MINI-REVIEW

On the Translocation of Proteins across the Chloroplast Envelope

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Abstract

Most of the chloroplast proteins are coded for in the nucleus and are synthesized in the cytosol from where they are subsequently transported into the different chloroplast compartments. The structural properties of the N-terminal extensions (transit peptides) of these nuclear-coded precursor proteins are discussed as well as the energy requirements for their translocation and the involvement of receptor proteins and that of other (ATP-dependent) factors.

Introduction

Many of the proteins in eukaryotic cells, destined for specific membrane-bound compartments, are coded for by nuclear genes and are synthesized in the cytoplasm as higher-molecular-mass precursor proteins. Since the sites of synthesis and catalytic function of these nuclear-coded proteins are located in different cell compartments, a transport step of these precursor proteins across the various organelle membranes is required in order to reach their final destination, e.g., mitochondria, chloroplasts, peroxisomes, or the endoplasmic reticulum where the precursor proteins are processed to their mature sizes by specific proteases.

In the case of chloroplasts there are six compartments to which the precursor proteins can be translocated, namely, the outer and the inner

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ROUTES OF NUCLEAR ENCODED POLYPEPTIDES TO CHLOROPLASTS

nuclear (genomic) DNA
        |
        ↓
    polyA$^+$mRNA
        | translation on
        | 80 S ribosomes
        ↓
precursor proteins
        |
        ↓
IMPORT INTO CHLOROPLASTS   — ENVELOPE   outer envelope membrane
                                                intermembrane space
                                                inner envelope membrane

                                  — STROMA SPACE

                                  — THYLAKOID   thylakoid membrane
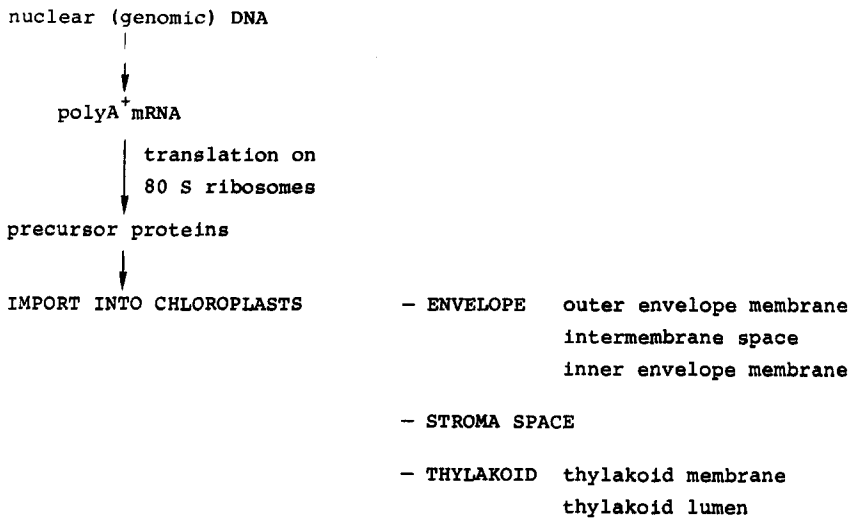   thylakoid lumen

Fig. 1. Routes of nuclear-coded proteins destined for chloroplasts.

envelope membrane, the intermembrane space, the stroma, the thylakoid membrane, and the thylakoid lumen (Fig. 1). For metabolites which are exchanged between the cytosol and the chloroplast stroma, the inner membrane is the only permeability barrier (Heldt and Flügge, 1987). Stromal proteins, however, have to be transported across both envelope membranes, and luminal proteins in addition also across the thylakoid membrane. For aspects on protein translocation into chloroplasts which are not covered in detail here, the reader is referred to recent reviews (e.g., Schmidt and Mishkind, 1986; Lubben et al., 1988; Keegstra et al., 1989).

Structural Properties of Transit Peptides

Chloroplast precursor proteins possess N-terminal extensions named transit peptides which direct the proteins to chloroplasts and contain (most of) the information for a correct sorting. Ample evidence has been accumulated that transit peptides of different nuclear-coded chloroplast proteins are capable of targeting an attached foreign passenger protein to chloroplasts albeit with variable efficiency, indicating that structural features of the mature part of the protein are also relevant (van den Broeck et al., 1985; Schreier et al., 1985; Lubben and Keegstra, 1986; Smeekens et al., 1987;

Lubben *et al.*, 1989). On the primary sequence level the transit peptides of different precursor proteins destined for chloroplasts and also for mitochondria exhibit practically no homology, suggesting that most likely structural features are crucial for recognizing the precursor by the import machinery (see, e.g., Keegstra *et al.*, 1989). Common structural elements of a great number of mitochondrial and chloroplast stroma and thylakoid transit peptides have recently been worked out (von Heijne *et al.*, 1989). According to this study mitochondrial transit peptides contain two structural domains: first an amino-terminal and positively charged amphiphilic α-helix which is supposed to interact with lipids and to direct the precursor protein across the mitochondrial membrane and down the membrane potential gradient; second, a carboxy-terminal domain with different amphiphilic properties. On the other hand chloroplast transit peptides consist of three distinct domains: an uncharged amino-terminal part, a central non-amphiphilic part, and a carboxy-terminal amphiphilic β-strand next to the cleavage site. In addition, most of the chloroplast transit peptides possess a lower arginine/serine ratio as compared with mitochondrial transit peptides.

Proteins destined for the thylakoid lumen (e.g., plastocyanin or proteins of the photosynthetic oxygen-evolving complex) contain in addition a carboxy-terminal apolar region (thylakoid transfer domain). These proteins are first translocated across the envelope into the stroma where they are processed by the stromal peptidase and are subsequently directed across the thylakoid membrane into the lumen whereby a second processing catalyzed by the thylakoid peptidase occurs (Smeekens *et al.*, 1986; Hagemann *et al.*, 1986; Smeekens and Weisbeek, 1988; Kirwin *et al.*, 1989). This process resembles that of mitochondrial proteins destined for the intermembrane space or the outer surface of the inner membrane and that of exporting proteins across the plasma membrane in prokaryotes (see Hartl *et al.*, 1989).

Very little is known about the biogenesis of the chloroplast envelope. Most of the envelope membrane proteins are obviously coded for by nuclear genes (Flügge, 1982). Only recently did the first sequence of an inner envelope membrane protein, the phosphate translocator, became available (Flügge *et al.*, 1989). Analysis of the transit peptide of this precursor protein revealed properties which are quite distinct from those of other chloroplast proteins: (i) It contains three charges within the first ten amino acid residues, (ii) an amphiphilic β-strand is not present in proximity to the cleavage site, indicating that this cannot be a feature common to all chloroplast precursor proteins being recognized by the stromal peptidase; and (iii) it is enriched in arginine (high arginine/serine ratio). Finally, it contains an N-terminal positively charged amphiphilic α-helix with a high hydrophobic moment (amino acid residues 10–27; Fig. 2). Such an amphiphilic α-helix can also be

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              L   A   S
        A      \  |  /      L
     A     \  18 11 22      /   A
        \   25       15    /
        14                26
    P  —  21             19  —  F
    T  — 18    ⌒    12  —  I
        — 17         23  —
      —   24 13   27 16   R
      •  K   / 28  \    R  •
         E   /  |  \    P
        •   A   R   S
              •
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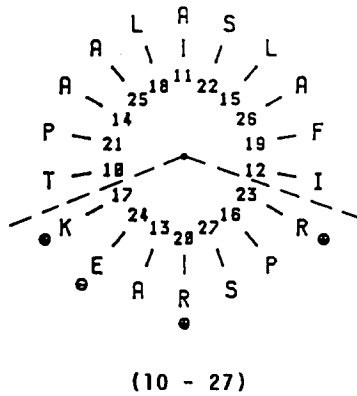
(10 – 27)

Fig. 2. Helical wheel projection of the amnio-terminal region of the phosphate translocator transit peptide (amino acid residues 10–27).

detected in the N-terminal part of the transit sequence of the 37-kD inner envelope membrane protein (unpublished results).

It is not yet known whether the presence of an N-terminal amphipathic structure, which was previously thought to be restricted to mitochondrial precursor proteins, may be also a common feature of chloroplast inner envelope membrane proteins. In any case the characteristics of the transit sequence of the chloroplast phosphate translocator are more mitochondrial than chloroplastic. However, in spite of their similarity to mitochondrial transit peptides, the transit peptide of the phosphate translocator and also that of the 37-kD inner envelope membrane polypeptide are able to direct the proteins to their correct compartment, the inner envelope membrane (see below).

Energy Requirements for Protein Translocation into Chloroplasts

Translocation of precursor proteins across biological membranes requires a suitable energy source. In all systems studied so far, the hydrolysis of ATP has been shown to be essential, e.g., for protein translocation across the bacterial membrane (Chen and Tai, 1985; Geller et al., 1986), the endoplasmic reticulum (Rothblatt and Meyer, 1986; Hansen et al., 1986; Waters and Blobel, 1986; Schlenstedt and Zimmermann, 1986), the mitochondrial membrane (Pfanner et al., 1987), peroxisomes (Imanaka et al., 1987), and also the chloroplast envelope (Grossman et al., 1980; Flügge and Hinz, 1986; Pain and Blobel, 1987; Schindler et al., 1987; Theg et al., 1989).

In the case of chloroplasts, Chua's group was the first to study the energy-dependent translocation of proteins into pea chloroplasts

(Grossman *et al.*, 1980). They observed a light-dependent import of proteins into chloroplasts which was reduced under dark conditions but could be increased by externally added ATP. The authors concluded that protein import was energized by stromal ATP which could be generated either by photosynthetic phosphorylation or, in the dark, by ATP which is transported into the chloroplasts via the chloroplast ATP translocator. In spinach chloroplasts, however, little or no dark inhibition of protein import was observed which can, in retrospect, be explained by the presence of ATP in the *in vitro* translation mix used for the import studies. By using ATP-free translation products which were obtained by ammonium sulfate precipitation and subsequent dialysis of the precursor proteins, we could show that protein import into spinach chloroplasts under dark conditions was absolutely dependent on MgATP (Flügge and Hinz, 1986). In the absence of ATP, virtually no import activity was obtained. In the light, substantial translocation could be observed even in the absence of added ATP and uptake could be increased by adding higher concentrations of ATP (2–5 mM) (Fig. 3). Protein import could not be promoted in the presence of non-hydrolyzable analogues of ATP, indicating that the hydrolysis of ATP was essential for protein translocation (Flügge and Hinz, 1986; Schindler *et al.*, 1987; Pain and Blobel, 1987). In the dark, protein import could also be
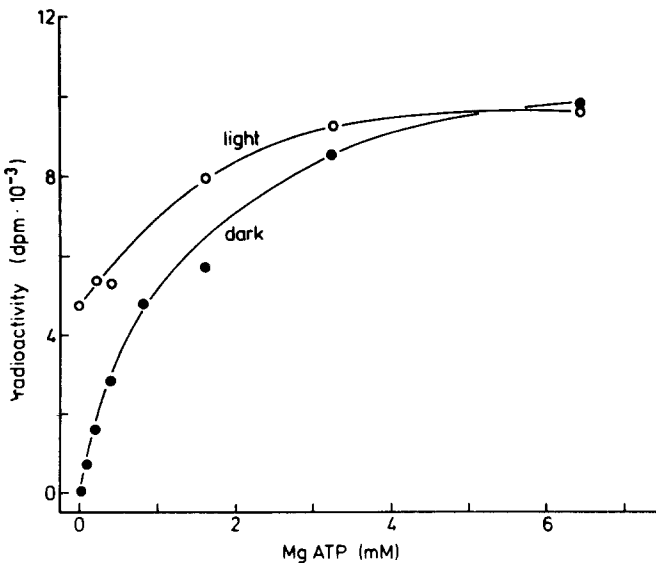

Fig. 3. Import of a chloroplast precursor protein (pSSU) into spinach chloroplasts is dependent on light or, in the dark, on externally added ATP. Import reactions (0.66 mg chl/ml) were allowed to proceed for 10 min at 25°C and were terminated by silicon oil layer filtering centrifugation. Data from Flügge and Hinz (1986).

promoted by providing a system enabling the generation of stromal ATP, e.g., by the addition of triosephosphate/phenazine methosulfate (Flügge and Hinz, 1986) or triosephosphate/oxaloacetate (Schindler et al., 1987; Pain and Blobel, 1987). Thus, protein import into chloroplasts is strictly dependent on ATP either generated in the light or, in the dark, on externally added or indirectly imported ATP. If the stromal ATP level was decreased by the presence of either energy-transfer inhibitors (phlorizin, which does not affect the electrochemical gradient) or by ionophores, protein import in the light was inhibited but this inhibition could be relieved by the addition of ATP which, however, could not restore the transmembrane ΔpH (Grossman et al., 1980; Flügge and Hinz, 1986; Pain and Blobel, 1987; Schindler et al., 1987; Theg et al., 1989). Thus, ATP is the only energy source required for protein translocation into chloroplasts.

The fact that protein import into chloroplasts relies only on ATP and not on a component of the protonmotive force (pmf) ensures that the import process can be sustained during the dark period, when energy from the pmf is not available but ATP levels are high enough to support protein translocation (Flügge and Hinz, 1986). In other systems, e.g., mitochondria and bacteria, ATP and additionally the membrane potential are required for protein translocation (Pfanner and Neupert, 1985; Eilers et al., 1987; Bakker and Randall, 1984), but in contrast to chloroplasts the energization of these systems is not subjected to a diurnal rhythm but is only dependent on energy-rich substrates.

The question where the site of ATP utilization is located can be addressed by experimentally manipulating the ATP levels inside and outside the chloroplasts. ATP external to the outer envelope membrane can be removed by ATP traps (e.g., hexokinase/glucose or fructose-6-phosphate kinase/fructose-6-phosphate) or by apyrase, an ATP- and ADP-hydrolyzing enzyme, whereas the chloroplastic ATP level can be either increased by externally adding triosephosphate/phenazine methosulfate (or oxaloacetate) or the stroma can be depleted of ATP by adding glycerate which is imported into chloroplasts and transformed to 3-phosphoglycerate at the expense of ATP.

In spinach chloroplasts external ATP could only be completely removed by apyrase but not by hexokinase/glucose. This result is presumably due to the presence of an envelope-bound myokinase generating ATP from ADP (Murakami and Strotmann, 1978). Since in the dark, protein import is strictly dependent on externally added ATP (Fig. 3), import into darkened chloroplasts was only partially inhibited by hexokinase/glucose but completely abolished in the presence of apyrase (Fig. 4). In the light, however, external ATP can be totally removed without affecting protein import into chloroplasts. From these results and that of other groups (Grossman et al., 1980;

A   IMPORT OF pLHCP

B   IMPORT OF pRUBP-SSU
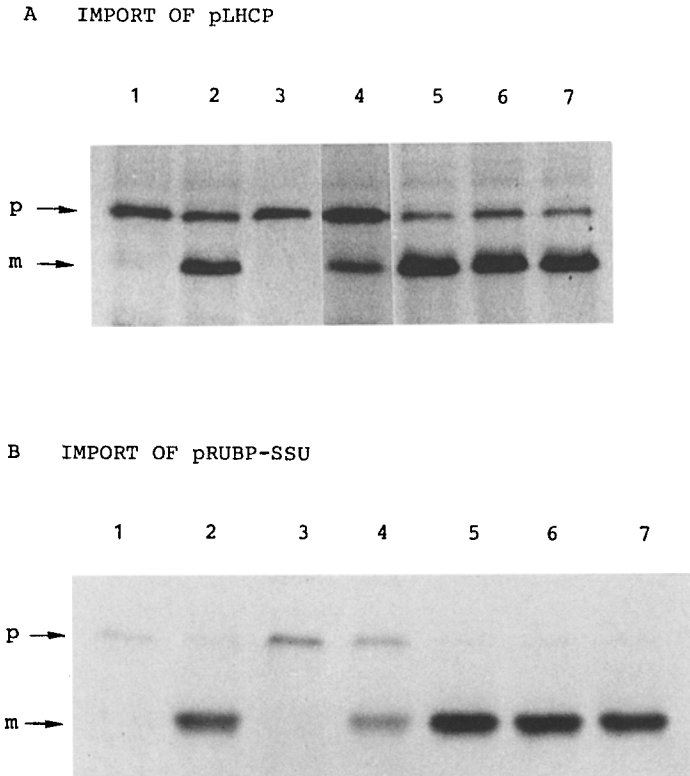
Fig. 4. Effect of the presence of external ATP traps on the import of pLHCP (A) and pSSU (B) into spinach chloroplasts. Lanes 1–4, dark conditions; lanes 5–7, light conditions. Chloroplasts were preincubated for 15 min at 25°C with 2 mM ATP (lanes 2–4) and 2 U apyrase (lanes 3 and 6) or 20 U hexokinase/10 mM glucose (lanes 4 and 7). Lanes 1 and 5, no addition. Import reactions were started by the addition of radiolabelled precursor proteins and terminated by silicone oil layer filtering centrifugation. Fluorograms of dried SDS-polyacrylamide gels are shown.

Pain and Blobel, 1987; Theg *et al.*, 1989), it is tempting to conclude that ATP is required inside the chloroplasts to energize protein import, but actual evidence indicates only that the import of a transport-competent precursor protein is obviously independent of ATP present outside the outer envelope membrane (Flügge and Hinz, 1986; Hinz and Flügge, 1988; Flügge *et al.*, 1989). Therefore either the intermembrane space and/or the stroma may represent the actual site of ATP utilization. The results of Schindler *et al.* (1987) demonstrating that protein import into pea chloroplasts was inhibited in the presence of hexokinase/glucose even in the presence of an internal ATP-generating system are somehow contradictory. But the internal ATP levels were not monitored in these experiments and the possibility that the

chloroplasts were depleted of stomal ATP by hexokinase or/and the presence of ammonium sulfate from the enzyme preparation cannot be excluded.

By manipulation of the ATP levels on both sides of the inner envelope membrane, and by further inhibiting the activity of the inner envelope ATP translocator, we could show that protein import was correlated with low stromal but a high external (i.e., intermembrane) ATP concentration. This indicated that the intermembrane space might play an important role in energizing protein translocation (Flügge and Hinz, 1986). Theg *et al.* (1989) concluded from similar experiments that internal, not external, ATP drives protein import into pea chloroplasts. In addition, they provided kinetic evidence for this conclusion. Import of precursor protein started immediately upon addition of the precursor protein if the chloroplasts were preincubated in the presence of ATP but only after a lag phase when ATP and the precursor protein were added together. This time lag was interpreted as being due to the transport of ATP into the stroma where it is then used to drive protein import. We have obtained the same results with spinach chloroplasts but could not find any evidence for a kinetic limitation of ATP transport exerted by the ATP translocator. These experiments, however, clearly indicate that another ATP- and time-dependent step besides that of actual ATP-dependent energization is involved in protein translocation into chloroplasts.

A requirement for ATP has recently been shown for the insertion of pLHCP into thylakoids and the transport of a luminal protein across the thylakoid membrane. These results were obtained using either isolated thylakoid membranes or a reconstituted system (Fulson and Cline, 1988; Kirwin *et al.*, 1989). This would imply that, in the intact chloroplast system, stromal ATP is needed for efficient membrane insertion and translocation of thylakoid membrane proteins.

Import of Envelope Membrane Proteins

As outlined above, previous work has focussed on the import of stroma and thylakoid proteins. Recently we have determined the first complete amino acid sequence of an inner envelope membrane protein from its *c*DNA sequence (Flügge *et al.*, 1989). This polypeptide represents the chloroplast phosphate translocator which catalyzes the export of fixed carbon out of the chloroplasts in exchange with inorganic phosphate (Flügge and Heldt, 1984). Interestingly, its amino acid sequence does not show any homology to metabolite transport systems from mitochondria or bacteria, suggesting that chloroplast translocators may represent a special class of transport proteins.
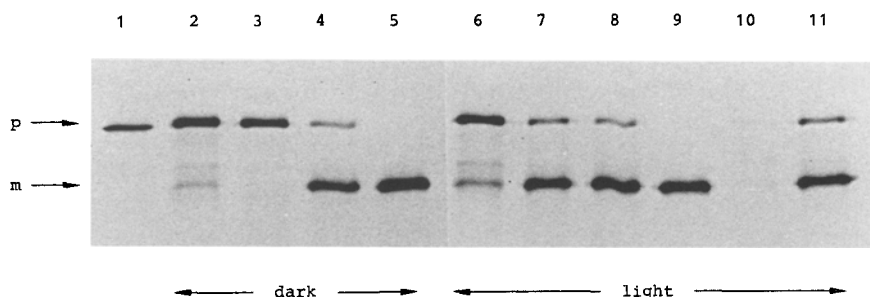
Fig. 5. Energy-dependent insertion of the phosphate translocator protein into envelope membranes. Lane 1, phosphate translocator precursor protein. Lanes 2–5, dark conditions; lanes 6–11, light conditions. Import assays contained the import buffer and, in addition: lane 2, 2 mM adenosine 5′[β, γ-methylene]triphosphate; lane 3, 2 mM ATP and 2 U apyrase, lanes 4 and 5, 2 mM ATP; lanes 6 and 7, 5 μM CCCP, 5 μM valinomycin, and 5 mM ATP (lane 7); lanes 8 and 9, no addition; lane 11, 2 U apyrase. Lane 10: Chloroplasts were treated with thermolysin prior to import. After import, samples 5 and 9 (lanes 5 and 9) were further treated with thermolysin. Envelope membranes were partially purified from the import assays and analyzed by SDS-PAGE. A fluorogram of the dried SDS-polyacrylamide gel is shown. Data from Flügge *et al.* (1989).

The import of the *in vitro* synthesized precursor protein (MW 42.2 kD) into chloroplasts has been studied in some detail (Flügge *et al.*, 1989). The energy requirements for insertion of the phosphate translocator precursor protein resemble those for importing stromal and thylakoid proteins (Fig. 5): import which can be driven by light or, in the dark, by externally added ATP is linked to processing and insertion of the translocator into the inner envelope membrane. This leads to a protease-resistant mature protein. The site of ATP hydrolysis driving the translocation of the transport-competent precursor protein is located behind the outer envelope membrane, i.e., either in the intermembrane space and/or in the stroma. Binding and translocation of the phosphate translocator precursor protein is completely abolished by pretreatment of the chloroplasts with small amounts of the protease thermolysin, which specifically digests outer envelope membrane proteins (Cline *et al.*, 1984). Presumably, proteins of the outer envelope function as receptors and are apparently involved in the import process. The question arises how the detailed import pathway of that inner envelope membrane protein is like. Possibly the phosphate translocator is first translocated across both envelope membranes into the stroma, processed to an intermediate or to its mature size, and subsequently redirected into the inner envelope membrane. However, we could not find any experimental evidence supporting this view. Preliminary results suggest that import does not proceed via the stroma but that processing and integration into the envelope are events occurring in parallel.

By cloning and sequencing the first outer envelope membrane protein, information on the import pathway of such a protein became available
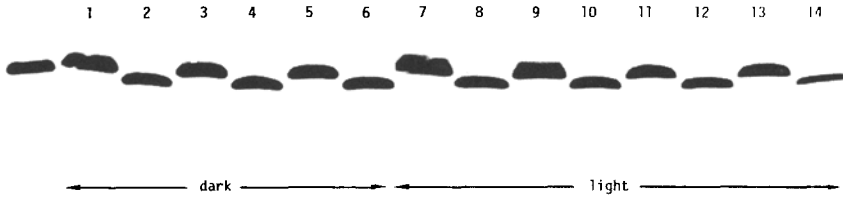
Fig. 6. Insertion of the 6.7-kD outer envelope membrane protein (E 6.7) into chloroplasts. Lanes 1–6, dark conditions; lanes 7–14, light conditions. Import was studied in the presence of 2 U apyrase (lanes 1, 2 and 9, 10), 2 mM ATP (lanes 3, 4), 3 μM CCCP and 3 μM valinomycin (lanes 5, 6 and 11, 12). Lanes 7 and 8, no addition. Lanes 13 and 14: Chloroplasts were treated with thermolysin prior to import. After import chloroplasts were incubated without (lanes 1, 3, 5, 7, 9, 11, and 13) or with thermolysin (lanes 2, 4, 6, 8, 10, 12, and 14). The further experimental conditions were identical to that described in the legend to Fig. 5. The lower-molecular-weight species obtained after protease treatment is a proteolytic breakdown product of unprocessed E 6.7 (Salomon *et al.*, 1990).

(Salomon *et al.*, 1990). This protein represents a major component of the outer envelope membrane and has an apparent molecular weight of 10 kD (Douce *et al.*, 1984). However, its true molecular weight, deduced from the amino acid sequence, is only 6.7 kD. Integration of this protein (E 6.7) into the outer envelope membrane differs from that of other chloroplast proteins in three ways (Fig. 6): first, it does not have a cleavable transit sequence as it is the case for mitochondrial outer membrane proteins (Gasser and Schatz, 1983). Second, its integration into the outer envelope occurs both in the light and in the dark even in the absence of ATP or of an electrochemical gradient, i.e., it is energy independent. Third, digestion of protease-sensitive components which are involved in binding and translocation of other chloroplast proteins (see below) does not affect the effectiveness of binding and integration of E 6.7. Nevertheless, the membrane insertion process exhibits some membrane specificity, indicating that the protein may contain an internal addressing signal and/or that nonproteinaceous components (e.g., lipids) may be involved. Worth noting is that E 6.7 is a rather small protein with a hydrophobic central part. It has yet to be proven whether the requirements described above for its membrane insertion are similar to other outer envelope membrane proteins. This will be possible as soon as other cDNA clones become available.

ATP Utilization during Protein Translocation; Protein Binding and Receptor Proteins

Binding of precursor proteins to the target membrane is the first step in translocating transport-competent proteins into membrane-bound
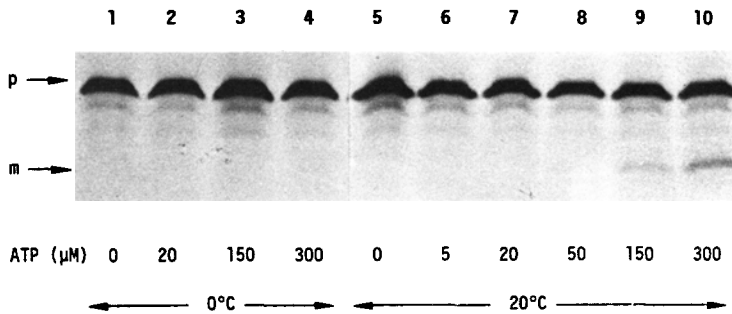
Fig. 7. Effect of ATP on binding and import of pSSU into spinach chloroplasts. Lanes 1–4, 0°C; lanes 5–10, 20°C. Intact spinach chloroplasts (0.2 mg chl/ml) were preincubated with increasing concentrations of ATP as indicated for 10 min at the given temperatures (dark conditions). The import reaction was started by the addition of radiolabelled precursor protein which had been freed of ATP by ammonium sulfate precipitation and subsequent dialysis. The incubations were terminated after 10 min by silicone oil layer filtering centrifugation. Chloroplasts were subsequently analyzed by SDS-PAGE fluorography.

compartments. In the case of other membrane systems (e.g., bacterial plasma membrane, mitochondria) the binding of precursor proteins to the membrane appears to be independent of ATP. However, Olsen *et al.* (1989) demonstrated recently an energy requirement for precursor binding to the envelope membrane of pea chloroplasts. They showed that binding of precursor proteins was stimulated by low concentrations of ATP (50–100 μM) which was utilized either in the stroma or the intermembrane space. An ATP requirement for binding of precursor proteins to chloroplasts cannot however, be generalized, since in spinach chloroplasts an ATP-dependent binding is not observed (Fig. 7). Here, binding of the precursor protein occurs equally well (and almost spontaneously) at 0°C and at higher temperatures even in the complete absence of ATP which had been removed by ammonium sulfate precipitation and subsequent dialysis of the precursor protein. Translocation, however, is temperature dependent and is only initiated at ATP concentrations higher than about 150 μM and at higher temperatures.

For the actual protein translocation process the energy obtained from ATP hydrolysis could be utilized by three main and mutually compatible ways.

1. ATP could be used to maintain the precursor protein in a transport-competent unfolded conformation, a process mediated by ATP-dependent cytosolic factors. This idea was first proposed by Rothman and Kornberg (1986) and is supported by the observation that protein translocation across the membranes of the endoplasmic reticulum, the bacterial plasma membrane, and that of mitochondria requires the presence of soluble ATP-dependent factor(s). In the bacterial system, GroEL, a heat-shock protein,

SecB, and the trigger factor were shown to be involved in stabilizing proteins in conformations competent for translocation (Lecker et al., 1989). In mitochondria this process is presumably mediated by cytosolic heat-shock proteins of the hsp70 family and/or other ATP-dependent cytosolic factors which are also involved in protein transport across the endoplasmic reticulum (Chirico et al., 1988; Deshaies et al., 1988; Zimmermann et al., 1988). In support of this view is the observation that import of precursor proteins into mitochondria is strictly dependent on cytosolic ATP but becomes independent of external ATP (but not of the membrane potential) if the precursor proteins were already denatured by pretreatment with urea (Eilers et al., 1988; Ostermann et al., 1989). Although the ATP requirement in the cytosolic compartment can be bypassed by this treatment, a second ATP-dependent mitochondrial component, the constitutively expressed heat-shock protein hsp60, located in the mitochondrial matrix, was shown to be necessary for folding the imported proteins and for their assembly into oligomeric complexes (Ostermann et al., 1989). Taken together, the above-mentioned proteins are involved in protein translocation across different cell membranes by binding to proteins either prior to or after the translocation step. They belong—together with the Rubisco subunit binding protein (which is closely related to groEL)—to the group of molecular chaperones which are believed to generally promote proper oligomeric protein assembly (Ellis and Hemmingsen, 1989).

The question arises as to whether cytosolic (ATP-dependent) components are also involved in protein translocation into chloroplasts. One difference between protein import into chloroplasts and mitochondria is that ATP is the sole energy source for importing proteins into chloroplasts (see above) and that this ATP is obviously utilized behind the outer envelope membrane. Removal of external ATP does not affect protein import into chloroplasts as long as ATP is provided inside the chloroplasts by photosynthetic phosphorylation (Fig. 4). This obviously argues against the requirement for ATP and/or an ATP-dependent cytosolic factor involved in protein translocation into chloroplasts. We have speculated that if an ATP-dependent component was actually required for keeping the precursor protein in a transport-competent conformation, then it might be located in the envelope membrane. This activity might be energized from the intermembrane space, thus rendering protein translocation independent of a cytosolic and ATP-dependent factor (Hinz and Flügge, 1988; Flügge et al., 1989). However, one has to bear in mind that when chloroplasts are depleted of external ATP (e.g., Fig. 4) only the import of these precursor proteins is observed which are still transport-competent even in the presence of apyrase. Precursor proteins, normally obtained from in vitro translation systems, may contain an ATP-dependent activity that can maintain the synthesized

precursor proteins in a translocation-competent form (Zimmermann *et al.*, 1988). Rigorous removal of ATP by pretreatment of the *in vitro* synthesized precursor proteins with apyrase or by ammonium sulfate precipitation/ dialysis indeed reduces substantially the effectiveness of protein import also into chloroplasts (unpublished observations). This indicates that the transport competence of part of the precursor proteins has been lost by these treatments. Evidence that a distinct and transport-competent conformation of a chloroplast precursor protein is important for import has been obtained by the observation that the import of the 5-enolpyruvyl-shikimate-3-phosphosynthase precursor protein into chloroplasts can be prevented by specific binding of the inhibitor glyphosate to the enzyme–substrate complex (della Cioppa and Kishore, 1988). This finding is similar to that of a pioneering experiment by Eilers and Schatz (1986) showing for the first time that binding of the inhibitor methotrexate to the DHFR moiety, which had been attached by gene fusion to a mito-chondrial signal peptide, led to a folded and transport-incompetent conformation and thus blocked import into mitochondria. Moreover, recent observations by Waegemann *et al.* (1990) revealed that import of pLHCP, which had been isolated from *E. coli* transformants, could only be obtained in the presence of soluble (ATP-dependent) components present in the leaf extract, probably cytosolic factors. Thus the participation of ATP-dependent cytosolic factors involved in unfolding and/or preventing misfolding of precusor proteins cannot be ruled out for protein import into chloroplasts.

In addition, a soluble stromal factor has recently been shown to be required for efficient insertion of pLHCP into the thylakoid membrane (Fulson and Cline, 1988). Possibly its function resembles that of hsp60 required inside the mitochondrial matrix for stabilizing conformation of proteins to be assembled into supramolecular complexes. It is tempting to speculate that the presence of hsp60-like proteins and/or other ATP-dependent stromal factors may be essential for assisting in the folding and assembly of chloroplast-imported proteins into supramolecular chloroplast protein complexes (e.g., Rubisco or complexes involved in photosynthetic electron transport and photophosphorylation).

2. ATP could be utilized by a transmembrane system functioning as a protein translocase which couples the energy derived from ATP hydrolysis to the translocation of the precursor protein across the membrane. In support of this view are recent findings by Wickner's group (Lill *et al.*, 1989) demon-strating that in *E. coli* a peripheral membrane protein, SecA, functions as a membrane-dependent ATPase which couples the hydrolysis of ATP to the translocation of a precursor protein, proOmpA, across the membrane by a so far unknown mechanism.

3. ATP could serve as an energy source for the phosphorylation of a component of the translocation apparatus, thus triggering the import reaction. Recently we obtained evidence that the phosphorylation of a 51-kD protein (P51) located in the outer envelope membrane is presumably involved in protein import into chloroplasts (Hinz and Flügge, 1988). Several lines of evidence support this conclusion. First, it could be shown that pyridoxal-5'-phosphate (PLP) inhibits protein translocation into chloroplasts by modifying (a component of) the import machinery and that the inhibition of protein import was closely correlated with the increase of the phosphorlylation state of P51. The level of phosphorylated P51 represents most likely a balance between the activities of a protein kinase and a protein phosphatase which is apparently impaired by the presence of PLP and affects the overall regulation of protein import into chloroplasts. Second, the concentration-dependent inhibition of protein import by the ATP affinity analogue p-fluorosulfonyl-benzoyl-5'-adenosine, a protein kinase inhibitor, was shown to be linked to the decrease in the phosphorylation of P51. Third, internally generated ATP (by light or by the addition of triosephosphate/phenazine methosulfate) was shown to be able to compete specifically with externally added γ^{32}P-ATP for the phosphorylation of P51, demonstrating that P51 as an outer envelope membrane protein can effectively be phosphorylated by stormal ATP as well. This indicates that a distinct level of ATP can be built up in the inter-membrane space, presumably due to a spatial proximity between the ATP/ADP translocator and the translocation apparatus (Flügge and Hinz, 1986). Interestingly, this is possible in spite of the presence of chloroplast porin molecules in the outer membrane (Flügge and Benz, 1984) which allow rapid equilibration between the intermembrane space and the cytosol. These findings further demonstrate that the intermembrane space may be involved in the energization of protein import into chloroplasts. Fourth, treatment of the chloroplasts with low concentrations of the protease thermolysin which is unable to penetrate the outer envelope and therefore does not affect inner envelope membrane proteins (Cline et al., 1984) lead to a loss in protein import activity linked to a removal of the phosphorylation site of P51. Finally, it was demonstrated that binding of a precursur protein (the transit peptide of pSSU linked to the mature part of dihydrofolate reductase, a cytosolic enzyme) produced a marked and specific increase of P51 phosphorylation, an effect which was not exerted by the mature form of the precursor protein.

Phosphorylation of P51 occurred with an apparent K_M (ATP) of only about $5 \mu M$ which is severalfold less than that obtained for protein import (Flügge and Hinz, 1986). Possibly, two separate ATP-dependent processes both occurring behind the outer envelope membrane are involved in protein translocation as reflected by the different demands of ATP. One process

with the high-affinity binding site for ATP is presumably linked to the phosphorylation of P51 as a putative component of the translocation apparatus possibly functioning as a receptor protein. The conformational changes associated with the phosphorylation of the import machinery could possibly trigger the movement of the translocated protein through the membrane.

As outlined above, low concentrations of ATP are required for binding of precursor proteins to pea chloroplasts (Olsen *et al.*, 1989). Although a demand of ATP for precursor binding to spinach chloroplasts could not be observed, the question remains to be answered whether ATP-dependent phosphorylation in spinach chloroplasts and ATP-dependent percursor binding to pea chloroplasts reflect only different aspects of a common mechanism. The other process with a K_M (ATP) of about 0.9 mM (Flügge and Hinz, 1986) appears to reflect the ATP requirement for the translocation itself possibly being equivalent to the requirement of a membrane potential for mitochondrial protein import and/or for stabilizing and folding the proteins catalyzed by chaperone-like activities (see above). The precise role of ATP in translocation of proteins into chloroplasts, however remains rather elusive.

Taken together, but elucidating the possible role(s) for ATP in energizing protein import into chloroplasts we have found some evidence that an ATP-dependent phosphorylation might functionally be related to the process of binding and/or translocation of precursor proteins. In addition, these experiments suggest that a 51-kD outer envelope membrane protein may be part of the import apparatus, possibly being a putative receptor protein or a protein intimately associated with the targeting apparatus.

Evidence that proteinaceous components of the outer envelope membrane may function as protein receptors, required for binding and/or translocation, has mainly been obtained from observations that protein import is sensitive to pretreatment of chloroplasts with proteases, e.g., thermolysin (Cline *et al.*, 1985; Flügge and Hinz, 1986; Flügge *et al.*, 1989). The identification and isolation of (components of) the translocation apparatus are aggravated by the fact that receptor proteins represent only minor components of the envelope membrane. Friedman and Keegstra (1989) performed receptor–ligand binding studies and calculated the number of pSSU receptor binding sites to be about 1500–3500 per chloroplast. Thus, the receptor might represent only about 0.3% of the total envelope membrane protein. Indeed, the amount of P51 as analyzed by SDS-PAGE lies in this range (unpublished results).

Two other attempts have been made to identify such putative chloroplast import receptors. Cornwell and Keegstra (1987) used a precursor protein which was modifed by a cross-linking reagent and subsequently

coupled to chloroplasts by photoactivation. This approach led to a labelling of an 86-kD conjugate from which the molecular weight of the putative receptor was calculated to be 66,000. However, a 66-kD membrane protein has not yet been identifed as a component of the envelope membrane. Blobel's group (Pain *et al.* 1988) has used anti-idiotypic antibodies raised against binding sites of peas pSSU in order to identify putative receptor binding sites. Immunogold electron microscopy studies using this anti-idiotypic antiserum suggested that the putative import receptor is located in distinct patches of the envelope membrane where both membranes are in close apposition, suggesting that protein import into chloroplasts might occur via contact sites as has been shown for mitochondria (Schleyer and Neupert, 1985). The identification of the putative import receptor as being the major 30-kD envelope membrane protein, however, is not indisputable. Obviously, the authors were not aware of the fact that the major 30-kD envelope membrane protein represents the phosphate translocator protein (see above) which is, however, located in the inner envelope membrane (Flügge and Heldt, 1984). A more careful analysis of the identification experiments including phosphate translocator-specific probes led the authors to suggest that the identified 30-kD polypeptide represents a protein different from the phosphate translocator (personal communication, Joyard and Douce, 1988). Another attempt to identify a putative receptor protein by cross-linking experiments (Kaderbhai *et al.*, 1988) led to the labelling of the phosphate translocator as the major 30-kD component of the envelope membrane, thus corroborating the observations reported by Pain *et al.* (1988). These authors propose that the phosphate translocator is also involved in receptor functioning, but their data are not conclusive and do not reveal any support for this suggestion. In summary, the identification of putative receptor proteins in the envelope membrane has led to different results, and direct evidence for the involvement of any of the identified polypeptides in receptor function is still incomplete.

Final Remarks

It is clear from the evidence presented here that protein import into chloroplasts shares more features in common with protein transport in other membrane systems than had been previously thought (Keegstra *et al.*, 1989). Although direct evidence is still fragmentary, it is conceivable that both cytosolic and stromal (ATP-dependent) components are involved in either keeping the precursor proteins (and, in addition, the imported and processed intermediates destined for the thylakoid lumen) in a transport-competent conformation or in assisting in the assembly of imported proteins into

multimeric complexes. Work on the identification of such components as well as that of membrane constituents involved in translocation, however, is only at the very early stage.

A main difference between protein import into chloroplasts and that into mitochondria is that, in chloroplasts, only ATP is required for energizing protein import. Thus, protein import into chloroplasts is not subjected to a diurnal rhythm which could occur if the import was dependent on light-induced membrane energization.

The role of ATP is energizing protein import, however, is rather elusive. Besides being presumably required as a substrate for putative cytosolic and stromal factors, ATP might, in part, be used for the phosphorylation of a membrane component which is possibly part of the translocation apparatus. Apart from this, the hydrolysis of ATP is required and used for the translocation process itself. However, the question how these two processes are coupled waits to be answered. Only one more question remains to be addressed: How is it that the interaction of the translocated protein with components of the envelope and the thylakoid membrane provides the information for the correct ultimate location of the protein into the different chloroplasts compartments?

Many gaps remain in our understanding of the import process, and future work will definitely add additional complexity to the general picture of protein translocation as outlined above.

Acknowledgments

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