

SHORT COMMUNICATION

A functionally active protein import complex from

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Isolated outer chloroplast envelope membranes were solubilized by digitonin and separated on linear sucrose density gradients. A membrane complex was recovered from the gradients and exhibited characteristics of a protein import apparatus, i.e. the interaction of the complex with the precursor polypeptides depends on the presence of a transit sequence, ATP and protease-sensitive components. Furthermore, translocation intermediates detected in the organellar system are also found after interaction of the precursor polypeptide with the isolated import complex.

Introduction

A great number of chloroplast proteins are of cytosolic origin and are imported into the organelle in a post-translational event (Keegstra *et al.*, 1989). A challenging question is how precursor proteins are recognized and translocated through the outer and inner chloroplast envelope membranes. The vast majority of precursor proteins seem to follow a common route of translocation, requiring a transit peptide, receptor, and ATP. However, little is known about the membrane components which mediate single steps in the process or their supramolecular arrangement (Cornwall and Keegstra, 1987; Hinz and Flügge, 1988; Kaderbhai *et al.*, 1988). Recent studies have demonstrated that it is possible to isolate a membrane complex from either mitochondria (Kiebler *et al.*, 1990) or chloroplasts (Waagemann and Soll, 1991) which seems to be involved in protein import. The functional capacity of these membrane complexes was, however, not demonstrated. Here, we report the isolation of a membrane complex from purified outer chloroplast envelopes which is active in recognition and insertion of precursor polypeptides.

Outer envelope membranes from pea chloroplasts were mildly solubilized by digitonin after incubation with either the precursor form of the small sub-unit of Rubisco (pSSU) or the mature form (SSU) (EC 4.1.1.39). Analysis of the detergent-treated membranes by sucrose density centrifugation revealed the presence of free pSSU and SSU at the top of the gradient while only pSSU was able to associate with outer envelope membrane components and thus migrated to a higher density in the sucrose gradient (Figure 1). The requirement of a transit sequence for the specific interaction of the outer envelope membrane with a polypeptide destined for the inside of the organelle is clearly demonstrated and extends data obtained earlier (Waagemann and Soll, 1991).

To assess the functional capacity of this putative import complex, we solubilized outer envelope membranes with 0.5% digitonin followed by sucrose density centrifugation. Fractions 3–7 from the top of the gradient and 14–19 (0.45 M sucrose), respectively, were pooled and subsequently incubated with ³⁵S-labelled pSSU in the presence of Mg-ATP. Partially solubilized and unsolubilized membrane fragments could be recovered from the pellet fraction. The assays were loaded on a second 5–20% (w/v) sucrose density gradient and spun at 250 000 g for

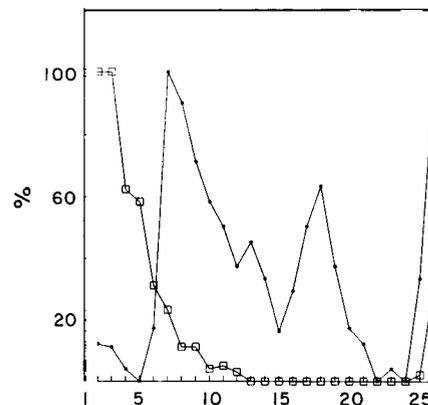


Figure 1. Isolated outer envelope membranes interact with pSSU but not SSU.

Purified outer envelope membranes from pea chloroplasts were incubated with ³⁵S-labelled pSSU (■) or SSU (□), respectively (Waagemann and Soll, 1991). Envelope membranes were recovered from the incubation mixture by centrifugation through a sucrose cushion and washed once. Membranes were solubilized and fractionated by a sucrose density gradient (Waagemann and Soll, 1991). Radioactivity was determined by liquid scintillation counting.

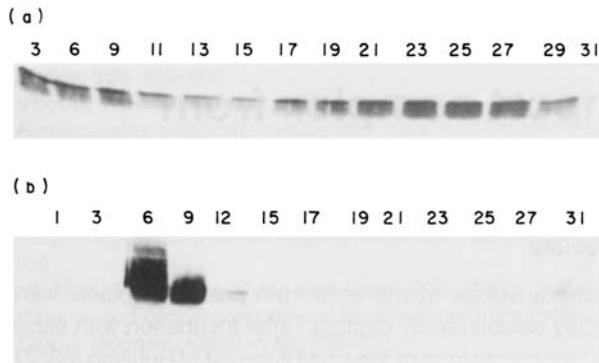


Figure 2. The translocation complex is present only in fractions 14–19 but not in fractions 3–7 of a typical sucrose density gradient as described in Figure 1.

Envelope membranes were solubilized and separated by sucrose density gradient centrifugation. The gradient was fractionated into 25 aliquots. Fraction 3–7 (b) and 14–19 (a) were combined, diluted twofold and incubated with ³⁵S-labelled pSSU. The mixture was layered on top of a second 5–20% (w/v) sucrose density gradient and centrifuged overnight. The gradient was fractionated from the top and proteins analysed by SDS-PAGE, silver staining and fluorography. A fluorogram is shown, numbers on top indicate fraction numbers of the second gradients.

14 h. Only the constituents of fractions 14–19 of the first gradient showed the ability to interact with pSSU and shift its mobility to higher density (Figure 2). pSSU did not interact with proteins present in fractions 3–7, and consequently the precursor remained in the upper part of the gradient (Figure 2), as did pSSU translation product which was loaded on a mock sucrose density gradient containing buffer instead of solubilized envelope components (data not shown).

Post-translational protein uptake into mitochondria and chloroplasts share a number of characteristics common to both systems (Hartl *et al.*, 1989; Keegstra *et al.*, 1989), i.e. a target or transit sequence, proteinaceous receptors exposed on the organellar surface, and hydrolysis of ATP for translocation. The isolated import apparatus from outer envelope membranes exhibits the same characteristics as the organellar system *in vitro*. Interaction between the putative import complex was detectable only if the precursor form pSSU, but not the mature protein SSU, was used (Figure 3a). These data indicated the presence of a transit sequence-dependent component: the receptor in the complex. Mild protease treatment of outer envelope membrane vesicles prior to digitonin treatment and isolation of the complex from the first sucrose density gradient resulted in a pronounced loss of interaction capacity of the complex with pSSU (Figure 3b). Thermolysin-treated complexes showed only a residual binding capacity of 30%, indicating that the untreated complex could indeed contain a receptor for pSSU. Polypeptides which are proteolytically degraded by thermolysin include OEP 86 and OEP 34, both of which are present in the isolated complex one and complex two (Waegemann and Soll, 1991; and data not shown).

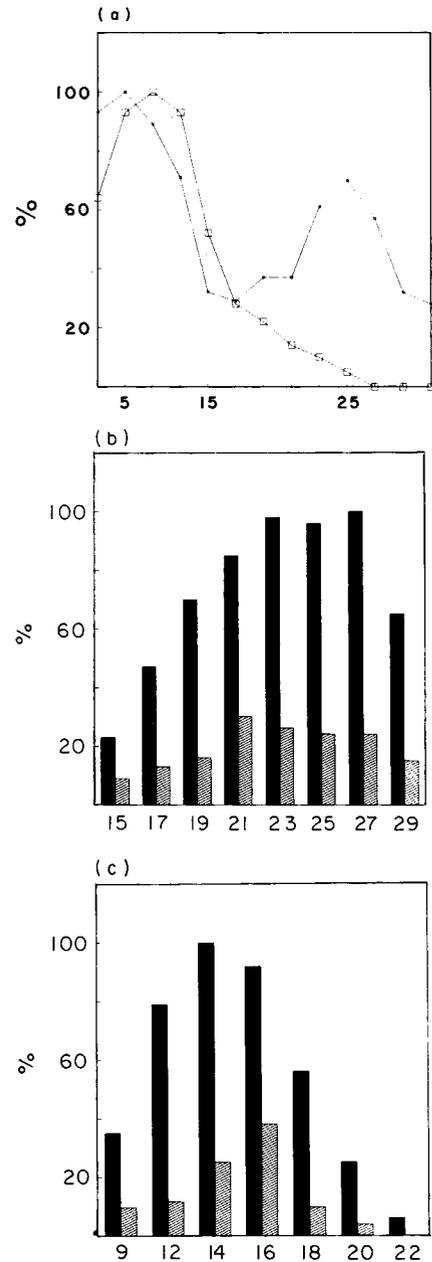


Figure 3. Requirements for an isolated complex from outer chloroplast envelope to interact with pSSU.

(a) Interaction of the isolated complex depends on the presence of a transit sequence (■ pSSU; □ SSU). Results were quantified by laser densitometry of the exposed X-ray film.

(b) Outer envelope membranes were treated (▨) or not treated (■) with 1 μg thermolysin per 10 μg envelope protein. Membranes were recovered, washed, solubilized and incubated with pSSU as above. Results were quantified as above.

(c) pSSU translation product was treated (▨) or not treated (■) with apyrase. The centrifugation time was 4 h instead of overnight.

Protein translocation into chloroplasts requires the hydrolysis of ATP (Flügge and Hinz, 1986; Schindler *et al.*, 1987). To investigate the ATP requirement of pSSU/import complex interaction, the pSSU translation product was depleted of ATP by the action of apyrase (Hwang and

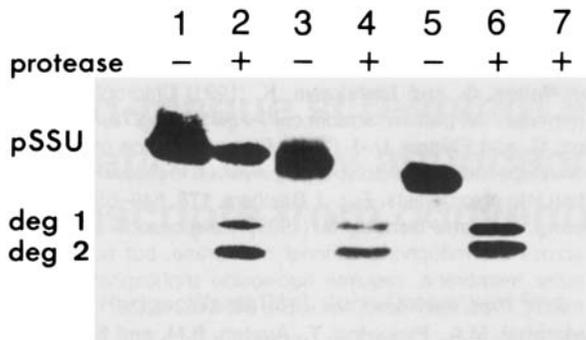


Figure 4. pSSU forms a protease-protected complex with envelope polypeptides.

pSSU bound to outer envelope membranes (lanes 1, 2), complex one (lanes 3, 4) or complex two (lanes 5, 6, 7) was treated in the absence (lanes 1, 2, 3, 4) or presence of thermolysin (lanes 2, 4, 6). Lane 7, as for 6 but with addition of 0.5% Triton X-100 and 0.05% SDS. Samples were analysed by SDS-PAGE and fluorography.

Schatz, 1989). The removal of ATP resulted in a concomitant loss of pSSU bound to the complex (Figure 3c). Inclusion of a non-hydrolysable ATP analogue, adenyli-mido-diphosphate (2 mM), in an ATP-depleted pSSU sample did not lower the amount of pSSU in the complex significantly (data not shown).

In a recent study (Waegemann and Soll, 1991) translocation intermediates of pSSU were found using either intact chloroplasts or envelope membranes (see also Figure 4, lanes 1 and 2). Identical protease-protected translocation intermediates pSSU deg 1 and deg 2 could be detected (i) if envelope membranes were loaded with pSSU, solubilized by digitonin, separated by sucrose density gradient centrifugation and treated with protease (Figure 4, lanes 3 and 4), or (ii) if the isolated complex was incubated with pSSU separated on the second sucrose density gradient and treated with protease (Figure 4, lanes 5 and 6). Protease treatment in the presence of 0.5% Triton X-100 and 0.05% SDS resulted in the complete digestion of the protease-protected translocation intermediates pSSU deg 1 and deg 2 (Figure 4, lane 7, and data not shown). Furthermore, neither the precursor polypeptide itself nor pSSU obtained from the top of a sucrose gradient (Figure 2a, e.g. fraction 6) are protease resistant *per se* (data not shown). The results clearly indicate that the protease-protected fragments obtained by thermolysin treatment of the different membrane fractions are true translocation intermediates, providing further evidence that the isolated membrane fraction represents a large part of the translocation machinery of chloroplasts. The protease-protected peptides deg 1 and deg 2 are probably no longer interacting with components at the surface of the complex but it could be that they are trapped in a proteinaceous pore or channel which is formed by the constituents of the import apparatus.

The polypeptide pattern of complex two as analysed by

SDS-PAGE and silver staining is not altered significantly by the second sucrose density gradient in comparison to complex one (data not shown). Hsc 70 is present in the outer envelope and also in the first and second complex, as determined by immunoblot analysis (Marshall *et al.*, 1990; Waegemann and Soll, 1991; data not shown).

Discussion and conclusions

The data presented in this communication demonstrate for the first time that it is possible to isolate a protein translocation apparatus as a functionally active unit from organelles such as chloroplasts, mitochondria or peroxisomes. The isolated membrane complex exhibits identical biochemical characteristics to the organellar system, demonstrating that it can be used as a bona fide system to study early events in the import pathway. The import apparatus is made up of a limited number of polypeptides, two of which, OEP 86 and OEP 34, are sensitive to protease, a feature common to a putative receptor. Chloroplastic transit sequences have been postulated to form a random coil and would therefore require guidance by molecular chaperones, e.g. hsc 70, during their passage through the chloroplast envelope membranes (von Heijne and Nishikawa, 1991). Hsc 70, a prominent component in the enriched import apparatus fraction, could act as such a chaperone and coat a proteinaceous pore formed by other constituents. The import pathway of precursor proteins into chloroplasts would therefore be distinct from that of mitochondria in a second important characteristic, which, in contrast to that in chloroplasts, requires a membrane potential (Hartl *et al.*, 1989; Hinz and Flügge, 1986; Keegstra *et al.*, 1989; Schindler *et al.*, 1987).

Experimental procedures

Solubilization of outer envelope membranes

Outer envelope membranes were isolated from pea chloroplasts as previously described (Keegstra and Youssif, 1986; Waegemann and Soll, 1991). Envelope membranes equivalent to 100 µg protein were mildly solubilized in 25 mM Mops-KOH (pH 7.2), 1 mM EDTA and 0.5% (w/v) digitonin (Kiebler *et al.*, 1990) for 10 min at room temperature. The suspension was layered on top of a continuous 5–20% (w/v) sucrose density gradient and centrifuged for 4 h at 330 000 *g* in a swinging bucket rotor (Waegemann and Soll, 1991). The gradients were fractionated from the top into 200 µl aliquots. In general, fractions 14–19 were combined and diluted at least twofold with 25 mM Mops-KOH (pH 7.2), 5 mM MgCl₂ and, if not otherwise indicated, 500 µM ATP. Labelled precursor protein (10 µl) was added and incubated with the gradient fractions for 10 min at room temperature. The mixture was layered on a second 9 ml continuous 5–20% (w/v) sucrose density gradient (final volume 12 ml) and spun overnight at 250 000 *g* in a swinging bucket rotor. Gradients were fractionated from the top into 400 µl aliquots. Protein was precipitated by 10% trichloroacetic acid.

Polypeptides were analysed by SDS-PAGE (Laemmli, 1970), silver staining (Ansorge, 1982) and fluorography (Bonner and Laskey, 1974).

Incubation of envelope membranes with pSSU

Envelope membranes were incubated with pSSU as described by Waegemann and Soll (1991). Membranes were separated from the incubation mixture by centrifugation through a sucrose cushion and washed once prior to thermolysin treatment or solubilization. Protease treatment of envelope membranes either before or after incubation with precursor protein was carried out at 1 µg protease per 10 µg envelope protein at 25°C for 90 sec in the presence of 0.5 mM CaCl₂ and 5 mM MgCl₂. The reaction was stopped by the addition of excess EDTA. Membranes were repurified as above.

pSSU containing complex one and complex two were treated with thermolysin at 5 µg ml⁻¹ for 90 sec at 25°C. The reaction was stopped and analysed further as described above.

Acknowledgements

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