COMMENTARY

Import of proteins into chloroplasts

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Introduction

Chloroplasts are the sites of photosynthesis in eukaryotic plants and are able to harvest solar energy for the synthesis of carbon skeletons. They, like mitochondria, are enclosed by two membranes, the outer and the inner envelope. The latter is the actual permeability barrier between the cytosol and the chloroplast stroma and the site of different metabolite translocators that coordinate the metabolism between compartments (Heldt and Flügge, 1987). Distinct from the envelope membranes are the energy-transducing thylakoid membranes that are located within the chloroplasts, and which enclose the thylakoid lumen. Stroma and thylakoid proteins each account for about 50% of the total chloroplast protein, whereas the two envelope membranes represent less than 1%. In spite of the chloroplasts' semi-autonomy the majority of the chloroplasts' proteins and also those of other cell compartments are coded for in the nucleus. These nuclear-coded proteins are synthesized in the cytosol, normally with N-terminal extensions called transit peptides, and are subsequently transported into the chloroplasts where they are processed to their mature sizes by specific proteases. Mechanisms must therefore exist to ensure that these proteins are (1) correctly targeted to chloroplasts and (2) subsequently localized into the correct chloroplast compartment: namely, the outer or inner envelope membrane, the intermembrane space, the chloroplast stroma, the thylakoid membrane or the thylakoid lumen. Major aspects of protein transport have been reviewed quite recently (Schmidt and Mishkind, 1986; Lubben et al. 1988; Keegstra et al. 1989). This Commentary will focus on the basic phenomena of this topic and will also present recent findings that extend the view of the reviews cited above.

Protein targeting

It has been suggested that the transit peptides of proteins destined for the different cell compartments contain (most of) the information for correct targeting and sorting. This has been deduced from observations that transit peptides of different nuclear-coded mitochondrial or chloroplastic proteins can direct an attached foreign passenger protein to its respective organelle and even into its correct subcompartment (for reviews, see Hartl et al. 1989; Keegstra et al. 1989). Attempts have been made to localize specific regions within the transit peptide that might be involved in the targeting process. Sequence analyses of a limited number of chloroplast transit peptides suggested that they contain three blocks of rather highly conserved amino acids (Karlin-Neumann and Tobin, 1986); however, a more extensive analysis of a greater number of different transit peptides revealed that they have practically no significant sequence homologies (von Heijne et al. 1989). Even transit peptides of the same stromal or thylakoid protein from different plant species contain only a limited amount of sequence homology and this is apparently also true for envelope membrane proteins (unpublished results). These observations have led to the conclusion that it is likely that distinct structural features are essential for recognition of the precursor by the translocation apparatus. Common structural domains contained in most stromal and thylakoid transit peptides are: (1) an uncharged amino-terminal part; (2) a central non-amphiphilic region; and (3) a carboxy-terminal amphiphilic y-strand in close proximity to the cleavage site (von Heijne et al. 1989). Mitochondrial transit peptides, on the other hand, contain an N-terminal and positively charged a-helix that is thought to interact with the lipid bilayer and to direct the precursor protein, according the membrane potential across the mitochondrial inner membrane. Such amphipathic structures appear to be absent in stromal and thylakoid proteins; however, they can be detected in the N-terminal parts of the two inner-envelope membrane precursor proteins that have been analyzed so far: the phosphate translocator and the 37000 Mr polypeptide (unpublished results). Thus, in contrast to what had been thought previously, these structural elements are not only restricted to mitochondrial transit peptides. The question as to whether these structures are common features shared by all inner envelope membrane proteins should be answered when more information on the sequences of other envelope polypeptides becomes available.

Proteins destined for the thylakoid lumen, such as plastocyanin or proteins of the photosynthetic oxygen-evolving complex, are transported not only across the two envelope membranes but also across the thylakoid membrane. Import of these proteins obviously occurs via the stroma and is linked to two successive processing events (Smeekens et al. 1986; Hageman et al. 1986; Smeekens and Weisbeek, 1988; Kirwin et al. 1989). The first is catalyzed by the soluble stroma processing peptidase yielding an intermediate that is directed into the thylakoid lumen, where it is processed to its mature size by the thylakoid-processing peptidase (TPP) residing in the thylakoid mem-

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brane with the active site facing the lumen. Interestingly, the transit peptides of these precursor proteins contain, in addition to the three structural elements mentioned above that are common to all precursor proteins (the envelope transfer domain), a carboxy-terminal apolar region (thylakoid tranfer domain). The import pathway of luminal proteins resembles that of mitochondrial proteins destined for the intermembrane space and that of protein export across the plasma membrane in prokaryotes ('conservative pathway'). In support of this view is the recent finding that TTP and the bacterial leader peptidase possess identical reaction specificities (Halpin et al. 1989).

Structural analyses of transit peptides as well as deletion studies have provided some information on structure/function relationships; however, it is still unclear how the information contained in the transit peptide (and, possibly, also in the adjacent protein) is precisely used for the correct targeting and sorting of the translocated protein into the different organelle subcompartments.

### Binding of precursor proteins to chloroplasts

Binding of a precursor protein to the outer surface of a membrane is the first step in the overall process of translocation across the membrane. Generally, binding appears to be energy-independent; however, a requirement for small amounts of internal ATP has recently been reported for the binding of different precursor proteins to pea chloroplasts (Olsen et al. 1989). The possibility cannot be excluded that the actual site of ATP utilization during precursor binding is located in the intermembrane space where the ATP-dependent phosphorylation of a putative component of the translocation apparatus also occurs (Hinz and Flugge, 1988; see below). Possibly, this process reflects the apparent ATP requirement for binding.

Binding of precursor proteins to biological membranes, including the chloroplast envelope, is thought to be receptor-mediated. Evidence that proteinaceous components of the outer envelope membrane may function as protein receptors has been obtained from observations that binding of precursor proteins is sensitive to pretreatment of the chloroplasts with the protease thermolysin, which specifically digests outer envelope membrane proteins (Cline et al. 1985; Hinz and Flugge, 1988; Flugge et al. 1989). Attempts have been made to identify such receptor proteins, which are thought to represent only minor components of the envelope; roughly 0.003% of the total chloroplast protein (Friedman and Keegstra, 1989). Cornwell and Keegstra (1981) used a modified precursor protein that was cross-linked to chloroplasts by photoactivation. This procedure led to labelling of an 86K (K = 10^3 M.) conjugate from which the molecular weight of the putative receptor was calculated to be 66K. Pain et al. (1988) identified the major 30K envelope polypeptide as the receptor protein, by using an anti-idiotypic antibody directed against a precursor binding site. Although these observations have apparently been corroborated by Kaderbhai et al. (1988), the identified 30K polypeptide is the component of the inner envelope membrane (Flugge and Heldt, 1984; Flugge et al. 1989). Therefore, the identified '30K import receptor' should represent a membrane component distinct from the phosphate translocator.

We have used another approach for identifying the putative receptor protein (Hinz and Flugge, 1988). By elucidating the possible roles for ATP in energizing protein import into chloroplasts (see below) we have found some evidence that (an ATP-dependent phosphorylation of) a 51K outer envelope membrane protein might be part of the import machinery. Possibly, this protein represents a putative receptor protein or a protein intimately associated with the targeting apparatus. However, direct evidence for the participation of the 51K protein, and also for that of the other identified polypeptides, is still missing.

### Translocation of proteins into the chloroplasts and its energy requirement

Translocation of proteins destined for either the chloroplast stroma or the thylakoid membrane is linked to the transport across two membranes, the outer and the inner envelope membrane. In mitochondria, matrix proteins are also transported across two membranes and it has been proposed that import occurs via contact sites between both membranes, i.e. at distinct regions where the outer and the inner membrane are in close apposition (Schleyer and Neupert, 1985; Pon et al. 1989). This would imply that import is not subdivided into successive transport processes across two distinct membranes but occurs in only one step. Recent experiments by Pain et al. (1988) have led to the suggestion that protein import into chloroplasts might also occur at envelope membrane junctions.

The translocation process of proteins across biological membranes generally requires the input of energy. This may be needed to energize the membrane by either an ion gradient or a membrane potential to drive the movement of the proteins across the membrane. In the case of chloroplasts, an H+ gradient (inside alkaline) is established across the envelope membrane due to the light-driven proton transport into the thylakoids, but the presence of a membrane potential has not yet been demonstrated and is rather unlikely. Also ATP could serve as an energy source, either per se, or by energizing the membrane via an ATP-dependent ion pump. Early investigations showed that ATP is required for protein translocation into chloroplasts (Grossman et al. 1980). A more extensive study of the energy dependency of the import of ATP has indeed the only energy source required for protein translocation into chloroplasts. Also neither a component of an electrochemical gradient, which is required for mitochondrial and bacterial protein transport, nor the only partially characterized envelope ATPase appears to be involved in the energization of protein translocation into chloroplasts (Flugge and Hinz, 1986; Schindler et al. 1987; Pain and Blobel, 1987; Thøger et al. 1989). ATP can promote protein import either when generated inside illuminated chloroplasts by photosynthetic phosphorylation, or when it is added externally, in the dark. This holds for the import of all chloroplastic stromal and thylakoid precursor proteins so far analyzed (Keegstra et al. 1989) as well as for that of two precursor proteins destined for the inner envelope membrane, the phosphate translocator (Flugge et al. 1989) and the 37K polypeptide (unpublished results). However, import of a 6.7K outer envelope membrane protein occurs independently of ATP (and is also receptor-independent) (Salomon et al. 1990) but it remains an open question whether the insertion of other outer envelope membrane proteins also follow this pathway.

It is not yet clear where the actual site of ATP hydrolysis
promoting import into chloroplasts is located. Apparently, translocation of transport-competent precursor proteins is independent of cytosolic ATP (ATP outside the outer envelope membrane). Our experiments suggest that the intermembrane space may play an important role in the energization of protein import (Flügge and Hinz, 1986; Hinz and Flügge, 1988) but other groups have suggested that the stromal space is the actual site of ATP hydrolysis (Pain and Blobel, 1987; Theg et al. 1989). Possibly, energization of protein import into chloroplasts is achieved by a combined action of events in both compartments.

The ATP required for the actual protein translocation process into chloroplasts could be utilized in three main and mutually compatible ways, all of which might occur.

1. ATP could be used as a substrate for an ATP-dependent protein translocase that couples the energy derived from ATP hydrolysis to the movement of the protein across the membrane. Although this is feasible, direct experimental evidence for such an ATP-driven protein translocase has not yet been obtained in chloroplasts, but is likely to occur in bacteria (Lill et al. 1989). However, the precise mechanism that links ATP hydrolysis and protein translocation is still unknown.

2. ATP could be utilized for the phosphorylation of a component of the translocation apparatus. The conformational or topological changes associated with phosphorylation could trigger the movement of the protein across the membrane. It is not yet known whether this happens in other membrane systems, but experiments from our laboratory suggest that the phosphorylation of a 51K outer envelope membrane component may be linked to protein translocation into chloroplasts (Hinz and Flügge, 1988). The ultimate identity of this 51K polypeptide is not established but it might represent a receptor protein or a component intimately associated with the import machinery.

3. ATP could be used as a substrate for cytosolic factors that maintain the precursor protein in an unfolded and transport-competent conformation. This possibility obviously occurs in other membrane systems (Lecker et al. 1989; Chirico et al. 1988; Deshaies et al. 1988; Zimmermann et al. 1988). Proteins involved in this process are in part related to heat-shock proteins and belong to the group of molecular chaperones that are believed to assist generally in correct oligomeric protein assembly (Ellis and Hemmingsen, 1989). Recent observations showed that these ATP-dependent proteins are not only required outside the organelle but also internally. For example, in the matrix space of the mitochondria, heat-shock proteins (hsp60) are required for those proteins that are assembled into supramolecular complexes (Ostermann et al. 1989).

Are cytosolic and stromal (ATP-dependent) factors involved in protein translocation into chloroplasts

As outlined above, the import of proteins into chloroplasts requires the hydrolysis of ATP in the intermembrane space and/or in the stroma. This observation obviously argues against a requirement of cytosolic (ATP-dependent) factors. It has been speculated that if an ATP-dependent component were actually required for keeping the precursor protein in a translocation-competent conformation then it might be located in the envelope membrane, there being energized from the intermembrane space (Hinz and Flügge, 1988). However, it should be noted that under conditions where external ATP is removed by dialysis, or by the presence of ATP-consuming enzymes, only the import of those precursor proteins that are still transport-competent occurs. Import efficiency is significantly lowered by prolonged dialysis of the precursor proteins prior to import or by rigorous removal of intrinsic ATP by treatment of the precursor proteins with the ATP-and ADP-hydrolyzing enzyme apyrase (unpublished results). Direct evidence that a distinct and transport-competent conformation of a chloroplast precursor protein was essential for import was first obtained by della-Cioppa and Kishore (1988), who showed that binding of the inhibitor glyphosate to the 5-enolpyruvylshikimate-3-phosphate synthase–substrate complex prevented its import. Furthermore, Waegemann et al. (1990) demonstrated that soluble (ATP-dependent) factors are required for the import of a thylakoid membrane protein that had been expressed and purified from Escherichia coli transformants. These experimental findings led to the suggestion that (ATP-dependent) cytosolic factors may also be involved in protein translocation into chloroplasts, although evidence for this is only fragmentary.

As mentioned above, it has been recently demonstrated that, in mitochondria, heat-shock proteins (hsp60) residing inside the organelle are involved in ATP-dependent folding and assembly of newly imported proteins (Ostermann et al. 1989). It appears very likely, that soluble stromal factors also participate in the overall process of protein translocation into chloroplasts. Conceivably, these factors may assist in conferring transport-competence to proteins that have already been translocated across the envelope membrane but are further destined to the thylakoid lumen. In addition, these factors may be essential for stabilizing the conformation of those imported proteins that are to be assembled into multimeric protein complexes, e.g. polypeptides of the photosynthetic electron transport and the phosphorylation machinery. Indeed, a soluble stromal factor has been shown to be required for efficient insertion of a component of the photosystem II complex (Fulson and Cline, 1988). Moreover, it has been demonstrated very recently that several imported chloroplast proteins can form stable high-molecular-weight complexes with a chloroplast chaperonin known as Rubisco large subunit binding protein (Ellis and van der Vies, 1988). It is therefore possible that this protein may have the more general function of mediating the folding and/or the assembly of chloroplast proteins upon import (Lubben et al. 1989).

Concluding remarks

Although the detailed picture of protein transport into chloroplasts is still incomplete, it is becoming evident that this import process shows a higher degree of similarity to that in other membrane systems than had been previously thought. A deeper understanding requires, however, the identification and isolation of the distinct components involved in protein translocation and, ultimately, their functional reconstitution. This work has just been started.

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References


