Characterization of the protein import apparatus in isolated outer envelopes of chloroplasts

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Summary

Isolated outer envelope membrane from pea (*Pisum sativum* L.) chloroplasts can be used *in vitro* to study binding and partial translocation of precursor proteins destined for the inside of the organelle. Efficient binding to a receptor protein on the outside of the membrane vesicle and generation of a translocation intermediate depends strictly on the presence of ATP. Protease treatment of the translocation intermediate demonstrates its insertion into the membrane. The membrane-inserted precursor protein cannot be extracted by 1 M NaCl and is also NaOH resistant to a large extent. Mild solubilization of outer envelope membranes by detergent resulted in the isolation of a complex which still contained the precursor protein. We have identified a constitutively expressed homologue hsc 70 as part of this membrane complex. Antibodies against hsp 70 (inducible heat shock protein 70) were able to immunoprecipitate the complex bound precursor protein. A second protein of 86 kDa molecular weight (OEP 86) from the outer envelope membrane was also identified as a major component of this complex.

Introduction

Chloroplasts and mitochondria are of dual genetic origin. This means that a large number of organellar polypeptides are coded for on nuclear genes and their synthesis occurs on cytosolic ribosomes, mostly as larger precursor proteins (Hartl *et al.*, 1989; Keegstra *et al.*, 1989). An essential step in the biogenesis and viability of the organelles is the continuous transport of these polypeptides from their cytosolic origin to their organellar destination. Current studies have revealed a sequence of import events common to almost all nuclear-coded chloroplast proteins, e.g. cytosolic chaperones are involved to retain import competence (Waegemann *et al.*, 1990), ATP hydrolysis is necessary for complete membrane translocation (Flügge and Hinz, 1986; Schindler *et al.*, 1987; Theg *et al.*, 1989) and seems necessary for binding (Olsen *et al.*, 1989),

and translocation is then followed by processing and assembly (Robinson and Ellis, 1984). An intriguing question is how a precursor protein crosses the outer and inner chloroplast envelopes and which membrane proteins are involved in this complex process. In contrast to comparable studies using mitochondria from yeast or *Neurospora* (Hartl *et al.*, 1989) no translocation intermediates have been obtained so far for the chloroplast transport event. Import intermediates are essential to identify and study components of the translocation machinery (Baker and Schatz, 1991; Pfanner *et al.*, 1991). Crosslinking studies using intact chloroplasts have suggested that outer envelope proteins of 66, 52 and 30 kDa might be involved in the import pathway (Cornwall and Keegstra, 1987; Kaderbhai *et al.*, 1988). Different approaches using either anti-idiotypic antibodies (Pain *et al.*, 1988) or differential phosphorylation of envelope polypeptides (Hinz and Flügge, 1988) have also implied polypeptides of 30 and 51 kDA to be involved in the import process. To date no detailed information about the function of these proteins is available or that which is available is controversial (Flügge *et al.*, 1989; Schnell *et al.*, 1990).

In this work we have developed a system to study the early events in binding and translocation of precursor proteins into chloroplasts using only isolated outer chloroplast envelope membranes, which are able to bind specifically to the precursor protein and partially insert it into the transport apparatus. A membrane complex having the precursor protein bound to it was subsequently isolated and shown to contain constitutively expressed homologue hsc 70 and an outer envelope protein OEP 86.

Results

Comparison of the chloroplast translocation process with that of isolated outer envelope membranes

To establish the outer chloroplast envelope membrane translocation system we had to compare its properties with those of intact, purified organelles (Figure 1). Chloroplasts import precursor proteins, e.g. pSSU (precursor form of the small subunit of Rubisco SSU) in an ATP-dependent manner (Figure 1a). Chloroplasts were left on ice for 1 h in complete darkness prior to the import experiment to deplete internal ATP (Theg *et al.*, 1989). Furthermore, pSSU translation product was treated with apyrase, an ATP-hydrolysing enzyme, to remove ATP from the translation mix. Import experiments were carried out in the dark. ATP was added exogenously when appropriate.
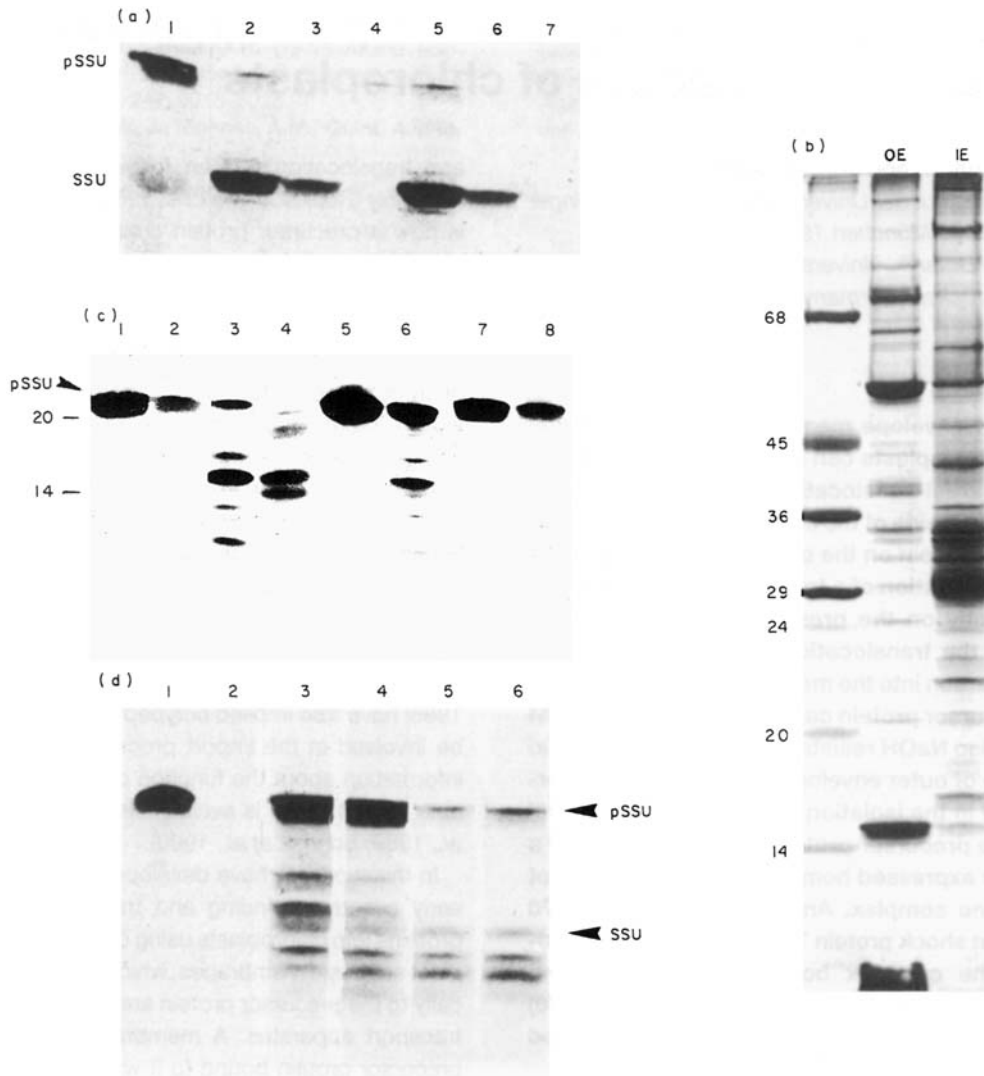
Figure 1. Comparison of pSSU translocation into chloroplasts and envelopes.

(a) pSSU is translocated into chloroplasts and processed to mature SSU in a protease-protected manner. Binding and translocation of pSSU depends on proteinaceous components on the chloroplast surface and on ATP. Lane 1, translation product; lane 2, import and processing of pSSU by intact chloroplasts; lane 3, chloroplasts were treated with the protease thermolysin prior to the import assay; lane 4, pSSU translation product was ATP-depleted through the action of apyrase prior to the import reaction; lanes 5–7, as lanes 2–4, respectively, but treated with thermolysin after termination of the import assay.

(b) Silver-stained SDS–PAGE demonstrating the polypeptide composition of inner envelope membranes (IE) and outer envelope membranes (OE).

(c) Interaction of pSSU with outer and inner envelope membranes. pSSU was incubated with isolated outer envelopes (lane 1), or outer membranes pre-treated with thermolysin (lane 2). Lane 3, as lane 1 but treated with thermolysin after re-isolation of the membranes. Lane 4, as lane 3 but membranes were solubilized by detergent prior to protease treatment. Lanes 5 and 6, as lanes 1 and 3 but incubations were carried out at 4°C instead of 25°C. Lanes 7 and 8, interaction of pSSU with outer or inner membranes, respectively.

(d) Translocation intermediates appear to be time- and temperature-dependent. Incubation of intact chloroplasts with pSSU in the presence of 100 μM ATP. Lane 1, 4°C, 5 min; lane 2, as lane 1 but chloroplasts were treated with thermolysin after the incubation. Lanes 3–6, chloroplasts were incubated with pSSU at 25°C for 10 min in the presence of 100 μM ATP and left at 4°C for an additional 0, 20, 30 and 40 min, respectively. Only thermolysin-treated samples are shown.

Under these conditions translocation of pSSU into chloroplasts depended completely on exogenously supplied ATP (Figure 1a, lanes 2 and 4). The imported precursor protein was processed to its mature form SSU and was protease-protected inside the organelle (Figure 1a, lanes 2 and 5). Protease-sensitive receptors which are exposed on the cytosolic leaflet of the outer chloroplast envelope are responsible for recognition of pSSU and translocation initiation (Keegstra *et al.*, 1989). If chloroplasts were treated with the protease thermolysin prior to the import assay, binding and import was greatly diminished (Figure 1a, lanes 2, 3, 5 and 6). Under import conditions, i.e. high ATP, the amount of pSSU bound to the chloroplast surface was low since the translocation event was rapid. In the absence

of ATP very little pSSU was found at the chloroplast surface, corroborating the results of Olsen *et al.* (1989) that early events in translocation localized at the outer chloroplast envelope depend on ATP.

Inner and outer envelope membranes from pea chloroplasts were isolated and purified by established methods (Keegstra and Youssif, 1986). Outer and inner membranes were pooled separately and repurified to obtain a homogenous membrane population (see Experimental procedures). Isolated envelope membranes occur only as closed vesicles as demonstrated by electron microscopy (Douce *et al.*, 1984). They were stored at −80°C and retained their biological activity for several years. The polypeptide composition of the envelope preparations was controlled by SDS–PAGE to demonstrate its purity (Figure 1b). In principle, isolated membrane vesicles can occur in two orientations, i.e. outside-out or inside-out. By using different techniques such as aqueous two-phase partitioning, electron microscopy, immunofluorescence and limited proteolysis we are able to show that the outer envelope membrane vesicles, also used in the present work, are >90% outside-out (Waegemann, manuscript in preparation). We conclude from this that the proteinaceous receptors present in the outer envelope are still exposed to the outside, as in the intact organelle, and are accessible for precursor polypeptides. Consequently, outer envelope membranes specifically bind pSSU (Figure 1c, lane 1). When envelope membranes were treated with a protease prior to the experiment, the amount of pSSU found to interact with the membrane vesicles dropped to about 20% of the control (Figure 1c, lane 2). Inner envelope membranes interacted only poorly with pSSU (Figure 1c, lanes 7 and 8), further indicating the validity of our approach. Inner envelope membrane preparations are contaminated by outer membrane proteins to about 10–15% while much purer outer membrane populations can be obtained (Douce *et al.*, 1984; Keegstra and Youssif, 1986). This could be due to the separation techniques of inner and outer envelope membranes (see Experimental procedures). One can speculate that binding of precursor proteins to inner envelope preparations is due to outer membrane proteins of the import apparatus located in contact sites which were partly isolated in the inner membrane preparation.

When outer envelopes were re-isolated after incubation with pSSU and subsequently treated with the protease thermolysin, we observed protease-protected pSSU and four proteolytic breakdown products, pSSU deg 1–4, pSSU deg 1 and 2 are of about 17 and 15 kDa, respectively, and pSSU deg 3 and 4 are of about 13 and 10 kDa, respectively (Figure 1c, lane 3). None of these forms corresponded in size to authentic mature SSU (data not shown). pSSU and pSSU deg 1–4 were almost exclusively found in the membrane fraction if the incubation mixture

was separated into a membrane pellet and supernatant by centrifugation after thermolysin treatment (data not shown). The appearance of protease-protected pSSU and pSSU deg 1–4 was probably due to their insertion into the translocation apparatus of the outer envelope. If the membranes were solubilized by detergents, i.e. 0.5% Triton X-100 and 0.5% SDS, pSSU and pSSU deg 1, 3 and 4 were completely degraded, pSSU deg 2 was partially degraded. Experiments carried out at 4°C indicate that binding was increased (Figure 1c, lane 5). The precursor protein seems to interact with components of the import apparatus in such a way at 4°C that renders more of the precursor form protease-protected than at 25°C. At the same time, the amount of pSSU deg 1–4 was decreased compared to assays carried out at 25°C. These results indicated a sequence of steps in the binding and insertion process of pSSU into the translocation machinery which could be distinguished by the accessibility of the precursor protein to external protease. This is in accordance with binding experiments using intact chloroplasts (Figure 1d). pSSU bound to the chloroplast surface could be found when chloroplasts were incubated with pSSU in the presence of 100 μM ATP at 4°C for a short period (5 min). Under these conditions, insertion into the membranes and translocation was slow and precursor polypeptides accumulated on the outside of the organelle (Figure 1d, lane 1). The surface-bound precursor was completely degraded by protease treatment (Figure 1d, lane 2). However, pSSU deg 1–4 could be observed when intact chloroplasts were incubated with pSSU under identical conditions as for isolated outer membranes, i.e. 100 μM ATP, 10 min at 25°C followed by prolonged incubation at 4°C prior to further treatment (Figure 1d, lanes 3–6). The prolonged incubation period was supposed to mimic the time we needed to repurify the outer envelope membranes after the experiment. While chloroplasts could be purified through a Percoll cushion and washed in 5–7 min prior to thermolysin treatment, the similar procedure using envelopes takes about 1 h (see Experimental procedures). pSSU deg 1–4 could already be detected after the first incubation for 10 min at 25°C; after longer periods at 4°C only pSSU deg 3 and 4 were still visible (Figure 1d, lanes 5 and 6), indicating that the precursor protein was interacting differently with the translocation apparatus.

Interaction of pSSU and outer chloroplast envelopes requires ATP

Import studies using intact organelles indicated two different ATP-requiring steps, low ATP (100 μM) seems necessary for binding (Olsen *et al.*, 1989), high ATP (1–3 mM) is required for efficient translocation rates (Theg *et al.*, 1989). Though we have not elucidated the role of ATP in binding and penetration of the import apparatus in
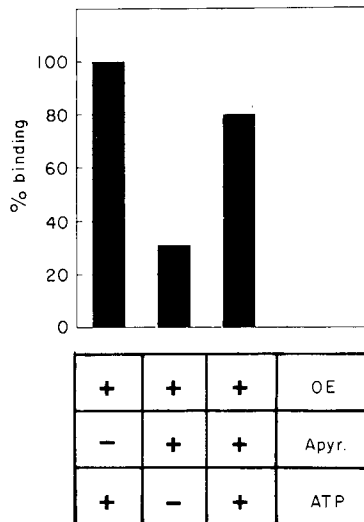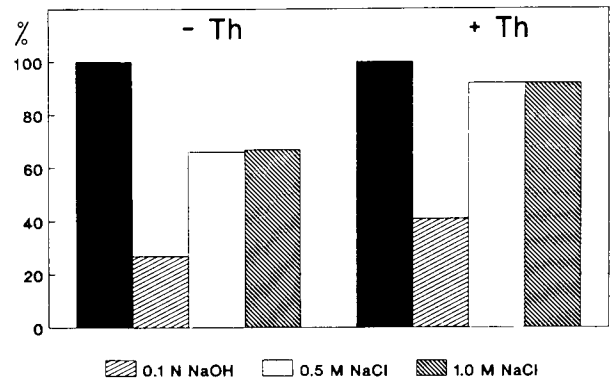
Figure 2. ATP is required for pSSU–envelope interaction.
ATP was removed from the translation mixture by treatment with apyrase (apyr., lane 2). Lane 3, excess ATP (5 mM) was added to an apyrase-treated sample, to ensure that sufficient ATP was present during the incubation period.


Figure 3. Envelope–pSSU interaction is salt resistant.
Outer envelope membranes were incubated with radiolabelled pSSU. After re-isolation, the membranes were treated with different concentrations of NaCl or NaOH either before thermolysin (−Th) or after thermolysin (+Th) treatment. The amount of pSSU recovered in the pellet fraction is shown. pSSU and pSSU deg 1–4 were added together in samples treated with thermolysin. Thermolysin removes about 20–25% surface-exposed pSSU. The 100% value in the experiment plus thermolysin is therefore lower than the corresponding value without thermolysin.

detail, it is evident from the results presented in Figure 2 that ATP is a prerequisite for the interaction of pSSU with outer envelopes. Omission of ATP from the incubation mixture and simultaneous removal of ATP from the translocation mixture containing pSSU using apyrase reduced the level of membrane-associated pSSU to 25% of the control levels. Re-addition of ATP to an apyrase-treated sample resulted in the restoration of association capacity. These findings strongly indicate an ATP-dependent step in protein translocation into chloroplasts which is localized at the cytosolic leaflet of the outer chloroplast envelope.

Tight interaction between pSSU and outer envelope

The results presented in Figure 1c indicate that pSSU is partly inserted into the translocation machinery of the outer chloroplast envelope. To obtain further evidence for this finding we examined the strength of interaction between pSSU and the membrane components by extraction with high concentration of NaCl or NaOH (Figure 3). About 70% of pSSU, bound to the outer envelope, was resistant to washes with 0.5 or 1 M NaCl and the precursor protein was recovered with the membrane fraction. After an alkaline wash using 0.1 M NaOH, 25–30% of pSSU remained in the membrane pellet, demonstrating the tight interaction between membrane components and precursor protein. When protease-sensitive pSSU, e.g. nonspecifically bound pSSU, was removed from the membrane surface by thermolysin treatment the percentage of pSSU and pSSU deg 1–4 which was resistant to 1 M NaCl or 0.1 M NaOH increased to more than 90 and 40% precursor polypeptide recovered in the membrane fraction,

respectively (Figure 3). From the data presented in Figures 1c and 3 we assume that a large portion of pSSU not only interacts with surface-exposed receptor proteins but that it has proceeded on its way through the membrane.

Isolation of a membrane complex containing pSSU

The strong interaction between pSSU and components of the outer envelope suggested that mild detergent solubilization of the membrane could be used to isolate a membrane complex involved in protein translocation. This was found to be the case. Outer envelope membranes, which were loaded with pSSU (see Experimental procedures), were partially solubilized by 0.5% (w/v) digitonin. The mixture was then separated by centrifugation on a continuous sucrose density gradient. The distribution pattern of radioactivity in the gradient showed a peak around fraction 16 (Figure 4a). Fractions were analysed by SDS–PAGE and fluorography (Figure 4b). Radioactivity present in the top fractions of the gradient was due to unbound pSSU and free methionine, as also determined by control gradients which were loaded with translation mixture containing pSSU (Figure 4b). pSSU bound to membrane components migrated to higher density in the sucrose gradient and was first detectable at about fraction 10. In accordance with the results obtained by liquid scintillation counting (Figure 4a) the highest amount of pSSU was found between fractions 15 and 18 (Figure 4b). Complex-bound pSSU dropped to a minimum around fraction 23. Solubilization of envelope membranes by digitonin was not complete, but membrane-bound pSSU could be recovered from the pellet of the sucrose density gradient (Figure 4b). When envelope membranes were
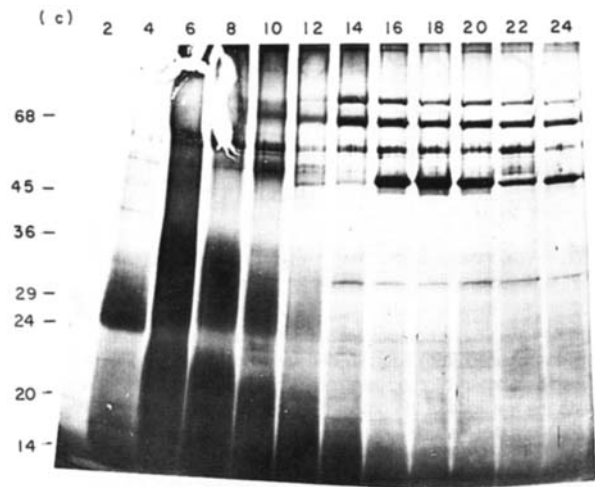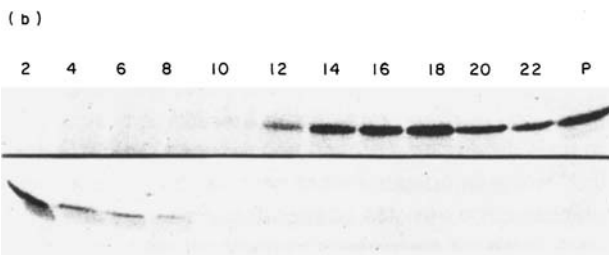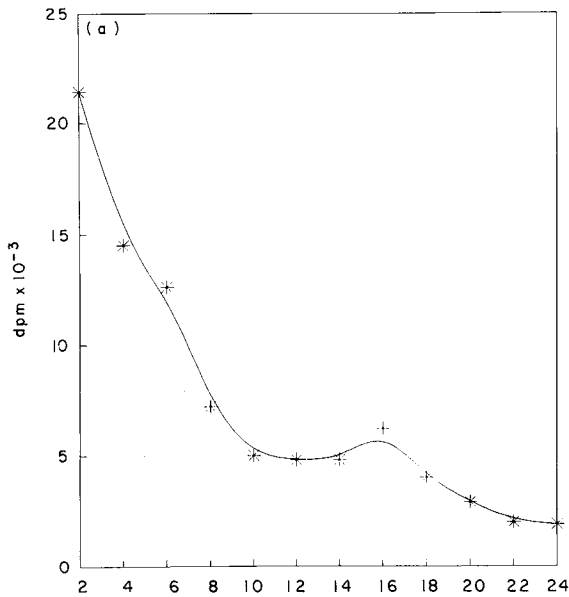
Figure 4. Solubilization of outer envelope membranes.
Envelope membranes which had been incubated with pSSU were solubilized with digitonin and analysed after centrifugation on a continuous sucrose density gradient.
(a) Distribution of radioactivity throughout the gradient was determined by liquid scintillation counting.
(b) Radiolabelled pSSU was determined by SDS–PAGE and fluorography of fractions obtained from a sucrose density gradient as in (a) (upper half). The fluorogram presented in the lower part shows the distribution of pSSU in a sucrose gradient which was loaded with pSSU translation mixture only.
(c) Polypeptide pattern of fractions from a similar gradient as in (a). Proteins were analysed by SDS–PAGE and visualized by silver staining.
Numbers on top or bottom indicate the fraction number. Numbers on the left of (c) indicate the size of molecular weight markers in kDa. P denotes the pellet fraction recovered from the gradient.

treated with thermolysin prior to pSSU binding and membrane solubilization, the level of pSSU found in the peak fractions of the sucrose gradient was around 20–30% of non-treated controls (data not shown). This was in good correlation with the results shown in Figure 1c.

Subsequently, we analysed the polypeptide pattern of the gradient fractions by SDS–PAGE and silver staining. The polypeptidè composition of those fractions which contained pSSU was found to be distinct and different from that of total envelope membranes and the top fractions of the sucrose gradient (compare Figures 4c and 1b). High molecular weight polypeptides in the range between 50 and 90 kDa were very prominent in this complex. Less abundant were polypeptides in the region between 32 and 40 kDa, while low molecular weight proteins could not be detected in pSSU-containing fractions. These polypeptides were confined to the top fractions of the gradient and the pellet. When we compared the polypeptide pattern of control fractions with those obtained from envelope membranes treated with thermolysin, no obvious changes were observed with one exception.

A high molecular weight protein (denoted by an arrow in Figure 5) was no longer detectable, instead a new polypeptide (denoted by an asterisk in Figure 5) appeared, which probably represents a proteolytic breakdown product.

Identification of two proteins in the complex

Outer envelope membranes showed a typical membrane composition including a certain set of marker proteins. One of these proteins had a size of around 86 kDa and was susceptible to thermolysin treatment (Cline *et al.*, 1984; Joyard *et al.*, 1983). Antibodies which we have raised against the outer envelope protein of 86 kDa (OEP 86) recognized the high molecular weight component in fractions 15–18 of the sucrose gradient (Figure 5). OEP 86 was not present in fractions obtained from the top of the gradient. The putative breakdown product of OEP 86 mentioned above was also recognized by the antibody and hence assigned to OEP 86 (Figure 5).

Evidence has accumulated (Marshall *et al.*, 1990; Soll,
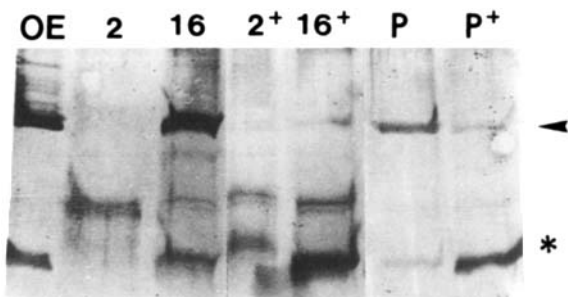
Figure 5. OEP 86 is a component of the translocation complex.
Fractions obtained from sucrose density gradients as in Figure 4 were analysed by Western blotting using purified IgGs against OEP 86. Numbers indicate the fractions of the gradient which were used for the analysis. P denotes the pellet fraction recovered from the sucrose gradient. Envelope membranes were either treated (+) or not treated by thermolysin prior to the experiment. The arrow indicates the position of OEP 86; the asterisk denotes the position of the putative proteolytic fragment.
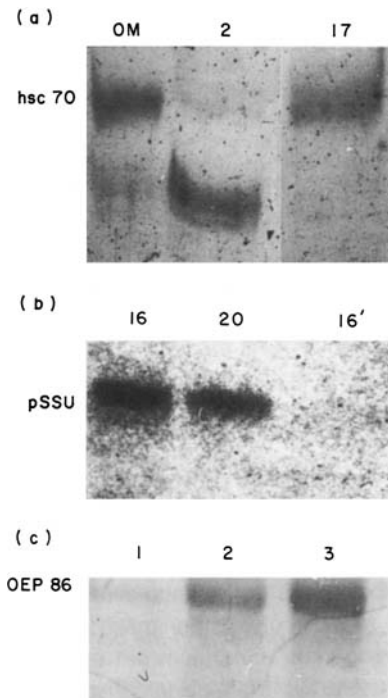
Figure 6. Outer-envelope-localized hsc 70 is part of the import apparatus and interacts with pSSU.
(a) Outer envelope membrane (OE) and fractions 2 and 17 from a sucrose density gradient as shown in Figure 4a were probed by Western blotting using an hsp 70 antibody.
(b) Immunoprecipitation of complex-bound pSSU using hsp 70 antibodies was carried out from fraction 16 and 20 of a standard sucrose density gradient or from a sucrose density gradient loaded with pSSU translation product only, 16′ (compare with Figure 4b).
(c) Immunoprecipitation of complex-bound OEP 86 using hsp 70 antibodies was carried out from fractions 14–18 of a standard sucrose density gradient. OEP 86 was detected after precipitation by SDS–PAGE and Western blotting. Lane 1, 300 μl combined fractions were treated with pre-immune serum; lanes 2 and 3, 300 μl and 900 μl fractions, respectively, were treated with immune serum.

unpublished data) that the outer chloroplast envelope contains at least one hsc 70 homologue which could function in the translocation process of precursor proteins through the envelope membranes as proposed by von Heijne and Nishikawa (1991). Using an antibody against hsp 70 (Neumann *et al.*, 1987) we identified an hsc 70 homologue in the isolated complex which was not present in fractions at the top of the sucrose gradient or in control gradients which were loaded with pSSU translation product only (Figure 6a and data not shown). pSSU could be immunoprecipitated from the sucrose gradient fractions containing the precursor protein using hsp 70 antibodies (Figure 6b), indicating the close association of precursor and chaperone. In another control experiment, pSSU translation mixture was loaded on a sucrose density gradient and used for immunoprecipitation (Figure 6b). No pSSU was detectable in immunoprecipitates of fraction 16 of this gradient. This suggests that hsc 70 homologue localized in the outer chloroplast envelope is an important component of the protein translocation machinery. OEP 86 could be immunoprecipitated from fractions 14–18 of the sucrose density gradient by hsp 70 antibodies (Figure 6c), further indicating that OEP 86 and hsc 70 are members of the import complex.

Discussion

We have developed a protein translocation system in this study which is independent of intact organelles but uses outer envelope vesicles isolated from intact pea chloroplasts. Such a system offers a number of advantages.
(i) For the first time it was possible to obtain translocation intermediates and therefore we should be able to analyse distinct steps in the binding and translocation pathway. Until now it was only possible to distinguish between binding of the precursor to chloroplasts and complete insertion into the organelle (Keegstra *et al.*, 1989).
(ii) The outer envelope membranes used in this study represent a purified organellar compartment which makes up less than 0.5% of the total chloroplast protein but which should contain all of the precursor binding capacity and at least part of the translocation apparatus (Douce *et al.*, 1984). Attempts to identify or isolate proteins involved in precursor recognition, binding or translocation should be greatly facilitated in this partially purified and enriched system.

A number of criteria were used to establish the outer envelope membranes from pea chloroplasts as a bona fide protein translocation system. First, in a separate study (Waegemann, manuscript in preparation) we found that the isolated outer envelope membranes consist largely (>90%) of outside-out vesicles, which is essential for the present work. Secondly, binding of pSSU to isolated outer envelopes depends on surface-exposed protease-

sensitive components. Thirdly, ATP is required for binding and insertion into the outer envelope. Fourthly, we observed that pSSU, which had been synthesized in a reticulocyte lysate system at 37°C under false co-factor conditions, was not competent for import into chloroplasts (data not shown). No binding of this import-incompetent precursor to isolated envelopes was observed, demonstrating that only competent pSSU interacts in a specific manner with the outer envelope membrane localized import system. Fifthly, identical translocation intermediates can be isolated from the organellar and the isolated membrane translocation system in a time- and temperature-dependent manner (Figure 1c and d). To our knowledge this is the first time that translocation intermediates have been reported for chloroplasts. pSSU deg 1 and 2 disappear after longer incubation times in the organellar system, suggesting that pSSU has moved to a different location in the import machinery. This is not the case in the membrane system where pSSU deg 2 is the major protease-protected precursor fragment. This observation could indicate that the isolated outer envelope membrane system does not contain the complete translocation apparatus. Due to the isolation procedure of envelope membranes (Douce *et al.*, 1984; Keegstra and Youssif, 1986), a set of polypeptides which could be part of the translocation apparatus but are localized in the inner envelope, are not present in our outer envelope membrane system which we describe in this paper. Further experiments will reveal the function of those proteins localized in the inner membrane.

Our results clearly position one ATP-requiring step at an early stage in the cascade of import steps (see also Olsen *et al.*, 1989). ATP might be required for the release of pSSU from cytosolic factors such as hsc 70 and simultaneous handing-over to an envelope receptor/hsc 70 complex (Figure 7). Whether ATP is hydrolysed by a cytosolic or an envelope protein remains to be elucidated (Hartl *et al.*, 1990). hsc 70 is present not only in the outer chloroplast envelope (Marshall *et al.*, 1990; Soll, unpublished data) but also in a precursor-containing complex which we were able to isolate in the present work. It was proposed (von Heijne and Nishikawa, 1991) that chloroplast transit peptides are designed to interact with different chaperones in succession during their import pathway. Our results add initial experimental evidence to this hypothesis and could pinpoint mechanistic differences between mitochondrial and chloroplastic import pathways. Though pSSU could be immunoprecipitated by hsp 70 antibodies from the isolated import complex, direct evidence for the interaction of hsc 70 with pSSU should be provided by chemical crosslinking studies (Kang *et al.*, 1990; Scherer *et al.*, 1990; Wiedmann *et al.*, 1989).

Interaction of pSSU with the import apparatus was very tight; only surface-exposed thermolysin-sensitive precursor proteins could be removed by 1 M NaCl. About
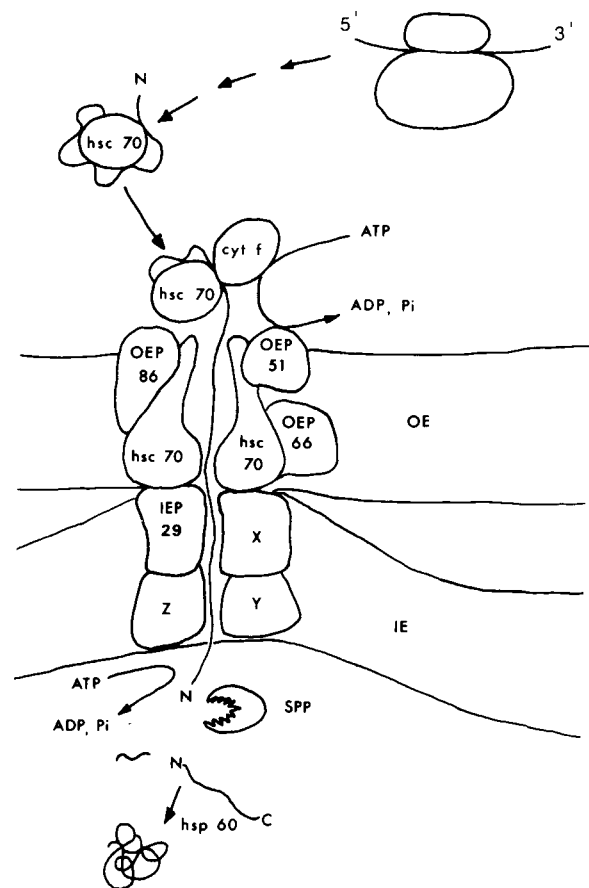
Figure 7. Working model of protein translocation into chloroplasts. The scheme comprises proteins which have been suggested to function in the import process in papers mentioned in the text and in the present work. The arrangement of polypeptides does not necessarily represent the *in-vivo* situation.

Abbreviations: cyt f, cytosolic factor; SPP, stromal processing protease.

25–30% of the precursor protein was loosely attached to the membrane, probably to the receptor, or non-specifically bound and salt-sensitive. If these precursor polypeptides were removed from the surface by protease the remaining pSSU was salt-resistant and about 40% pSSU was not extractable from the membrane by NaOH. In the light of this tight interaction of a large portion of pSSU we tried to isolate a translocation complex contained in the outer envelope by mild solubilization of the membrane. Radiolabelled pSSU was used as a marker for such a complex. The pSSU–membrane complex described should be at least part of the chloroplast translocation apparatus. It was only isolated in experiments which contained all components of the assay system in a functionally active manner. When we used an import-incompetent precursor (see above) or protease-treated envelopes, radiolabel was not present in the complex or was greatly diminished. In this study we have not yet tried to assign a certain function to the members of the complex,

with the exception of hsc 70 (see above). However, we have identified a second protein of the complex, OEP 86. This outer envelope protein is thermolysin-sensitive. A proteolytic fragment was detectable in the complex and in protease-treated membranes only a small proportion of OEP 86 was not degraded as determined by immuno-logical methods. This portion corresponds well with the remaining binding capacity of protease-treated mem-branes. We must, however, emphasize that until now direct evidence is lacking that OEP 86 is acting as a receptor for pSSU or is of similar function. The antibodies used in this study recognized the native protein only very poorly. Further experiments are needed to demonstrate directly which of the polypeptides we detected on the silver-stained SDS–PAGE (Figure 4c) are part of the import apparatus localized in the outer envelope.

A number of proteins have been suggested to be part of the import machinery, namely, polypeptides between 50 and 55 kDa and between 62 and 67 kDa (Cornwall and Keegstra, 1987; Hinz and Flügge, 1988; Kaderbhai *et al.*, 1988). Proteins of similar molecular size are present in the isolated complex which led us to develop a working model of the import complex (Figure 7). Recently, a major 30 kDa polypeptide of the envelope membranes was postulated to be the import receptor (Pain *et al.*, 1988). More recently, the cDNA of this putative receptor protein was sequenced (Schnell *et al.*, 1990) and found to be identical to the cDNA of the triose phosphate-3-phosphoglycerate phosphate translocator of the inner chloroplast envelope (Flügge *et al.*, 1989). Analysis of the isolated complex by Western blotting using an antibody against the phosphate trans-locator indicated the absence of this inner envelope protein from our preparations (data not shown). Furthermore, outer envelope fractions devoid of phosphate translocator protein bind pSSU, while inner envelope membrane frac-tions, where the phosphate translocator is the major protein, do not bind pSSU to a significant extent (Flügge and Soll, manuscript in preparation; Figure 1c). These data suggest that the phosphate translocator and the receptor for chloroplastic precursor proteins are not identical poly-peptides.

Experimental procedures

Isolation of chloroplasts and separation of envelope membranes

Chloroplasts were isolated from pea leaves (*Pisum sativum* L., cv. Golf) and purified by centrifugation through Percoll (Pharmacia, Uppsala, Sweden) gradients as described by Schindler *et al.* (1987). Isolation of outer and inner chloroplast envelope mem-branes was started from purified organelles equivalent to 200 mg of chlorophyll. Preparation and separation of envelope membranes was achieved by methods described in Keegstra and Youssif (1986). In brief, purified organelles were resuspended in hyper-tonic (0.65 M) sucrose solution and the suspension homogenized by 30 strokes in a Dounce tissue grinder. The sucrose concentra-tion in the suspension was lowered to 0.3 M sucrose by dilution with 10 mM Tricine–KOH buffer pH 7.9. Thylakoids were removed from this mixture by low speed centrifugation (4250 g for 10 min) and a crude envelope pellet was then obtained by high-speed centrifugation (200 000 g for 1 h). The crude envelope preparation was separated into inner and outer membranes by centrifugation on a discontinuous sucrose gradient (120 000 g for 5 h) employing concentrations of 1, 0.8 and 0.46 M sucrose in Tricine–KOH pH 7.9. Outer and inner membranes were recovered separately from the gradient and concentrated by centrifugation. Several mem-brane preparations were pooled and further purified on linear sucrose gradients (0.6–1.2 M sucrose buffered with 10 mM Tricine–KOH pH 7.9), as described by Keegstra and Youssif (1986), from which the highly purified membrane populations were recovered and stored in aliquots at −80°C.

Import into purified pea chloroplasts

Throughout this study the radiolabelled precursor protein (pSSU) of SSU was used. It was synthesized by *in-vitro* transcription–translation as described by Salomon *et al.* (1990). The clone containing pSSU-cDNA is described by Lubben and Keegstra (1986). Import assays into chloroplasts were performed for 10 min in the dark under dim safe light in a medium containing 2% bovine serum albumin, 10 mM methionine, 20 mM potassium gluconate, 10 mM NaHCO$_3$, 3 mM MgCl$_2$, 330 mM sorbitol, 50 mM Hepes-KOH (pH 7.6), and intact organelles equivalent to 15 µg of chloro-phyll. Chloroplasts were recovered from the import mixture by centrifugation through a 40% Percoll cushion as described by Schindler *et al.* (1987) and washed once in 50 mM Hepes-KOH (pH 7.6), 330 mM sorbitol and 3 mM MgCl$_2$. Reaction products were analysed by SDS–PAGE (Laemmli, 1970), followed by fluorography (Bonner and Laskey, 1974) using an Agfa MR 800 intensifying screen and Kodak X-Omat AR X-ray film.

Translocation of pSSU into isolated envelope membranes

Purified envelope membranes equivalent to 10 µg protein (Lowry *et al.*, 1951) were washed once in 25 mM Hepes-KOH (pH 7.6), 5 mM MgCl$_2$ (buffer A). Binding and membrane insertion was assayed in 25 mM Hepes-KOH (pH 7.6), 3 mM MgCl$_2$, 1% bovine serum albumin, 0.1 mM ATP (buffer B) and radiolabelled pSSU for 10 min at 25°C in a final volume of 25 µl. Envelope membranes were recovered by centrifugation (250 000 g for 20 min) through a 500 µl 0.2 M sucrose cushion in buffer A and finally washed once in buffer A. Envelope membrane vesicles were equally stable at 25°C and 4°C during the incubation period. SDS–PAGE and fluorography were performed as above. Protease treatment of envelope membrane vesicles either before or after translocation assays was carried out using thermolysin at a final concentration of 5 µg protease per 100 µg envelope protein for 2 min on ice in 25 mM Hepes-KOH (pH 7.6), 2 mM MgCl$_2$ and 0.25 mM CaCl$_2$ (buffer C). Proteolysis was stopped by the addition of excess (10 mM) EDTA. Membranes were recovered by centrifugation and washed once in buffer A supplemented with 0.1 mM EDTA. In some instances membranes were solubilized in 0.5% Triton X-100 and 0.5% SDS prior to the protease treatment.

Extraction of outer envelope membranes with 0.1 M NaOH or NaCl was done for 5 min on ice at 10 µg protein in a volume of 50 µl. The mixture was separated into pellet and supernatant by centrifugation, the latter was then precipitated by 20% trichlor-acetic acid prior to SDS–PAGE.

Apyrase treatment

pSSU mRNA was translated in a reticulocyte lysate system. The translation mixture was treated with apyrase (Sigma) to remove ATP. For chloroplast import, the translation mixture was incubated with 10 U ml^{-1} for 15 min at 25°C and for pSSU–envelope interaction the translation mixture was treated with 2 U ml^{-1} for 15 min at 25°C.

Solubilization of outer envelope membranes

Outer envelope membranes (100–125 μg protein) were incubated with radiolabelled pSSU in 100 μl buffer B as described above. Unbound pSSU was removed by centrifugation through a sucrose cushion. The membrane pellet was partially solubilized by 0.5% (w/v) digitonin in 25 mM Mops-KOH (ph 7.2) and 1 mM EDTA (buffer D) for 10 min at room temperature (Kiebler *et al.*, 1990). The total fraction was layered on top of a continuous 5–20% (w/v) sucrose gradient and centrifuged for 4 h at 330 000 *g* in a 5 ml swinging-bucket rotor. The sucrose gradient contained 25 mM Mops-KOH (ph 7.2), 1 mM EDTA, 0.2% digitonin and 0.05% Triton X-100. The gradients were manually fractionated from the top into 25 aliquots of 200 μl. Radioactivity in each fraction was determined by liquid scintillation counting. SDS–PAGE and fluorography of TCA-precipitated fractions was as above. For immunoprecipitation, the samples in question were incubated with protein-A–Sepharose carrying the respective immunoglobulins. The immunoprecipitates were washed three times in 25 mM Mops (pH 7.2), 75 mM NaCl, 1 mM EDTA and 0.5% digitonin (Kiebler *et al.*, 1990) prior to SDS–PAGE and fluorography.

Miscellaneous

Chlorophyll was determined by the method of Arnon (1949). X-ray films were quantified by densitometry using a laser scanner (LKB-Ultroscan XL, Beckenham, UK). Silver staining of SDS gels was as described by Ansorge (1982). Western-blotting was as in Towbin *et al.* (1979). IgGs were purified as described by Harlow and Lane (1988).

Acknowledgements

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