DUAL TARGETING OF GLUTATHIONE REDUCTASE TO MITOCHONDRIA AND CHLOROPLASTS

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Abstract

As a consequence of the presence of both mitochondria and chloroplasts in plant cells there is a higher sorting requirement in a plant cell than in a non-plant cell. Reflecting this, protein import to mitochondria and chloroplasts has been shown to be highly specific. However, there is a group of proteins which are encoded by a single gene in the nucleus, translated in the cytosol and targeted to both mitochondria and chloroplasts. These proteins are referred to as dual targeted proteins. The first protein shown to be dual targeted was pea glutathione reductase (GR). The focus of this thesis is the targeting properties of the dual targeted protein glutathione reductase.

In order to overcome the limitations with traditional *in vitro* import systems we have developed an import system for simultaneous import of precursor proteins into mitochondria and chloroplasts (dual import system). The chloroplastic precursor of the small subunit of ribulose bisphosphate carboxylase/oxygenase (SSU) was mis-targeted to pea mitochondria in a single import system, but was imported only into chloroplasts in the dual system. The dual GR reductase precursor was targeted to both mitochondria and chloroplasts in both the single and dual import system.

We have investigated the targeting and processing properties of the GR targeting signal. Using N-terminal truncations we have demonstrated that the GR targeting signal has a domain organisation. Our results show that the C-terminal is sufficient for chloroplast import, the internal part required for mitochondrial import and the N-terminal part contain a “fine-tuning” function. Furthermore, we have constructed a range of point mutations on the GR signal sequence, changing positive amino acid residues and stretches of hydrophobic amino acid residues. Overall single mutations had a greater effect on mitochondrial import than on import into chloroplasts. We have also shown that the recognition of the GR processing site differs between MPP and SPP. Single amino acid substitutions in the vicinity of the processing site clearly affected processing by MPP while processing by SPP showed low sensitivity to single mutations.
List of Publications for the thesis


Additional Publications


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<th>Abbreviation</th>
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<tr>
<td>AIP</td>
<td>Arylhydrocarbon receptor-interacting protein</td>
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<td>AKAP</td>
<td>Protein kinase A anchor protein</td>
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<td>AOX</td>
<td>Alternative oxidase</td>
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<td>At</td>
<td><em>Arabidopsis thaliana</em></td>
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<td>CAT</td>
<td>Chloramphenicol acetyltransferase</td>
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<td>EM</td>
<td>Electron microscopy</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<td>GIP</td>
<td>General import pore</td>
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<td>GR</td>
<td>Glutathione reductase</td>
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<td>Hsp70</td>
<td>Heat shock protein 70</td>
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<td>Hsp90</td>
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<td>IM</td>
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<td>Intermembrane space</td>
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<td>MPP</td>
<td>Mitochondrial processing peptidase</td>
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<td>MSF</td>
<td>Mitochondrial import stimulating factor</td>
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<td>mtHsp70</td>
<td>Mitochondrial Hsp70</td>
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<td>NMR</td>
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<td>OM</td>
<td>Outer membrane</td>
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<td>PAM</td>
<td>Presequence translocale-associated motor</td>
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<td>PBF</td>
<td>Presequence binding factor</td>
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<td>Presequence protease</td>
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<td>SAM</td>
<td>Sorting and assembly machinery</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<td>SET</td>
<td>Serial endosymbiotic theory</td>
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<td>SPP</td>
<td>Stromal processing peptidase</td>
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<td>SSU</td>
<td>Small subunit of ribulose bisphosphate carboxylase/oxygenase</td>
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<td>TF</td>
<td>Targeting factor</td>
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<td>Translocase of the inner envelope membrane of the chloroplast</td>
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<td>TOM</td>
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<tr>
<td>TPR</td>
<td>Tetratricopeptide repeat</td>
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THE STRUCTURE OF MITOCHONDRIA AND CHLOROPLASTS

Cellular structure

A plant cell as we know it today shares many features with other eukaryotic organisms such as mammals and fungi. It contains a nucleus harbouring most of the genetic material as well as a range of membrane bound organelles. Despite many similarities, the plant cell has some unique characteristics, maybe the most prominent being that they are able to manufacture their own food by using sunlight to convert water and carbon dioxide to carbohydrates.

A plant cell is approximately 10 to 100 µm in diameter and if has a spherical shape if grown in isolation, but when surrounded by other cells they are pressed in to a polyhedron and adjacent cells are often connected via the plasmodesmata. Each cell is enclosed by a rigid cell wall, which contains about 90 % carbohydrates and 10 % proteins. Inside the cell wall there is a plasma membrane (plasmalemma) which contains proteinaceous receptors and various transporters. Besides the nucleus, the plant cell contains the two energy converting organelles; plastids and mitochondria. Plant cells do not have lysosomes but their vacuole carries out virtually the same functions. Young plants usually contain numerous small vacuoles which merge to form a lager vacuole in the mature plant. The vacuole occupies a large part of the cell volume and plays an important role in maintaining cell turgor.

The mitochondrion

Virtually all eukaryotic cells contain mitochondria, and an average plant cell contains several hundred with an approximate diameter of about 1 µm. The shape can be oval, spherical or an elongated worm like structure. Most work concerning mitochondrial structure and morphology has been done in non-plant organisms. The mitochondrion contains two major compartments, the inter membrane space (IMS) and the matrix, bound by two membranes, the outer membrane (OM) and the inner membrane (IM). The OM contains large amounts of the pore forming protein porin, which renders it permeable.
to low molecular mass proteins (less than 10 kDa) and ions. The IM on the other hand is highly impermeable and molecules and ions have to pass through specific translocators. The IM comprises a much larger surface than the OM and is invaginated to fold the cristae which enclose the highly concentrated soluble matrix. Electron microscopic (EM) tomography has revealed that the cristae are connected to the inner boundary membrane via tubular structures termed crista junctions, which have an approximate diameter of 28 nm (Frey and Mannella 2000)

The plant mitochondrial proteome

The accumulation of nucleic acid data and recent advances in protein identification technology have enabled us to gain information about the mitochondrial proteome. Two-dimensional electrophoresis studies indicate that about 500-1500 protein spots can be resolved from plant mitochondrial samples (Bardel et al. 2002; Kruft et al. 2001; Millar et al. 2001), although the non-redundant set of proteins is probably lower. This is in agreement with proteomic studies from mammalian (Taylor et al. 2003a) and yeast mitochondria (Kumar et al. 2002). Taylor et al. were able to identify 615 mitochondrial proteins from human heart tissue using mass spectrometry (Taylor et al. 2003b). They were able to assign a function to 81% of the identified proteins with a significant proportion involved in respiration, signaling, RNA, DNA and protein synthesis, ion transport and lipid metabolism.

In another study Sickmann et al. were able to identify 750 mitochondrial proteins from the yeast, Saccharomyces cerevisiae, and based on mitochondrial proteins described in the literature they calculated this set to compromise about 90 % of the total mitochondrial proteome (Sickmann et al. 2003).

Although plant mitochondria are thought to resemble mammalian and yeast mitochondria they have some additional functions such as uncoupled bypasses of the electron transport chain and the synthesis of lipids and vitamins (Bartoli et al. 2000; Gueguen et al. 2000; Rebeille et al. 1997). There have been several attempts to identify the protein content of mitochondria from Arabidopsis (Kruft et al. 2001; Millar et al. 2001) and pea (Bardel et al. 2002) using two-dimensional electrophoreses coupled to mass spectrometric methods. These reports each identified 40-90 proteins. However, the use of gel based technologies
has several limitations, the most prominent being the difficulty to separate hydrophobic proteins and to detect low abundant proteins. To overcome this Heazlewood et al. undertook a systematic LC-MS/MS study of *Arabidopsis* mitochondria which allowed direct analysis of trypsin digested proteins without any prior gel separation. Combining the LC-MS/MS data with the gel based data generated a non-redundant set of 416 proteins where 407 were nuclear encoded and the remaining 9 were encoded in the mitochondrial genome. The identified proteins were assigned to different functional groups where most proteins were related to energy (24%), metabolism (19%) or protein fate (13%) (Heazlewood et al. 2004).

**The chloroplast**

Plant cells contain plastids which all originate from proplastids found in meristematic cells. During plant development the proplastids will differentiate to form three major groups of plastids, the green chloroplasts, the colored chromoplasts and the colorless leucoplasts. The most important and abundant plastids are the chloroplasts, which are mainly found in the mesophyll of green leaves. Chloroplasts are the second largest compartment in the plant cell and take up about 16% of the total cell volume. Chloroplasts are usually lens-shaped and are about 5-8 µm in diameter and 1 µm in length.

Chloroplasts are surrounded by two membranes, the outer envelope membrane which is permeable to molecules up to 10 kDa, but not to macromolecules such as bigger proteins and nucleic acids. The inner envelope membrane is a permeability barrier and molecules can only be transported across the membrane through specific translocaters. Chloroplasts also contain an internal system of membranes made up of thylakoids vesicles. Thylakoids are often stacked together and form flattered sacs called grana, which are connected by the non-stacked stroma lamellae. The membranes enclose three distinctive compartments, the inner membrane space, the stroma space and the thylakoid lumen.

**The chloroplastic proteome**

The chloroplast proteome has been estimated *in silico* using software tools
like TargetP and ChloroP (Emanuelsson et al. 2000; Emanuelsson et al. 1999). More than 3600 proteins are predicted to be targeted to the chloroplast (Baginsky and Gruissem 2004). However, the prediction programs can be erroneous since they are based on transit peptide prediction and transit peptides are not very well conserved (Bruce 2001). Furthermore there are examples of chloroplastic proteins without canonical transit peptides (Miras et al. 2002) which therefore can not be detected by prediction programs. A big challenge in proteomic studies is to identify both high and low abundant proteins. This is particularly difficult in compartments like the thylakoid membranes, in which the photosynthetic apparatus and its hydrophobic chromophores dominate. Friso et al. used a combination of different fractionation methods in an attempt to identify as many proteins as possible associated with the thylakoid membranes in Arabidopsis (Friso et al. 2004). In combination with the results from a previous study (Peltier et al. 2002) they were able to identify 198 proteins of which 76 (39%) were integral membrane proteins. Theoretical predictions of the luminal proteome from Arabidopsis chloroplasts estimated it to contain approximately 80 proteins. 2-D gels followed by both MALDI-TOF mass spectrometry and amino-terminal microsequencing identified 36 proteins in the lumen of Arabidopsis which is in agreement with studies from Spinach (Schubert et al. 2002). LC-MS/MS studies have identified at least 100 proteins in the envelope membranes many of which are ion and metabolite transporters, proteins involved in fatty acid, glycerolipid, vitamin and pigment metabolism, components of the protein import machinery and proteases (Ferro et al. 2003).

EVOLUTION OF THE PLANT CELL

Origin of the plant cell

Both chloroplasts and mitochondria were once free-living bacteria and the modern eukaryotic cell is a descendant from an endosymbiotic event. The endosymbiotic hypothesis was proposed by Margulis et al. 1970 and a few years later named the serial endosymbiotic theory (SET). The SET states that mitochondria and chloroplasts are direct descendants of eubacterial ancestors that were engulfed by a host cell, probably already containing a nucleus (Lang 1999).
Phylogenetic data suggest that an ancient α-protobacterium is the ancestor of mitochondria and was taken up by a host cell more than 2 billion years ago (Dyall et al. 2004). The α-protobacterium Rickettsia prowazekii, an obligate intracellular parasite and the causative agent of epidemic typhus is more closely related to the mitochondrial genome than any other microbe studied so far and is thought to be the closest living relative of the proto-mitochondria (Andersson et al. 1998). R. prowazekii has a small genome of 1.1 million base pairs (bp) encoding 834 proteins, which is less than a fourth of the gene content in the well studied γ-protobacterium Escherichia coli (4288 protein-coding genes) (Blattner et al. 1997). Gene structure is frequently conserved between bacterial and mitochondrial genomes, for example the genes rplKAJL and rpoBC are organized in an identical manner in R. prowazekii and in the mitochondrial genome of Reclinomonas americana, a freshwater protozoan. Sequence data from several other α-proteobacteria further support the idea of a protobacterial ancestor of mitochondria (Adams and Palmer 2003). Although genetic data points towards a monophylogenetic origin of mitochondria the progenitor is probably not R. prowazekii, although it might be another free-living bacteria prone to a parasitic life style.

Both phylogenetic and fossil data indicate that the chloroplast was taken up in an endosymbiotic event after the mitochondrion. In a first primary endosymbiosis about 1.5 billion years ago an ancient cyanobacterium was engulfed by a mitochondria-containing eukaryote. The plastids that have arisen from this primary endosymbiosis are surrounded by two membranes and are found in land plants, glaucocystophytes and in red and green algae (Gray 1999). Plastids were further distributed throughout the plant kingdom via a secondary endosymbiotic event where a heterotrophic eukaryote was fused with a primary algae already containing a primary plastid. Secondary plastids are found in lineages such as apicomplexa, dinoflagellates and ciliates and are characterized by the presence of three or four surrounding membranes (Archibald and Keeling 2002).

**Reduction of the organellar genomes**

As a remnant of once being free-living prokaryotes both mitochondria and chloroplasts still maintain their own genomes. Today organellar genomes are heavily reduced, encoding less than 5 % of the protein content of the respective organelle. During the
course of evolution a massive gene reduction of the organellar genome has occurred whereby the majority of the genes have been lost or transferred to the nucleus. As a consequence of becoming an intracellular organelle, many genes were expendable and therefore lost from the organellar genome, while the function of other genes was replaced by nuclear genes. Additionaly, a considerable number of genes have been transferred to the nucleus. Functional gene transfer ceased approximately 600 million years ago in animals and the majority of sequenced animal mitochondrial genomes contain the same 13 protein coding genes (Boore 1999). However, in plants and particular in angiosperms, gene transfer is a frequent and ongoing event (Adams et al. 2002).

A frequently asked question is what drives gene transfer. One popular hypothesis is Mullers’s ratchet which states that the asexual reproduction of mitochondria and chloroplasts can lead to a faster accumulation of deleterious mutations (Berg and Kurland 2000; Kurland 1992). Chloroplasts and mitochondria have a higher volume-based redox activity, hence the production of oxygen free radicals is higher (Allen and Raven 1996; Martin and Palumbi 1993). An increase in oxygen free radicals has been shown to lead to an increase in mutation rate and therefore it would be advantageous to relocate genetic material from the organelles to the nucleus. However, this might not be true for plant mitochondria where the substitution rate is lower than for nuclear DNA (Wolfe et al. 1987). As photosynthetic plants have three genomes and hence the need for three sets of gene expression apparatus, it has also been argued that having all genes in one location would be more economical and favourable to the organism (Berg and Kurland 2000).

Why then have organelles retained some genes when the majority have been transferred? There are two major explanations, one being the ‘hydrophobicity hypothesis’, which states that many hydrophobic, membrane embedded proteins are difficult to transfer (von Heijne 1986). Indeed many of the proteins still encoded in organellar genome are hydrophobic, membrane spanning proteins, and it has been shown that reduction in hydrophobicity was crucial for gene transfer in some cases (Daley et al. 2002). However, many genes encoding for hydrophobic proteins have been transferred and the expressed proteins are successfully imported back to the organelle. The second explanation, the CORR theory (Co-location for Redox Regulation) was proposed in 1992 (Allen 1992;
Allen 1993). This theory proposes that there is a direct correlation between coding location and gene expression, and that organellar control over certain genes would give a fitness advantage for the organism.

**TARGETING SIGNAL SEQUENCES**

**Evolution of targeting signal sequences**

To become activated the transferred gene has to acquire several regulatory elements for proper expression and in most cases this includes the gain of a targeting signal sequence to direct the precursor protein back to the correct compartment. There are several ways by which the acquisition of a targeting signal sequence can occur. One way is by associating with pre-existing organellar genes and examples of this are the rps genes from various plant species. For some transferred genes the flanking regions give no clue to where the targeting signal sequence came from although strategically located introns indicate that some exon shuffling has occurred (Daley et al. 2002; Nugent and Palmer 1991; Wischmann and Schuster 1995). One case of exon shuffling is glyeraldehyde-3 phosphate dehydrogenase from which three N-terminal exons were moved to cytochrome c1 and those three exons evolved to become the mitochondrial targeting signal sequence of cytochrome c1 (Long et al. 1996). Given the diversity in targeting signal sequences, it is also possible that some have formed de novo. It has also been suggested that some proteobacterial and cyanobacterial proteins carry rudimentary features of mitochondrial and chloroplastic targeting signals respectively, such as basic and amphipathic extensions at their amino terminal end and that those sequences might have functioned as targeting signal sequences for the first transferred genes (Lucattini et al. 2004).

**Features of targeting signal sequences**

The great majority targeting signal sequences from plant mitochondrial are between 20-60 amino acids long (Zhang and Glaser 2002) which is longer than targeting signals from animals and fungi. However, they can vary substantially in length, from 13 amino acids to 136 amino acids. Chloroplastic targeting signal sequences are considerably longer than
mitochondrial ones and can vary between 13 to 146 amino acids with an average of 58 residues (Zhang and Glaser 2002).

The amino acid composition of plant mitochondrial and chloroplastic targeting signal sequences is remarkably similar in that it is rich in hydrophobic, hydroxylated and positively charged amino acid residues, and low in acidic residues. Serine residues are clearly over represented (16.2 % for mitochondrial and 19.5 % for chloroplastic) which is in contrast to the serine content of mitochondrial presequences in yeast (7 %), mammals (3 %) and Neurospora crassa (10 %) (Glaser and Dessi 1999; Peeters and Small 2001).

Both NMR experiments and in silico analysis have shown that mitochondrial presequences have a propensity to form amphiphilic α-helices in the N-terminal portion of the presequence (Chupin et al. 1995; Karslake et al. 1990; von Heijne 1986), and substitution of amino acids in the predicted amphiphilic α-helix has shown that this structure is important for targeting to mitochondria (Roise et al. 1988). Structural data are available mainly for presequences from non-plant species of which most are less than 26 amino acid residues long. Mitochondrial presequences have been shown to adopt a helical conformation in the presence of membrane model systems such as micelles or organic solvents while being unstructured in aqueous environments. Recently, the first NMR structure of a mitochondrial presequence from higher plants, the Nicotiana plumbaginifolia F₁β, was reported (Moberg et al. 2004). In bicelles, the F₁β presequence adopted three α-helices, an N-terminal amphipathic helix, a small helix in the middle region and a C-terminal helix, separated by unstructured internal domains (Moberg et al. 2004).

Although different mitochondrial presequences do not share any sequence similarity there are some amino acids that are more abundant around the cleavage site and hence might be needed for processing. Mitochondrial presequences can be divided into three groups based on the cleavage site. Two major groups representing 38% and 42% of presequences contain an arginine either at position –2 or –3 relative to the cleavage site, and have a loosely conserved motif around the cleavage site, Arg-X ↓ Ser-Thr/Ser-Thr and Arg-X-Phe/Thr ↓ Ala/Ser-Thr/Ser/Ala, respectively. A third group includes presequences lacking a conserved arginine in the vicinity of the cleavage site (Zhang et al. 2001).
Initial studies indicated that chloroplastic targeting peptides contained three major homologous blocks of amino acids (Karlin-Neumann and Tobin 1986; Schmidt and Mishkind 1986; von Heijne et al. 1989), but this was later shown not to be valid for targeting peptides as a group. However, in general they contain three distinct regions, an uncharged N-terminal domain, a central domain lacking acidic residues and a C-terminal domain with the potential to form an amphiphilic β-strand (Claros and Vincens 1996; von Heijne et al. 1989). The secondary structure of targeting peptides is predicted to be mainly random coil (Pilon et al. 1992; Theg and Geske 1992), although later data suggest that chloroplastic targeting peptides in a hydrophobic solution or inserted into micelles have the ability to form an α-helical structure (Bruce 2000). Most data concerning the structure of chloroplastic targeting peptides come from proteins in *Chlamydomonas reinhardii*, a lower photosynthetic eukaryote, which has much shorter targeting peptides than higher plants. The only NMR structure available of a higher plant targeting peptide is *Silenes* ferredoxin, which is mainly unstructured with the ability to form two helical domains when introduced into micelles (Wienk et al. 1999).

It has been suggested that chloroplastic targeting peptides contain a loosely conserved motif around the processing site, Val/Ile-X-Ala/Cys ↓ Ala, and that most signal sequences contain a basic residue within the 7 most C-terminal amino acids of the targeting peptide (Gavel and von Heijne 1990). Extensive logos of 277 chloroplast targeting peptides with an experimentally mapped cleavage site confirmed the high abundance of Val in position –3 and Ala in position –1 (Zhang and Glaser 2002).

**PROTEIN IMPORT INTO MITOCHONDRIA**

The vast majority of mitochondrial proteins are encoded in the nucleus and synthesized in the cytosol and hence have to be imported into the organelle. This is facilitated by a cooperation between the newly synthesized precursor protein, cytosolic chaperones and proteinaceous components located in the outer and inner mitochondrial membrane. The protein import machinery of mitochondria has been studied extensively for decades and the majority of the work has been done in *S. cerevisiae* and *N. crassa* using biochemical and genetic approaches (for review see Truscott et al. 2003a). The plant
mitochondrial import machinery has been characterized in *S. tuberosum* and *A. thaliana* (Jansch et al. 1998; Lister et al. 2002; Murcha et al. 2003; Werhahn et al. 2001). Although sharing several features, the plant mitochondrial import machinery differs in some respects from the import machinery in yeast.

**Figure 1.** The plant mitochondrial import machinery. Model of the import of precursor proteins into mitochondria and components of the translocation apparatus.

**Interaction of mitochondrial proteins with cytosolic factors**

Generally mitochondrial proteins are more hydrophobic than cytoplasmic proteins and nascent polypeptides have to be protected against aggregation and misfolding on their route to the mitochondrial surface (Claros et al. 1995). There are two mechanisms working to minimize this; interaction of polypeptides and cytosolic chaperones or coupling of protein translation and translocation (Beddoe and Lithgow 2002).
It has traditionally been assumed that the majority of mitochondrial proteins are translated on ribosomes distant from the mitochondrial surface and then imported in a post-translational manner (Honlinger et al. 1995; Reid and Schatz 1982; Wienhues et al. 1991). This is mainly based on the observation that in vitro synthesized precursor proteins can be imported into mitochondria. To prevent misfolding or aggregation and to keep the newly synthesized precursor protein in an import-competent conformation, they interact with a range of cytosolic chaperones. Using two-hybrid screening Yano et al. were able to identify an arylhydrocarbon receptor-interacting protein (AIP) interacting with Tom20. Using an in vitro import assay they were also able to show that AIP binds specifically to mitochondrial preproteins. Furthermore, in cultured cells the overexpression of AIP stimulated import of preornithine transcarbamylase and depletion of AIP with RNA interference impaired import. They concluded that AIP forms a ternary complex together with Tom20 and the preprotein, and functions as a cytosolic chaperone to prevent mitochondrial precursor proteins from aggregation (Yano et al. 2003).

There is a range of other cytosolic chaperones described in the literature such as the presequence binding factor (PBF), the targeting factor (TF), the mitochondrial import stimulating factor (MSF). Since first being reported more than a decade ago not much attention has been paid to these proteins.

Already in the 1970’s it was demonstrated that translationally active ribosomes containing mRNA for mitochondrial proteins were accumulating on the surface of yeast mitochondria (Kellems et al. 1974). Several more recent reports support the idea that a co-translational process is involved in the import of some mitochondrial proteins (for review see (Lithgow 2000). One example is fumarase, which in yeast is found both in the cytosol and in mitochondria, and could only be co-translationally translocated into the mitochondrial matrix using in vitro assays (Knox et al. 1998).

Marc et al. preformed a genome-wide analysis to characterize the mRNA encoding mitochondrial proteins and showed that almost half of the studied mRNA’s were translated in the vicinity of the mitochondrial surface. Interestingly, it seems like proteins of prokayotic origin are mainly translated in a co-translational manner while proteins of eukaryotic origin are post-translationally imported (Marc et al. 2002).
**Translocase of the outer membrane (TOM)**

Initial recognition and translocation of precursor proteins earmarked for mitochondria are accomplished by the TOM complex. The TOM complex has a dual function in protein import. Firstly, via the action of import receptors TOM recognizes mitochondrial precursor proteins that have been translated in the cytosol (Brix et al. 1999). Secondly, it forms the import channel through which the precursor proteins are translocated across the outer membrane. Isolation of the TOM complex from yeast, mammals and plants indicates that the overall structure is rather well conserved between different organisms. However, it appears that the plant complex differs from its counterparts in yeast and mammals with respect to some of the receptor components.

**Import receptors**

In yeast the main receptors for protein recognition are Tom70, Tom20 and Tom22 (Abe et al. 2000; Brix et al. 1999; Kunkele et al. 1998), and are named according to their apparent molecular mass as determined by SDS-PAGE. Tom70 is attached to the outer mitochondrial membrane via an N-terminal hydrophobic membrane anchor and has a large hydrophilic domain exposed to the cytosol (Hines et al. 1990). It is the major receptor for precursor proteins that contain internal targeting signals, such as members of the metabolite carrier family. Tom70 does not only serve as an import receptor but can also function as docking point for cytosolic chaperones (Young et al. 2003). Despite several biochemical characterizations Tom70 has not been found as a component of the mitochondrial outer membrane in plants (Werhahn and Braun 2002). Furthermore, no homologue with significant sequence similarity could be identified in the *Arabidopsis* genome (Lister et al. 2003). Although some of the carrier proteins in plants contain an N-terminal targeting signal, 50-70 % of the carrier family in the *Arabidopsis* genome are predicted to contain internal signals (Millar and Heazlewood 2003). The absence of Tom70 in plants is therefore intriguing since a carrier import pathway has been demonstrated (Lister et al. 2002). Recently, Chew et al. identified an N-terminally anchored protein on the plant mitochondrial outer membrane, mtOM64, showing sequence similarity to the chloroplastic receptor protein Toc64. It is possible that
mtOM64 could substitute for the missing Tom70 in plant mitochondria (Chew et al. 2004).

Tom20 is the main receptor used by proteins containing an N-terminal cleavable presequence and thus utilizing the general import pathway. The structure of the cytosolic domain of rat Tom20 in complex with a peptide derived from the rat mitochondrial aldehyde dehydrogenase transit peptide has been solved and shows that the transit peptide binds in an apolar groove in Tom20 (Abe et al. 2000). Although sharing some sequence similarity, the plant Tom20 differs from Tom20 in mammals and fungi in its topology. While yeast Tom20 is anchored via its N-terminal region the plant protein seems to be attached via the C-terminal domain. A common feature that seems to emerge for plant genes is that they exist in gene families and there are at least four isoforms of Tom20 in Arabidopsis (Lister et al. 2003; Werhahn et al. 2001). The third receptor of the outer membrane is Tom22, and as well as Tom20 it is involved in the recognition of precursor proteins containing N-terminal targeting peptides. Tom22 probably interacts with the targeting peptide via ionic forces where the positively charged surface of the targeting peptide binds to the negatively charged Tom22 (Brix et al. 1997). Tom22 is a multifunctional protein, which is required for the high level organization of the TOM complex and is stably associated with import channel protein Tom40. It binds preproteins through both its cytosolic domain and its intermembrane space domain (van Wilpe et al. 1999). Although Tom22 is highly conserved between fungi and metazoan the plant protein differs substantially in size. The plant Tom22 homologue has a transmembrane segment and a trans domain equivalent to Tom22 but lacks the cytosolic acidic cis domain and is about 9 kDa in size. It has been suggested that the difference is due to the unique environment in plants where both mitochondria and chloroplasts are present and it is possible that this protein is involved in the targeting specificity (Macasev et al. 2000; Macasev et al. 2004).

**The sorting and assembly machinery (SAM)**

Three proteins of the SAM complex in yeast mitochondria are known, Sam50 and Sam35 which are essential proteins, and Sam37. The SAM-complex plays an important role in
the assembly of outer membrane β-barrel proteins such as Tom40 and porin. Sam50 contains a β-barrel domain, which is conserved from bacteria to man (Kozjak et al. 2003). The bacterial homologue Omp85 is involved in transport of either proteins or lipids to the outer membrane of gram-negative bacteria (Genevrois et al. 2003; Voulhoux et al. 2003), and it is possible that mitochondria have retained this conserved domain for import and assembly of β-barrel proteins. Sam35 is a peripheral membrane protein that is exposed on the mitochondrial surface (Milenkovic et al. 2004). When the SAM-complex was purified a fourth subunit Mdm10, a β-barrel protein, was found (Meisinger et al. 2004). Mdm10 has been previously identified by its role in maintaining mitochondrial distribution and morphology (Sogo and Yaffe 1994). Whereas the three subunits of the SAM core complex, Sam35, Sam37 and Sam50, are generally required for the biogenesis of β-barrel proteins of the outer membrane, Mdm10 plays a specific role in the assembly of the TOM-complex (Pfanner et al. 2004).

**The general import pore (GIP)**

Subsequent to interaction with receptors the precursor proteins are transferred to the general import pore (GIP), which consists of the channel forming protein Tom40 and three small proteins, Tom5, Tom6 and Tom7 (Hill et al. 1998; Kunkele et al. 1998). Tom5 functions as a link between the receptor proteins and the general import pore. Directly after interaction with Tom22 the precursor proteins are transferred to Tom5, which mediates their insertion into Tom40. Despite its small size Tom5 is an integral membrane protein with its negatively charged N-terminal portion exposed to the cytosol (Dietmeier et al. 1997). Tom40 is a pore-forming integral membrane protein which consists mainly of β-sheet structures and forms a cation-selective high conductance channel (Ahting et al. 2001). All mitochondrial proteins, which cross the outer membrane are imported via the Tom40 pore (Ahting et al. 2001). Each Tom40 molecule contains two to three channels where each channel has an approximate diameter of 22 Å, which is sufficient for the passage of a single α-helical segment or a loop (Kunkele et al. 1998). Tom40 is the only Tom protein that is essential for cell viability under all growth conditions (Baker et al. 1990).
The small subunits Tom6 and Tom7 do not interact with precursor proteins during protein import, rather they modulate the stability of the Tom components (Alconada et al. 1995; Honlinger et al. 1996). Tom6 has been proposed to support the cooperation between the receptors, in particular Tom22 and the general import pore (Alconada et al. 1995; Dekker et al. 1998; van Wilpe et al. 1999). Lack of Tom7 stabilizes the GIP complex and hence it was suggested that Tom7 plays a role opposite to that of Tom6 by exerting a destabilizing affect on the association of Tom components (Honlinger et al. 1996).

Translocation across the outer mitochondrial membrane is unidirectional and the movement across the membrane is thought to be promoted by electrostatic interactions, the so-called “acid chain hypothesis” (Komiya et al. 1998). A presequence containing protein successively interacts with at least five different binding sites on its way across the membrane – Tom20, the cytosolic domain of Tom22, Tom5, Tom40 and the intermembrane space domain of Tom22. Several of these receptor proteins contain negatively charged patches, and it has been suggested that the positively charged presequence is recognised by increasing affinity along the import pathway (Dietmeier et al. 1997; Komiya et al. 1998). However, Muto et al. reported that the presequence interacts with Tom20 via hydrophobic patches showing that forces other than ionic are crucial for interactions of the presequence with Tom proteins (Muto et al. 2001). This led to the re-naming of the “acid-chain hypothesis” to the “binding-chain hypothesis”.

**Translocase of the inner membrane (TIM)**

After guidance across the outer membrane by the chain of TOM proteins, preproteins interact with one of two TIM complexes in the inner membrane – the TIM23 complex and the TIM22 complex. The TIM23 complex mediates the translocation of proteins into the matrix, whereas the TIM22 complex is required for the insertion of some polytopic proteins into the inner membrane (Jensen and Dunn 2002).
The TIM23 complex

The majority of presequence-containing proteins destined for the matrix use the TIM17:23 channel to pass the inner membrane. After release from the TOM complex the protein first interacts with the membrane spanning protein Tim50, which exposes a large domain to the IMS. After interaction with Tim50 the precursor protein is transferred to Tim23 and Tim17, two essential integral membrane proteins that form a core complex of 90 kDa (Dekker et al. 1997) Jensen and Dunn (2002). Electrophysiological studies using mitochondrial IM fractions have shown that Tim23 and Tim17 form the pore through which proteins are translocated into the matrix (Lohret et al. 1997). In addition to the membrane domain Tim23 has an extension protruding across the IMS to the OM which is thought to link the outer and inner mitochondrial membrane (Donzeau et al. 2000). The role of Tim17 in yeast is not clear but based on its sequence similarity to Tim23 it is likely to function in channel formation as well (Jensen and Dunn 2002). The TIM17:23 translocase seems to be highly conserved among all eukaryotes (Rassow et al. 1999). In *A. thaliana* there are three genes present for each of TIM23 and TIM17. Although being homologous to their yeast counterparts AtTim23 and AtTim17 differ in their topology. In plants it seems like Tim17 rather than Tim23 contains a C-terminal extension that protrudes into the IMS and it has been suggested that this extension links the outer and inner mitochondrial membrane (Murcha et al. 2003).

The majority of presequence carrying proteins are imported into the matrix by the combined action of the TIM23 translocase and the presequence translocase-associated motor PAM. For decades it has been thought that the ATP dependent import motor consists of three essential proteins; the peripheral inner membrane protein Tim44, the chaperone mtHsp70, and the nucleotide exchange factor Mge1. Tim44 recruits mtHsp70 in its ATP-bound form, which has an open peptide-binding site (Schneider et al. 1994) (Schneider et al. 1996). After binding to the emerging precursor chain, ATP is hydrolysed, the peptide binding site closes and mtHsp70 is released from Tim44. As the precursor chain moves into the matrix mtHsp70 is released and this requires the nucleotide exchange factor Mge1 (Schneider et al. 1996). Mge1 removes the bound nucleotide and allows regeneration of the ATP-bound form and hence opening of the
peptide-binding site (Bolliger et al. 1994; Ikeda et al. 1994; Nakai et al. 1994). Recently two new essential co-chaperones have been identified, Pam18 (Tim14) and Pam16 (Tim16). Pam18 belongs to the J-protein family and stimulates the ATPase activity of mtHsp70 (D'Silva et al. 2003; Mokranjac et al. 2003; Truscott et al. 2003b). Pam16 is involved in the recruitment of Pam18 to the TIM23 complex (Frazier et al. 2004; Kozany et al. 2004).

Two models for the action of the import motor have been proposed and most likely both mechanisms co-operate to translocate precursor proteins across the membrane. In the Brownian ratchet (trapping) model Hsp70 acts as an arresting component of a ratchet which allows only forward movement of the polypeptide chain (Neupert and Brunner 2002). In this way retrograde movement of the polypeptide chain is prevented and spontaneous forward movement can be transduced into vectorial transport by cycles of successive mtHsp70 binding (Neupert and Brunner 2002). In the other model, the power stroke model, mtHsp70 has a more active role in which, in concert with Tim44 and Mge1 it pulls the precursor protein inwards to the matrix. After binding to the incoming presequence mtHsp70 undergoes a conformational change. By this repeated cycle of ATP-dependent binding mtHsp70 will function as a motor pulling the precursor into the matrix (Matouschek et al. 1997).

**The TIM22 complex**

A second translocon exists in the mitochondrial inner membrane, called the TIM22 complex. The TIM22 complex is responsible for the import of polytopic IM proteins such as the carrier proteins and the membrane imbedded members of the TIM23 complex. Tim22 was first found as a protein homologue to Tim23 and Tim17 in the yeast genomic DNA sequence (Sirrenberg et al. 1996). Like Tim23 and Tim17, Tim22 has four TM segments and is positioned with its C- and N- terminus facing the IMS. Tim22 forms a large aqueous pore 11 to 18 Å wide (Kovermann et al. 2002). Import via the TIM22 complex requires a membrane potential but does not appear to use ATP (Jensen and Dunn 2002). The TIM22 complex consists of two additional integral membrane proteins, Tim54 and Tim18 (Kerscher et al. 1997). Neither Tim54 nor Tim18 appear to play an essential role in protein import since gene disruption of TIM18 is not lethal (Kerscher et
al. 2000; Koehler et al. 2000). Although the exact role for Tim54 and Tim18 are unknown, both proteins are required for formation or stability of the TIM22 complex (Jensen and Dunn 2002).

For its function the TIM22 complex also requires the action of a family of small proteins called the small Tims (Adam et al. 1999; Koehler et al. 1998a; Koehler et al. 1998b; Sirrenberg et al. 1998). Tim12 is associated with the TIM22 complex while Tim9 and Tim10 are found both as a part of the TIM22 complex and as a soluble 70 kDa complex in the IMS. Tim8 and Tim13 form a complex similar in size to the Tim9-Tim10 complex in the IMS (Koehler et al. 1999). The small Tim proteins are thought to shuttle imported proteins from the TOM complex in the OM to the TIM22 complex in the IM. When the precursor protein emerges from the Tom40 pore it binds to the Tim9-Tim10 complex which functions in a chaperone-like manner, and binds to hydrophobic regions of the precursor (Paschen et al. 2000). In contrast to the Tim9-Tim10 complex the Tim8-Tim13 complex is not essential and only a few substrates require this complex, one of which is Tim23 (Paschen et al. 2000).

**The mitochondrial processing peptidase (MPP)**

After translocation of the precursor protein the matrix-targeting signal is no longer necessary and is protolytically removed by the mitochondrial processing peptidase MPP (Arretz et al. 1994; Braun et al. 1992; Eriksson 1992; Hawlitschek et al. 1988). MPP removes presequences in one proteolytical step from precursor proteins that are fully translocated as well as precursors in transit. MPP was originally purified from the mitochondria of *N. crassa* (Hawlitschek et al. 1988), *S. cerevisiae* (Yang 1988) and rat (Kleiber et al. 1990; Ou et al. 1989), and later from the potato (Braun et al. 1992), spinach (Eriksson 1992) and wheat (Braun et al. 1995). In yeast and mammals MPP has been shown to consist of two structurally related subunits, α-MPP and β-MPP. The α subunit is responsible for peptide binding and the β subunit for catalysis (Luciano and Geli 1996). The enzyme exists as a soluble hetrodimer of about 100-110 kDa (Glaser and Dessi 1999). In contrast to the situation in yeast and mammals the plant enzyme is intergrated as core subunits in the membrane bound cytochrome bc1 complex facing the
mitochondrial matrix (Braun and Schmitz 1995; Glaser and Dessi 1999). It has been shown that the translocation channel and the MPP/ bc1 are located separately in the inner membrane of plant mitochondria. Using chimeric constructs consisting of a mitochondrial presequence and dihydrofolic reductase (DHFR) it has been shown that the presequence is not processed directly upon exposure to the matrix, rather the precursor must be translocated some distance beyond the cleavage site. This result implicates the lack of a direct link between precursor translocation and processing (Additional publication 1).

The removed presequences are potentially harmful to the structure and function of mitochondria as they can penetrate and disrupt biological membranes and must therefore be rapidly degraded. This is carried out by a matrix located metallopeptidase, the Presequence Protease, PreP (Stahl et al. 2002).

**PROTEIN IMPORT INTO CHLOROPLASTS**

As for mitochondria, more than 95 % of chloroplastic proteins are encoded by genes located in the nucleus and have to be transported to, and imported into the chloroplast. This transport is aided by cytosolic factors and proteinaceous components located in the outer and inner chloroplastic envelope. As chloroplasts are the most recent organelle in the modern eukaryotic cell and several post-translational import systems were already in operation, such as those of mitochondria, peroxisomes and the plasma membrane (Kunau 2001; Neuhaus and Rogers 1998; Pfanner and Geissler 2001), the chloroplastic import machinery therefore, had to develop unique features to ensure organelle specificity. Over the past decade, a variety of biochemical techniques, using pea chloroplasts as a model system, have been used to identify and characterize the protein components of the chloroplastic import apparatus (Perry and Keegstra 1994; Schnell et al. 1994). The availability of *Arabidopsis* genomic sequence data has led to the use of more molecular-genetic strategies and has enabled the functional study of the import apparatus *in vivo* (Jarvis and Soll 2001).
Interaction of chloroplastic proteins with cytosolic factors

As for mitochondrial proteins, newly synthesized chloroplastic proteins are prone to aggregation and non-productive interactions, and hence need to be protected in the cytosol. Several, but not all chloroplastic transit peptides contain a motif, which can be phosphorylated on a serine or threonine residue (May and Soll 2000). The consensus for the phosphorylation motif, \((P/G)X_n(K/R)X_n(S/T)X_n(S^*/T^*)\) (where * indicates the phosphorylated residues), resembles the motif for binding of 14-3-3 proteins. 14-3-3 proteins belong to a ubiquitous eukaryotic protein family of regulatory proteins, which serve as molecular chaperones mediating protein-protein interactions (Aitken et al. 1992).
The precursor proteins are phosphorylated subsequently to synthesis by a protein kinase. The phosphorylated precursor interacts with the 14-3-3 protein together with Hsp70 to form a hetero-oligomeric cytosolic guidance complex. The guidance complex is then targeted to the appropriate receptor of the outer envelope membrane. Although not being necessary for import into chloroplasts the formation of a guidance complex will result in a 4-fold higher import efficiency for some proteins than for free precursor proteins. Before the precursor protein can be translocated it has to be dephosphorylated by a phosphatase. The model of a guidance complex is supported by the notion that a 14-3-3 protein in the wheat germ translation system can co-immunoprecipitate two different chloroplast proteins in a transit peptide dependent manner. Furthermore, interaction between the 14-3-3 protein and the precursor could only be detected when the transit peptide was phosphorylated on a serine residue within the predicted 14-3-3 binding motif (May and Soll 2000). Besides forming a guidance complex with 14-3-3 proteins, phosphorylated precursor proteins bind to receptors at the outer envelope membrane with a higher affinity than non-phosphorylated (May and Soll 2000). Therefore it has been proposed that a cycle of phosphorylation/dephosphorylation could be used as a regulatory mechanism, which converts the protein from low import competent to a highly import competent form. A recent study, however, found that point mutations within the 14-3-3 binding site of several proteins did not affect protein targeting efficiency or specificity (Nakrieko et al. 2004).

**Translocase of the outer envelope membrane (TOC)**

As for the TOM complex of mitochondria the TOC translocon is involved in both recognition of precursor proteins and translocation of precursor proteins across the outer envelope membrane. Once a precursor protein arrives to the surface of the chloroplast a highly specific recognition process is initiated in which both lipid and protein components of the outer envelope membrane are involved (Jarvis and Soll 2001). Following recognition, the precursor protein is translocated through the TOC complex in an energy-dependent process (Jarvis and Soll 2001). Four TOC components have been described to date, namely Toc159, Toc34, Toc75 and Toc64 which are named according to their predicted molecular masses (Jarvis and Soll
Toc159 has been regarded as the major point of contact for precursor proteins arriving to the import apparatus and has therefore been thought to be the main chloroplast import receptor (Hirsch et al. 1994; Kessler et al. 1994; Perry and Keegstra 1994). The Toc159 protein has a three domain structure: an N-terminal acidic domain (A-domain), a central GTP-binding domain (G-domain) and a C-terminal membrane anchor domain (M-domain) (Chen et al. 2000). Toc159 belongs to a class of GTP-binding proteins and possesses characteristic GTP-binding site motifs within its central domain. In Arabidopsis, four homologues to pea Toc159 have been identified (Hiltbrunner et al. 2001).

Toc34 belongs to a same class of GTP-binding proteins as Toc159, and shares homology with Toc159 even outside of the conserved GTP-binding site motifs (Kessler et al. 1994). Toc34 is anchored to the outer envelope membrane by its carboxy-terminal tail, and most of the protein is exposed to the cytosol (Seedorf et al. 1995). Recently it has been suggested that Toc34 acts as an initial receptor (Becker et al. 2004b). Two different Toc34 homologues exist in Arabidopsis and are referred to as atToc33 and atToc34 (Gutensohn et al. 2000; Jackson-Constan and Keegstra 2001; Jarvis et al. 1998).

Toc75 forms the pore through which precursor proteins are translocated across the outer membrane and is the most abundant protein of the chloroplast outer envelope membrane (Joyard et al. 1983; Keegstra et al. 1984). Toc75 is predicted to be a β-barrel protein with 16 transmembrane β-sheets (Sveshnikova et al. 2000). It forms a cation-selective high-conductance ion channel (Hinnaah et al. 1997), which is approximately 25 Å wide at the entrance and 15-17 Å wide inside the channel. This is wide enough to accommodate a polypeptide stretch that still retains some secondary structure (Hinnaah et al. 2002). Four different Toc75 homologues exist in Arabidopsis, and as for Toc159 and Toc34 one isoform is dominantly expressed (Jackson-Constan and Keegstra 2001).

The role of the fourth TOC component, Toc64, is less well-defined. Toc64 contains three tetratricopeptide motifs in the cytosolic part of the protein (Sohrt and Soll 2000), which are involved in protein-protein interactions. Similar motifs have been found in the mitochondrial receptor Tom70 and it has been suggested that Toc64 has a similar role to that of Tom70 in recognition of hydrophobic precursor proteins (Soll and Schleiff 2004).
Recently a new Toc component was identified, Toc12. Toc12 is an outer envelope protein exposing a soluble domain into the intermembrane space. Toc12 contains a J-domain and stimulates the ATPase activity of DnaK (Becker et al. 2004a).

**Translocase of the inner envelope membrane (TIC)**

After translocation across the outer envelope membrane via the TOC complex the precursor protein is transferred to the TIC complex of the inner envelope membrane. The translocation across the inner envelope membrane requires ATP hydrolysis (Flugge and Hinz 1986), which is probably needed for the action of molecular chaperones in the stroma (Kessler and Blobel 1996; Nielsen et al. 1997). The TIC translocase is a multi-subunit complex which consists of Tic110, Tic62, Tic55, Tic40, Tic22 and Tic20 (Soll and Schleiff 2004). Relatively little is known about the role of these proteins and there are some disagreements in the literature. It has been suggested that the discrepancies are due to the existence of two or more TIC complexes as in the case of the TIM translocase in mitochondria (Soll and Schleiff 2004). Tic110 is the most abundant of the TIC components and has one or two transmembrane segments in its amino-terminal region (Kessler and Blobel 1996; Lubeck et al. 1996). Tic110 is thought to constitute the pore through the inner envelope membrane and can form a cation-selective channel in lipid bilayers (Heins et al. 2002). Besides Tic110 the two most abundant subunits are Tic62 and Tic55, which both have the potential to catalyse electron transfer reactions (Caliebe et al. 1997; Kuchler et al. 2002). Tic55 contains an iron-sulphur centre and a mononuclear iron binding site. Tic62 has a conserved NAD(P) binding site and its carboxy terminal, exposed to the stroma, can interact with ferredoxin NAD(P) reductase. Tic62 and Tic55 are implicated in redox regulation of protein import through the TIC complex (Soll and Schleiff 2004). Tic40 is an integral membrane protein which is tightly associated with Tic110 (Stahl et al. 1999). The exact function of Tic40 is not known but it shares some sequence similarity with the Hsp70-interacting protein (Hip) in its C-terminal domain (Chou et al. 2003). Hip is a mammalian co-chaperone that regulates nucleotide exchange by Hsp70 (Frydman and Hohfeld 1997; Hohfeld et al. 1995) and it is possible that Tic40 has a similar role in regulating the chaperones involved in driving chloroplast protein import. Tic22 is localized to the inter-envelope space and has been
suggested to have a role in the coordination of TOC and TIC functions or in transport of proteins across the inter-envelope space (Kouranov et al. 1998; Soll and Schleiff 2004). After interaction with Tic22 the precursor protein is transferred to Tic20, which is an integral membrane protein with four putative transmembrane segments (Kouranov et al. 1998). Tic20 shares some sequence similarity with the mitochondrial import component Tim17 (Rassow et al. 1999) and has been proposed to participate in channel formation.

**The stromal processing peptidase (SPP)**

Immediately upon arrival in the stroma, precursor proteins are proteolytically processed in order to remove their transit peptide by the stromal processing peptidase (SPP). It has been shown that SPP removes transit peptides from an array of chloroplastic precursor proteins (Richter and Lamppa 1998). The enzyme was first purified from pea chloroplast extract using the chloroplastic protein LHCP as an affinity ligand (Oblong and Lamppa 1992). SPP is a soluble, monomeric enzyme of about 100 kDa and dependent on metal ions for its activity (Oblong and Lamppa 1992). The enzyme contains an inverted zinc-binding motif, which is characteristic of members of the metallopeptidase family M16, such as pitrilysin, insulin-degrading enzymes, PreP and the β-subunit of the mitochondrial processing peptidase (Rawlings and Barrett 1995; VanderVere et al. 1995). SPP initially recognizes a precursor by binding to the transit peptide and then removes it in a single endoproteolytic step. The mature part is then released while the transit peptide remains bound to SPP. After initial processing by SPP a second cleavage is performed and the transit peptide is converted to a subfragment form (Richter and Lamppa 2003). The trimmed transit peptide is further degraded by the same metallopeptidase, PreP, as for presequences in mitochondria (Bhushan et al. 2003; Moberg et al. 2003; Stahl et al. 2002).

**DUAL TARGETING**

As a consequence of the presence of both mitochondria and chloroplasts in a plant cell there is a higher sorting requirement than in non-plant cells. Reflecting this, protein import to mitochondria and chloroplasts has been shown to be highly specific in vivo
The vast majority of proteins that are involved in similar biological functions in more than one organelle are encoded by distinct genes for each organelle. However, an increasing number of studies have shown that some proteins are targeted to more than one compartment (Peeters and Small 2001). Proteins that are encoded by a single gene in the nucleus and targeted to both mitochondria and chloroplasts are referred to as dual targeted proteins (Peeters and Small 2001). The first protein to be reported as dual targeted was glutathione reductase (GR) from pea in 1995 (Creissen et al. 1995). Since then, more than 20 dual targeted proteins have been identified and it is expected that there will be many more.

Specificity of import and mis-targeting of proteins

Mitochondria originated much earlier than chloroplasts and thus the chloroplasts arose in cells that already contained an efficient system for targeting to mitochondria. To maintain specificity of import, chloroplastic import systems and import signals had to evolve unique features to avoid unnecessary or lethal mis-targeting between organelles. Interestingly, plant mitochondrial presequences differ from other eukaryotic mitochondrial presequences, since they are longer and richer in serine residues (Glaser et al. 1998). Furthermore, the outer mitochondrial proteins Tom20 and Tom22 in plants have greatly diverged from their eukaryotic counterparts (Macasev et al. 2000). The sequence of Arabidopsis Tom22 is different from Tom22 in non-plant species and this could possibly have been a response to the arrival of chloroplasts, so that the two import systems would not interfere with each other (Macasev et al. 2000). Although being specific there are reports of mis-targeting of proteins. Hurt et al. have shown that the targeting signal of ribulose 1,5-bisphosphate carboxylase/oxygenase from C. reinhardtii supported import of yeast cytochrome oxidase subunit IV into yeast mitochondria (Hurt 1986). Furthermore, the mitochondrial presequence of the yeast cytochrome oxidase subunit Va can function both as a mitochondrial and chloroplastic targeting peptide in transgenic tobacco plants (Huang et al. 1990). The chloroplastic PsaF protein from C. reinhardtii was shown to be imported in vitro into both spinach and C. reinhardtii mitochondria (Hugosson et al. 1995). The transit peptide of the chloroplast
inner envelope protein, triose-3-phosphoglycerate phosphate translocator (TPT) coupled to reporter CAT was imported into plant mitochondria in vitro but not in vivo (Brink et al. 1994) (Silva-Filho et al. 1997). More recent studies have reported mis-targeting of chloroplastic proteins into pea mitochondria in vitro such as the small subunit of Rubisco (SSU) and ferrochelatase-I (Fc-I). Notably, the mis-targeting of SSU and Fc-I seems to be a property of pea mitochondria as soybean and Arabidopsis mitochondria did not display this activity (Lister et al. 2001). Furthermore, it has been demonstrated that a range of chloroplastic proteins could be imported into pea mitochondria with the same efficiency as into chloroplasts, including plastocyanin, the 33-kDa photosystem II protein, Hcf136 and coproporphyrinogen III oxidase (Cleary et al. 2002). In contrast to the situation for mitochondria no cases of mis-targeting of mitochondrial proteins to chloroplasts have been reported. (Cleary et al. 2002).

**Dual targeting to mitochondria and chloroplasts**

The majority of proteins that are targeted to more than one compartment are localized in mitochondria and chloroplasts. There are two ways in which a single gene can provide a product that is targeted to both mitochondria and chloroplasts; either through an ambiguous targeting signal or via a twin targeting signal (Peeters and Small 2001). The ambiguous targeting signal arises from genes encoding single precursor proteins carrying a targeting signal that is recognized by the import apparatus of both organelles (Small et al. 1998). Twin-targeting signals may arise from alternative forms of transcription initiation, translation initiation, splicing or post-translational modifications all resulting in the production of multiple precursor proteins, each possessing different targeting information (Danpure 1995). As a result each precursor protein has a different targeting signal located at its N-terminus. It has been shown that when two targeting signals are put in tandem the most N-terminal will dictate the destination of the protein (de Castro Silva Filho et al. 1996). One protein utilizing a twin targeting signal is protoporphyrinogen oxidase II (protox-II), an enzyme necessary for the biosynthesis of chlorophyll in the chloroplasts and for haem in chloroplasts and mitochondria. Protox-II has two in-frame initiation codons, hence two different proteins are made by the means of alternative translation in which the longer form is imported into chloroplasts and the shorter into
mitochondria (Watanabe et al. 2001). Dual targeting of the THI1 protein, an enzyme of the thiamine biosynthesis pathway, also uses two alternative translation sites, and as for protox-II the longer form directs the protein to chloroplasts and the shorter to mitochondria (Chabregas et al. 2001). Monodehydroascorbate reductase (MDAR) is dual targeted to both mitochondria and chloroplasts by the use of multiple transcription initiation sites. Obara et al. showed that two MDAR mRNAs are produced from a single gene and give rise to two different proteins in which the longer is transported to mitochondria and the shorter to chloroplasts (Obara et al. 2002).

The vast majority of proteins dual targeted to mitochondria and chloroplasts have an ambiguous targeting signal (Peeters and Small 2001). Many of these proteins are involved in gene expression in the organelles such as several aminocyl-tRNA synthetases, RNA polymerase, methionine aminopeptidase and a peptidyl deformylase (Peeters and Small 2001). Other dual targeted proteins are involved in the protection against oxidative stress such as pea GR (Creissen et al. 1995) and several other enzymes from the ascorbate-glutathione cycle. Ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR) and glutathione reductase (GR) from A. thaliana have been shown to be dual targeted to both organelles in vivo and in vitro (Chew et al. 2003). Recently, a targeting peptide degrading zinc metallopeptidase (PreP) from A. thaliana was shown to be dual targeted to both mitochondria and chloroplasts (Bhushan et al. 2003; Moberg et al. 2003). An ambiguous targeting signal possesses features of both mitochondrial and chloroplastic targeting signals and evidence from in vitro work suggests that the import pathway used by dual targeted proteins is the same as for other imported precursor proteins (Peeters and Small 2001). Peeters and Small compiled 19 ambiguous targeting signals and compared their features with a large set of mitochondrial and chloroplastic targeting signals. They concluded that dual targeted peptides contain classical features of both mitochondrial and chloroplastic targeting signals but they contain fewer alanine residues and a greater abundance of phenylalanine and leucine, suggesting that they are more hydrophobic (Peeters and Small 2001). Some dual targeted proteins seem to have a domain structure in which different domains regulate the import to the organelles. The pea GR targeting signal has a triple domain structure in which two domains are responsible for the targeting to the respective
organelles and the third, most N-terminal domain harbours a fine-tuning function which controls the distribution between the organelles (Paper II). RNA polymerase RpoT2 also has a domain structure in which the N-terminal region is responsible for chloroplastic targeting and the C-terminal portion for mitochondrial targeting (Hedtke et al. 2000). Furthermore, the dual targeted zinc metallopeptidase PreP has a reversed domain structure in which the N-terminal domain is necessary for mitochondrial import and the C-terminal domain mediates chloroplastic targeting (Bhushan et al. 2003).

**The Dual import system**

Targeting of proteins to mitochondria and chloroplasts can be studied using both *in vitro* and *in vivo* approaches. *In vivo* approaches use an intact cellular system and reflect the *in vivo* targeting capacity of a signal. However, *in vivo* approaches have limitations: (i) chimeric constructs usually contain passenger proteins and therefore the role of the mature protein is ignored; (ii) the investigated proteins are usually over-expressed at very high levels with great variations in expression, which can affect targeting; (iii) no kinetics or efficiency of targeting can be assessed; and (iv) dissection of the mechanism involved in protein recognition and import is not possible. *In vitro* approaches can overcome these limitations but they have other disadvantages instead; (i) a lack of an intact cellular system: (ii) it is possible to get incorrect targeting which is not seen *in vivo*; and (iii) competition between organelles is absent.

In order to overcome the limitations of the *in vitro* import system and to elucidate the mechanisms involved in dual targeting we have developed a novel *in vitro* dual import system for simultaneous targeting of precursor proteins into mitochondria and chloroplasts (Paper I). We used purified organelles that were mixed, incubated with precursor protein and re-purified after import. This allowed the determination of the targeting specificity of import when both mitochondria and chloroplasts are present. The *in vitro* dual import system also allowed the use of authentic precursors so that the role of the mature part could be assessed.

In order to examine the fidelity of protein import we carried out *in vitro* import experiments using both single and dual import systems. We investigated import into pea leaf mitochondria and chloroplasts and soybean mitochondria with precursor proteins of
the soybean mitochondrial alternative oxidase (AOX), the pea chloroplast small subunit of RUBISCO (SSU) and the pea dual targeted glutathione reductase (GR). In the single import system SSU was imported into isolated chloroplasts while AOX was only imported into isolated mitochondria. The dual targeted protein GR was imported into both mitochondria and chloroplasts. Additionally, SSU was mis-targeted into isolated pea mitochondria and cleaved to the same apparent molecular mass product as in chloroplasts. This import was transit peptide dependent as the mature protein of SSU alone could not be imported. In contrast, in the dual import system SSU was imported only into chloroplasts. This shows that the dual import system can overcome the limitation with single import systems as it abolishes mis-targeting of chloroplast precursor observed in pea leaf mitochondria. It should be emphasised that under the same experimental conditions the dual targeted protein GR was imported into both mitochondria and chloroplasts.

In order to investigate the targeting capability of the dual targeting signal of GR we have constructed chimeric constructs between the GR targeting signal and the mature proteins of AOX, FAd and SSU and investigated import of these constructs in the single and dual import system. In both import systems, the GR targeting signal supported import of mature FAd into mitochondria and chloroplasts whereas the mature AOX could be imported only into mitochondria. There was efficient targeting of the mature SSU under the control of the GR targeting signal into chloroplasts in both of the systems, however this construct was only imported into mitochondria using the single system whereas the dual system did not support import of GR(p)-SSU(m). From these results we conclude that the GR targeting signal has the ability to direct proteins to both mitochondria and chloroplasts. However, this ability seems to be selective and dependent of the nature of the passenger protein.
The glutathione reductase targeting signal

The precursor protein of pea GR is dual targeted to mitochondria and chloroplasts by means of an N-terminal targeting signal of 60 amino acid residues, which has been described as an ambiguous targeting signal (Creissen et al. 1995) Peeters and Small 2001). It has been shown that the dual targeting capability of pea GR to both mitochondria and chloroplasts is dependant on the targeting peptide by, in vivo targeting of a fusion protein between pGR and phosphinothricin acetyl transferase (Creissen et al. 1995) and in vitro studies with chimeric constructs containing the GR signal coupled to a reporter protein or different mitochondrial or chloroplastic proteins (Paper I) (Cleary et al. 2002). The GR targeting signal possesses features of both mitochondrial and chloroplastic targeting signals. It has a high serine content (17 %), an abundance of aliphatic amino acid residues, an overall positive charge and an uncharged N-terminus. In the GR targeting signal the most positively charged N-terminal residue, arginine, occurs first at position 20, which is unusual for mitochondrial presequences but common for chloroplastic transit peptides. When compared with chloroplastic transit peptides, mitochondrial presequences are shorter and contain a higher content of positively charged...
residues in the N-terminal portion (Zhang and Glaser 2002). The GR targeting signal is successfully identified as a mitochondrial targeting signal by MitoProt (Claros and Vincens 1996) and as a chloroplastic targeting signal by ChloroP (Emanuelsson et al. 1999), programs that are single location predictors. However, TargetP (Emanuelsson et al. 2000) and Predotar, designed to predict intracellular targeting ability, predict the GR targeting peptide as a solely chloroplastic targeting signal. In line with this observation, in silico removal of the uncharged N-terminal portion of the GR targeting signal increased the probability for the peptide to function as a mitochondrial presequence and accordingly decreased the probability to function as chloroplastic transit peptide. However, removal of 30 N-terminal amino acids, halving the targeting signal, decreased the predicted targeting ability to the extent that none of the available prediction programs gave a confident prediction for either destination.

**Domain structure of glutathione reductase targeting signal**

*In silico* predictions using programs, such as TargetP and Predotar suggest that the GR targeting signal has a domain structure in which different regions of the targeting signal are important for targeting to mitochondria or chloroplasts. In order to determine whether the predictions reflect the situation in situ, we created deletion constructs and performed *in vitro* import assays into isolated mitochondria and chloroplasts (Paper II). When the different deletion constructs; GRΔ2-16 and GRΔ2-30, were incubated with mitochondria and chloroplasts, the same 55 kDa mature form as for wild type GR was produced. However, the import efficiency of the constructs varied depending on the number of N-terminal amino acid residues removed. GRΔ2-16 import was stimulated into mitochondria to 160 % compared to wild type, whereas the removal of 16 N-terminal amino acids did not significantly affect import into chloroplasts. As predicted, import of GRΔ2-30 was decreased into both mitochondria and chloroplasts. Mitochondrial import was completely inhibited while chloroplastic import was reduced by 65 %. These results are in line with the domain model of targeting signal organization for mitochondrial protein import proposing that the information for targeting to mitochondria is housed in the N-terminal domain. However, our findings show that substantial import into chloroplasts can be achieved with the C-terminal part of the targeting signal.
Furthermore, the results show that the 16 amino acid portion of the targeting signal has an inhibitory effect on mitochondrial targeting and that the region between Tyr17 and Pro30 in the GR targeting signal allowed mitochondrial import. This data indicates that the GR targeting signal has evolved a dual targeting signal with the C-terminal part being sufficient for chloroplast import, the internal part required for mitochondrial import and the most N-terminal part housing a “fine tuning” function. Removal of N-terminal residues seems to have an inhibitory effect on translocation into chloroplasts, but not for targeting or binding, since the amount of bound but not imported precursor increased 2-3-fold for the shorter construct in comparison to wild-type GR. In contrast, binding to mitochondria was not affected for the G\(\Delta\)2-16 mutant and decreased by approximately 35 % for the G\(\Delta\)2-30 mutant. Measurements of the valinomycin sensitivity of GR import into mitochondria revealed that import of GR wild-type was partially membrane potential insensitive. However, the two deletion constructs, G\(\Delta\)2-16 and G\(\Delta\)2-30, were imported in an almost complete membrane potential dependent manner. This might imply that the shorter constructs were completely passed through the membranes and imported into the matrix while some of the longer GR wild-type was accumulating in the intermembrane space. It may also reflect different requirements for membrane potential for the longer and shorter constructs as observed with pb2-barnase constructs (Huang et al. 2002). Precursors with short presequences required membrane potential, whereas those that were long enough to reach the matrix and mitochondrial Hsp70 were imported in the absence of membrane potential.

The presence of a second methionine residue in the GR targeting signal at position 5 raised the possibility that the import of GR into mitochondria and chloroplasts was a result of two different translation products, one starting from the first methionine and a shorter starting from the second methionine. Four N-terminal amino acid residues were removed in order to investigate the role of the second methionine residue in the targeting signal. Removal of the four first amino acids did not affect import into pea mitochondria or chloroplasts. This negated the possibility of an essential role of the second methionine residue in the targeting capacity.

GR is important in scavenging of reactive oxygen species in both the chloroplast and the mitochondria where ROS are produced. In pea leaves 77 % of the total activity of GR has
been localised to the chloroplast and 3 % to the mitochondria (Edwards 1990). There is no detailed information regarding GR protein distribution in chloroplasts and mitochondria but a great excess of the chloroplast GR protein is expected as there is about ten fold excess of chloroplast protein over mitochondrial protein in leaf cells. A mechanism regulating the distribution of GR precursor between mitochondria and chloroplasts is required. This study suggests that the mechanism for controlling targeting, hence import of GR, into the two organelles may be located within the targeting signal itself. We propose that the 16 residue N-terminal region functions to dampen the rate of import into mitochondria.

![Domain structure of GR targeting signal. The C-terminal part is sufficient for chloroplast import, the internal part required for mitochondrial import and the N-terminal part housing a “fine-tuning” function.](image)

**Figure 4.** Domain structure of GR targeting signal. The C-terminal part is sufficient for chloroplast import, the internal part required for mitochondrial import and the N-terminal part housing a “fine-tuning” function.

**Import of glutathione reductase**

To characterize the signal responsible for dual targeting we further examined the targeting signal of pea GR. We investigated if the determinants for dual targeting of GR into mitochondria and chloroplasts and if changes of amino acid residues affect targeting only to one, or to both organelles (Paper III). In this and earlier studies we noticed that GR is imported into mitochondria in a partially membrane independent manner. This suggests that at least some of the precursor protein has not crossed the inner membrane. In order to assess the intraorganellar location of imported GR we ruptured the outer membrane after import to allow access of added protease to intermembrane space components. Some imported GR was still protease protected under these conditions.
indicating that some protein had been imported across the inner membrane. We calculated that 30-50% of GR was imported to the intermembrane space. This dual intraorganellar location is consistent with studies in *Phaseolus* which suggested that up to 50% of the activity could be located in the intermembrane space (Iturbe-Ormaetxe et al. 2001). Using site-directed mutagenesis we changed positive residues and groups of hydrophobic residues in the targeting signal. Arginine residues were changed to negative residues or glycine whereas lysine residues were changed simply to abolish the positive charge. Any group of 3 hydrophobic residues was changed to non-hydrophobic residues to disrupt an amphiphilic structure that might be present. All changes were analysed with TargetP and notably the targeting prediction remained largely unchanged. Since the import kinetics into chloroplasts are different from mitochondria we checked the import kinetics for several of the mutated proteins. It was clear that all mutations showed typical chloroplastic kinetics with import saturated after 5-10 min. However, it was apparent that the amount of import after 30 min was similar to that of 10 min. We used import assays of 25 min in order to standardize with mitochondrial import. Using single *in vitro* import systems it was evident that changing individual amino acid residues had a greater inhibitory effect on mitochondrial import compared to chloroplastic import. Substitution of arginine residues in the C-terminal (R-2) and middle region (R-28) had the greatest inhibitory effect on mitochondrial import, in which R-2 inhibited mitochondrial import by more than 50% and R-28 by as much as 80%. In contrast, changing lysine residues (K-17, K-34) had a more similar inhibitory effect on mitochondrial and chloroplastic import. Furthermore, mutation of the N-terminal hydrophobic region of the targeting signal (AMA-57) almost completely abolished mitochondrial import whereas chloroplastic import was still 60% compared to wild-type GR. Chloroplastic import was most powerfully inhibited by changing hydrophobic residues in the N-terminus, as PL-53QC almost completely abolished chloroplastic import, and LFF-38QYY and FPF-32SQC reduced chloroplastic import to below 40%. R-41G and LFF-38QYY also substantially reduced both mitochondrial and chloroplastic import. In summary, mutations of positive or hydrophobic residues in the N-terminal or middle portion of the targeting signal inhibited both the mitochondrial and chloroplastic import but mutations in the most C-terminal region had the strongest effect on mitochondrial import. A
construct, in which the N-terminal and C-terminal region were removed (GRA2-16, A30-52) could only be imported into mitochondria if 8 C-terminal residues were left. This is in agreement with the notion that the R-2 mutation inhibited mitochondrial import. In order to investigate if the in vitro situation reflects the in vivo situation we created constructs in which GFP was linked to either the targeting signal of GR or the full length protein. We used soybean suspensions and tobacco leaves to investigate the targeting of GR in vivo. The targeting signal of GR, as well as the full length protein, only produced a typical chloroplastic pattern when it was used to direct GFP. Therefore we conclude that although pea GR has been convincingly shown to target phosphinothricin acetyl transferase to both organelles (Creissen et al. 1995) it does not appear to be able to achieve this with GFP as a passenger protein. The lack of mitochondrial import could be due to a number of reasons. Firstly, the GR targeting signal may not be sufficient to support import of GFP to mitochondria. In a study of the targeting properties of three different dual targeting signals it has been shown that they vary in their ability to target different passenger proteins to mitochondria, despite the fact that under the same conditions they all supported import of their native passenger into both organelles (Chew 2003). A similar case was observed with aminomidazole ribonucleotide synthetase from cowpea, which despite being dual localised in mitochondria and plastids was not capable of targeting GFP to mitochondria or plastids (Goggin et al. 2003). Alternatively, targeting of GR-GFP to mitochondria may be below the limit of detection. We further investigated the effect of various mutations on chloroplastic import in vivo. We chose constructs mutated at hydrophobic regions since these mutations had the greatest effect on chloroplastic targeting in vitro. All mutated versions examined had disrupted targeting to plastids as determined by the pattern of GFP targeting. The distinctive of plastidic pattern was lost when compared with the chloroplastic control, Toc64. Clearly, mutations that affect chloroplastic import in vitro have a similar effect in vivo. Furthermore, we performed import into two organelles simultaneously using a dual import system. Using the dual import system we could study the effect of mutations on both mitochondria and chloroplast under the same conditions. Although a few differences were noted the overall results from dual import was in agreement with the results from single organelle import in that single mutants had a greater effect on mitochondrial import. In this study we used
several different approaches to study organellar import. We found that although no approach gives the complete picture by itself, all are in a general agreement.

Processing of glutathione reductase

Dual targeting peptides not only have to be recognised by the import apparatus of two different organelles, they also have to be properly cleaved inside the organelle. The majority of mitochondrial presequences are cleaved by MPP (Eriksson 1992) and most chloroplastic transit peptides by SPP (Richter and Lamppa 1998). The recognition elements for processing by MPP or SPP are not fully understood. However, it has been shown that an arginine is frequently located in position -2 or -3 upstream of the cleavage site in mitochondrial presequences (Zhang et al. 2001). For processing with SPP, an alanine in position -1 and a valine in position -3 are often found. In order to establish the recognition determinants for processing of GR we performed processing experiments with the different deletions as well as single amino acid residues mutations (Paper IV).

$\text{GR}^{\Delta 2-16}$ did not affect stromal processing while processing by the MPP/bc$_1$ complex was increased three fold compared to wild-type. In contrast to the import results, for $\text{GR}^{\Delta 2-30}$ processing by both the MPP/bc$_1$ complex and stromal extract were significantly increased. Processing with the MPP/bc$_1$ complex was increased as much as six fold, whereas stromal processing was increased by a moderate 50%. Processing efficiency is a reflection of the ability of the precursor to access the binding cleft of the processing peptidase. Furthermore, bend promoting residues are frequent in order to improve access to the processing site. The access may be limited by peptide length, i.e. a shorter targeting signal will access the catalytic site more easily and thus be processed more efficiently. Mukhopadhyay et al. showed that the efficiency of cleavage decreased dramatically as the number of residues on the N-terminal side increased (Mukhopadhyay et al. 2002). Our findings are in line with these results as reducing precursor length resulted in a more significant increase in processing efficiency with both MPP and with SPP.

Amino acid residues in the vicinity of the processing site, as well as positive residues throughout the targeting signal have been shown to play a role in processing. Therefore, a series of point mutations were created. The mutated proteins can be divided into two groups: i) those with changes in the vicinity of the processing site and ii) those with
changes of positive residues throughout the targeting signal. In the first group, three amino acid residues on each side of the processing site were mutated to amino acids with different properties. Both isolated stroma and recombinant SPP processed all mutated constructs with efficiency similar to the wild-type construct, regardless of position or property of the amino acid introduced. However, when the same mutated constructs were incubated with the isolated MPP/bc₁ complex, mutations at position -1 had the greatest effect, reducing processing activity by more than 90%. Mutations at position -2 and +1 reduced processing by almost 60%. Furthermore, mutations in position +2 reduced processing by about 50%. Mutations at position -3 and +3 had no significant effect on processing. From these results we concluded that amino acid residues in the proximity of the processing site, in the range of -2 to +2 are involved in the recognition of the cleavage site by MPP. As no effect was observed with single mutations on the SPP processing activity, we made double and triple mutations upstream of the cleavage site. The bulky phenylalanine was introduced in position -3 and -1 instead of valine and alanine, respectively, and a negatively charged aspartate was introduced in position -2 instead of arginine. Stroma and the recombinant SPP catalysed processing were inhibited by 33% and 52% with the double mutant and by 70% and 67% with the triple mutant, respectively. These results show that great changes around the cleavage site are required to affect SPP catalysed processing activity and indicate preference for small uncharged and positively charged residues upstream of the cleavage site. In the second group, in which positive amino acid residues were changed throughout the targeting signal, none of the mutations had any significant effect on processing by stroma. When processed by the MPP/bc₁ complex no significant change was seen for N-terminal mutants. Changing R-28 to an uncharged residue, R-28G, did not effect processing by MPP/bc₁ complex whereas changing to a negative residue, R-28D, inhibited processing with about 50%. When L-17 was changed to asparagine (L-17N) no difference was seen, however, changing to a threonine (L-17T), another uncharged amino acid, inhibited processing by 45%. Besides R-2, there are two additional positive charged amino acid residues, H-8 and R-7, within the eight most C-terminal residues. When both of these residues were changed to uncharged amino acid residues and incubated with MPP/bc₁ complex processing efficiency was reduced by almost 70%. These results show that positive charges
throughout the GR targeting signal have significance for recognition by MPP, but not by SPP. Positively charged amino acid residues might be required for binding of the presequence to the negatively charged cleft of the MPP/bc$_1$ complex.
CONCLUDING REMARKS

To date approximately 25 different proteins have been reported as dual targeted (Peeters and Small 2001) Silva-Filho 2003). This might just be the tip of the iceberg and there might be a significant proportion of proteins, which utilize this solution. It should also be noted that a large number of the studies were carried out using A. thaliana. A wider, more representative analysis of gene products from different plant species would provide a better understanding. We know that glutathione reductase is dual targeted in both pea and Arabidopsis and it would be of interest to broaden this study to a range of different plant species.

Confirming that similar proteins in different compartments are encoded by the same gene is not always a trivial exercise. The most common ways to study dual targeted proteins are by preforming in vitro or in vivo imports. However, both these methods have their limitations and one must be careful with interpretation of results. We have developed a dual import system that will overcome some of the disadvantages with traditional import assays. In our system we preform in vitro import with both mitochondria and chloroplasts present simultaneously. This allows us to study targeting of native precursor proteins with competition between organelles. Furthermore, this system abolishes mis-targeting of chloroplastic precursor proteins to mitochondria which occurs in single in vitro assays.

We have shown that the targeting signal of the dual targeted protein glutathione reductase has a domain structure in which the most N-terminal part regulates the amount of protein imported to each of the organelles. A range of constructs could be made to further investigate this issue. We have initiated this work by creating deletion mutations that only contain the central domain of the glutathione reductase targeting signal. If this portion by itself can support import into mitochondria it would further strengthen the domain structure hypothesis. It would also be of great interest to take a more global approach. Is the domain structure a general feature for dual targeting proteins or is it unique to glutathione reductase and a few more proteins?

An interesting experiment would be to solve the structure of the GR targeting signal. So far no structure of a dual targeted signal has been solved. Most likely there is not a separate pathway for import of dual targeted proteins but they use the general import
pathways to the respective organelles. A structure of GR in complex with import receptors from mitochondria and chloroplasts would help in elucidating the mechanisms for recognition. Surprisingly, using secondary structure prediction programs no α-helix in the N-terminal part is predicted for GR. Since it is known that an amphiphilic α-helix is of importance for at least mitochondrial targeting it would be of great interest to see what kind of secondary structure is present in the GR targeting signal.
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