**Internal ATP Is the Only Energy Requirement for the Translocation of Precursor Proteins across Chloroplastic Membranes**

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The energy requirements for the import of nuclear-encoded proteins into isolated chloroplasts have been re-investigated. We have shown that, in contrast to protein import into mitochondria, the translocation of the precursors to ferredoxin, plastocyanin (prPC) and the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (prSS) across all chloroplastic membranes is independent of a protonmotive force and requires only ATP. This extends previous works in which investigations were limited to prSS and demonstrates that our results are probably general to all chloroplastic protein precursors. Our results are particularly interesting for the import of prPC, since in addition to the two envelope membranes, this protein must traverse the energy-transducing thylakoid membranes en route to its proper location in the thylakoid lumen. This lack of involvement of a protonmotive force, specifically of a transmembrane electric potential, demonstrates that separate mechanisms operate during the import of proteins into chloroplasts and mitochondria.

We also examined the question of whether ATP is utilized inside or outside of chloroplasts during protein import. Previous attempts to resolve this question have resulted in conflicting answers. We found, by two independent approaches, that ATP for protein import is utilized inside chloroplasts. The implications of these results on the possible mechanisms of protein import into chloroplasts are discussed.

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Chloroplasts and mitochondria are unique among subcellular organelles in that they contain their own DNA and are capable of semiautonomous protein synthesis. Nonetheless, most of their complement of proteins are encoded by nuclear genes, translated on cytoplasmic ribosomes, and post-translationally imported into the organelles (see Refs. 1 and 2 for recent reviews). It has been suggested that protein import into these two organelles occurs via a common mechanism (3, 4).

The process of organellar protein import requires energy. Both ATP and an energized inner membrane are required for protein import into mitochondria, and experiments suggest that the ATP is utilized outside the organelle (5–8). In chloroplasts, both early (9, 10) and recent (11–13) experiments demonstrated that only ATP, but not an electric field, is required for the import of one protein, the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (SS), across the two envelope membranes into the stroma. The latter publications also addressed the site of ATP utilization for import of prSS but obtained conflicting results. Whereas Flugge and Hinz (12) and Schindler et al. (11) concluded that ATP is required outside the stromal space, Pain and Blobel (13) concluded that ATP is utilized inside chloroplasts.

Considerably more information is available concerning the mechanism of protein import into mitochondria than into chloroplasts (cf. Ref. 1). It is therefore tempting to draw conclusions about chloroplastic protein import from the mitochondrial literature. In order to do so, however, the similarities and differences between these processes in the two organelles must be understood. Since the utilization of energy is fundamental to the mechanisms used for protein translocation across the respective membranes, we have re-examined the role of energy in the import of precursors into chloroplasts. Our experiments revealed a number of differences between the chloroplastic and mitochondrial import mechanisms. In the preceding accompanying paper (14) we show that, unlike mitochondria (cf. Ref. 14), chloroplasts require ATP to bind precursors prior to the translocation step. The present paper documents that neither the electrical nor protonic component of a protonmotive force is required for translocation of protein precursors across chloroplastic membranes. This is true even for prPC, a protein which must cross the energy-transducing thylakoid membrane in addition to the two envelope membranes before reaching the lumen. We also examined the site of ATP utilization for protein import by two independent techniques. Our results indicate that ATP supporting import is utilized inside, rather than outside, chloroplasts. The consequences of this result for the choice of possible mechanisms of protein import into chloroplasts is discussed.

**MATERIALS AND METHODS**

Intact chloroplasts were isolated, and protein precursors were synthesized from cloned genes (prFD (15), prPC (15), prSS (16)) and gel filtered as detailed in the preceding paper (14). Import reactions were performed at 25 °C at pH 8.0 in a medium containing 330 mM sorbitol and 50 mM K-HEPES (import buffer) with chloroplasts at 0.33 mg Chl/ml. Other additions are indicated in the figure legends. The experiments shown in Figs. 3 and 5 were performed and worked

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1 The abbreviations used are: SS, small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase; pr as a prefix, precursor; Chl, chlorophyll; FD, ferredoxin; PC, plastocyanin; HEPES, N-hydroxyethylpiperazine- N'-2-ethanesulfonic acid; o-ATP, periodate-oxidized ATP.
Energetics of Protein Import into Chloroplasts

up as in Ref. 14. Other experiments were performed in 400-μl poly-
ethylene microcentrifuge tubes in a 60-μl suspension layered over
silicone oil (Wacker AR 200) floating on 1 M perchloric acid. Reac-
tions were terminated by centrifuging the intact chloroplasts through
the oil into the acid thylakoids released from broken chloroplasts
removed in the top phase and only intact chloroplasts penetrated
the oil. The tubes were frozen at -80 °C, then sliced at the oil/
aqueous interfaces. The precipitated radiolabeled proteins that pel-
lated in the acid phase, which included precursors bound to the outside
of the chloroplasts and mature-sized translocated polypeptides inside
the chloroplasts, were resuspended in 0.5 M Tris buffer, diluted with
SDS-containing sample buffer, subjected to polyacrylamide gel elec-
trophoresis, and visualized by fluorography.

In the experiment depicted in Fig. 4, the ATP content of the intact
chloroplasts (i.e. ATP released into the perchloric acid supernatant)
and of the original assay medium above the oil was analyzed using
the luciferin-luciferase assay. Luminescence was detected in a scint-
tillation counter (Beckman model LS 7800 operated in the single
photon detector mode) and calibrated by the addition of known
amounts of ATP to each sample. In experiments such as these, the
internal chloroplastic ATP content corresponds directly to the ATP
measured when no exogenous ATP has been supplied to the samples.
The analysis of internal ATP is considerably more complicated when
ATP has been added exogenously. In this case, the amount of ATP
passing through the silicone oil layer with water bound to the external
surface of the chloroplasts must be accounted for. This bound water
typically represents a volume equal to that contained within the
chloroplastic envelopes (data not shown), making the correction for
externally carried ATP untenable. Thus, we have not reported the
chloroplastic ATP contents of those samples in Fig. 4 to which
exogenous ATP had been added.

All chemical reagents were purchased from commercial vendors.
Luciferin-luciferase was purchased from LKB (ATP monitoring kit
1243-102), prepared as in Ref. 17, and used in the buffer recom-
mended by the manufacturer. Silicone oil was kindly provided by
Gudrun Windfuhr of the Stauffer-Wacker Silicones Corp., Adrian, MI.

RESULTS

A Protonmotive Force Is Not Required for Protein Import
into Chloroplasts—Previous investigations have shown that
ATP, but not a protonmotive force, is required for the import
of prSS into the stroma (9, 11-13). This conclusion was based
on the lack of inhibition by ionophores of ATP-dependent
prSS import into dark-adapted chloroplasts. In view of the
known requirement for an electric field across the inner
mitochondrial membrane for protein import into mitochon-
dria, we felt that the nature of the energy requirement for
chloroplastic protein import deserved reinvestigation. In par-
ticular, we recognized that prSS is translocated across only
the two envelope membranes into the stroma. These mem-

branes are not expected to carry much of a protonmotive
force, since energy transduction in chloroplasts occurs using
the thylakoid membrane. If the chloroplastic and mitochon-
drial situations were similar, one might expect a protonmotive
force requirement for the translocation of proteins across the
thylakoid membrane, but not necessarily for transport across
the envelope membranes. We, therefore, investigated the effect
of ionophores on the import of prPC, which is known to be
targeted to the thylakoid lumen (15).

The effects of ionophores at specific concentrations on the
energetics of isolated thylakoids have been well characterized
(see Ref. 18 for a review). Somewhat higher concentrations of
ionophores may be required to produce the same effects in
intact chloroplasts (19), although this has not usually been
considered a problem. Recently, Nishio and Whitmarsh (20)
reported that as much as 50-fold higher concentrations of
valinomycin and gramicidin are required to accelerate the
decay of the thylakoid transmembrane electric field in intact
chloroplasts compared with naked thylakoids. In order to
avoid the possibility that we would not add a sufficient con-
centration of a particular ionophore to dissipate the targeted
energetic component of membrane, we titrated the amount of
nigericin required to inhibit light-mediated protein import
into intact chloroplasts. As shown in Fig. 1, nigericin inhibited
light-mediated precursor binding by 63% and translocation
into intact chloroplasts by 86% at concentrations below 0.5
μM. This amount of nigericin can then be assumed to be
sufficient to uncouple electron transport from ATP synthesis
at the high Chl concentrations used in our experiments.
Furthermore, since other commonly used ionophores, i.e. vali-
nomycin and gramicidin, affect thylakoid functions in the
same concentration range as does nigericin, we can expect that
similar concentrations of those compounds will also be
effective in intact chloroplasts.

The experiments depicted in Fig. 2 and the bottom panel of
Fig. 3 demonstrate that the ATP-dependent import of prPC
into chloroplasts in the dark was unaffected by nigericin,
valinomycin, or gramicidin at concentrations as high as 5 μM.
In addition, these ionophores did not affect ATP-mediated
import of prSS or prFD (Fig. 3, top two panels). These

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\[ \text{membrane transport in chloroplasts.} \]
ionophores (at 5 μM) should have completely dissipated any proton gradients (nigericin, gramicidin) or electric fields (valinomycin, gramicidin) present across any of the chloroplastic membranes (18). We conclude, therefore, that a protonmotive force is not required for the import of precursors across the chloroplastic envelope membranes, or for the translocation of PC across the energy-transducing thylakoid membrane.

**ATP Is Utilized Inside Chloroplasts to Support Protein Import: Manipulation of Stromal and Extrachloroplastic ATP Levels**—Three studies have recently addressed the question of the side of the envelope membranes at which ATP is required during protein import into chloroplasts (11-13). In each instance, the internal and external ATP concentrations were experimentally manipulated, and the resultant effect on protein import was determined. The results of such experiments have led two groups to conclude that ATP is required inside chloroplasts (13). In only one case was the ATP content of the chloroplasts actually measured under conditions similar to those used for the import reactions (12).

The side of the membrane on which ATP is required has a profound influence on the types of mechanisms one can postulate to govern protein import into chloroplasts. Because of the ambiguities noted in the previous studies, we felt this problem warranted further examination. Our approach was to manipulate the ATP content of chloroplasts as in the earlier studies, but in addition, to measure, where possible, the actual ATP concentrations of the internal (stromal) and external (extrachloroplastic) phases in the same samples that were analyzed for protein import. This is feasible when the import assays are terminated by centrifuging intact chloroplasts through silicone oil into perchloric acid; the chloroplasts rupture upon entering the acid, leaving ATP in the acid solution and precipitated protein as a pellet in the bottom of the tube.

The results of one such experiment are shown in Fig. 4. Here the import of prSS was measured under conditions which establish different levels of internal and external ATP. For each condition, the amount of SS imported was determined at 3, 6, and 9 min and is represented by the three bars in each group, respectively. The ATP concentration in the external medium, and in those samples to which ATP had not been exogenously added, the level of ATP inside the chloroplasts, was also determined (numbers along the top of the figure). The first group shows that SS was imported very poorly when no additions were made to the chloroplasts. Direct measurements indicated that the internal ATP level was in fact low, and no ATP could be detected in the external medium. ATP was added to the second group, and SS import was observed to proceed at a considerable rate.

The data represented by the third group in Fig. 4 were obtained in an attempt to increase the internal ATP concentration while simultaneously depleting the external medium of ATP. The combination of dihydroxyacetone phosphate, oxaloacetate and inorganic phosphate (dihydroxyacetone phosphate/oxaloacetate) allows the Calvin cycle to run backward past the step at which ATP is normally utilized in the conversion of 3-phosphoglycerate to 1,3-bisphosphoglycerate, thereby generating ATP in the stroma (11, 12). The presence of glucose and hexokinase (glu/hex), the latter of which is too large to penetrate the outer envelope membrane, ensures that no ATP is present in the external medium. ATP measurements showed that the internal ATP level was elevated approximately 3-fold by the addition of dihydroxyacetone phosphate-oxaloacetate, while ATP remained undetectable in the external solution. Under these conditions SS import proceeded at a rate even higher than that observed with added

**Fig. 3.** Ionophores have no effect on the ATP-mediated import of prSS, prFD, and prPC into chloroplasts in the dark. All samples contained 5 mM ATP; when present, the concentration of ionophores was 5 μM.

**Fig. 4.** Manipulation of internal and external ATP levels and its effect on prSS import into chloroplasts in the dark. The three bars in each of the six groups represents the extent of prSS import occurring after 3, 6, and 9 min, respectively. The numbers above the bars refer to an average determination of the ATP content of the chloroplasts at the three time points in units of nmol/mg Chl; n.d. = not determined. The extent of prSS import and the external ATP concentrations and internal chloroplastic ATP contents were determined in the same samples as described under “Materials and Methods.” Results of the ATP measurements are written above the appropriate samples across the top of the figure; out = external ATP concentration in mM (0.00 represents [ATP] < 0.01 μM). 1 mM ATP corresponds to 3 μmol of ATP/mg Chl, in = internal ATP in nmol/mg Chl. Where indicated, the concentrations of the added reagents were: ATP, 1 mM; dihydroxyacetone phosphate (DHAP), 1 mM; oxaloacetate (OAA), 1 mM; glu = glucose, 10 mM; hex = hexokinase, 2 units in 60 μl; glyc = glycercate, 10 mM; 10 mM inorganic phosphate was also added to group 3.
ATP (second group). (Measurements of hexokinase activity on the same day revealed that the enzyme was indeed active; data not shown.)

In order to deplete the stroma of ATP, glycerate, which is taken up into the stroma and phosphorylated inside chloroplasts by glycerate kinase at the expense of internal ATP (12), was added to the samples represented by the fourth group in Fig. 4. At the same time, exogenous ATP was provided. It was anticipated that under these conditions the extrachloroplastic concentration of ATP would be high while the stromal ATP level would be low. However, measurements of the actual ATP levels revealed that glycerate caused a depletion of the external ATP to approximately one-fourth of the original concentration. We currently have no explanation for this effect. Compared with the third group, the rate of protein import in these samples was substantially reduced. However, the low rate of import cannot be due solely to the lower external ATP levels since the third group displayed lower import rates despite the complete absence of any external ATP. Thus, the third and fourth groups, when taken together, provide strong evidence that protein import is supported by ATP inside, rather than outside, the chloroplasts.

The fifth and sixth groups in Fig. 4 show SS import when ATP was absent from both the internal and external phases, or when ATP was present in both phases, respectively. The ATP levels in the first and fifth groups of samples reflect the background levels inside dark-adapted chloroplasts. Thus, the lack of protein import in the absence of added ATP is a consequence of the lack of internal ATP in those samples. The rate of protein import in the presence of both internal and external ATP (group 6) was similar to that observed when only internal ATP was present (group 3). Similar results were obtained using prPC and prFD (data not shown), indicating that the internal ATP requirement is probably general for all chloroplastic protein precursors.

**ATP Is Utilized Inside Chloroplasts to Support Protein Import: Kinetic Evidence—**In a second approach to the question of the site of ATP utilization during protein import into chloroplasts, we have taken advantage of the observation that the initial rates of PC import are higher in the light than in the dark, it must be transported into the chloroplasts on the adenylate translocator (21) before it is available for use in protein import. If this explanation is correct, we reasoned that the initial rate of protein import in the dark should be increased by preloading chloroplasts with ATP prior to the addition of precursor.

The results of the experiment depicted in Fig. 5 are in agreement with this hypothesis. Protein import began almost immediately upon addition of prPC when the chloroplasts were preincubated for 15 min in the presence of ATP, and after only a short lag phase when import was driven by illumination. In contrast, when the precursor was added to the chloroplasts at the same time as ATP, a significantly longer lag phase was observed, and the overall rate of import was somewhat lower. Interpretation of these kinetic results might be confounded by the fact that PC is transported into the thylakoid lumen in two steps (15). However, similar results were obtained for the mechanistically less complicated import kinetics of prFD (data not shown).

In the experiment shown in Fig. 6, chloroplasts were preincubated with 0–5 mM ATP for 15 min. At the end of the preincubation period, precursor and additional ATP were added simultaneously such that the ATP concentration at the beginning of the import reactions was 5 mM for all samples. The reactions were terminated at 4 min, a point at which the rate of import is still nearly linear. Clearly, the amount of precursor taken up by the chloroplasts in 4 min was correlated with the amount of ATP present inside the chloroplasts at the time the precursors were added. These results were obtained with prPC, prSS (Fig. 6), and prFD (not shown), indicating that this phenomenon, the dependence of the import kinetics on the concentration of ATP present inside the chloroplasts at the onset of the import reaction, is probably not restricted to any particular precursor.

Fig. 7 shows the results of an experiment in which an attempt was made to slow the entry of ATP into the chloroplasts using o-ATP, a partial inhibitor of the envelope adenylate translocator (12). We reasoned that if the relatively slow penetration of ATP into the chloroplasts was responsible for the lag in protein import, this lag might be extended by slowing the transport of ATP even further. As shown in Fig. 7 (open symbols), the lag in the import of prPC was eliminated.
when chloroplasts were preincubated with ATP. The data depicted by the closed symbols represent the results of the same experiment when o-ATP was added concomitantly with the precursor. It can be seen that when protein import depended on the transport of ATP across the envelope membranes o-ATP caused an additional lag and decrease in the rate. When the chloroplasts were preloaded with ATP before the addition of precursor and o-ATP, the initial rate of prPC import was similar to that observed in the absence of o-ATP. This indicates that o-ATP had no significant effect on the rate of protein import per se. Taken together, the experiments of Figs. 5–7 provide evidence, independent of that presented in Fig. 4, that ATP inside the chloroplasts is responsible for protein import into the organelle.

**DISCUSSION**

In both this and the accompanying paper (14), we have re-examined the role of energy in the process by which cytoplasmically synthesized precursor polypeptides are imported into chloroplasts. Whereas the preceding paper focused on a previously unrecognized requirement of ATP for efficient binding of precursors to the outer surface of chloroplasts, we have in this paper addressed questions dealing with the translocation step. Although we have not attempted to distinguish between the binding and translocation steps in this paper, we suggest that the energy requirements studied here apply to the translocation step. In particular, we have demonstrated that a proton motive force plays no role in the translocation and localization of proteins destined for the stroma or lumen compartments, and that ATP within the chloroplasts can be the sole source of energy supporting protein translocation.

In mitochondria, protein import to the matrix requires that an electric field be established across the energy-transducing inner mitochondrial membrane. The analogous membrane in chloroplasts is the thylakoid membrane. However, unlike the inner mitochondrial membrane, thylakoids utilize a transmembrane pH gradient rather than an electric field to drive ATP synthesis (cf. 18). Thus, if the chloroplastic and mitochondrial import systems are analogous, one might expect that a proton motive force would be required for transport of proteins across the thylakoid membrane and that the component used might be a proton gradient. Our finding that a proton motive force is not required for the translocation of prPC to the thylakoid lumen (Figs. 1–3) points to one clear difference between the mechanisms used to translocate proteins across the membranes of chloroplasts and mitochondria.

The question of the site of ATP utilization for the import of proteins into the chloroplast stroma was a matter of controversy which we sought to settle. Like others, we performed experiments in which the internal and external chloroplastic ATP levels were manipulated and examined the resultant effect on protein uptake. Our results do not agree with those published by Schindler et al. (11) which showed that conditions resulting in high internal and low external levels of ATP inhibited protein import. On the contrary, we found that those very conditions allowed protein uptake to occur at high rates (Fig. 4). In addition, our results apply to several precursors (data not shown). The reasons for the discrepancy between our results and those of Schindler et al. are at present unknown.

Flugge and Hinz (12) also concluded that ATP is required outside chloroplasts for protein import. In addition to measuring the effects of different ATP manipulations on protein import, they also measured separately the effects of those treatments on the internal ATP level itself. They found import to be correlated with low internal and high external ATP levels. We can offer a number of possibilities for the discrepancies between their results and ours. First, we found some variability in the ability of glycerate to act as an effective internal trap for ATP. We have assumed that this reflects intrinsic variability in the rates of glycerate uptake and phosphorylation compared with the rate of transport of ATP on the adenylate translocator. Flugge and Hinz’s measurements of ATP levels and protein import were apparently carried out as different experiments, allowing for the possibility that glycerate produced different results on different days. Second, Flugge and Hinz observed that the external ATP trap alkaline phosphatase inhibited prSS import and interpreted this to mean that ATP was required outside the chloroplasts. We also obtained this result using alkaline phosphatase (data not shown). However, they point out that alkaline phosphatase is not specific for the hydrolysis of ATP and in fact probably did not act in their experiment by removal of external ATP. This argument was based on their observation that neither hexokinase/glucose nor fructose-6-phosphate kinase/fructose-6-phosphate, both efficient external ATP traps, significantly affected the import of prSS. Notably, this is precisely the result published by Pain and Blobel (13), and obtained by us (Fig. 4, third group) which led us to conclude that ATP is required inside the chloroplasts for protein import. Since we measured both the internal and external ATP levels under these conditions in a number of experiments, and since protein import was measured in the same sample used for ATP determinations, we feel confident in interpreting this portion of our experiment, i.e. protein import can occur at high rates when the internal level of ATP is elevated and the external medium is completely devoid of ATP. This possibility that external ATP may also be involved in the import process cannot be ruled out by our experiments since the combination of glycerate plus ATP did not always inhibit import completely (Fig. 4, fourth group). However, complete inhibition was observed on a number of occasions (not shown), and we believe that our results are best explained on the basis of a variability in the efficiency with which glycerate can act as an internal trap for ATP. Taking the results of Fig. 4 with the kinetic experiments presented in Figs. 5–7, we feel the data strongly support the conclusion that internal, not external, ATP drives protein import into chloroplasts.

It should be noted that our data do not preclude the possibility that ATP is utilized in the space between the two chloroplastic envelope membranes. This idea was recently embraced by Hinz and Flugge (22) who correlated the transient phosphorylation of a protein located in the outer enve-
lope membrane with protein import. However, an examination of the relative amounts of ATP required for phosphorylation of the envelope protein (22), precursor binding to chloroplast surfaces (14), and protein import (12) suggests that the transient phosphorylation event characterized by Hinz and Flugge may be more closely related to the binding of precursors than to their translocation. Furthermore, our kinetic experiments (Figs 5–7) cannot be readily explained on the basis of ATP utilization for protein translocation in the intermembrane space. Thus, we believe the evidence most readily favors the hypothesis that the ATP involved in the translocation of proteins into chloroplasts is located in the stroma.

It is interesting to consider where in the overall picture of protein translocation across biological membranes the process of protein import into chloroplasts fits. In principle, one might expect different mechanisms for protein translocation across membranes to be distinguishable on the basis of their respective energy requirements. The secretion of proteins from bacteria depends strictly on the presence of ATP but may be enhanced by a protonmotive force (23). The import of certain proteins into mitochondria requires both ATP and an membrane potential across the inner mitochondrial membrane (1, 5–8). Among these processes which probably strictly require ATP (or NTP) but no component of a protonmotive force are the post-translational import of proteins into the endoplasmic reticulum (24), and separately, their subsequent movement to (25) and through the Golgi apparatus (26), and the post-translational uptake of polypeptides into chloroplasts (9, 11–13, this work), nuclei (27), and peroxisomes (28). Finally, an energy requirement for the translocation of proteins from the mitochondrial matrix to the intermembrane space has yet to be demonstrated (29, 30). Strictly speaking, the same can be said of the translocation of luminal-directed proteins across the thylakoid membrane from the stroma, although our experiments do not rule out an involvement of ATP in that process. The wide diversity of energy requirements for different protein translocation processes would appear to preclude the notion that all translocation systems will be found to operate by the same fundamental mechanism, as has been suggested (3, 4).

The unexpected differences between protein import into chloroplasts and mitochondria deserve further discussion. These organelles are similar in that they both probably entered prokaryotic cells as endosymbionts, both contain their own genomes and protein synthesizing machinery, both contain energy-transducing membranes consisting of similar components, and cytosolic proteins targeted to both organelles contain amino-terminal transit peptides with somewhat similar properties. On the other hand, a number of observations point to differences in the mechanisms used to import cytosolic proteins into those organelles. The transit peptides of chloroplast-targeted precursors, although rich in hydroxylated and basic amino acids, as are the mitochondrial presequences, show no predicted propensity to form amphipathic helices. The amphipathic tendencies of mitochondrial transit peptides are thought to play a key role in the import of the attached passenger proteins into that organelle (31).

Added to this are the earlier (9, 11–12) and present works which demonstrate that the energy requirement for the import of proteins into chloroplasts and mitochondria are different in at least three respects. First, the initial binding of the precursors to the outside of the chloroplasts depends on ATP (14), whereas binding to mitochondria does not (cf. Ref. 14). Second, an electric field is required in addition to ATP for the import of most mitochondrial precursors (5–8), while ATP alone suffices to support import into chloroplasts (Figs 1–3, (11, 12)). Finally, experiments suggest that ATP is utilized outside mitochondria for protein import (7, 8), whereas our experiments (and those in Ref. 13) indicate that the ATP which drives protein uptake into chloroplasts is inside the organelle (Figs 4–7). At present it is not possible to decide whether these differences between protein import into mitochondria and chloroplasts represent variations of a common mechanism or whether they indicate that fundamentally different mechanisms operate in the two organelles. They do, however, underscore the danger in relying too heavily on the larger mitochondrial literature to interpret results obtained with chloroplasts.

One implication of our finding that internal ATP drives protein import into chloroplasts concerns the possibility that ATP is utilized to unfold precursors in the external medium prior to their binding and translocation into the stroma. In 1986 Eilers and Schatz (32) postulated that mitochondrially targeted precursors unfold prior to and during import into those organelles and that the unfolding is mediated by ATP. Their postulate was based in large part on the ability of methotrexate to inhibit the import of a chimeric construction containing mouse dihydrofolate reductase as a passenger protein. This hypothesis has gained wide acceptance as consistent evidence has accumulated from a number of laboratories over the past 2 years (cf. Ref. 1). Recently, della-Cioppa et al. (33) interpreted their finding that glyphosate partially inhibited the import of the precursor to 5-enolpyruvylshikimate-3-phosphate synthase into chloroplasts in a similar fashion; they postulated that chloroplastic precursors also must unfold (or at least undergo a conformational change) prior to their import. Our finding that internal ATP drives import would appear to preclude the possibility that precursor unfolding under the influence of cytosolic ATP is a critical step in the transport of proteins into chloroplasts.

It is difficult to postulate a mechanism through which internal ATP mediates both the binding of precursors to the external surface of the outer envelope membrane and their subsequent translocation. It may be more likely that separate ATP-dependent mechanisms govern the binding and translocation steps and that the ATP utilized respectively resides in different locations within the chloroplasts. The most attractive hypothesis at present concerning the way in which ATP is utilized during import was put forth by Schindler et al. (11) and Flugge and Hinz (12) and posits that ATP is needed for the transient phosphorylation of some component of the import machinery. Transient phosphorylation of proteins in isolated chloroplastic envelopes has indeed been observed (34), and more recently, a precursor-dependent transient phosphorylation of an outer envelope protein in intact chloroplasts has been described by Hinz and Flugge (22). Whether this protein is part of the molecular import machinery and whether its phosphorylation is in fact required for precursor binding or protein translocation remain interesting problems to be addressed in the future.

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