ATP Is Required for the Binding of Precursor Proteins to Chloroplasts*

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One of the first steps in the transport of nuclear-encoded, cytoplasmically synthesized precursor proteins into chloroplasts is a specific binding interaction between precursor proteins and the surface of the organelle. Although protein translocation into chloroplasts requires ATP hydrolysis, binding is generally thought to be energy independent. A more detailed investigation of precursor binding to the surface of chloroplasts showed that ATP was required for efficient binding. Protein translocation is known to require relatively high levels of ATP. As little as 50–100 μM ATP caused significant stimulation of precursor binding over controls with no ATP. Several different precursors were tested and all showed increased binding upon addition of low levels of ATP. Nonhydrolyzable analogs of ATP did not substitute for ATP, indicating that ATP hydrolysis was required for binding. A protonmotive force was not involved in the energy requirement for binding. Other (hydrolyzable) nucleotides could substitute for ATP but were less effective at stimulating binding. Binding was stimulated by ATP generated inside chloroplasts even when an ATP trap was present to destroy external ATP. We conclude that internal ATP is required for stimulation of precursor binding to chloroplasts.

Nuclear-encoded chloroplastic proteins are synthesized as higher molecular weight precursors in the cytoplasm and posttranslationally imported into the organelle (1). Transport is initiated by a specific binding interaction between the precursor protein and a component of the transport apparatus, probably a proteinaceous receptor, on the surface of the chloroplastic envelope (2–4). Next, the precursor polypeptide is translocated across the envelope; the exact mechanism for this step is largely unknown. Following import, the precursor is proteolytically processed to its mature size, localized, and assembled into a functional macromolecular complex (5).

Protein import into chloroplasts is energy dependent (2, 6–10). Several groups have shown that the hydrolysis of MgATP is required; a transmembrane electrochemical potential across the chloroplastic envelope is not involved in translocation (6–10). More recently, the question of whether ATP for import is required inside or outside chloroplasts has been addressed. Flugge and Hinz (8) and Schindler et al. (7) concluded that ATP is required outside the stromal space, possibly in the intermembrane space of the envelope (8). However, Pain and Blobel (9), using approaches similar to the other groups, concluded that protein transport requires the hydrolysis of ATP inside chloroplasts. In a companion paper (10), we have re-examined this controversy. Using a combination of direct ATP measurements and kinetics of import, we provide evidence that internal ATP is required for chloroplastic precursor import (10).

Energy requirements for translocation of precursor proteins into mitochondria have been more extensively studied (11–15). Both ATP and a transmembrane potential are required for import of mitochondrial precursor proteins. ATP hydrolysis is needed outside the inner membrane. In many instances, binding of precursor proteins to mitochondria is also energy dependent. A transmembrane potential is required for some precursors to bind to mitochondria (13, 16), while others (e.g. ATPase β-subunit) may instead require ATP for the binding step (15, 17). One popular hypothesis proposes a role for ATP in unfolding the precursor in the cytosol prior to or during precursor binding and translocation into mitochondria (12, 13, 17–19).

An energy requirement for binding of precursor proteins to chloroplasts, however, has not been previously demonstrated. In this paper we provide evidence that ATP hydrolysis is required for binding to chloroplasts using precursors to ribulose-1,5-bisphosphate carboxylase small subunit (SS), ferredoxin, and plastocyanin. A membrane potential is not needed, in the presence of ATP, for binding. The ATP for precursor binding is required inside chloroplasts, either in the stromal space or in the intermembrane space between the envelope membranes.

EXPERIMENTAL PROCEDURES

Materials—ATP (Mg2+ and Na+ salts), AMP-PCP, AMP-PNP, valinomycin, A23187, carbonyl cyanide m-chlorophenylhydrazone, and gramicidin were obtained from Sigma. CTP, GTP, UTP, and ADP (all Na+ salts) were obtained from Pharmacia LKB Biotechnology Inc. Nigericin was from Calbiochem. [3H]Leucine, approximately 140 Ci/mmol, was purchased from Du Pont–New England Nuclear. Wheat germ was the gift of General Mills Inc. The plasmid phe85 (20), containing the gene for pea SS, was provided by Dr. A. R. Cashmore. The plasmid containing the gene for pea plastocyanin precursor was provided by D. Last and Dr. J. Gray. The pea ferredoxin precursor clone was isolated by Dr. W. Thompson's lab and subcloned into an expression vector by Dr. D. Murray and Dr. B. Kohorn before it was provided to us.

* The abbreviations used are: SS, small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase; prSS, precursor to SS; Hepes, 4-(2-hydroxyethyl) P-piperazineethanesulfonic acid; DHAP, dihydroxyacetone phosphate; AMP-PCP, adenylyl-(β,γ-methylene) diphosphate; AMP-PNP, 5′-adenylyl-imidodiphosphate.

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Chloroplast Isolation—Intact chloroplasts were isolated from 10- to 15-day pea (Pisum sativum var. Little Marvel) seedlings as described (21). Isolated chloroplasts were suspended in import buffer (330 mM sorbitol, 50 mM Hepes/KOH, pH 8) at a concentration of 1 mg chlorophyll/ml (1-3 x 10⁸ chloroplasts/ml) and kept on ice in the dark until use.

Precursor Preparation—The full length precursor polypeptides used were obtained from SP6 in vitro expression vectors which were linearized with an appropriate restriction enzyme. Transcription with SP6 polymerase was as described (22). Triticum-labeler precursor proteins were synthesized in a cell-free wheat germ system (2).

Following translation, the precursors were centrifuged through Sephadex G-25 to remove small molecules, especially ATP, from the translation mixture. The G-25 columns were prepared in 1-ml disposable syringes, with glass fiber filters at the bottom and top, after equilibration of the beads in import buffer. The syringes were held in 15-ml Falcon tubes during the centrifugation steps. The columns were washed three times with import buffer by centrifugation at 1700 g, 2 min at speed, and then treated with 2% bovine serum albumin in import buffer (1700 x g, 2 min at speed). A 200-μl aliquot of 30% translation mixture was loaded onto the column, gel-filtered by centrifugation at 1700 x g for 2 min at speed, and collected in microcentrifuge tubes below the columns.

The relative efficiency of the G-25 columns at removing small molecules from the translation mixture was monitored by measuring free versus incorporated ['H]leucine (total versus trichloroacetic acid-precipitable radioactivity), as well as direct ATP measurements (10), before and after gel filtration.

Binding and Translocation Assays—All reactions contained 50 μl of chloroplasts (1 mg of chlorophyll/ml), filtered translation mixture equivalent to about 10⁷ dpm trichloroacetic acid-precipitable radioactivity (5-20 μl), and import buffer in a final volume of 150 μl. Other additions were as noted in the figure legends. Binding and translocation assays differed only by the amount of ATP present during the assays. Unless otherwise noted, all reactions were performed for 10–15 min in the dark at room temperature. Following binding (or translocation), samples were prepared for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described by Cline et al. (2).

Quantitation—Analysis of bound (organelle-associated precursor) and translocated (mature-sized) proteins was as described (2) except that ['H]-labeled proteins were extracted from sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel slices by rehydration in 0.25 M urea and translocated (mature-sized) was computed from the dpm in extracted gel bands, the specific radioactivity of leucine, and the number of chloroplasts/mg of protein (usually about 2 x 10⁹).

RESULTS

Removal of ATP from the Translation Mixture—ATP and an ATP-regenerating system are present in the cell-free wheat germ translation reaction mixtures. In order to study the energetics of binding, it was necessary first to remove this ATP from the translation mixture. To accomplish this, translation mixtures were passed through Sephadex G-25 columns, as described under "Experimental Procedures." Table I shows typical results when a solution containing prSS translation mixture was passed through a Sephadex G-25 column. An aliquot of the filtered precursor was assayed for ATP and for free versus incorporated leucine (Table I). Virtually all of the free leucine was removed by this procedure; only acid-precipitable radioactivity remained in the filtered precursor translation product. At least 98-99% of the initial ATP was also removed, leaving a final ATP concentration in the filtered translation mixture of about 0.1 μM. When the other components of a binding reaction (import buffer, chloroplasts, reagents) were added, this level of ATP was diluted to less than 10 nM.

ATP Is Required for Precursor Binding to Chloroplasts—The capacity of filtered precursors to function in the binding and translocation steps of import was examined. As expected,

**TABLE I**

| % ['H]Leucine | ATP (μM) |
|--------------|---------|
| Before gel filtration | 890 |
| Incorporated | 100 |
| After gel filtration | 18 |

* 100% = 774,779 dpm/μl, corresponding to 2.38 pmol of leucine/μl.

**FIG. 1. ATP requirement for binding.** Intact pea chloroplasts (equivalent to about 50 μg of chlorophyll) were incubated with ['H]leucine (3200 molecules/chloroplast) and ATP (0-1000 μM) in the dark for 15 min. Chloroplasts were then repurified on a 40% Percoll cushion, washed once with import buffer, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis/fluorography. Panel A shows a photograph of the fluorogram. Panel B shows the results of quantitative analysis of the number of molecules bound (prSS) or translocated (SS) per chloroplast in each assay (see "Experimental Procedures").

filtered precursors lacking ATP were not imported into chloroplasts in the dark (Fig. 1), whereas they could be imported in the light (data not shown). Nevertheless, it was expected that filtered precursors would bind to chloroplasts in the dark. However, very little binding was actually observed (Fig. 1, lane 1). Several possible explanations for the poor binding...
were investigated, and it was found that addition of ATP to filtered precursors stimulated binding (Fig. 1). At very low ATP levels (1–10 μM), precursor binding was low, but binding increased steadily with increasing ATP, reaching a maximum level of binding at about 75–100 μM ATP. (At levels of ATP which support precursor binding, but not translocation, 6–10% of the prSS presented binds to chloroplasts.) Mature SS appears at higher ATP concentrations, where ATP levels were sufficient to support translocation. The low ATP requirement for precursor binding has not been observed before because import reactions have been performed with enough ATP present to support both binding and translocation (2, 6–9).

The binding seen in these experiments is physiologically significant, as judged by the ability of bound precursors to be translocated in the presence of additional ATP. Between 60–75% of gel-filtered prSS bound in the presence of 75 μM ATP in the dark was subsequently translocated upon the addition of 1 mM ATP (data not shown). Bound prSS was protease sensitive; translocated SS was protease resistant. At least 90% of the prSS bound in assays containing 100 μM ATP (enough to support binding but not translocation) remained bound to the chloroplasts during three washes (data not shown), regardless of whether or not the wash buffer included 100 μM ATP. Therefore, binding of prSS to chloroplasts was not readily reversible.

The ATP requirement for binding to chloroplasts was not limited to prSS. A significant stimulation of binding by low levels of ATP was observed for precursors for SS, ferredoxin, and plastocyanin (data not shown). In contrast, energy requirements for binding apparently differ between mitochondrial precursors (13, 16, 17, 23; also see discussion below).

*Nonhydrolyzable ATP Analogs Cannot Substitute for ATP*—AMP-PCP and AMP-PNP, two nonhydrolyzable analogs of ATP, did not stimulate precursor binding when included in binding assays in the absence of ATP (Fig. 2). Even high concentrations of the analogs were insufficient to support either binding or translocation. When ATP and AMP-PNP were added in equimolar amounts to the binding assays, ATP was unable to support binding (data not shown). Binding and translocation were restored to near control levels when the ATP concentration was at least 10 times greater than the concentration of nonhydrolyzable analog.

*Other Hydrolyzable Nucleotides Can Substitute for ATP but Are Less Effective*—The effect of other hydrolyzable nucleotides (CTP, GTP, UTP, and ADP) on chloroplastic precursor binding was also examined. Each nucleotide stimulated prSS binding above control (no ATP) levels, but only about 60–70% as well as ATP (Table II). Similar results were obtained with the precursor to pea ferredoxin (data not shown). Some of this effect may be due to contaminating ATP in the nucleotide preparations. Since only a small amount of ATP is needed to increase binding, only low concentrations of ATP would need to be present in the other nucleotide preparations in order to affect binding. To check this possibility, an external ATP trap (hexokinase/glucose) was added to the binding assays with CTP or GTP. All contaminating ATP should have been removed by the trap, so any observed effects on binding would be due to CTP or GTP alone. Results from these experiments showed that binding was lower in the presence of the trap than without it (data not shown) but could not account for all of the stimulation of binding seen with CTP or GTP.

*A Protonmotive Force Is Not Involved in Precursor Binding to Chloroplasts*—It seemed possible that an electrical potential across the chloroplast envelope may have contributed to the observed ATP requirement for binding. To test this, isolated intact chloroplasts were incubated with several ionophores prior to precursor binding. Chloroplasts were preincubated with A23187 (a divalent cation/H+ antiporter) or nigericin (a H+/K+ antiporter) to collapse the pH gradient, valinomycin (a K+ uniporter) to dissipate the membrane potential, and gramicidin (a channel former), carbonyl cyanide n-chloro-phenylhydrazone (a H+ uniporter), or nigericin + valinomycin to collapse the total proton motive force. In the presence of ATP, each of the above reagents had very little effect on prSS binding (Fig. 3).

**Fig. 2.** Effect of nonhydrolyzable analogs of ATP on precursor binding. The experimental conditions were similar to those in Fig. 1, except that each assay received either ATP, AMP-PCP, or AMP-PNP at the concentrations (micromolar) indicated. Panel A shows a photograph of the fluorogram. Panel B shows the results of quantitative analysis of the number of molecules bound (prSS) or translocated (SS) per chloroplast in each assay (see "Experimental Procedures").

**Table II**

| Nucleotide | % ATP Control |
|-----------|---------------|
| ATP       | 100           |
| CTP       | 69            |
| GTP       | 55            |
| UTP       | 64            |
| ADP       | 67            |

Binding assays were performed as described in Fig. 2. All nucleotides in each experiment were tested at the same concentration (50 μM in two experiments, 75 and 100 μM in one experiment each). The numbers represent the average of all four experiments.
To each assay. Half of the samples also received ATP (final concentration 100 μM). Chloroplast suspensions were incubated in the dark in the presence and absence of ATP. The effect of various ionophores on precursor binding was similar to those in Fig. 1, except that the binding assays were incubated for 10 min and contained the indicated levels of glycerate to remove internal ATP. Thus, glycerate, by depleting the chloroplast stroma of ATP, indirectly inhibited precursor binding. Although high concentrations of glycerate were unable to completely block binding, the general effect was clear. When glycerate was present in addition to externally added ATP, the extent of ATP-dependent stimulation of binding was less pronounced than when glycerate was absent (Fig. 5). On the other hand, when ATP was generated inside chloroplasts by DHAP, in the presence of an external ATP trap, binding was significantly stimulated (Fig. 4). These results demonstrate that internal, rather than external, ATP supports precursor binding to chloroplasts.

**DISCUSSION**

The energy dependence for transport of precursor proteins into chloroplasts has been known for many years (6) and has been recently reconfirmed by several investigators (2, 7–10). Many of these investigators observed binding, and indeed, Cline et al. (2) focused on the binding step. It was assumed in those studies that binding did not require energy because binding occurred under conditions where there was not enough ATP to support translocation. In fact, the data in most of these earlier reports is consistent with our conclusion that binding requires a level of ATP approximately 10-fold lower than is required for translocation. Cline et al. (2) blocked translocation by preventing ATP synthesis in the light with nigericin (a K⁺/H⁺ ionophore); this block could be overcome with exogenous ATP added separately or even present in the translation mixture. They solved this latter problem by diluting their translation mixture sufficiently such that the ATP present would support binding, but not translocation. As shown in Table I, nonfiltered precursor mixtures contain enough ATP (900 μM) to support binding, even after dilution in the reaction assay (it would still be greater than 100 μM ATP). Thus, gel filtration of the translation mixture to remove ATP was crucial to our demonstration of an ATP requirement for binding.

Flugge and Hinz (8) and Schindler et al. (7) removed ATP from their translations by ammonium sulfate precipitation of the proteins followed by dialysis. The ATP requirement for binding was not seen by Flugge and Hinz (8) because they generally conducted import reactions in the light or in the dark in the presence of 0.3–3 mM ATP. Careful inspection of

**Fig. 3.** The effect of various ionophores on precursor binding in the presence and absence of ATP. Intact chloroplasts (equivalent to 50 μg of chlorophyll) were incubated in the dark with the ionophores (5 μM) indicated for 5 min. Precursor was then added to each assay. Half of the samples also received ATP (final concentration 100 μM). Chloroplast suspensions were incubated in the dark for an additional 15 min. Each assay was prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed as described in Fig. 1. The results are normalized to controls containing 100 μM ATP alone (100% corresponds to about 260 molecules prSS/chloroplast). Each lane represents the average from three experiments.

**Fig. 4.** Precursor binding to chloroplasts in the presence of internally generated ATP or exogenously added ATP. The experimental conditions were similar to those in Fig. 1, except that the binding assays were incubated for 10 min. The assays using DHAP to generate internal ATP also contained 1 mM oxaloacetate and 10 mM P; plus an external ATP trap consisting of 2 units of hexokinase and 10 mM glucose/assay. prSS, ATP; SS, AT; prSS, DHAP + hexokinase/glucose; SS, DHAP + hexokinase/glucose.

**Fig. 5.** Precursor binding to chloroplasts in the presence or absence of an internal ATP trap. The experimental conditions were similar to those in Fig. 1, except that the binding assays were incubated for 10 min and contained the indicated levels of glycerate. The results are normalized to controls containing 100 μM ATP. Careful inspection of added external ATP caused decreasing levels of binding (Fig. 5). Thus, glycerate, by depleting the chloroplast stroma of ATP, indirectly inhibited precursor binding. Although high concentrations of glycerate were unable to completely block binding, the general effect was clear. When glycerate was present in addition to externally added ATP, the extent of ATP-dependent stimulation of binding was less pronounced than when glycerate was absent (Fig. 5). On the other hand, when ATP was generated inside chloroplasts by DHAP, in the presence of an external ATP trap, binding was significantly stimulated (Fig. 4). These results demonstrate that internal, rather than external, ATP supports precursor binding to chloroplasts.

precursor binding, two sets of experiments were performed. In one set, ATP was generated inside chloroplasts in the dark by the addition of the Calvin cycle intermediates DHAP, oxaloacetate, and P; (7, 9, 10). To remove ATP that may be translocated out of chloroplasts, a chloroplast impermeable ATP trap consisting of hexokinase and glucose (7, 9, 10) was also included. Thus, only internal ATP should be present in these experiments. Although ATP was not measured in the binding experiments, measurements made on similar assays confirmed that these conditions produced high internal and low (or no) external ATP (10). Binding of prSS in response to increasing concentrations of DHAP (Fig. 4) was similar to that observed with increasing concentrations of added ATP. Measurements of hexokinase activity on the same day confirmed that the enzyme was active (data not shown).

In the second set of experiments, ATP was present outside chloroplasts, while internal ATP levels were lowered by the action of glycerate kinase on exogenously added glycerate (8, 10). Increasing concentrations of glycerate in the presence of...
their data reveals that binding did not occur in the dark without added ATP. Schindler et al. (7) did not show enough of the autoradiograms to allow an evaluation of precursor binding in their energetics experiments; only the translocated form was shown. Pain and Blobel (9) did not remove ATP (or the ATP regenerating system) from the translation. However, it was diluted so much in the assays that the ATP contributed by translation mixture would not have been enough to support binding. In the dark, with no added ATP, they saw neither binding nor translocation; addition of 1 mM ATP restored translocation.

In our experiments, variable amounts of residual binding were always seen in the absence of added ATP (compare lane 1, Figs. 1 and 2). This may be due to differing endogenous levels of ATP in the isolated intact chloroplasts. This interpretation is supported by the observation that addition of glycerate to the binding reactions in the absence of added ATP further decreased the level of residual binding (Fig. 5), presumably by removing endogenous ATP from the isolated chloroplasts. Another possibility is that precursor bound in the absence of ATP is not bound to receptors on the envelope surface but rather is associated with membrane lipids in a nonspecific, non-ATP-mediated fashion.

At high ATP concentrations (Figs. 1 and 2, A, lane 4), the level of binding decreased, apparently due to translocation of bound precursor. This was seen frequently during the course of this study (Figs. 1 and 2) and underscores the physiological significance of the observed binding. Occasionally, binding would level off and remain constant at high ATP, even in the presence of increased translocation. It is possible that the precursor concentration in those experiments was greater than in others due to higher translation efficiency, so that precursor availability was not a limiting factor.

The energy requirement for chloroplastic precursor translocation is specific for ATP (7, 9). The unexpectedly broad nucleotide specificity for binding (Table II) may mean that different ATP-utilizing enzymes (with different nucleotide specificities) are involved in binding and translocation into chloroplasts. Alternatively, it may be explained by the fact that the other nucleotides (CTP, GTP, UTP, ADP) are transported into chloroplasts more slowly than ATP. Once inside they could be rapidly converted (by phosphotransferases and adenylate kinase) to ATP which would then stimulate precursor binding.

The site of ATP hydrolysis for binding was difficult to determine unequivocally. We were unable to completely block binding with high concentrations of glycerate (to remove internal ATP) (Fig. 5). It is possible that glycerate transport is less efficient than ATP translocation, so more ATP was inside the chloroplasts than the glycerate (via glycerate kinase) could remove. Alternatively, glycerate kinase may not function optimally in the dark. Regardless of the cause of the limitation of glycerate as an internal ATP trap, it was clear that when ATP was added externally, in the presence of glycerate, stimulation of binding was not as great (Fig. 5).

When ATP was generated inside chloroplasts by DHAP (with oxaloacetate and P0), in the presence of an external ATP trap, binding was stimulated (Fig. 4). However, with only internal ATP present, the level of binding was still less than that seen by adding exogenous ATP (Fig. 4). Although unlikely, we were unable to rule out the remote possibility that DHAP and glycerate act directly to stimulate and inhibit, respectively, precursor binding (Figs. 4 and 5). A more likely explanation is that ATP is required in the intermembrane space between inner and outer chloroplast envelope membranes, as suggested by Flugge and Hinz (8, 24) for translocation. Then binding would be less with internally generated ATP because the external ATP trap (Fig. 4) would decrease ATP levels in the intermembrane space. Our data, although not able to fully support this possibility, are not sufficient to rule out a requirement for ATP in the intermembrane space. Although it is clear that external ATP is not required, we cannot distinguish between a stromal and an intermembrane space requirement for ATP.

The ATP requirement for precursor binding to chloroplasts may represent another important difference between mitochondrial and chloroplastic protein transport mechanisms. There is some conflict concerning the precise energy requirement for precursor binding to mitochondria, so it is difficult to make detailed comparisons. Pfanner and Neupert (25), Eilers et al. (14), and Verner and Schatz (18) all concluded that energy is not required for the initial binding of precursors to the mitochondrial membrane, although a transmembrane potential is needed for insertion into the membrane. Eilers et al. (16) concluded that a membrane potential, but not ATP, is required for the precursor-membrane interaction, i.e. binding. Finally, Pfanner et al. (17) showed that binding of the ATPase P0 β-subunit precursor to mitochondria requires NTP, but is independent of a transmembrane potential. Whether the energy requirements for binding vary from one precursor to another or whether the different investigators are looking at different steps remains to be clarified.

The single point on which these papers agree is that ATP is required outside the mitochondria, possibly for unfolding of the precursor (12, 14, 17–19, 25, 26). However, the ATP requirement for both binding and translocation into chloroplasts seems to be internal (Fig. 4; Refs. 9, 10), although some controversy still exists about this (7, 8). It is difficult to imagine how ATP required inside the chloroplasts can act to cause precursor unfolding (or even stabilize or maintain precursors in an unfolded state) outside of the chloroplasts before the precursor interacts with the envelope membrane.

Precursor recognition and specificity is also commonly considered to be conferred at the binding step. It is difficult to imagine how internal ATP would be directly involved in an interaction between precursor and a component of the outer envelope. For that reason, an intermembrane space requirement for ATP is more attractive, mechanistically, in trying to explain how ATP mediates chloroplastic precursor binding. Perhaps ATP causes (by phosphorylation or otherwise) a conformational change in a proteinaceous component of the chloroplast envelope which results in productive precursor binding. Chloroplast envelope membranes are known to have protein kinase activity (27, 28). Such a protein kinase could utilize ATP to phosphorylate the bound form of the precursor or a proteinaceous component (receptor) of the transport apparatus in the chloroplast envelope. The latter possibility seems more likely, given the internal (or intermembrane space) requirement for ATP. Alternatively, the ATP may be required for some kind of stromal ATP-binding protein (molecular chaperone) which in turn binds to the transport apparatus in the membrane, causing a conformational change that allows specific binding to occur. These may be more plausible suggestions if one considers that binding may take place at contact sites between the inner and outer chloroplast envelope membranes (4, 29–31). Binding to contact sites in mitochondria has also been discussed (23, 25, 26).

To thoroughly understand the mechanism of chloroplastic protein import, it will be necessary to dissect the process and study each step separately. The energetics of binding as discussed here provide a way of easily separating binding from translocation. The data presented in this report do not address
the question of the role of ATP in binding. Further work will be needed to develop more testable mechanistic models of the role(s) of ATP in protein transport into chloroplasts.

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