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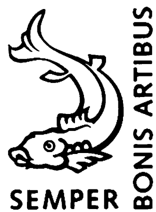

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Analysis of the Chloroplast Protein Import Machinery*

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Summary

Chloroplast destined precursor proteins bind to distinct areas on the organellar surface as visualized by immunogold decoration. Binding seems to occur predominantly in regions where the space between outer and inner envelope membranes appears dense in electron microscopic pictures. When complete protein translocation is blocked by antibodies, which bind specifically to the precursor protein, this precursor can still be partially translocated into the organell, as deduced from its maturation by the stromal processing peptidase. It remains, however, sensitive to exogenous protease, thus indicating that the preprotein spans both membranes while in transit through the plastid envelopes. Chloroplast envelope polypeptides that are involved and in close proximity to the precursor protein in the translocation event are identified by chemical crosslinking. Crosslinking experiments, using different translocation systems, i.e. intact organelles, outer envelope vesicles and an isolated import complex, gave identical labelled products. These crosslinked polypeptides are likely candidates for active participants in the import process.

Key words: Pisum sativum L., chloroplast envelope, protein transport, crosslinking.

Abbreviations: SSU = small subunit of ribulose-1,5-bisphosphate carboxylase-oxygenase; pSSU = precursor of SSU; Omp A = outer membrane protein A from *E. coli;* pSOmp A = hybrid form between the transit sequence of SSU and Omp A protein.

Introduction

The majority of plastid proteins are nuclear coded, synthesized in the cytosol and translocated posttranslationally into the organell. The sequence of steps has been well documented and described in general terms, i.e. (i) most proteins are synthesized as precursors with an N-terminal extension called transit peptide, (ii) the transit peptide is necessary and sufficient to direct the precursor to the organellar surface, (iii) productive interaction with protease sensitive receptors on the chloroplast envelope surface requires the hydrolysis of ATP, (iv) in a distinct step translocation into the organell occurs, again requiring the hydrolysis of ATP, (v) the transit sequence is cleaved off in the stroma by a specific processing peptidase, and (vi) further events include intra-organellar sorting, folding and assembly (for recent reviews see de Boer and Weisbeek, 1991; Soll and Alefsen, 1993; Theg and Scott, 1993).

One intriguing problem in this series of events is how a hydrophilic macromolecule passes through the two chloroplast envelope membranes and which envelope components are involved in this process. A number of different approaches have been used so far to identify single members of the chloroplast import apparatus. Antiidiotypic antibodies that mimic parts of the transit sequence of pSSU pointed to a 30 kDa envelope polypeptide as a receptor (Pain et al., 1988); however, this protein later proved to be the phosphate-triosephosphate translocator of the inner envelope (Flügge et al., 1989). Increased phosphorylation of a 51 kDa protein of spinach chloroplast envelopes upon precursor binding indicated the participation of this protein in the import process (Hinz and Flügge, 1988). Further putative components, which could be active in translocation, were identified by studies using chemical crosslinkers to attach the radiolabelled precursor, e.g. pSSU covalently to its next neighbours

* Dedicated to Prof. Dr. H. K. Lichtenthaler on the occasion of his 60th birthday.

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in the import apparatus. A 62 kDa envelope protein was the major crosslink product found by Cornwell and Keegstra (1987), while Kaderbhai et al. (1988) identified a 54 kDa polypeptide that most likely represents the large subunit of rubisco. None of the above proteins was studied further to our knowledge.

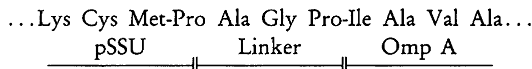
Only recently it became possible to dissect the translocation process into binding, partial translocation and complete import by manipulating exogenous parameters such as temperature, ATP and lipid composition (Olsen et al., 1989; Waegemann and Soll, 1991; Kerber and Soll, 1992). In addition, by using chloroplast outer envelope vesicles or a partially enriched import complex isolated from outer envelopes in conjunction with intact chloroplasts, we were able to define more precisely the localisation of the precursor in the import machinery. Using this knowledge as background we undertook this study to identify polypeptides involved in protein translocation into chloroplasts.

Material and Methods

cDNA-clones and in vitro-transcription-translation

The cDNA clones for proOmp A and pSSU used in this study are described by Freudl et al. (1985) and Lubben and Keegstra (1986). The hybrid protein pSOmp A was constructed using a Nru I-Bgl II fragment of the proOmp A cDNA, which was subsequently subcloned into pUC 18 (United States Biochemicals, Cleveland, USA). This DNA fragment was removed from the pUC 18 vector by Pst I-EcoR I digestion and ligated in frame with the DNA coding for the pSSU transit sequence present in the vector pGEM 3 (Promega, Madison, USA) after Pst I-EcoR I digestion. The resulting DNA was sequenced by the method of Sanger et al. (1977). The fusion site of pS with Omp A has the following amino acid composition

...Lys Cys Met-Pro Ala Gly Pro-Ile Ala Val Ala...



The pSSU or pSOmp A cDNAs were transcribed using SP6-RNA polymerase and the RNA was translated in a reticulocyte lysate system in the presence of [^{35}S] methionine as outlined in Salomon et al. (1990).

Precursor binding and translocation

Chloroplasts equivalent to 15 µg chlorophyll were incubated in 100 µL import buffer (330 mM sorbitol, 3 mM $MgCl_2$, 10 mM methionine, 10 mM $NaHCO_3$, 20 mM potassium gluconate, 2 % bovine serum albumin, 50 mM Hepes-KOH, pH 7.6) at 25 °C for 10 min in the dark in the presence of up to 5 µL translation product. Binding conditions refer to 100 µM ATP while import conditions were present at 3 mM ATP. After completion of the translocation experiment intact chloroplasts were recovered through a 40 % Percoll cushion, washed once and used for further treatment (Waegemann and Soll, 1991).

Isolated chloroplast outer envelope membranes equivalent to 10 µg protein were incubated with pSSU under conditions as described above except that 0.2 % bovine serum albumin was used. After completion of the binding experiment membranes were layered on top of a 200 mM sucrose cushion (500 µL) and separated from unbound pSSU by centrifugation at 300,000 × g for 20 min. The membranes were washed once and used for further analysis (Waegemann and Soll, 1991).

Immunogold decoration

Intact chloroplasts were incubated with pSOmp A under binding conditions. They were subsequently incubated in import buffer with Omp A antiserum at a final dilution of 1 : 100. Reisolated organelles were treated with protein-A conjugated to 8 nm colloidal gold for 1 h at room temperature in 330 mM sorbitol, 0,5 % bovine serum albumin, 3 mM $MgSO_4$ and 50 mM Hepes-KOH, pH 7.6. Chloroplasts were recovered by centrifugation (1,500 × g, 1 min) and treated with 2.5 % glutardialdehyde in 260 mM cacodylat, pH 7.6, and 2 mM $MgSO_4$. The chloroplasts were embedded, sectioned, and viewed as previously described (Alefsen and Soll, 1993).

Chemical crosslinking experiments

Precursor protein was bound to intact chloroplasts or outer envelope membranes as described above and subsequently incubated with N-hydroxysuccinimidester derived crosslinking agents (Pierce, Rockford, USA) for 5 min on ice. The coupling reactions were terminated by the addition of excess lysine and glycine, each at 10 mM final concentration. In case the second crosslinking step was due to photolysis of an azido group, the reaction mixtures were exposed to UV-light at 254 nm for 2 min at a distance of 3 cm using a Camag Typ 29200 lamp.

Isolation and subfractionation of chloroplasts

Intact purified chloroplasts were obtained from pea leaves as described in Waegemann and Soll (1991). Outer chloroplast envelope membranes were isolated by the method of Keegstra and Youssif

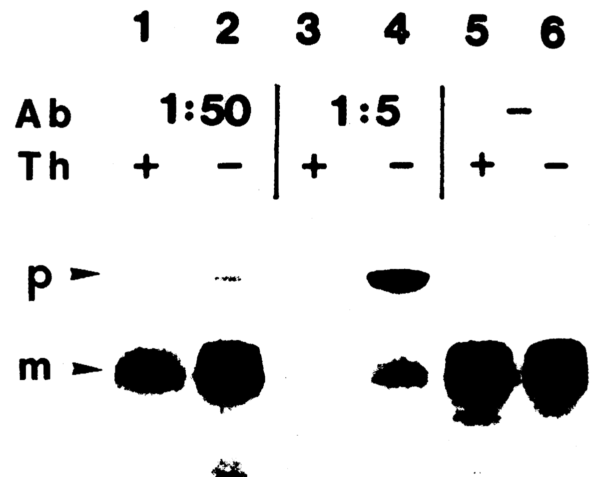


Fig. 1: Protein translocation into chloroplasts is inhibited by a precursor specific antibody. The hybrid protein pSOmp A (p) was incubated with intact chloroplasts under binding conditions (lanes 1–6). Chloroplasts were reisolated and incubated without or with different concentrations of Omp A antiserum (Ab, a 1 : 50 or 1 : 5 dilution of the serum was used, lanes 1–4); subsequently import conditions were established by raising the ATP concentration to 3 mM (lanes 1–6), yielding the processed form Omp A*(m). After completion of the import assay chloroplasts were treated with$^{(+)}$ or without$^{(-)}$ thermolysin (Th).

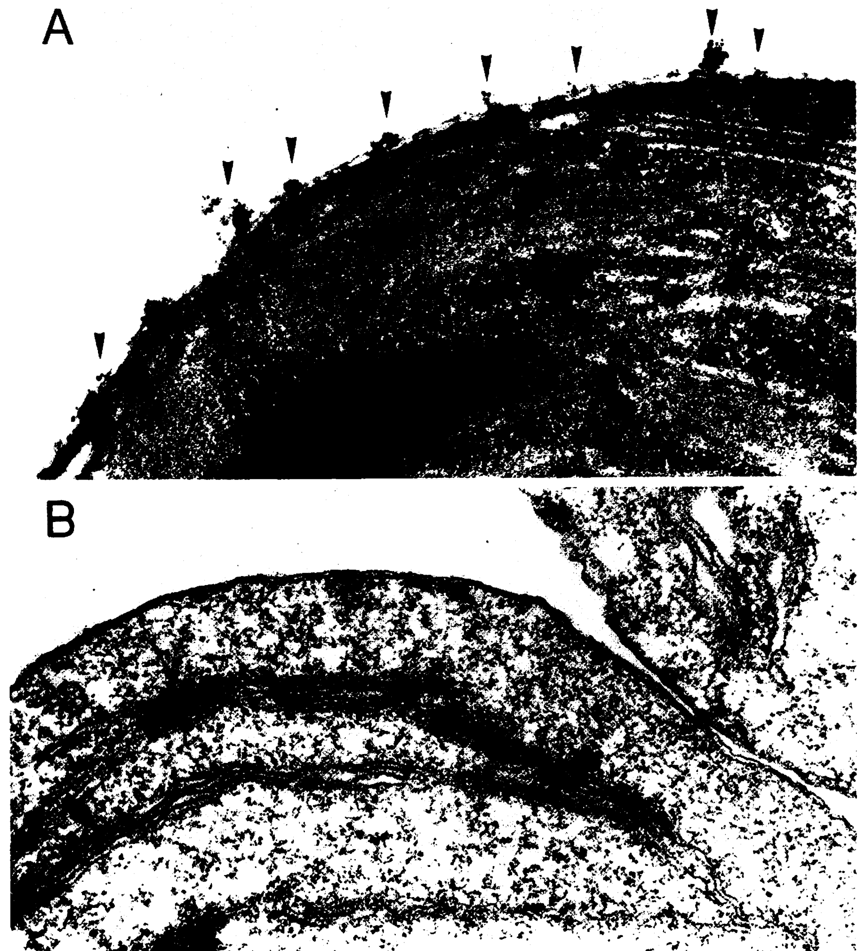


Fig. 2: Chloroplast surface bound precursor polypeptides can be visualized by immunogold decoration. (A) Intact chloroplasts were incubated with pSOmp A under binding conditions, reisolated, incubated with Omp A antiserum and immundecorated with protein A conjugated to 8 nm colloidal gold prior to fixation as in Material and Methods. (B) As in A except that no precursor protein was added.

(1986) after treatment in hypertonic sucrose solution. Outer envelope membranes either with or without bound pSSU were solubilized in 0.5% digitonin in 20 mM potassium phosphate, pH 7.3, and 1 mM EDTA (Waegemann and Soll, 1991). The suspension was layered on a 5–20% linear sucrose gradient and centrifuged for 4 h at 330,000 x g. The gradient was fractionated from the top into 25 fractions of 200 µL and used for further analysis.

Miscellaneous procedures

Protein was determined using bovine serum albumin as a standard (Lowry et al., 1951). Polyacrylamide gel electrophoresis and fluorography were as in Laemmli (1970), and Bonner and Laskey (1974). Chlorophyll was measured as in Arnon (1949).

Results

The chloroplast is surrounded by two membranes, the outer and the inner envelope, which are distinct entities having a unique polypeptide and lipid composition, respectively (Douce et al., 1984). We therefore asked the question, can the translocation process occur simultaneously through both membranes and can these import sites be visualized by immunogold decoration? To study this, a fusion protein was constructed consisting of the transit sequence and the first methionine of the mature part of SSU and the precursor of the outer membrane protein A from *E. coli* (proOmp A) starting at amino acid eight. A polyclonal antibody that recognized the C-terminal end of Omp A was available, and we reasoned that since translocation proceedes from the N-terminus of a polypeptide, the C-terminal sequence should still be exposed on the surface of the chloroplast, even if translocation had already partly proceeded. The pSOmp A protein is readily bound to intact organelles under binding conditions and can be chased into the organelle by raising the ATP concentration to 3 mM. The processed form Omp A*(m) is detected protease protected inside chloroplasts (Fig. 1, lanes 5, 6). Control experiments showed that pSOmp A translocation depended on the hydrolysis of ATP, receptors and temperature as described for other plastidal precursor proteins (not shown). In a parallel binding experiment with pSOmp A chloroplasts were subsequently incubated with Omp A antibodies prior to establishing translocation conditions by raising the ATP concentration. More pSOmp A remained bound to the chloroplast surface (Fig. 1, lane 1–4), indicating that complete import was hindered by the IgG chains interacting with Omp A. At the same time mature Omp A* was detectable inside the organelles; however, in this case the

mature form (m) was largely sensitive to exogenous protease. This indicates that the C-terminal parts of the polypeptide were still exposed on the cytosolic leaflet of the outer envelope, while the N-terminal part of the polypeptide had moved into the stroma to such an extent that it became accessible to the stromal processing peptidase (the exact processing site of pSOmp A was not determined). These results show that a precursor protein can span both chloroplast envelope membranes simultaneously while being in transit into the organelle.

Precursor polypeptides bound to the chloroplast surface under conditions that do not allow complete translocation but favour binding and partial translocation, i.e. low ATP and low temperature, can be visualized by immunogold decoration of the intact organelles (Fig. 2A). Again an antiserum was used that recognized the C-terminal end of Omp A; this part of the polypeptide should still be exposed to the cytosolic side of the envelope membrane under the conditions applied. The chloroplast outer surface is rather densely labelled by gold particles (Fig. 2A). In most instances, labelling is visible in regions where the intermembrane space between the envelope membranes appears dense, indicating a tight coupling between the import-machineries of both membranes, corroborating our findings in Fig. 1. Control experiments using either protease pretreated chloroplasts to remove receptor polypeptides (not shown) or a regime that included everything except the pSOmp A precursor (Fig. 2B) revealed that immunogold decoration was specific, i.e. dependent on receptor polypeptides and precursor protein. No gold grains are detectable inside the organelle or as nonspecific background.

The results described in Fig. 1 and 2 clearly indicated that conditions can be established that allow stopping the translocation event after binding but before complete import into the organelle (see also Waegemann and Soll, 1991). Under these circumstances the precursor polypeptide should be in close vicinity to the components of the import apparatus. This interaction can be experimentally fixed by connecting the two partners covalently by bifunctional crosslinking agents, thus allowing the identification of constituents of the chloroplast import-machinery. When chloroplast bound pSSU was crosslinked to intact organelles a number of labelled products could be detected in the fluorograms (Fig. 3, lane 3), ranging in molecular size from 30 kDa to around 200 kDa. If we substracted the molecular weight of pSSU (21 kDa) from the most prominantly labelled products, educt sizes of about 10–15 kDa, 25–30 kDa, 45–50 kDa, 70–75 kDa and 85–90 kDa were detected. Most of the labelled proteins were sensitive to exogenous thermolysin, indicating that indeed crosslinking of pSSU occurred with outer envelope proteins (Fig. 3, lane 4). Control experiments showed that the appearance of crosslinked products depended on the presence of intact chloroplasts (Fig. 3, lanes 1, 2), exogenous ATP for binding (lanes 5, 6) and protease sensitive components on the chloroplast surface (lanes 7, 8).

Purified outer envelope membranes from pea chloroplasts can be used as a bonafide system to study early events in precursor protein binding and translocation (Waegemann and Soll, 1991). We therefore wanted to see which of the crosslink products established in Fig. 3 was most likely localized

Fig. 3: The precursor of SSU interacts specifically with components of the chloroplast surface as detected by chemical crosslinking. Chloroplasts were incubated with pSSU (p) under binding conditions; i.e. 100 μM ATP, reisolated and subjected to a treatment with bis(sulfosuccinimydyl)suberat. Lanes 1, without chloroplasts; 3, complete assay; 5, as 3 except in the absence of exogenous ATP; 7, as 3 except that chloroplasts were pretreated with thermolysin (Waegemann and Soll, 1991). Even numbers are as odd numbers respectively, except that the assay was treated with the protease thermolysin after completion of the crosslinking experiment. A fluorogram is shown. Numbers on the left indicate molecular weight markers in kDa.

in this membrane compartment. Outer envelope membranes were therefore treated with a chemical crosslinker and subsequently incubated with pSSU. A distinct set of labelled products was found (Fig. 4). As for intact chloroplasts crosslink products were predominantly in the range of 30–35 kDa, 45–50 kDa, 70 kDa, and 80–90 kDa molecular size, resulting in educt sizes of 10–15 kDa, 25–30 kDa, 45–50 kDa and 70–75 kDa. Crosslink products of very high molecular size ≥ 100 kDa are less pronounced in isolated envelope membranes compared with the intact organelles, however clearly detectable (compare Fig. 3, lane 3 and Fig. 4, lane 2). The exact cause for this is not clear, but could be due to technical reasons, e.g. the isolation and fractionation procedure of outer chloroplast envelopes.

Further purification of the outer envelope localized import machinery can be achieved by mild detergent solubilisation in the presence of digitonin followed by sucrose density centrifugation (Soll and Waegemann, 1992). This regime results in the enrichment of a functionally active protein translocation complex, which is composed of a specific and limited subset of outer chloroplast envelope proteins; however, it is not known which of the constituent polypeptides

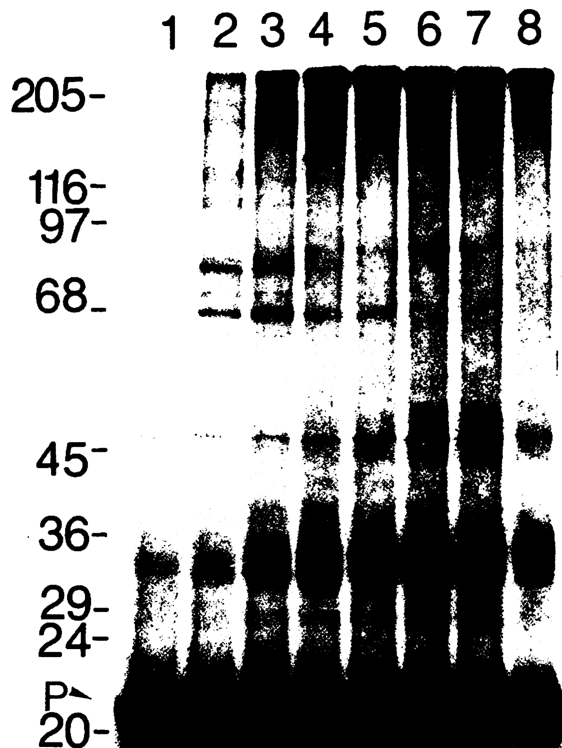


Fig. 4: Outer envelope membrane proteins form crosslinked products with pSSU. Purified outer envelope membranes were incubated with increasing concentrations of sulfosuccinimydyl-6-(4′-azido-2′ nitrophenylamino)hexanoate, repurified and subjected to crosslink experiments using pSSU (p) translation product. Lanes 1–8, envelope membranes were incubated with 0, 0.05, 0.2, 0.25, 0.5, 1, 2, 5 mM crosslinker, respectively.

interact closely with pSSU in this complex (Waegemann and Soll, 1993). The precursor protein is inserted and bound tightly to this complex. Crosslinking of pSSU to components of this complex should constrict the number of labelled polypeptides even further and demonstrate more clearly their involvement in the translocation event. Envelope membranes that contained bound pSSU were therefore solubilized with digitonin, fractionated by sucrose density gradients and each fraction subjected to crosslinking (Fig. 5). We could detect three prominent labelled crosslink products of molecular size around 32–34 kDa, 68–70 kDa and 90 kDa only in those fractions (16–20) that contained the membrane complex. The calculated educt sizes of the target envelope proteins were as in the other systems described above, around 12–14 kDa, 45–50 kDa and 75 kDa. Since these polypeptides were found in all three translocation systems, they are most likely directly involved in the import event.

Discussion

We have identified a distinct subset of chloroplast envelope proteins as likely candidates for participants of the chloroplast import machinery. These envelope proteins have molecular sizes of about 12–14 kDa, 45–50 kDa and 70–75 kDa, and were detected in translocation systems, fractionated to different extents. Covalently crosslinking of pSSU to intact chloroplasts, i.e. the standardized organellar system, yielded a greater number of labelled products than the isolated envelope membrane system. However, some of the most prominent products are found in both systems, which might represent intermediate translocation stages, where precursor proteins accumulate under the *in vitro* conditions. In addition, the even further fractionated and purified chloroplast import complex gave rise to a very limited subset of crosslinked proteins, again homologous to the more complex systems, i.e. chloroplast and envelope vesicles. These three envelope proteins are prominent and should therefore be in close proximity to pSSU during the translocation process. High molecular weight crosslink

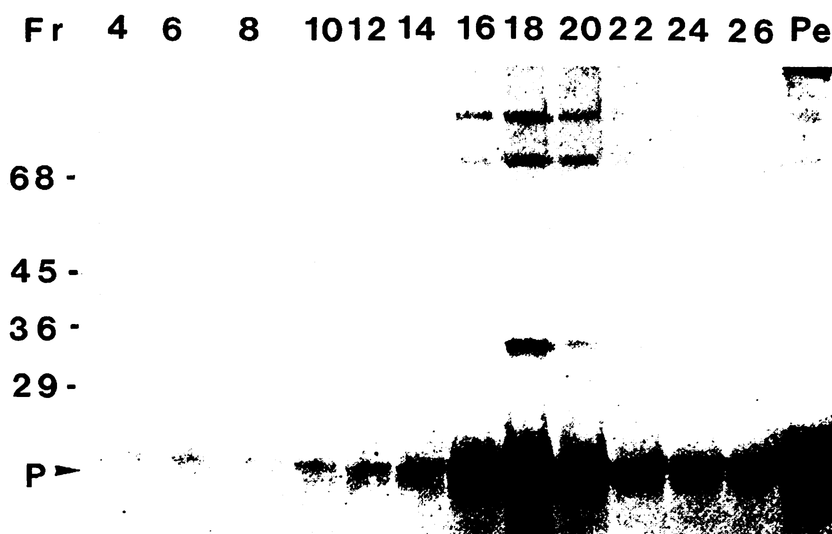


Fig. 5: A partially enriched membrane complex of the outer envelope yields specific crosslink products with pSSU. Purified outer envelopes were incubated with radiolabelled pSSU (p), reisolated, solubilized with digitonin and fractionated on a linear sucrose density gradient. Twenty six fractions (Fr) of 200 µL were recovered and used (even numbers) in the crosslink experiments in the presence of 0.5 mM sulfosuccinimydyl-6-(4′-azido-2′ nitrophenylamino)hexanoate. Membrane material recovered in the pellet fraction (Pe) was also used in the crosslinking experiment.

products ≥ 100 kDa obtained with intact organelles are less pronounced in purified envelope vesicles and barely detectable in experiments using the isolated import complex. This could be due to the following reasons: (i) these crosslinks represent proteins involved in later steps in the translocation pathway, which can not occur in the further fractionated systems, and (ii) these crosslinks are products of more than two proteins, i.e. further chloroplast proteins that might be unrelated to the translocation process but in proximity to the desired target also become attached and yield an oligomeric crosslinked complex; this reaction is repressed in the more purified translocation systems. The assignment of exact molecular weight educt sizes is difficult, because we do not know how these kind of non-linear polypeptides behave in a SDS-polyacrylamide gel.

Using different approaches and methods, outer envelope proteins of 86 kDa, 75 kDa, 70 kDa and 34 kDa were already implicated to be involved in the import process (Waegemann and Soll, 1991; Waegemeann and Soll, 1993). Crosslinking experiments were carried out under binding conditions, which allow only the partial movement of pSSU into the translocation apparatus, but not complete import into the organelle (Olsen et al., 1989; Waegemeann and Soll, 1991). Large portions of pSSU remain on the outside of the chloroplasts. Therefore, most of the labelled crosslinked products are protease sensitive in intact organelles, indicating that the conditions we choose for our experiments favoured the covalent attachment of pSSU to outer envelope proteins, which are at least in part exposed to the cytosolic side of the chloroplast. It is difficult to assign a certain function of chronological order in which these proteins act in the translocation event. We have tried to covalently attach pSSU to phospholipase C treated chloroplasts, which hinders the movement of the receptor bound precursor into the translocation apparatus (Kerber and Soll, 1992) and should result preferentially in a labelled receptor. However, these attempts were not successful, since the phospholipase C treated chloroplasts were too fragile to endure the crosslinking procedure. In connection with the published polypeptide composition of the isolated chloroplast import apparatus (Soll and Waegemann, 1992) our data presented in this work further strengthen the notion that OEP 86, OEP 75, OEP 70, OEP 34 and OEP 12 are, beyond others, involved in the import process and should be analysed further.

We could show that the hybrid protein pSOmp A, which can be imported normally into chloroplasts, is stopped from full translocation by an antiserum that preferentially recognized the C-terminal part of Omp A. This protein was processed by the stromal processing peptidase, but was still sensitive to exogenous protease, indicating that a precursor protein can span the outer and inner envelope membranes, thus engaging both translocation machineries simultaneously. Under these conditions the outer and inner envelope membranes seem to be «connected» by material that appears dense in electron microscopic pictures and might originate from the interenvelope space. Attempts to isolate such contact sites of the outer and inner envelope have failed so far (Pain et al., 1988; Schnell and Blobel, 1993). We therefore favour a dynamic model, i.e. separate translocation units in the outer and inner envelope membrane engage, maybe with the help of interenvelope space proteins, after a precursor protein initiates a productive translocation cycle after binding. Similar models have been proposed for mitochondria (Hartl et al., 1989) and while this work was in progress, also for chloroplasts (Schnell and Blobel, 1993). Detailed reconstitution studies are, however, necessary to validate this model.

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Note added in proof

While this manuscript was under review a report appeared (Perry, S. E. and K. Keegstra: Envelope membrane proteins that interact with chloroplastic precursor proteins. The Plant Cell *6*, 93–105, 1994) that also identifies, by chemical crosslinking, OEP 86 and OEP 75 as polypeptides interacting with pSSU.

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