Plant Mitochondria

With Emphasis on RNA Editing and Cytoplasmic Male Sterility

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33. Protein Import into Chloroplasts: an Outline of Early Events in the Translocation Pathway

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Summary

The import of cytoplasmically synthesized proteins into chloroplasts requires at least the interaction between the precursor protein and the import apparatus located in the plastidal envelope membranes. In this review we summarize the present knowledge of the events in which components of the plastid envelope membranes are directly involved, i.e. binding of the precursor to the organellar surface and translocation across the envelope membranes. The requirement of soluble cytosolic components for the import process is discussed. Furthermore we describe the import routes of proteins destined for the outer chloroplast envelope and compare it to that of polypeptides localized inside the plastid.

Introduction

The majority of the plastid proteins (1) among them most of the envelope proteins (2) are encoded by the nuclear genome. As a consequence, the biogenesis of chloroplasts requires the transport of the cytoplasmically synthesized polypeptides into the organelle. This involves a system of selective intracellular sorting and an efficient import machinery into or across the organellar membranes. In a general outline, chloroplast protein transport can be described by the following events: All imported chloroplast proteins are initially synthesized on cytosolic polysomes as higher molecular weight precursor proteins with positively charged amino-terminal presequences that have been shown to contain the

essential targeting information (3-5). The precursors are transferred from the cytosol to the organelle via a posttranslational mechanism. After recognition by proteinaceous receptors on the outer envelope membrane surface, the proteins are translocated into and across the envelope membranes. ATP hydrolysis is required at least at two distinct steps during the import pathway while a membrane potential necessary in mitochondrial protein translocation is not requisite in plastids (6, 7). Low concentrations (5-100 μM) ATP are required for binding (8), but higher concentrations (0,2-2 mM) to completely translocate the precursor protein into the plastid (9-12). Inside the organelle the N-terminal transit peptides are removed by a stromal processing protease. The proteins are sorted to their final subplastidal destination and if required assembled to functional units (for details see 4, 5, 13 and references therein). Protein transport and routing inside the chloroplast have been reviewed in more detail in Schmidt and Mishkind (14), Lubben et al. (15), Mishkind and Scioli (16), Keegstra et al. (17), Smeekens et al. (13) and Joyard et al. (18). We will therefore focus on the description of the chloroplast envelope localized translocation process.

Import competency of precursor proteins

One main problem in protein transport consists of the mechanism by which large and maybe already partially folded polypeptides cross the hydrophobic environment of biological membranes. Considering that protein import into chloroplasts and mitochondria is a posttranslational event, at least some protein folding is likely to occur prior to the initiation of membrane transfer. Cytosolic factors should ensure formation or conservation of an import competent protein structure, since the specific penetration of tightly folded proteins through membranes seems improbable.

Della Cioppa and Kishore (19) could demonstrate that translocation of a tightly folded protein across the chloroplast envelopes is severely diminished. The import of the *in-vitro* synthesized chloroplast precursor 5-enolpyruvylshikimate-3-phosphate synthase (pEPSPS) into isolated chloroplasts was inhibited by the addition of its first substrate shikimate-3-phosphate and the competitive inhibitor and herbicide glyphosate instead of phosphoenolpyruvate. Thus the enzyme forms a stable ternary complex with the shikimate-3-phosphate and the herbicide that can not be translocated by the import machinery, whereas import of other precursor proteins was not hindered when both of the substrates were simultaneously present in the import assay.

The involvement of cytosolic factors in mediating an import competent protein structure is suggested from experiments performed with the precursor of the major light-harvesting chlorophyll a/b binding protein (pLHCP) that was overexpressed in Escherichia coli. Waegemann et al. (20) showed that at least two cytoplasmic components present in pea leaf extracts are necessary for efficient translocation of the purified pLHCP after its denaturation and unfolding in 8 M urea. The presence of these cytosolic factors renders the precursor of this hydrophobic membrane protein more susceptible to protease treatment probably due to a less tightly folded polypeptide conformation. Purified hsc 70 was also able to support the translocation of urea denatured pLHCP indicating the involvement of molecular chaperones of the hsc 70 familiy in this process (20) which corresponds with the findings that members of the hsp 70 family appear to facilitate

transport of proteins into different organelles by retarding the spontaneous folding process of the polypeptides by a so far incomplete understood mechanism which requires ATP hydrolysis (for recent reviews see 21-23). On the other hand cytosolic factors did not seem to be necessary for protein import of purified ferredoxin precursor, pFD, (24, 25) or plastocyanin precursor, pPC, (26) in isolated chloroplasts *in vitro*. Both proteins were taken up by the chloroplasts after dilution of the urea denatured proteins into the import mixture. It is unknown at present how these divergent results obtained *in vitro* reflect the *in vivo* situation. Maybe chaperones are required by all precursor proteins however at different times during the translocation process, e.g. in the cytosol or at the envelope. In fact hsp 70 homologues have been found to exist inside the chloroplast as well as in the chloroplast envelope of higher plants (27) and Euglena gracilis (28).

Binding of precursor proteins to the organellar surface

Once proteins are targeted towards the chloroplast they must interact with the plastid envelope which consists of rather closely spaced outer and inner membranes, separated by the intermembrane space. Productive binding of the precursor to the receptor requires ATP hydrolysis. ATP concentrations between 1-100 μM have been determined to support binding to isolated organelles (8), right-side-out envelope vesicles (29) and the isolated import apparatus (30).

In order to identify the sites of precursor binding we used immunogold labelling with a chimeric protein composed of the E. coli outer membrane protein A (ompA) and the presequence of the small subunit of ribulose-bisphosphate-carboxylase-oxygenase (pS). As seen on electron micrographs (Fig. 1 a and b), the precursor polypeptides (pSompA) are confined to certain distinct sites in the membrane. When chloroplasts were shrunken in hypertonic medium after labelling and before fixation the bound precursors are located at areas where the two envelope membranes are closely apposed indicating that both membranes might be contracted by the translocating precursor proteins.

Chloroplast import is proposed to be initiated by binding of the precursor polypeptides to proteinaceous receptors of the outer membrane because protease treatment of intact organelles (31) or right-side-out envelope vesicles (29) reduces binding of precursor polypeptides drastically. A limited amount of 1500-3500 binding sites per chloroplast was determined by Pfisterer et al. (32) using isolated envelope membranes, and by Friedman and Keegstra (33) using isolated intact chloroplasts. Precursor binding is a high affinity interaction with a dissociation constant of 6-10 nM. Competition studies carried out with synthetic transit peptides indicate that all precursor proteins examined so far use the same receptor species for binding. Peptides, homologue to parts of the pSSU transit sequence with a length of 20-30 amino acids inhibited binding with rather low affinities, i.e. between 2-40 μM peptide were necessary for 50 % inhibition (34, 35). Perry et al. (36) report that synthetic peptides corresponding to the central region of the pSSU transit sequence inhibit binding of authentic precursor proteins (pSSU, pLHCP, pFD and pPC), while peptides representing either the N- or C- terminal end of the pSSU transit peptide have little or no effect on binding but drastically reduce the translocation process. However, the obvious disadvantage of this approach is that these peptides represent only part of the transit

peptides, and it remains to be investigated if the properties of the entire chloroplast targeting domain can be understood simply as the sum of the properties of its constituent fragments. Experiments using purified pPC as authentic precursor polypeptides demonstrated that a 10-100 fold lower concentration was necessary for efficient competition of pPC, pSSU and pFD than in the case of synthetic peptides (26).

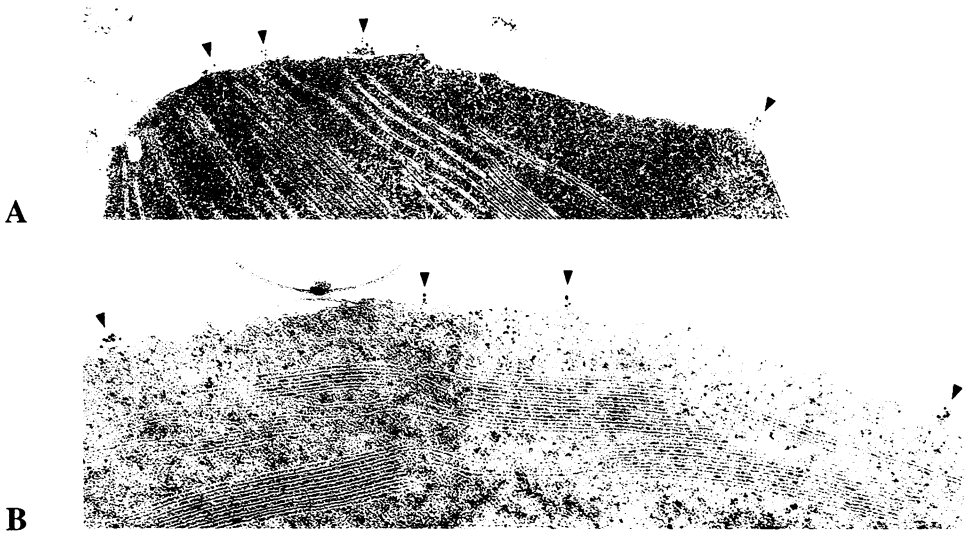

Fig.1. Distribution of binding sites on the chloroplast surface. Precursor proteins (pSompA) were bound to the chloroplast surface, and after reisolation of the organelles the bound precursor polypeptides were visualized by labelling with antibodies (anti-ompA) and protein A- gold (as indicated by arrows). Prior to fixation chloroplasts were shrunken in hypertonic buffer to separate outer and inner envelope membranes in some extent and reveal that protein binding sites are concentrated at distinct membrane contact sites. **(A)** magnification 112000 x, **(B)** magnification 117000 x.

Components of the translocation apparatus

The mechanism by which precursor proteins translocate the envelope membranes involves a number of as yet unidentified components. Different approaches have been used to identify polypeptides involved in the import process including: proteolytic digestion of the outer envelope membranes, chemical crosslinking studies, phosphorylation, use of synthetic peptides and solubilization of envelope membranes. Cornwell and Keegstra (37) used a heterobifunctional, photoactivatable crosslinking reagent to identify a 66 kDa chloroplast surface protein that was associated with pSSU binding, minor crosslink products were found at 25, 57 and 60 kDa (molecular weight (MW) data result after substracting the MW of the crosslinked precursor). In a comparable approach Kaderbhai et al. (38) observed similar crosslink products of 52 kDa (most likely the large subunit of Rubisco) and two

crosslink conjugates with inner envelope proteins of 30 kDa and 60 kDa. An entirely different approach was taken by Pain et al. (39), who raised antibodies against antibodies directed against a synthetic transit peptide of pea pSSU. These antiidiotypic antibodies which should mimick the binding properties of the transit peptide were shown to inhibit protein transport and reacted with a protease sensitive protein on the surface of intact chloroplast at regions where inner and outer membrane are in contact as also observed in our immunogold labelling experiment (Fig. 1). In western blotting experiments the antiidiotypic antibodies reacted with a 52 kDa protein present in the stromal compartment, representing the large subunit of Rubisco and the major 30 kDa envelope protein that was suggested to be the import receptor and an integral chloroplast envelope membrane protein. However, the major 30 kDa polypeptide in the envelope membrane is known to be the phosphate translocator (40). Schnell et al. (41) subsequently reported the isolation of a full length cDNA clone of their 30 kDa protein that was found to be very homologue to the phosphate translocator protein from spinach and identical to that from pea (42, 43). The authors therefore concluded that this 30 kDa envelope membrane protein serves as the import receptor for pSSU and not as the phosphate translocator. Their studies raise a series of questions as discussed by Joyard and Douce (44), Flügge et al. (45), de Boer and Weisbeek (5), Soll and Alefsen (46). It seems therefore likely that the protein import receptor remains yet to be identified.

Probably more than one protein is necessary for binding to and translocation across the envelope. For example a 51 kDa protein has also been implicated to be a component of the receptor complex since an increase in phosphorylation of this protease sensitive outer envelope protein was observed during precursor binding (47).

Recently it was demonstrated that it is possible to isolate a membrane complex loaded with pSSU from purified outer chloroplast envelopes (29, 30). After incubation with pSSU translation product and reisolation the outer envelope vesicles were solubilized by digitonin and subjected to fractionation on a linear sucrose gradient. The pSSU containing membrane complex consists of about 10 outer envelope polypeptides (MW 86, 75, 72, 64, 54, 52, 42, 34 kDa and minor components) which were identified either by molecular weight or immunological methods. Using the reverse order, an isolated membrane complex was subjected to binding assays and shown to be able to recognize pSSU in an ATP, protease sensitive and transit sequence dependent manner. Furthermore, translocation intermediates detected in chloroplasts were also found after interaction of the precursor polypeptide with the isolated import complex. It seems therefore possible to isolate large parts of the protein translocation apparatus from chloroplasts as a functional unit which is active in recognition and insertion of precursor polypeptides.

In addition to the proteinaceous components described above, envelope bound heat-shock proteins (27) and envelope membrane lipids (48) are proposed to be involved in the translocation process. A possible function of lipids in the import process can be assumed easily because envelope membranes have a unique lipid composition, containing high amounts of the phospholipids phosphatidylcholine and phosphatidyglycerol as well as the specific chloroplast galactolipids, monogalactosyldiacylglycerol and digalactosyldiacylglycerol and the sulpholipid, sulphoquinovosyldiacylglycerol. Recently Van't Hof et al. (48) reported that fragments representing different parts of the transit peptide of pSSU interact specifically with lipids typically present in the outer chloroplast envelope, indicating that transit peptide-lipid interactions could play a certain role in a productive chloro-

plast protein import pathway. In contrary Endo et al. (49) showed that the chloroplast-targeting domain of PC transit peptide has only low affinity for lipid bilayers consisting of galactolipids and phospholipids to mimick the lipid composition of chloroplast envelope membranes, but can form a helical structure in a hydrophobic environment. Since the PC transit peptide consists of the N-terminal positively charged hydrophilic chloroplast-targeting domain and the C-terminal hydrophobic thylakoid-transfer domain they suggest that the first interaction of pPC with the chloroplast surface might be mediated by the thylakoid transfer domain at the C-terminal part of the transit sequence. Subsequently the chloroplast-targeting domain might form a helical structure which in turn could be recognized by receptor proteins located at the chloroplast surface, thus indicating a conformation mediating influence of the lipid bilayers.

Furthermore Kerber and Soll (50) demonstrated that hydrolysis of phosphatidylcholine by phospholipase C (PLC) treatment inhibited almost completely import of the precursor protein into the organelle (Fig. 2, upper panel). At which step PLC blocks the translocation process was determined by carrying out the import assays under conditions

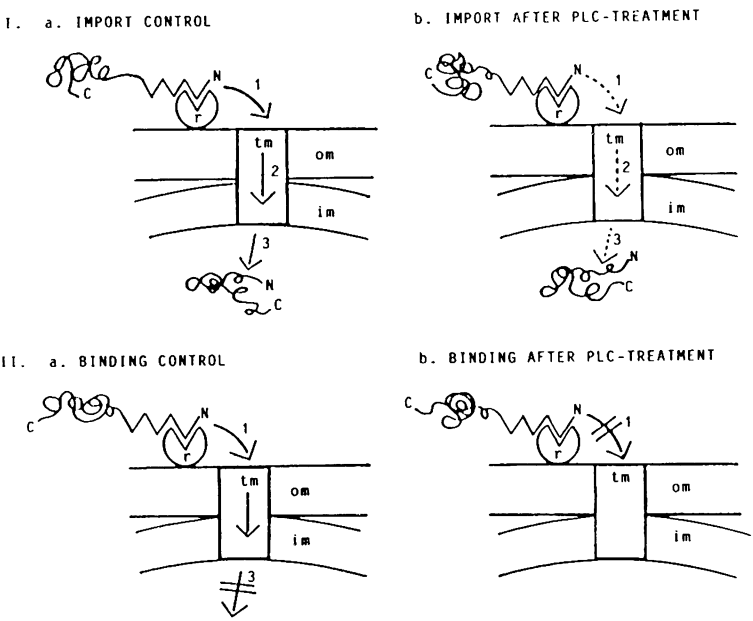

Fig. 2. Schematic representation of the effect of phospholipase C (PLC) treatment of intact chloroplasts on the interaction with pSSU under import and binding conditions, respectively. I. Untreated and PLC-pretreated chloroplasts were incubated with precursor proteins under import conditions. a. import control, b. import into PLC-pretreated organelles. II. Precursor proteins were bound to untreated and PLC-pretreated chloroplasts. a. binding control, b. binding to PLC-pretreated organelles. Membrane located translocation events: binding to (1), translocation through (2) and release (3) of the precursor protein from the translocation machinery (tm). Blocks in the translocation pathway are indicated by the crossed out arrows, hindrance is indicated by arrows in dashed lines. Om, outer envelope membrane, im, inner envelope membrane, tm, translocation machinery, r, receptor.

which allowed binding but not import, e.g. chloroplasts incubated with pSSU in the dark and in the presence of 10 μM ATP. In control experiments these conditions led to partial precursor insertion into the import apparatus which could be detected as protease-protected translocation intermediates after thermolysin digestion. Identical binding assays with PLC-pretreated chloroplasts however, did not provide any translocation intermediates while binding to the chloroplast surface was nearly as efficient as in control binding. These data indicate (50) that hydrolysis of phosphatidylcholine inhibits precursor protein translocation at a step after binding but before insertion into the import machinery (compare Fig. 2, panel II a and b) and give rise to the supposition that phosphatidylcholine is an essential constituent of the translocation complex described by Soll and Waegemann (30).

Additionally the isolated import complex (29) and the outer envelope membranes (27, 51) do contain hsc 70 homologues which could play a role in the translocation event. Von Heijne and Nishikawa (52) propose that chloroplast transit peptides are essentially devoid of regular secondary or tertiary motifs and that such unfolded structures are favorable for guidance of chaperones operating at different steps of the chloroplast protein import pathway. This may partially argue against the findings of Endo et al. (50). Supplementary members of the hsp 60 family, collectively termed chaperonin 60 (cpn 60) were identified in the chloroplast stroma (53) where they assist the folding and assembly of both imported and organellar synthesized proteins and only recently a cpn 10 protein was found in chloroplasts from pea and spinach that is functional as co-chaperonin in Rubisco folding (54).

Protein routing into the envelope membranes

The chloroplast envelope consists of rather closely spaced outer and inner membranes, separated by an intermembrane space. So far only little is known about import of proteins into the envelope membranes and due to a lack of authentic proteins nothing is known about transfer into the intermembrane space. So far only two inner envelope proteins have been cloned and their import behaviour has been analysed (42, 55). Like typical polypeptides destined for the inside of the organelle they possess cleavable target sequences, which are recognized by proteinaceous receptors on the organellar surface and require ATP for binding and transport into the inner envelope membrane. Obvious differences in the structure of the transit peptides, i.e. the ability to form an amphiphilic α-helix (43) and the absence of a β-strand domain typical for internal chloroplast proteins (56), suggest that certain details of the import mechanism are significantly distinct between stromal or thylakoid proteins and envelope proteins, respectively. Insertion of outer envelope proteins again differentiates strikingly from import of those destined for the inner plastid compartments. The three proteins studied so far, i.e. a 6,7 kDa spinach protein (57), a 14 kDa pea protein (58) and a hsp 70 spinach protein (51), do not possess a cleavable target sequence, do not require protease sensitive receptors on the organellar surface and do not require ATP for either binding or insertion into the outer envelope. Thus, several distinct uptake processes may exist, not only within the plant cell, but probably also within the chloroplast (59).

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