Protein import into chloroplasts requires a chloroplast ATPase

(cell-free translation/precursor of small subunit of pea ribulose-1,5-bisphosphate carboxylase/pea chloroplasts/ nonhydrolyzable ATP analogs/ ionophores)

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ABSTRACT We have transcribed mRNA from a cDNA clone coding for pea ribulose-1,5-bisphosphate carboxylase, translated the mRNA in a wheat germ cell-free system, and studied the energy requirement for posttranslational import of the [35S]methionine-labeled protein into the stroma of pea chloroplasts. We found that import depends on ATP hydrolysis within the stroma. Import is not inhibited when H+, K+, Na+, or divalent cation gradients across the chloroplast membranes are dissipated by ionophores, as long as exogenously added ATP is also present during the import reaction. Our data suggest that protein import into the chloroplast stroma requires a chloroplast ATPase that does not function to generate a membrane potential for driving the import reaction but that exerts its effect in another, yet-to-be-determined, mode. We have carried out a preliminary characterization of this ATPase regarding its nucleotide specificity and the effects of various ATPase inhibitors.

The in vitro import of cytoplasmically synthesized proteins into chloroplasts has been previously demonstrated. Translation of mRNA for a cytoplasmically synthesized polypeptide of the chloroplast stroma, the small subunit (S) of ribulose-1,5-bisphosphate carboxylase, yielded a large precursor (pS) (1), and posttranslational inclusion with isolated chloroplasts resulted in import of pS into the chloroplast stroma accompanied by conversion of pS into S (2, 3) by means of cleavage of an NH2-terminal transit sequence (4).

Import is an energy-dependent process (5). When the import reaction was carried out in the dark, addition of ATP stimulated uptake into the chloroplast. Likewise, illumination of the import reaction stimulated uptake, and this stimulation was abolished when uncouplers were present, again suggesting that ATP is required for import (5).

Recently, Cline et al. (6) succeeded in experimentally separating the import reaction into two steps. In the first reaction precursors were bound to the surface of ATP-depleted chloroplasts. In a second reaction much of the bound precursor was imported into the chloroplast, depending on exogenously added ATP.

In the present paper we have addressed the questions of how and where ATP is used to drive the import of pS into the chloroplast stroma.

MATERIALS AND METHODS

Subcloning and Phage T7 Transcription. A clone representing the complete coding sequence of pS was constructed from a partial cDNA clone, SS15, that lacks part of the NH2-terminal transit sequence (7) and a genomic clone, pUC3AE9. The plasmid pUC3AE9 contains two S gene segments; one is derived from the rbcS-3A gene (8) and contains the complete transit peptide of pS, and the other is derived from the rbcS-E9 gene (9) and contains the entire mature polypeptide (S) sequence, including two introns. A portion of pUC3AE9 containing the two introns but not the transit sequence was excised with Sph I and Kpn I and an identical Sph I-Kpn I fragment from SS15, which lacked the two introns, was substituted. The resulting clone contained the complete uninterrupted coding region of pS.

The pS coding DNA was excised from the pUC vector with Xba I and Cla I and ligated to pT7-1 cut with Xba I and Acc I. The resulting plasmid (pT7-pS) was purified on a sucrose gradient and linearized downstream from the gene with Pst I before transcription. In vitro transcription using phage T7 RNA polymerase (United States Biochemicals, Cleveland, OH) was done essentially according to supplier's assay conditions except that the nucleoside triphosphates and the cap analog diguanosine triphosphate [G(5')ppp(5')G] were used at 0.25 mM each. After transcription, the solution was extracted with phenol/chloroform and the mRNA was precipitated with LiCl. About 2 µg of transcript was obtained per µg of DNA template (as judged by A260).

Cell-Free Translation. The previously described wheat germ cell-free translation system (10) was supplemented with 10 µM S-adenosyl-l-methionine and contained 200 ng of mRNA and 2.8 µl of wheat germ extract per 10-µl translation mixture. The final concentrations of the other components of the translation mixture were adjusted to 43 mM Hepes–KOH at pH 7.5, 112 mM KOAc, 2.1 mM Mg(OAc)2, 0.25 mM spermidine, 3 mM dithiothreitol, 0.5 mM ATP, 20 µM GTP, 8 mM creatine phosphate, creatine kinase at 65 µg/ml, each of the 20 amino acids except for methionine at 26 µM, and [35S]methionine at 0.9–1.2 µCi/ml (1 Ci = 37 GBq). After 1-hr incubation at 25°C, the translation mixture was centrifuged for 22 min at 4°C in a Beckman Airfuge at 30 psi (140,000 × g), yielding a postribosomal supernatant (PRS) that contained the newly synthesized pS.

Posttranslational Import Assay. Intact chloroplasts were isolated from the leaves of 2- to 3-wk-old pea (Pisum sativum, Progress no. 9) seedlings and purified on a Percoll step gradient as described (11). Isolated chloroplasts were resuspended in 50 mM Hepes–KOH, pH 7.7/0.33 M sorbitol at a concentration of 2–4 mg of chlorophyll per ml. To deplete chloroplasts of their endogenous ATP, we incubated them in the dark for 15 min at room temperature prior to use in the import assay. The import reaction was carried out essentially as in ref. 5 with some modifications. The basic import assay mixture (300 µl) contained 0.5 µl of PRS (see above), preincubated chloroplasts equivalent to 100 µg of chlorophyll, bovine serum albumin at 100 µg/ml, and 50 mM Hepes–KOH, pH 7.7/0.33 M sorbitol/40 mM KOAc/2 mM Mg(OAc)2/1.5 mM dithiothreitol/10 mM methionine. In most instances, the import assay mixture was further modified as specified in the figures. The import reaction was

Abbreviations: S, small subunit of ribulose-1,5-bisphosphate carboxylase; pS, precursor of S; PRS, postribosomal supernatant; CF1, coupling factor; pfm, promotive force; CCCP, carbonylcyanide m-chlorophenylhydrazone.
carried out at room temperature for 30 min with occasional gentle mixing, either in the dark or in the room light as specified.

**Quantitative Analysis of Import.** After incubation, each import mixture was chilled on ice, diluted with 5 ml of cold 50 mM Hepes-KOH, pH 7.7/0.33 M sorbitol and centrifuged for 1 min at 4000 x g. The pelleted chloroplasts were lysed in 0.8 ml of 2 mM EDTA, pH 7.5, by vigorous mixing. Sodium chloride was added to a final concentration of 0.24 M and the lysate was centrifuged at 12,000 x g for 15 min. To the resulting supernatant, representing the stroma fraction, ice-cold trichloroacetic acid was added to a final concentration of 10%. The precipitate was analyzed by electrophoresis on 12% polyacrylamide gels in NaDodSO4 (12). The gels were fixed in 10% acetic acid/35% methanol (vol/vol), treated with Enlightning (New England Nuclear), dried, and exposed to Fuji x-ray film for 6-24 hr at ~80°C. An AMBIS β-scanner was used to quantitate the radioactivity in pS or S in dried gels.

**Protease Protection.** The procedure for protease protection was identical to that given above for the quantitative analysis of import, except that prior to lysis the pelleted chloroplasts were gently resuspended in a final volume of 300 μl of ice-cold 50 mM Hepes-KOH, pH 7.7/0.33 M sorbitol. Either 250 μg/ml each of trypsin and chymotrypsin or the same amount of proteases plus Triton X-100 to a final concentration of 0.3% was added. After incubation for 30 min on ice, the reaction mixture was diluted with 0.8 ml of cold 2 mM EDTA, pH 7.5 containing protease inhibitors (2.5 mM phenylmethylsulfonyl fluoride) and mixed vigorously.

**RESULTS**

**ATP Is Required for Import.** mRNA for S was obtained by *in vitro* transcription of cloned DNA (see Materials and Methods) and was translated in a wheat germ cell-free system. The major translation product was a polypeptide of 20 kDa (Fig. 1, lane 1) that was immunoprecipitable with antibody raised against purified S from pea (data not shown) and that represented the precursor of S, pS.

A small quantity (0.5 μl) of PRS from the cell-free translation was added posttranslationally to a 300-μl import reaction mixture containing chloroplasts isolated from pea leaves. The maximal ATP concentration contributed by the translation mixture in the import reaction was 0.8 μM. After incubation for import, chloroplasts were sedimented, lysed in 2 mM EDTA, and fractionated into a soluble (stroma) and a membrane fraction. Upon import into the chloroplast stroma, pS is converted into S by cleavage of its NH2-terminal transit sequence (4).

When the import reaction was carried out in the dark and without additional ATP (see above), less than 2% of pS was found to be translocated (Fig. 1, lane 2). When the import reaction was carried out either in the dark in the presence of 1 mM ATP (lane 3) or in the room light in the absence of exogenously added ATP (lane 4), about 65% of pS was translocated. In the room light and in the presence of exogenously added ATP, import of pS was 80% (lane 5). A control in which, after completion of import, the chloroplasts were incubated with proteases prior to lysis showed that virtually all of the S molecules in the stroma fraction were resistant to protease digestion (lane 6) but were degraded when Triton X-100 was present during the incubation with proteases (lane 7). Thus, the S molecules detected in the stroma fraction were, in fact, imported.

The results shown in Fig. 1 confirm and amplify the data reported by Grossman *et al.* (5). The virtually complete dependence of import on ATP demonstrated by the data in Fig. 1 provides an explanation for the considerable "back-

![Fig. 1](image-url)
reaction in the absence or presence of low (1 mM) or high (10 mM) concentrations of ATP (Fig. 3). In the absence of ATP (lanes 3 and 6) either of these two analogs caused considerable inhibition of the light-stimulated import (for control see lane 1). This inhibition was partially or completely abolished at low or high ATP concentrations (lanes 4, 5, 7, and 8).

Effect of Other Nucleotides. We have tested the effect of CTP, GTP, dATP, or ADP on the light-stimulated import in the absence or presence of ATP (Fig. 4). CTP (10 mM, data not shown) or GTP (10 mM) strongly inhibited light-stimulated import in the absence of ATP (lane 2), but inhibition was greatly relieved in the presence of 1 mM ATP (lane 3). The mechanism by which GTP or CTP inhibits the putative stroma ATPase involved in import remains to be determined. dATP (lanes 4 and 5) showed only a slight inhibition of light-induced import, suggesting that dATP might be able to substitute for ATP as a substrate. ADP (lanes 6 and 7) had no effect on light-induced import, presumably because it can be converted to ATP in the chloroplast stroma.

A Membrane Potential Is Not Required for Import. ATP hydrolysis in the chloroplast stroma could be used to generate a membrane potential across the inner chloroplast mem-

brane that, in turn, could drive import into the chloroplast stroma. A membrane potential across the inner chloroplast membrane could be generated either indirectly, by the CF₁‒F₀ ATPase (CF₁, coupling factor) in the thylakoid membrane, or directly, by a proton- or ion-translocating ATPase in the inner chloroplast membrane.

To determine whether ATP hydrolysis is employed to generate a protonotive force (pmf), we tested the effect of the protonophore carbonylcyanide m-chlorophenylhydrazone (CCCP) (18), which dissipates the total pmf. CCCP, in fact, completely inhibited the light-induced import (Fig. 5, lane 2 and 3). Most importantly, however, addition of ATP in the presence of CCCP restored import into the chloroplast (Fig. 5, lane 4). Thus, the exogenously supplied ATP can substitute for the endogenously synthesized ATP. Exogenously added ATP presumably enters the stroma via the ADP/ATP exchanger and, in the presence of CCCP—i.e., under conditions in which the pmf has been abolished—serves as a substrate for an ATPase other than the CF₁‒F₀ ATPase in the thylakoid membrane or a proton-translocating ATPase in the inner chloroplast membrane to accomplish import.

To investigate whether a K⁺ or Na⁺-translocating ATPase might be involved in import we tested nigericin and monensin, respectively. Cline et al. (6) had already demonstrated that light-induced uptake into chloroplasts is inhibited by nigericin and that uptake is restored by exogenously added ATP.

FIG. 4. Effect of some nucleotides on import. The import reactions were carried out in the light. Numbers on top indicate the concentrations in mM.

FIG. 5. A membrane potential is not required for import. The import reactions were carried out in the light. Chloroplasts (equivalent to 100 μg of chlorophyll in 200 μl) were first incubated with various ionophores for 15 min on ice in the room light. The import reactions were started by adding 100 μl of a master mixture containing all the other components of the basic import reaction. Numbers on top indicate the final concentrations in the import reaction of the ionophores in mM (CCCP, monensin, and A23187) or nM (nigericin) and of ATP in mM. Fluorographs comprising lanes 1–9 and 10–15 were from separate experiments.
ATP. The data shown in lanes 5–7 of Fig. 5 confirm the findings of Cline et al. (6). Nigericin dissipates a K⁺ gradient across the membrane by catalyzing an electroneutral exchange of K⁺ and H⁺ leading to a breakdown of the pH gradient (ΔpH) without affecting the membrane potential (Δψ) (19, 20). Thus, in the presence of nigericin at two different concentrations (10 or 100 nM, lanes 5 and 6, respectively) there was very little or no import. However, when ATP was present in addition to 100 nM nigericin, import was restored (lane 7). Monensin (19, 20), which acts like nigericin except that it is more selective for Na⁺, also inhibited import (lane 8). Again, import was restored when ATP was also present (lane 9). These data suggest that neither a K⁺ nor a Na⁺ gradient is involved in import.

Similar results were obtained with the ionophore A23187, which is predominantly selective for divalent over monovalent cations (19, 20). In the absence of ATP, light-induced uptake of pS was inhibited by A23187 in a dose-dependent manner (Fig. 5, lanes 12–14). The inhibition was virtually complete at 6.7 μM A23187 (lane 14) and it could be relieved by 1 mM ATP (lane 15). Inhibition of light-induced import by A23187 is in good agreement with the ability of the ionophore to dissipate the pH gradient and thereby to interfere with endogenous ATP production (19).

**Effect of Various ATPase Inhibitors.** Since ATP hydrolysis was obligatory for import, it was of interest to test various inhibitors of ATPases (Fig. 6). NaF and Na₃VO₄, 10 mM and 1 mM respectively, gave about 35–45% inhibition of light-induced pS uptake (lanes 2 and 4). In both cases the inhibition could be reversed by exogenously added ATP (lanes 3 and 5). The light-induced import of pS was inhibited by ~7% when 1 mM inorganic pyrophosphate (PPᵢ) was present in the import assay (lane 6). Higher concentration of PPᵢ (10 mM) led to about 20–30% inhibition (data not shown). A preincubation of PPᵢ with pyrophosphatase was able to restore translocation to the normal level (data not shown). The slightly inhibitory effect of PPᵢ was also abolished in the presence of 1 mM ATP (lane 7). These results are consistent with the much lower affinity of the adenine nucleotide transporter for PPᵢ than for ATP (21). Oligomycin (20 μM) was ineffective to inhibit light-induced translocation to an appreciable extent (<8%) (lane 8). Oligomycin is known to exert only a weak uncoupling effect in chloroplasts (22). Quercetin, a powerful inhibitor of the purified ATPase activity of the CF₁ from spinach chloroplasts (23), was only 20–25% inhibitory in the absence of ATP (lane 10) and had no effect in the presence of added ATP (lane 11).

**DISCUSSION**

Our data here suggest the existence of an ATPase located in (or facing) the chloroplast stroma that is required for protein import from the cytoplasm into the chloroplast stroma.

We reached this conclusion after we first extended earlier studies (5) and demonstrated that ATP is required for import into the chloroplast stroma (Fig. 1). When the posttranslational import reaction was carried out in the dark where no ATP was synthesized in the chloroplast stroma, import was found to be dependent on exogenously added ATP. When the import reaction was carried out in the room light, apparently enough ATP was endogenously synthesized to support the import reaction. However, light-induced import was further stimulated by exogenously added ATP, suggesting that the ATP synthesized endogenously during illumination (in the room light) might not have reached optimal levels for import.

We then proceeded to demonstrate that the required ATP acts from within the chloroplast, presumably in the chloroplast stroma (Fig. 2). This was done by adding substrates (dihydroxyacetone phosphate/oxaloacetic acid/P) so that chloroplasts could synthesize ATP in the dark (13). Substrate-stimulated chloroplasts were able to import. Addition of nonpenetrating ATP-consuming systems (glucose plus hexokinase or apyrase) did not abolish import, strongly suggesting that the ATP synthesized in the substrate-stimulated chloroplasts was not required on the outside of the chloroplast, where it could have been exported to the ADP/ATP exchanger of the inner chloroplast membrane. We cannot rule out the possibility that the ATP was exported and acted within the space between the inner and outer chloroplast membrane. However, we consider this highly unlikely as molecules of up to 8000 daltons are thought to rapidly equilibrate between the intermembrane space and the cytoplasm (14), presumably through large pores in the outer membrane.

Hydrolysis of ATP is required for import. Nonhydrolyzable ATP analogs were shown to inhibit light-induced import (Fig. 3). These analogs were most likely transported into chloroplast stroma in exchange for endogenous adenine nucleotides via the ADP/ATP exchanger (17) and then competitively inhibited ATP hydrolysis in the stroma.

ATP hydrolysis was not used to generate a H⁺, Na⁺, K⁺, or divalent cation (Ca²⁺, Mg²⁺, etc.) gradient across the inner chloroplast membrane (Fig. 5), suggesting that a cation motive ATPase is unlikely to be involved in the import reaction. In the presence of ionophores dissipating either the ΔpH alone or both ΔpH and Δψ components of the pmf, exogenously added ATP was still able to drive the import reaction. Thus a membrane potential does not appear to be required for import into the chloroplast stroma.

Therefore, the proton-translocating activity of the CF₁–F₀ ATPase in the thylakoid membrane is not directly involved in import. It is also unlikely that the ATPase activity of the CF₁–F₀ is responsible for ATP hydrolysis obligatory for protein import, since hydrolysis by the CF₁–F₀ ATPase would then have to be coupled to some reaction other than generating a pmf. Moreover, quercetin, a potent inhibitor of ATPase activity of spinach CF₁ (23), failed to inhibit import in the presence of added ATP (Fig. 6).

Likewise, a recently characterized ATPase in the inner chloroplast membrane (24, 25) does not appear to be a likely candidate for the ATPase activity required for import. The former is known to have a broad substrate specificity, whereas the latter appears to be inhibited by GTP (Fig. 4) and CTP (data not shown). Moreover, the inner membrane ATPase has been shown to be inhibited (60–70%) by fluoride.
or vanadate (25), which at a more or less similar concentration had no effect on import stimulated by exogenously added ATP (Fig. 6).

Thus, the ATPase activity required for import must be a hitherto uncharacterized ATPase. Since this ATPase is apparently not involved in generating a membrane potential, other modes of action have to be considered. There are several, certainly not mutually exclusive, possibilities. One possibility (12) is that ATP is hydrolyzed by a protein (a "translocase") that would act as a mechanochemical transducer by coupling the energy obtained from ATP hydrolysis to the movement of proteins across the chloroplast envelope. Other possibilities are that ATP is hydrolyzed by a kinase, by an adenyllyltransferase, or by adenylate cyclase. A kinase or an adenyllyltransferase could modify protein(s) necessary for translocation. Likewise, cAMP could be used to activate a cAMP-dependent kinase, which in turn could activate a protein(s) involved in translocation. Phosphorylation may also occur on the chains to be translocated. Transit sequences contain a substantial number of serine and threonine residues (7). Their phosphorylation could somehow be coupled to active transport by analogy to the photophosphotransferase system for sugar transport in bacteria (26).

Recently, other cell-free translocation systems in which translocation of proteins can occur posttranslationally have been analyzed as to their energy requirement. Similar to our results on in vitro import into chloroplast stroma, in vitro translocation across inverted vesicles of the bacterial plasma membrane (16) and microsomal vesicles of yeast endoplasmic reticulum (12, 27, 28) have been shown to require ATP but not a membrane potential. Only in vitro import into mitochondria appears to depend on both ATP and a membrane potential (29).

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