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Membrane Biogenesis and Protein Targeting

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Contents

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CHAPTER 24

Comparison of two different protein translocation mechanisms into chloroplasts

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1. Introduction

Chloroplasts are highly structured plant specific organelles. They possess three discrete membrane systems which differ in composition and function, i.e. the outer/inner envelope and the thylakoid membranes. In addition three solute spaces can be distinguished, i.e. the space between the envelope membranes, the stroma and the thylakoid lumen [1]. While most of the chloroplastic proteins, which are synthesized as precursors in the cytosol, seem to follow a common route of translocation into the organelle, proteins of the outer envelope, which is in direct contact with the cytosol, are inserted (imported) by a very different and distinct mechanism [2,3].

A typical polypeptide destined for the inside of the organelle, possesses a cleavable target sequence, retains a loosely folded conformation with the help of molecular chaperones, is recognized by proteinaceous receptors on the organellar surface, requires low concentration (μM) ATP for binding but high concentrations (mM) for complete translocation through the membranes [4,5]. Outer envelope polypeptides (OEP) studied so far, do not possess a cleavable target sequence, do not require protease sensitive receptors on the organellar surface and do not require ATP for either binding or insertion into the outer envelope [3,6].

2. Results and discussion

2.1. Import characteristics of pSSU and OEP 7

A typical import experiment for a plastidic precursor protein destined for the inside

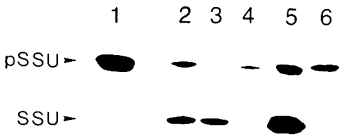

Fig. 1. Characteristics of pSSU import into chloroplasts. pSSU translation product (lane 1) is imported into chloroplasts and processed to its mature form (lane 2). SSU appears protease protected inside the organelle after thermolysin treatment (lane 3). Chloroplasts pretreated with thermolysin bind pSSU only to a very small extent in a non-productive way (lane 4). The import assay was either not depleted (lane 5) or depleted of ATP (lane 6) through the action of apyrase. Methods are described by Waegemann and Soll [15].

of the organelle is shown in Fig. 1. The precursor protein (pSSU) binds to the chloroplast outer envelope, it is subsequently translocated inside the organelle, processed to its mature form and protected against externally added protease. Chloroplasts pretreated by thermolysin, a protease which only digests surface exposed polypeptides [7,8], bind pSSU only to a very small extent in a non-productive manner [2]. Similar results are obtained if ATP is removed from the import incubation mixture by the ATP hydrolyzing enzyme apyrase. Binding is greatly reduced and import not observed, demonstrating the ATP dependence of binding as well as translocation [5].

The insertion (import) of OEP 7 seems to follow a quite distinct pathway. No shift in molecular weight can be observed between the translation product and the inserted form, demonstrating the absence of a cleavable transit sequence [3]. Translocation experiments carried out in the light, i.e. in the presence of ATP, show no greater OEP 7 translocation efficiency than those carried out in the dark, i.e. in the absence of ATP (Fig. 2). Neither apyrase treatment nor the simultaneous inclusion of a non-hydrolysable ATP analog, adenylylimidodiphosphate, influenced the yield of OEP 7

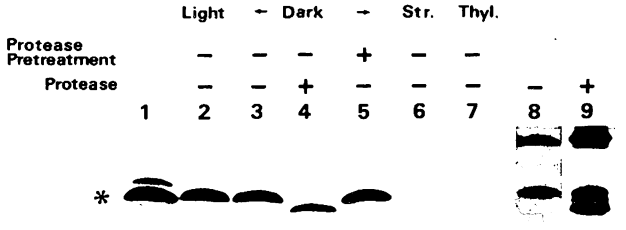

Fig. 2. Characteristics of OEP 7 import (insertion) into chloroplasts. OEP 7 translation product (lane 1) was incubated with intact chloroplasts either in the light (lane 2), i.e. presence of ATP, or in the dark (lane 3), i.e. absence of ATP (translation product was apyrase treated). Protease treatment after import yields a protease protected breakdown product (lane 4). Pretreatment of intact chloroplasts by thermolysin does not influence the efficiency of OEP 7 import (lane 5). OEP 7 is localized in the envelope membranes (lanes 6, 7). Lanes 8 and 9 show a silver stained gel of envelope membranes either not treated (lane 8) or treated (lane 9) with thermolysin. Protease treatment of imported OEP 7 and OEP 7 in situ gives identical proteolytic breakdown products (compare lanes 4 and 9). Methods are described by Salomon et al. [3].

insertion into the outer envelope membrane of chloroplasts. Translocation of OEP 7 followed by thermolysin treatment resulted in a lower molecular weight breakdown product identical to that found if envelope membranes were treated with protease (Fig. 2). OEP 7 contains only the N-terminal methionine, which is not removed by either maturation or thermolysin treatment. This clearly indicates that the N-terminus is exposed to the intermembrane space and therefore protease protected, while the C-terminus is on the cytosolic leaflet of the membrane and susceptible to external protease. Analysis of the amino acid sequence of OEP 7 corroborates these results and predicts only one membrane span [3]. The outline of this translocation route is supported by findings described in [6] where an identical insertion mechanism is described for an outer envelope protein of pea chloroplasts.

2.2. Specificity and mechanism of OEP 7 insertion

The import route outlined above for OEP 7 is very distinct and also differs from that described for proteins localized in the outer mitochondrial membrane, which require protease sensitive receptors and ATP for correct routing and efficient translocation [9]. Experiments were carried out to address the problems of specificity and mechanism of OEP 7 import. In an initial experiment, chloroplasts and mitochondria, both isolated from pea leaves, were incubated in the same import assay with OEP 7 translation product. After completion of the import reaction, chloroplasts were separated from mitochondria by differential centrifugation and each organelle type analyzed, respectively. The results (Fig. 3) demonstrate that OEP 7 binds to the surface of chloroplasts as well as mitochondria. Treatment of the organelles with thermolysin, however, clearly demonstrates that OEP 7 integrates only into the outer envelope of chloroplasts in the proper way but not into the outer membrane of mitochondria as judged from the protease protected breakdown product.

It has been shown that precursor proteins have to retain a loosely folded (transport competent) conformation in order to be translocated through the import apparatus of either mitochondria [9,10] or chloroplasts [4]. They do this with the help of

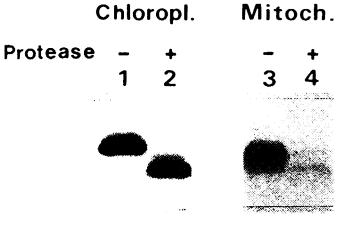

Fig. 3. OEP 7 specifically inserts into chloroplast but not mitochondrial membranes. Chloroplasts and mitochondria, isolated from pea leaves, were incubated in the same incubation assay with OEP 7 translation product. After completion of the reaction, organelles were separated by differential centrifugation and either not treated or treated by thermolysin prior to SDS-PAGE and fluorography.

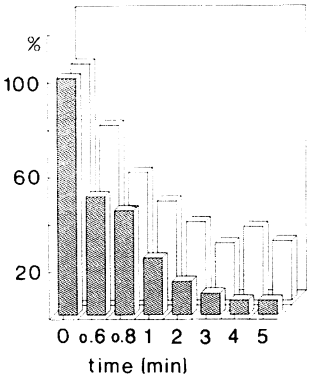

Fig. 4. Efficient insertion of OEP 7 into chloroplasts requires a special conformation. OEP 7 was synthesized either at 26°C (▨) or 37°C (□) in a reticulocyte lysate system. Only OEP 7 synthesized at 26°C inserts efficiently into the outer envelope of chloroplasts (not shown). OEP 7 synthesized at 26 °C (□) is more susceptible to trypsin treatment (units/ml) than OEP 7 synthesized at 37°C(□).

molecular chaperones, e.g. hsc70 [11,12]. We therefore addressed the question whether it was possible to distinguish between import competent and import incompetent OEP 7.

This was indeed found to be the case. OEP 7 synthesized in a reticulocyte lysate system at 37°C under improper cofactor conditions and prolonged reaction time did no longer insert into the outer envelope of chloroplasts (not shown). Trypsin treatment of import incompetent OEP 7 demonstrates that it is much less sensitive to protease than the import competent form of OEP 7 (Fig. 4). The data strongly indicate that OEP 7 like other precursor polypeptides needs to retain a special conformation until it has been inserted into the outer envelope membrane. The outer chloroplast envelope has a unique lipid and protein composition in comparison not only to other organellar membranes exposed to the cytosol but also to the other membrane systems of the chloroplast. Phosphatidylcholine for example is not present in thylakoids. Monogalactosyldiglyceride and digalactosyldiglyceride which are exclusively found in plastidal membrane systems are also major lipid constituents of the outer envelope [1]. The specificity of OEP 7 insertion into the outer envelope could therefore be, at least in part, due to the specific lipid composition.

Most likely the interaction of OEP 7 with other outer envelope proteins aids the insertion specificity. This can be deduced from experiments presented in Fig. 5. Purified chloroplast membranes, i.e. outer envelope, inner envelope and thylakoids, were incubated with OEP 7 translation product. Only the interaction of OEP 7 with outer envelope membrane vesicles, either pretreated with or without protease, resulted in the correct insertion of the protein into the membrane bilayer. OEP 7 also bound to the other chloroplast membranes but we could not detect the typical proteolytic breakdown product, indicating that OEP 7 was either surface exposed and thus protease sensitive or inserted incorrectly into the membranes.

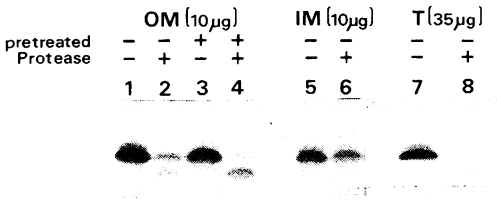

Fig. 5. OEP 7 integrates correctly only into isolated outer chloroplast envelopes. Chloroplasts were separated into outer/inner envelopes and thylakoids prior to incubation with OEP 7. All other manipulations are indicated on the top of each fluorogram. Methods as in Salomon et al. [3].

It has been shown that thylakoid membranes are isolated as outside-out vesicles [13]. The same was found to be the case for isolated outer envelope membranes [14]. These findings are important to interpret the described data correctly. Outside-out envelope vesicles were also used to study their interaction with pSSU, a normal precursor [15]. As in the organellar system, pSSU requires ATP and protease sensitive receptors to bind to the envelope surface (Fig. 6). The interaction between pSSU and the isolated envelope membrane does not halt at the binding stage but pSSU is partly inserted into the translocation apparatus as characterized by protease protected translocation intermediates [15]. Isolated outer envelope membranes therefore contain at least part of the chloroplast import machinery in a functionally active manner. Early events in binding and translocation can thus be analyzed in this isolated and partially purified system in vitro.

Our results indicated that the precursor was not translocated into the inside of the vesicle but was stuck in the transport apparatus. Solubilization of precursor loaded outer envelope membrane vesicles followed by sucrose density centrifugation resulted in the isolation of a membrane fraction with precursor protein still bound to it (Fig. 7)

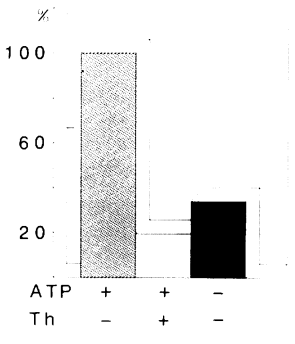

Fig. 6. Isolated outer envelope membrane vesicles represent a bonafide system to study pSSU binding. pSSU binding to isolated outer envelope was analyzed (column 1). Interaction is dependent on thermolysin (Th) sensitive receptors (column 2) and the presence of ATP (column 3). Results were quantified by laser densitometry of an exposed X-ray film. Methods as in Waegemann and Soll [15].

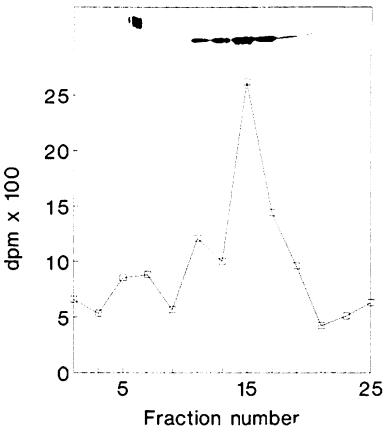

Fig. 7. A membrane complex loaded with pSSU can be isolated from outer envelope membranes. Purified outer envelopes were incubated with pSSU translation product, re-isolated, solubilized by digitonin and subjected to fractionation on a linear sucrose density gradient. pSSU distribution was determined by liquid scintillation counting (graph) or SDS-PAGE and fluorography (insert). Free pSSU stays on top of the gradient while complex bound pSSU migrates to higher density in the sucrose density gradient. Methods as described by Waegemann and Soll [15].

[15]. When the same experiment was carried out using OEP 7 translation product, no radiolabelled protein was detectable in fractions 14–18 of the sucrose gradient (compare Fig. 7) (not shown). This might indicate that OEP 7 does not enter the common import apparatus to be inserted into the outer envelope.

The membrane fraction recovered from the sucrose density gradient was shown to contain all the proteins necessary for a transit sequence and ATP dependent insertion of pSSU into the isolated complex [16]. The interaction of pSSU with the isolated complex also gave rise to the transport intermediates described for the chloroplast system. Isolation of an active import apparatus represents a major advantage to study the function of single components in the translocation event. So far we have identified an outer envelope localized hsc70 homologue and OEP 86 as constituents of the isolated import complex. The hsc70 homologue localized in the import apparatus could act in sequence with its cytosolic and stromal counterparts in an unidirectional import process [15,16].

The polypeptide composition of the isolated import apparatus together with results from crosslink studies imply the involvement, either direct or indirect, of a number of proteins in the translocation event. A schematic view of the different transport pathways into chloroplasts is depicted in Fig. 8.

The major envelope protein which was described as the master receptor for chloroplast protein import [17] and subsequently found to be identical to the phosphate-triose phosphate translocator of the inner envelope [18,19] is neither found in isolated outer envelope membranes which are active in pSSU recognition and insertion nor in the isolated import complex. Together with data presented in [20]

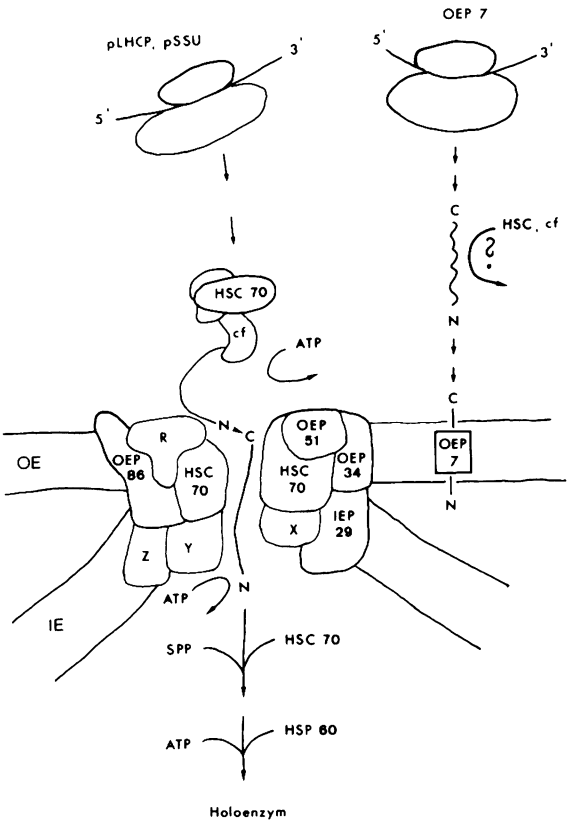

Fig. 8. Schematic view of two import pathways into chloroplasts. The scheme comprises proteins which have been implied to function in the import process in papers mentioned in the text. The number of proteins involved in the import and shown in the scheme is probably underestimated. We propose that the import apparatus forms a proteinaceous pore in the membranes which could be coated by hsc70 homologues to guide the passage of a precursor through the membrane. Other proteins of the complex are most likely also in close contact with the precursor protein on route to the inside of the organelle. This is not represented in the drawing. Much less is known about the insertion pathway of OEP 7. No envelope components have been identified so far which influence the insertion of OEP 7.

we conclude that a receptor for chloroplastic precursor proteins still remains to be identified.

Acknowledgements

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