or that it is associated with a supramolecular complex.

It is likely that cleavage by EP5 occurs within the transit peptide since this region is more exposed and hence more protease sensitive that the folded mature sequence. EP5 must recognise a specific cleavage site within the transit peptide since cleavage results in a single product. Similar specific cleavages occur with stromal endopeptidases EP2, 3 and 4 (Musgrove *et al.*, 1989).

If EP5 cleavage is N-terminal, sufficient transit peptide may be retained for recognition by TPP. The EP5 cleavage product may therefore be present only transiently before further cleavage, by TPP. Thus, EP5 cleavage products may not be observed using *in vitro* processing assays containing TPP. If this is correct, addition of TPP to completed EP5 assays in the presence of iodoacetate (to abolish EP5

activity) should produce processing of the EP5 cleavage product to the mature size.

Resolution of TPP and EP5 activity on sucrose pads suggests that EP5 activity is not essential for the maturation of PCi; it is more likely that EP5 is responsible for protein turnover within the thylakoid. Section III.2.E. describes how the transit peptide for thylakoid lumen protein precursors is possibly released into the lumen. This peptide must be broken down after cleavage, and EP5 may be involved in this process. It is likely that additional proteases exist within the thylakoid which will only become apparent under the correct isolation conditions, since investigation of this area was not exhaustive.

In conclusion, this work demonstrated the existence of an endopeptidase in the thylakoid which has different characteristics from TPP. It is possible that this endopeptidase (EP5) may be involved in the degradation of transit peptides released from thylakoid lumen protein precursors.

The observation of EP5 activity was made fortuitously during the purification of TPP. Thus, whilst investigation of EP5 represented a brief departure from the main aim of this thesis it was considered to be of interest to characterise a thylakoidal peptidase other than TPP.

IV : CONCLUSION AND FUTURE WORK

IV.1. CONCLUSION

The work presented in this thesis has been concerned with a detailed investigation of TPP isolated from pea thylakoids. The following conclusions can be made from this study.

(i) TPP is a hydrophobic integral thylakoid membrane protein with a molecular weight of less than 250 000. It is independent of major thylakoid membrane complexes and the active site resides on the lumenal (trans) side of the membrane.

(ii) TPP is not inhibited by diagnostic protease inhibitors but stimulated by the presence of EDTA and EGTA.

(iii) TPP displays no species specificity and is therefore capable of cleaving lumenal precursors proteins from a range of plant species. TPP is also able to cleave precursors of eukaryotic and bacterial origin and therefore displays similarities in reaction specificity to LEP.

(iv) TPP cleaves lumenal precursors <u>in vitro</u> at the predicted cleavage site.

IV.2. FUTURE INVESTIGATION

Work described in this thesis has partially characterised TPP; however, further investigation is required to produce a more extensive understanding of the involvement of TPP in chloroplast protein import. A discussion of possible further investigations is presented below.

(i) Purification of TPP

The purification protocol described in this thesis did not achieve purification to homogeneity. However, the possible involvement of additional proteins in the TPP cleavage mechanism could result in failure of attempts to purify TPP to such an extent. Evans *et al*. (1986) have attempted to purify signal peptidase (a membrane bound processing peptidase) from the E.R., which has resulted in the presence of several contaminating proteins at the final purification stage. These workers propose that signal peptidase is a multimeric protein and is therefore possibly associated with additional proteins involved in the translocation step. Matrix processing peptidase (MPP) of mitochondria is associated with an 'enhancing protein' cofactor (Hawlitschetk *et al*., 1988). Similar proteins in thylakoids associated with TPP could prove difficult to remove in order to achieve purification to homogeneity. The instability and poor recovery of

TPP activity in purification protocols discussed in Section III.1.B. also presents a substantial obstacle for purification to homogeneity.

An alternative approach to purification is affinity chromatography. It was observed in Section III.3.C. that inhibition of TPP activity can be produced by a signal sequence polypeptide. This is likely to be due to competitive inhibition between the signal sequence polypeptide and the precursor transit peptide for the active site of TPP. Synthesis of large quantities of this peptide would allow construction of an affinity column which may selectively bind TPP. Elution of TPP from such a column should yield a highly purified TPP preparation and permit identification of TPP on an SDS-PAGE gel. Such a highly purified preparation would be valuable for further investigation. Work described in Section III.3.B. investigated whether the same processing peptidase was responsible for maturation of different lumenal precursors by using partially purified TPP in hydroxyl apatite column eluates with <u>in vitro</u> assays. A more highly purified preparation of TPP resulting from affinity chromatography would provide more substantial evidence as to whether a common peptidase was involved in processing a range of lumenal precursors. Additionally a highly purified TPP preparation may provide sufficient TPP

to raise antibodies in rabbits which may be exploited as discussed below.

(ii) Investigation of TPP using Anti TPP Antisera

The potential use of affinity chromatography could yield a purified preparation containing sufficient TPP to raise antibodies in rabbits. These antibodies would be a valuable tool in two aspects of future research.

(a) Anti TPP antibodies could be employed to reveal whether TPP was encoded on the nuclear or chloroplastic genome. Proteins synthesised by chloroplasts can be radiolabelled by mixing isolated chloroplasts with ^{35}[S] methionine (Siddell and Ellis, 1975). Western blotting of SDS-PAGE gels of these radiolabelled proteins with anti TPP antisera could indicate whether TPP is chloroplast encoded. Alternatively, screening of a lambda GT11 cDNA expression library may permit isolation of a cDNA clone encoding TPP, which would indicate a nuclear origin. Nuclear encoding of TPP would raise the question as to how TPP is imported into chloroplasts and whether it is synthesised as a precursor capable of mediating its own maturation. Sequencing of a cDNA clone for TPP may permit further

comparison as to the similarities between TPP and LEP by comparing the sequences for both peptidases.

(b) Chloroplasts which develop from greening etioplasts are capable of protein synthesis (Siddell and Ellis, 1975). It is therefore interesting to speculate when these plastids are capable of processing lumenal precursors, i.e. when TPP occurs in an active form. The use of TPP antisera to western blot etiolated and greening tissue extracts run on SDS-PAGE gels could reveal aspects of the expression of TPP in chloroplasts.

(iii) The Thylakoid Import and Processing Mechanism

A greater understanding of the association between the import and processing mechanisms of lumenal precursors is required. This may be addressed by the use of fusion constructs in which transit peptides are fused on to non-thylakoidal proteins. Meadows et al. (1989) fused the transit peptide of pre-33KDa to the mouse cytoplasmic protein dihydrofolate reductase (DHFR) and reported import and processing during in vitro chloroplast import experiments. These workers suggest that the pre-33KDa transit peptide mediates import but suggest that part of the mature sequence may play a role.

In this case deletion of mature sequence would indicate what information was required for import.

As discussed in Section III.2.E. it is not known whether TPP cleavage occurs co-translocationally or post-translocationally. It would therefore be desirable to construct a transit peptide fusion protein which is incapable of translocation and thereby becomes locked in the thylakoid membrane. Processing of such a construct would suggest that TPP cleaved in a co-translocational mode. Inhibition of TPP activity *in vitro* could also indicate the timing of the cleavage event. A synthetic signal sequence which will inhibit TPP as described in Section III.4. may be used to determine when cleavage occurs. Kirwin *et al.* (1989) demonstrated that isolated thylakoids could import and process lumenal proteins *in vitro*. In similar assays inhibition of TPP by the synthetic signal sequence would indicate whether cleavage by TPP occurred co-translocationally or post-translocationally. This investigation would require introduction of the inhibitor into the lumen at a sufficient concentration to cause inhibition, this may require sonication as described in Section III.2.D. Extensive washing would be necessary to remove any inhibitor from the thylakoid membrane to prevent saturation of a putative receptor complex. A post-translocational cleavage mechanism would be indicated by the appearance of uncleaved precursors in the lumen.

As discussed in Section III.4.C. the amino acid sequence $^{-3}$Ala - x - Ala^{-1} in association with other constraints is likely to be involved in recognition of transit peptide cleavage site for TPP. However, further evidence is required to confirm this suggestion. Kuhn and Wickner (1986) isolated non-cleavable mutants to a precursor normally processed by LEP. These workers used mutants with single amino acid changes in residues at -3 and -1 relative to the cleavage site. Mutation or deletion of the DNA sequence at -3 and -1 of lumenal precursors would indicate the involvement of these residues in the recognition by TPP. Failure of TPP to cleave these precursors would suggest that the sequence was essential for TPP recognition. However, this would not eliminate the possibility of the presence of an additional constraint being required for recognition. Dierstein and Wickner (1986) propose that four amino acids a few residues upstream of the cleavage site are essential for LEP recognition of leader sequences. Similar mutational analysis may also be applied to this region in lumenal precursors. It has been proposed that the hydrophobic core of leader sequences is not involved in LEP recognition, however, this does not preclude the involvement of the hydrophobic core of transit peptides in TPP recognition. Mutational analysis may also be applied to this area. Robinson and Ellis (1985) used charged amino acid analogues to disrupt the presequence structure of stromal protein precursors cleaved by SPP. These workers replaced threionine residues with B hydroxy norvaline during *in vitro* translations, a similar substitution could be made with PCi

used in *in vitro* TPP activity assays to establish whether disruption of the hydrophobic core prevented recognition by TPP.

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