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Transport of Proteins into the Thylakoid Lumen—Stromal Processing and Energy Requirements for the Import of the Precursor to the 23-kDa Protein of PS II

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Transport of the precursor to the 23-kDa protein of photosystem II was examined by incubation of the precursor with isolated intact chloroplasts in the presence of ATP in darkness. An intermediary-sized form was accumulated in the stroma at 0.1–1 mM ATP. At higher concentrations of ATP (3.2–10 mM), the precursor was imported into the thylakoid lumen and processed to the mature form. The precursor was not imported even as far as the stroma in the absence of ATP. The intermediary-sized form that accumulated at low concentrations of ATP was imported into the thylakoid lumen and processed to the mature form when chloroplasts were subsequently incubated in the light. These observations indicate that the accumulated intermediary-sized form was suitable for further translocation and that the intermediary-sized form is a transport intermediate that occurs under natural conditions. Import of the protein into the thylakoid lumen, which was observed at the higher concentrations of ATP, was inhibited by the addition of nigericin or carbonylcyanide *m*-chlorophenyl hydrazine. The effects of these ionophores suggests that the translocation of the protein across thylakoid membrane requires a proton gradient across the membrane. The results together show that the protein is imported from the cytosol into the thylakoid lumen in discrete steps: ATP-driven translocation across envelope membranes, stromal processing to the intermediate, translocation of the intermediate across the thylakoid membrane and final processing to the mature protein within the thylakoids.

Key words: Chloroplasts — *Pisum sativum* L. — 23-kDa Protein — Protein transport — PS II — Transport intermediate.

The chloroplast proteins that are encoded by the nuclear genome are synthesized in the cytosol as larger precursors. These precursors include transit peptides at their amino terminals. The amino acid sequences of some of these transit peptides have been deduced from cDNA clones of various precursor proteins. These sequences lack any conserved motifs but have a common feature in terms of amino acid composition. Transit peptides of stromal or thylakoid-membrane proteins are generally rich in hydrophilic and basic amino acid residues. The transit peptides of thylakoid luminal proteins, such as plastocyanin and the three peripheral proteins of PS II, namely the 33-, 23-, and 18-kDa proteins, have additional clusters of hydrophobic amino acids at their carboxyl terminals (Keegstra and Olsen 1989). The functions of these transit peptides have been studied in experimental systems in vitro. Such systems consist of isolated intact chloroplasts and precursor proteins that are translated from RNAs transcribed from the corresponding cDNAs. The chloroplasts take up the precursors in an energy-dependent manner, and the import process that occurs in vivo is reproduced (Grossman et al. 1980, Flügge and Hinz 1986). Experiments with truncated or chimeric transit peptides in such a system have provided evidence that the hydrophilic regions serve as signals for targeting to the stroma (Clark et al. 1990, Smeekens et al. 1990) and that the hydrophobic cluster in the luminal precursor directs proteins to the thylakoid lumen (Smeekens et al. 1986, Ko and Cashmore 1989). A peptidase isolated from the stroma has been shown to cleave the hydrophilic region from a precursor to a luminal

Abbreviations: the 23-kDa, the 23-kDa protein of oxygen evolving complex; p23-kDa, precursor to the 23-kDa; CCCP, carbonylcyanide *m*-chlorophenyl hydrazine.

protein and to generate an intermediary-sized protein (Musgrove et al. 1989). It has been suggested that thylakoids recognize hydrophobic clusters, import intermediary-sized proteins and process them to mature forms. A peptidase activity that can carry out the final processing step has been found from the thylakoids (Musgrove et al. 1989).

Chloroplasts require energy for the import of proteins, as do other organelles that import proteins post-translationally. Light is a good source of energy for the import of proteins in experiments with isolated chloroplasts since chloroplasts convert light into various forms of usable energy. ATP has also been demonstrated to be a source of energy for the import of stromal proteins (Theg et al. 1989). Ionophores inhibit the light-driven import of thylakoid-lumen proteins by chloroplasts at the step where translocation from the stroma to the thylakoid lumen occurs, an indication that this step requires a proton-motive force (Mould and Robinson 1991, Cline et al. 1992).

It has been reported that full-size precursors for some of the luminal proteins can be transported to the lumen of isolated thylakoids without prior processing (Mould et al. 1991, Cline et al. 1992). These observations indicate that stromal processing is not obligatory for the translocation of luminal proteins across thylakoid membranes under the experimental conditions, but they do not rule out the possibility that such processing occurs in vivo. We have found in the present study that the precursor to the 23-kDa (p23-kDa) accumulated in the stroma in an intermediary-sized form. This form was able to traverse the thylakoid membrane, if an appropriate source of energy was available. Our results suggest that stromal processing does actually occur in vivo.

Materials and Methods

Plant material and isolation of chloroplasts—Pea (*Pisum sativum* L. cv. Usui) seedlings were grown in soil for 12–15 days under a cycle of 12 h of light at 18°C and 12 h of darkness at 12°C. Intact chloroplasts were isolated by the method of Morgenthaler and Mendiola-Morgenthaler (1976) and suspended in import buffer (330 mM sorbitol, 25 mM K-Tricine and 4 mM $MgCl_2$, pH 8.0) at 1 mg Chl ml^{-1}. To minimize the accumulation of starch, we isolated chloroplasts from young leaves at the end of a 12 h dark period.

Preparation of labeled precursors—Capped RNA was transcribed from full-length cDNA for the 23-kDa from pea by SP6 polymerase (Konishi et al., manuscript in preparation). The mRNA for the 23-kDa was translated in a wheat germ system in the presence of 370 kBq of [^{3}H]-leucine (4.4 TBq $mmole^{-1}$, Amersham International plc, Amersham, England). Small molecules including ATP were removed from the translation mixture by gel filtration or by ultrafiltration. Gel filtration was performed using Sephadex G-25, which had been equilibrated with the import buffer, by the method of Olsen et al. (1989). For ultrafiltration, the translation mixture was concentrated to less than 5 μl on a membrane filter (Ultrafree-C3; Millipore Corp., Bedford, MA, U.S.A.) and diluted to 100 μl with import buffer. This concentration-dilution process was repeated three times.

Import experiments—A 10 μl aliquot of a suspension of chloroplasts (10 μg Chl) and precursor protein (10 μl) were mixed and import buffer was added to 40 μl in a polypropylene tube. The mixture was incubated at 20°C for 60 min, unless otherwise specified, either with illumination from tungsten lamps (500 $\mu Em^{-2} s^{-1}$) or in darkness. CCCP, valinomycin or nigericin (Sigma, St. Louis, MO, U.S.A.) was added to the mixture at 5 μM. The experiments in darkness were performed in total darkness except during pipetting or mixing which were carried out under dim green light (0.03 $\mu Em^{-2} s^{-1}$). After incubation, the chloroplasts were recovered by centrifugation at 6,000 × *g* for 1 min, resuspended in 100 μl of import buffer that contains 1 mM $CaCl_2$, supplemented with 20 μl of a solution of 1 mg ml^{-1} thermolysin in import buffer and kept on ice for 20 min. Then the chloroplasts were recovered and washed with buffer that contained 330 mM sorbitol, 1 mM EGTA and 25 mM Tricine-KOH, pH 8.0. For fractionation into stroma and thylakoids, the chloroplasts were lysed with 100 μl of 10 mM Tricine-KOH, pH 8.0. After centrifugation at 12,000 × *g* for 3 min, the supernatant was collected as the stromal fraction, and protein was concentrated by precipitation upon the addition of an equal volume of acetone. The pellet of thylakoids was washed and resuspended in import buffer that contained 1 mM $CaCl_2$ and treated again with thermolysin. Conditions for SDS-PAGE and fluorography were those described by Minami and Watanabe (1984).

Results

ATP-mediated import of the 23-kDa—To confirm whether ATP alone can drive the import of the 23-kDa in our assay system, we added a series of different concentrations of ATP to the import reaction mixture. To exclude any supply of energy from photosynthesis, each reaction was carried out in darkness. In the presence of ATP at 3.2 mM or 10 mM, the 23-kDa was processed to the mature form (Fig. 1) which was localized in the thylakoid (Fig. 2). With ATP at 0.32 mM and 1.0 mM, imported protein was processed into an intermediary-sized form (Fig. 1) which was located in the stroma (Fig. 2). The intermediary-sized form was not detected when the import of the 23-kDa was driven by light (Fig. 3, light). No transport was detected in the absence of ATP (Fig. 1). These results indicate that the 23-kDa is translocated across the envelope membranes in

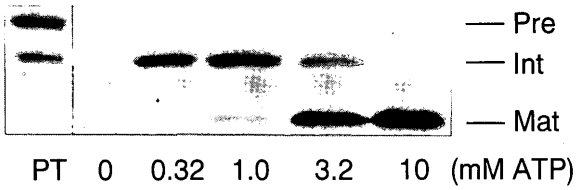

Fig. 1 Effect of concentrations of ATP on the import of the 23-kDa. The product of translation, the 23-kDa, was incubated with intact chloroplasts in the dark in the presence of ATP at the indicated concentrations. After incubation, the chloroplasts were treated with thermolysin, and proteins were separated by SDS-PAGE. Radioactivity of the imported proteins was detected by fluorography. Lane PT, the product of translation. Pre, the precursor form; Int, the intermediary-sized form; Mat, the mature form.

an ATP-dependent manner, as has been well characterized in the case of stromal proteins (Theg et al. 1989).

Effects of destruction of the proton gradient on import into the thylakoids—The intermediary-sized form of the 23-kDa could be generated either by limiting the supply of ATP or by withholding necessary sources of energy other than ATP. Chloroplasts can convert ATP to other form(s) of energy which may be necessary for the translocation of protein across the thylakoid membranes. The proton-motive force across membranes is one of the known requirements for the translocation of proteins across the inner membranes of bacteria and mitochondria. To reduce the proton-motive force across the chloroplast membrane, we added ionophores to the reaction mixture in the presence of 10 mM ATP. The intermediary-sized form of the 23-kDa accumulated in the presence of either CCCP or nigericin (Fig. 3). CCCP and nigericin reduce the proton gradient, acting as a proton transporter and a K^+-H^+ exchanger, respectively. The intermediary-sized form accumulated in the stroma, and the formation of the mature form was greatly reduced (Fig. 3, lanes S and T), as we

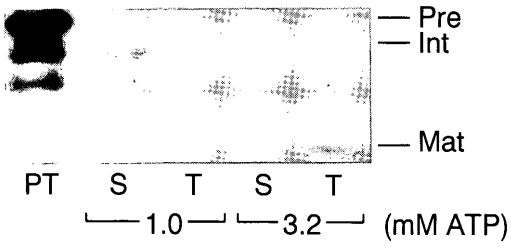

Fig. 2 Localization of the imported proteins in chloroplast subfractions. The 23-kDa was transported to chloroplasts in the presence of ATP at indicated concentrations. After treatment with thermolysin, the chloroplasts were fractionated into stromal (S) and thylakoid (T) fractions. See legends to Fig. 1 for abbreviations.

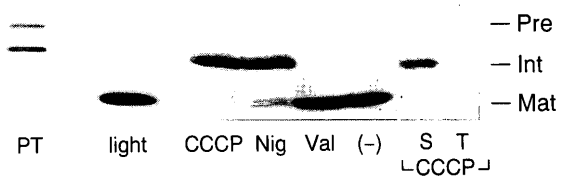

Fig. 3 Effects of ionophores on ATP-driven import. The 23-kDa was imported into chloroplasts at 10 mM ATP in the presence of various ionophores. Pre, the precursor form; light, products of light-driven import; Nig, nigericin; Val, valinomycin; (—), absence of ionophores. After import reaction in the presence of CCCP, the chloroplasts were fractionated into stromal (S) and thylakoid (T) fractions. See legends to Fig. 1 for abbreviations.

observed also with ATP alone at lower concentrations (Fig. 1). Valinomycin is a K^+ transporter and reduces the electrical potential across membranes. It did not have any effects on the transport of the 23-kDa, and the mature form of the protein was found in the thylakoid lumen (Fig. 3). These results indicate that a protein gradient across a membrane is required for the translocation of the protein across the thylakoid membrane. ATP drives the translocation across the envelope membranes.

Light-driven import of the intermediary-sized form into thylakoids—The intermediary-sized form of the 23-kDa protein could either be an incomplete or an inappropriate product of the import. If the former is the case, the intermediary-sized form should remain a substrate for further translocation and should move to the thylakoid lumen if a proton gradient is regenerated. To examine this possibility, we illuminated the chloroplasts that had accumulated the intermediary-sized form as a result of ATP-driven transport.

Chloroplasts were incubated with p23-kDa at 0.32 mM ATP in the dark for 20 min and then treated with thermolysin to remove proteins bound to their surfaces. The intermediary-sized protein was detected from the stroma (Fig. 4, pulse, lane S). Then the chloroplasts were incubat-

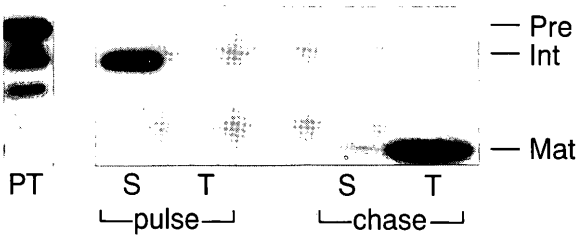

Fig. 4 Light-driven translocation of the intermediary-sized form into thylakoids. Intact chloroplasts were incubated with p23-kDa at 0.1 mM ATP for 20 min, and they were treated with thermolysin (pulse). Chloroplasts were then illuminated for 20 min (chase). See legends to Fig. 1 and 2 for abbreviations.

ed again in the light for a further 20 min. The intermediary-sized form was translocated across the thylakoid membrane and processed to the mature form (Fig. 4, chase, lane T). These data suggest that the intermediary-sized form of the 23-kDa retains the capacity to be translocated across the thylakoid membrane. Therefore, it is most likely a transport intermediate of the protein, and it may also be formed in vivo.

Taken together, our results suggest that ATP drives the import of p23-kDa to the stroma, where the precursor is processed to a transport intermediate. A proton gradient across thylakoid membrane is required for the translocation of this intermediate to the lumen.

Discussion

In our experimental system with isolated chloroplasts, p23-kDa was imported into the stroma in an ATP-dependent manner, and it accumulated there as a transport intermediate. The subsequent step of the import reaction, the translocation across the thylakoid membrane and the maturation of the protein, took place once a proton gradient had been regenerated in the thylakoids by illumination. These observations indicate that the import of the 23-kDa across the envelope membranes is driven by ATP and the import across the thylakoid membranes requires a proton-motive force. The proton gradient is normally generated by the proton pumping that is coupled with photosynthesis, but in our experimental system a reverse reaction of the ATP synthase may serve to pump protons into the thylakoids by hydrolyzing exogenously added ATP.

Our results clearly demonstrate the occurrence of a transport intermediate, which accumulated in the stroma when transport to the lumen was blocked. These observations provide firm evidence for the stromal processing of the precursors to luminal proteins and for discrete translocation systems in the envelopes and in the thylakoids. Mould et al. (1991) reported that a precursor to the 23-kDa could be translocated across isolated thylakoid membranes without prior processing in the stroma. Since our system with intact organelles more closely resembles the natural conditions, it is likely that the stromal processing of the precursors actually occurs in vivo prior to translocation across thylakoid membranes.

It is known that a proton-motive force is required for both the secretion of proteins across the inner membranes of bacteria (Bassford et al. 1991, Wickner et al. 1991) and translocation of proteins from the matrix to the intermembrane space of mitochondria (Pfanner and Neupert 1990, Baker and Schatz 1991). While these membrane systems and the thylakoids generate and maintain the proton-motive force in different ways, their protein transport systems have common properties: proteins are transported in the opposite direction to the direction of the proton-motive force; specific organelles or membrane vesicles isolated from cells can transport proteins independently of translation; and the precursor proteins have similar N-terminal extensions which act as signals for transport. These similarities seem to suggest that a single ancestral protein-transport system may be common to each creatures.

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