

Photosynthesis: from Light to Biosphere

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THE PROTEIN IMPORT MACHINERY OF CHLOROPLASTS.

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1. Introduction

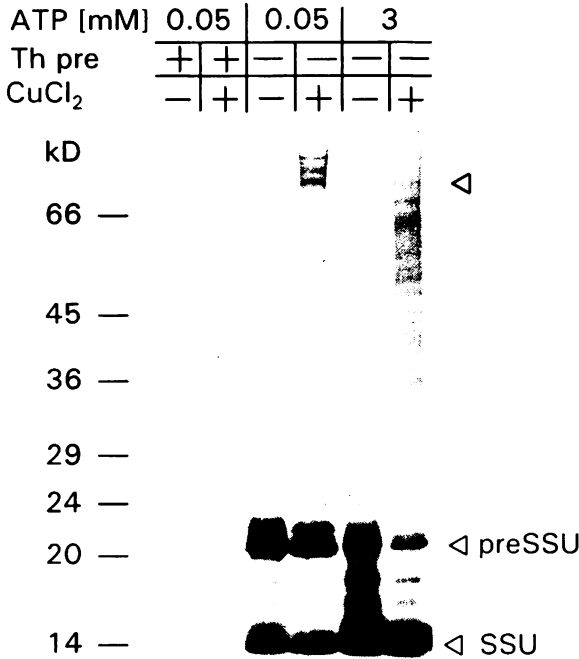
The vast majority of plastid localized protein are nuclear encoded, synthesized in the cytosol and imported into the organelle in a posttranslational event (1, 2). The polypeptides are synthesized in the cytosol in general with an NH₂-terminal extension, called pre- or transitsequence which is responsible for targeting, i.e. recognition of the proper organelle, and for keeping the precursor protein in a conformation which allows binding and translocation (2,3). The plastid directing presequence is cleaved off in the stroma by a stromal processing protease. After cleavage proteins are assembled into functional active units, or further sorting and transport signals are revealed, e.g. for translocation across the thylakoids, and start a new round of membrane translocation (4). Major chloroplast protein components, e.g. the small subunit of Rubisco, ferredoxin, LHCP, plastocyanin and the subunits of the oxygen evolving complex have been suggested to use the same recognition and translocation systems localized in the chloroplasts envelope membranes. The data indicate that one general import machinery exists in the plastid envelope membranes which is responsible for the bulk import of proteins into plastids (2, 3). Alternative pathways for a number of proteins exists and have been described, but these are mostly localized in the envelope membranes (5). This report will describe, how components of the chloroplast import machinery were identified in recent years, what might be their functions and how this could be regulated.

2. Results

Protein import into chloroplasts can experimentally be devided and halted at different steps. In the absence of exogenous ATP a precursor protein binds preferentially to protease sensitive chloroplast surface component, but remains in a completely protease accessible location (6). Upon raising the ATP concentration between 10-50 μ M the precursor becomes forwarded into the translocation apparatus which renders the precursor partially protease protected (7, 8). Raising the ATP concentration above 100 μ M

results in increasing yields of import. 1-3 mM ATP are sufficient for maximal import yields (9). Precursor protein import is also impaired or comes to a stop at 0°C even in the presence of ATP. These experimental manipulations have resulted in a number of well described translocation intermediates, which can be used to identify the envelope membrane components with whom the precursor protein interacts at a given *in vitro* condition (8, 10).

A)



B)

anti
OEP 75 PIS

Figure 1. Chloroplasts bound preSSU can be crosslinked to envelope proteins by CuCl₂. **A)** Intact chloroplasts either not treated or treated with the protease thermolysin (Th_{pre}, as indicated) were incubated with preSSU at 50 μM ATP for 5 min. Chloroplasts were reisolated and treated with 1 mM CuCl₂ for 5 min (when indicated). Crosslink products were analysed by SDS-PAGE under non reducing conditions and fluorography. **B)** Four crosslink experiments as in A, lane 4, were carried out in parallel. Chloroplasts were reisolated, pooled and solubilised by 0.5 % digitonin. Immunoprecipitation was carried out, using a preimmune- (PIS) or immune-serum to OEP75 (anti OEP75). The precipitate was analyzed by SDS-PAGE under reducing conditions and fluorography.

2.1 Identification of import machinery components by chemical crosslinking. Perry and Keegstra (6) have used a label transfer (¹²⁵J) thiol cleavable crosslinker bound to preSSU to label envelope proteins in close vicinity to the precursor proteins. Their studies showed that the outer

envelope protein OEP86 interacts with preSSU very early in the translocation process, while in the presence of ATP OEP75 became the preferentially labelled target. OEP86 is a protease sensitive constituent of the outer envelope while OEP75 is very protease resistant (11, 12). We have used the thiol oxidant CuCl_2 as a crosslinker (13). Upon oxidation by CuCl_2 the thiol groups can form inter- and intramolecular disulfide bridges (14, 15). The redox status of thiol-groups seems important for a productive precursor protein import process (16) and it was possible to show that protein import into chloroplasts is reversibly inhibited upon CuCl_2 oxidation (13). Indeed, when pea chloroplasts were incubated with preSSU at 50 μM ATP and subsequently treated with 1 mM CuCl_2 several crosslink products could be detected (Fig. 1A). One crosslink product at a molecular size between 90-95 kDa was especially prominent. This radiolabelled crosslink product could be immunoprecipitated specifically with an antiserum against OEP75 but not with the preimmune serum (Fig. 1B). The OEP75-preSSU disulfide bridge was formed neither in the absence of CuCl_2 nor under conditions which favour import, i.e. 3 mM ATP or which inhibit a precursor chloroplast interaction, i.e. when chloroplasts were pretreated with protease (Fig. 1A). These data show that thiol groups of preSSU and OEP75 are exposed during their interaction and in such close physical proximity that they can form a covalent connection via a disulfide bridge upon oxidation with CuCl_2 . CuCl_2 can thus be used a "zero Å" crosslinker. Non cleavable chemical crosslinkers have been used in a number of studies and lead to the identification of further proteins, e.g. OEP62 (17), OEP45, OEP34, OEP24 and OEP15 (18), which could be involved in precursor protein import into chloroplasts. The molecular size of the OEP's was an estimate after subtraction of the size of the precursor protein used and might not reflect exactly the correct size of the target protein. In another study translocation intermediates of the chimeric protein precursor OEE1-DHFR were generated and after chemical cross-linking and co-immunoprecipitation with specific antibodies shown to interact with OEP70 (an hsc70 homologue), OEP44 and the inner envelope protein IEP97 (19).

2.2 Isolation of the envelope localized import machinery and description of the constituents. Purified right-side out outer envelope membrane vesicles isolated from pea chloroplasts were solubilized by digitonin and fractionated by sucrose-density centrifugation. This procedure resulted in the enrichment of a membrane protein-complex which interacted specifically with preSSU in the presence of ATP but not with SSU (20). Furthermore translocation intermediates detected in intact chloroplasts were also found in this import complex after protease treatment. This indicated that not only a functionally active receptor but that also part of the translocation apparatus was retained actively in this isolated membrane-protein complex (8, 20). Furthermore preSSU can be crosslinked to OEP75 in the isolated complex by CuCl_2 (Fig. 2) corroborating our conclusion that the isolated envelopes and the import complex represent bonafide systems to study early events in protein translocation into chloroplasts (8, 20). Another labelled product at 50 kDa could represent a crosslink with OEP34, but was not further analyzed (Fig.2).

Major constituents of the isolated import complex are OEP86, OEP75, OEP70, OEP34 and OEP16 (8, 10, 20). OEP86 is very sensitive to exogenous added protease (11, 21). In addition FAB fragments of

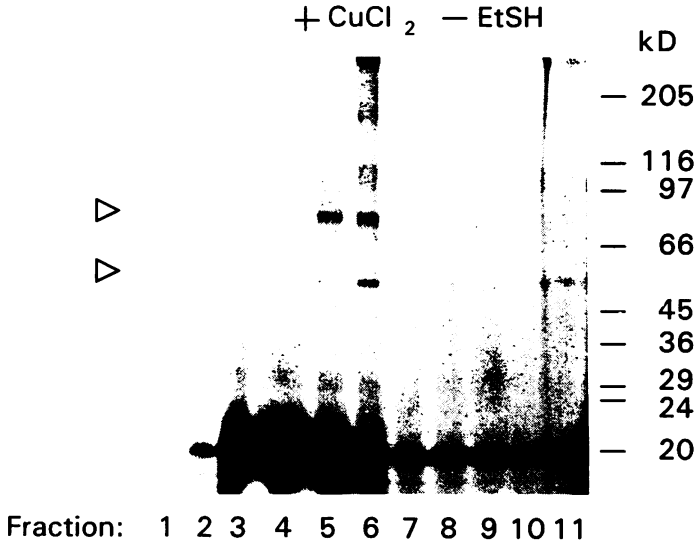


Figure 2. PreSSU interacts with OEP75 in the isolated import complex. Purified pea chloroplast outer envelope membranes were incubated with [35 S] labelled preSSU at 50 μ M ATP (8). Membranes were recovered by centrifugation through a sucrose cushion, washed once and solubilized with digitonin (8). The mixture was separated on a linear 5-20 % (w/v) sucrose density gradient and fractionated from the top (fraction 1) into 11 aliquots. Each sample was treated with 1 mM CuCl_2 for 30 min and further analysed by SDS-PAGE under non reducing. A fluorogram is shown.

antibodies to OEP86 could inhibit binding and import of preSSU into chloroplasts (21). Taken together these data indicate that OEP86 functions as a receptor for precursor proteins. The gene coding for OEP86 was isolated recently (21, 22) and it was demonstrated that OEP86 has specific and separate binding sites for ATP and GTP (21, 22, 23). Furthermore OEP86 is posttranslationally modified by ATP dependent protein phosphorylation (24), indicating a highly regulatable process of receptor-precursor protein interaction (25).

The gene coding for OEP34 was also recently cloned (22, 23). OEP34 represents a new type of GTP-binding (26) which shows outside the conserved GTP-binding domains no further homology to any known protein except to the NH_2 -terminal 30 kDa of OEP86 (21, 22). OEP34 is anchored into the outer envelope membrane by a carboxyterminal hydrophobic domain, while most of the polypeptide including the GTP-binding motifs protrude into the cytosol (23). In *E. coli* overexpressed and partially purified OEP34 exhibits GTPase activity (23). When outer envelope membrane proteins are separated on SDS-PAGE in the absence of mercaptoethanol or DTT a covalent association between OEP75 and OEP34 can be detected which is reversible by a reductant (23). In the presence of CuCl_2 a high

molecular weight complex is formed which contains as major constituents OEP86, OEP75 and OEP34 (13). OEP34 contains only one cysteine in its primary amino acid sequence. From the data mentioned above it can be concluded that OEP75 and OEP34 interact directly via thiol groups while OEP86 interacts with OEP75 but not with OEP34 via thiol groups or disulfide bridges. As deduced from the crosslinking data (6) and from the primary sequence data (12) OEP75 might represent part of the translocation pore. Due to the close interaction of OEP75 with OEP34 it can be envisioned that OEP75 is regulated by the GTP-binding properties of OEP34. We have also isolated the gene coding for OEP16 (Pohlmeyer and Soll, unpublished) which forms a major crosslink product with preSSU (18). The coding sequence for OEP16 shows no obvious sequence similarity to any other known proteins. OEP16 and OEP34 are like OEP86 posttranslationally modified by ATP-dependent proteinphosphorylation (23, 24, Soll, unpublished). In addition the sequence data known so far for components of the outer envelope translocation machinery show no homology to other proteins, indicating that a new translocation apparatus for plastids was developed during evolution. This seems especially due in comparison to constituents of the import machinery of the second semiautonomous cell organelles in plant cells, i.e. mitochondria. Mitochondria seem to import preproteins by a similar mechanism in plants, fungi and animals which is not influenced by guanosin-nucleotides or posttranslational modification.

Together these data lead to a mechanistic model of precursor protein import which is unique to plastids (Fig. 3).

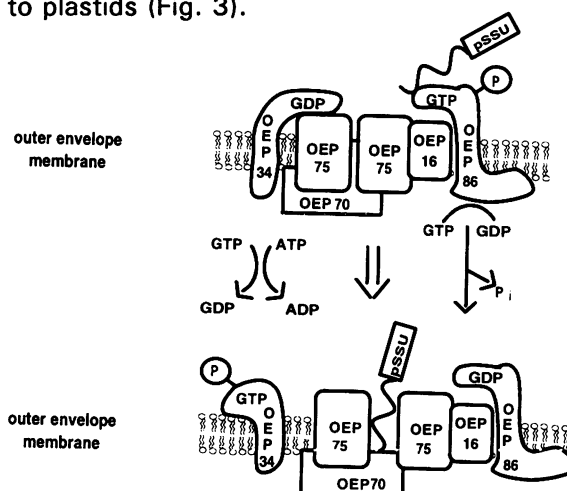


Figure 3. Hypothetical model of protein import into plastids. A chloroplast destined precursor protein, e.g. preSSU, interacts in an high affinity binding mode with OEP86. This could require either a guanosin nucleotide exchange or phosphorylation. To enable the precursor protein to move further in the translocation process the receptor-precursor protein affinity has to be changed by guanosin nucleotide exchange or dephosphorylation. In addition the productive interaction between the receptor and the translocation machinery might require one of the above mentioned

posttranslational modifications. The major constituent of the putative translocation pore OEP75 could be regulated via an GTP-GDP-exchange at OEP34 and change the open-close properties of the putative pore or its affinity to the precursor protein. The function of OEP16 is sofar unknown. OEP70 might function to keep the precursor in a translocation competent conformation or to pull the preprotein across the membrane.

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