A New Chloroplast Protein Import Intermediate Reveals Distinct Translocation Machineries in the Two Envelope Membranes: Energetics and Mechanistic Implications

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Abstract. Chloroplast protein import presents a complex membrane traversal problem: precursor proteins must cross two envelope membranes to reach the stromal compartment. This work characterizes a new chloroplast protein import intermediate which has completely traversed the outer envelope membrane but has not yet reached the stroma. The existence of this intermediate demonstrates that distinct protein transport machineries are present in both envelope membranes, and that they are able to operate independently of one another under certain conditions. Energetic characterization of this pathway led to the identification of three independent energy-requiring steps: binding of the precursor to the outer envelope membrane, outer membrane transport, and inner membrane transport. Localization of the sites of energy utilization for each of these steps, as well as their respective nucleotide specificities, suggest that three different ATPases mediate chloroplast envelope transport.

CHLOROPLASTS are double membraned organelles which acquire the majority of their proteins from the cytoplasm. Proteins destined for chloroplasts are encoded in the nucleus, synthesized on cytoplasmic ribosomes, and posttranslationally targeted to the plastid import apparatus by an NH_2-terminal extension called a transit peptide (see Theg and Scott, 1993; de Boer and Weisbeek, 1991 for reviews). After binding to a proteinaceous receptor on the outer envelope membrane (for a review see Gray and Row, 1995), the precursor traverses the two membranes to arrive in the stroma, where the transit peptide is immediately removed by a stromal processing peptidase (Reed et al., 1990). Precursors destined for the thylakoids contain a bipartite transit peptide; the most NH_2-terminal region is an envelope targeting domain which is cleaved by the stromal peptidase to expose a subsequent thylakoid-directing signal sequence. After targeting to the thylakoid lumen, this sequence is removed by a second peptidase, leaving the mature-sized protein (Theg and Scott, 1993).

Studies of the envelope transport process have revealed two distinct energetic requirements. The first is a low (<50 μM) ATP requirement which mediates the irreversible binding of chloroplast precursors to the outer envelope membrane. Other hydrolyzable nucleotide triphosphates (NTPs)[1] can substitute for ATP (Olsen et al., 1989). The ATP used for precursor binding is apparently hydrolyzed in the inner membrane space, rather than outside the chloroplast (Olsen and Keegstra, 1992). Recently, this step has additionally been shown to require GTP (Kessler et al., 1994), although the contribution of GTP hydrolysis to the binding energy requirement has not yet been elucidated. The second energetic requirement governs translocation of precursors into the stroma. This step, which encompasses both inner and outer membrane transport events, requires a high concentration of ATP which is hydrolyzed in the stromal compartment (Pain and Blobel, 1987; Theg et al., 1989; Leheny and Theg, 1994). No other (NTP) can substitute for ATP in this reaction (Pain and Blobel, 1987; Olsen et al., 1989). By analogy with mitochondrial models (Neupert et al., 1990), it has been suggested that chloroplast hsp70s may be the ATP-hydrolyzing component of the chloroplast transport apparatus (Soll and Waegemann, 1992; Tsugeki and Nishimura, 1993; Alefsen et al., 1994; Schnell et al., 1994; Wu et al., 1994). Unlike mitochondrial

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1. *Abbreviations used in this paper:* AMP-PNP, adenylyl-imidodiphosphate; DHAP, dihydroxycetone phosphate, NTP, nucleotide triphosphate; OAA, oxaloacetic acid; o-ATP, periodate oxidized ATP; OE17, 17-kD subunit of the photosynthetic oxygen-evolving enzyme complex; P36, phosphate translocator; SS, small subunit of ribulose bisphosphate carboxylase/oxygenase.

protein import, there is no $\Delta\psi$ requirement for chloroplast envelope transport (Theg et al., 1989).

We envision two possible models describing the chloroplast envelope transport event: either a protein crosses the two membranes in a single step at regions where they are closely appressed ("contact sites"), or a protein traverses the two membranes separately and sequentially through two distinct translocation machineries. These two models are not mutually exclusive; envelope membranes may contain import machineries that are capable of transporting proteins in either a coupled or uncoupled fashion, or the presence of a translocating polypeptide itself may cause the formation of a translocation contact site (Pfanner et al., 1992; Perry and Keegstra, 1994). The first evidence for protein translocation at contact sites was obtained through work with mitochondria. Schleyer and Neupert (1985) found that when mitochondrial protein import reactions were performed at low temperatures, or when protein uptake was hindered by the binding of a specific antibody to the precursor protein, membrane-spanning intermediates were formed. These intermediates were processed to their mature sizes, indicating that the transit peptide cleavage site had reached the matrix. At the same time, they remained susceptible to digestion by externally added proteases, suggesting that the bulk of the polypeptide remained outside the organelle.

In addition to studies of mitochondrial protein import at contact sites, it has been shown that the two mitochondrial membranes are capable of transporting some proteins independently. Mitochondria stripped of outer membranes retain the ability to import matrix-directed polypeptides, indicating that the inner membrane has a complete complement of import machinery components (Ohba and Schatz, 1987; Hwang et al., 1991; Glick et al., 1991; Segui-Real et al., 1993). Vesicles made from isolated outer membranes import cytochrome c heme lyase, a peripheral inner membrane protein (Mayer et al., 1993), but cannot import mitochondrial precursors which normally cross the inner membrane. This indicates that a component of the inner membrane, probably the recently described Isp45p/MIM44 (Kronidou et al., 1994; Schneider et al., 1994; Ungermann et al., 1995; Mayer et al., 1995), is essential for matrix proteins to traverse the outer membrane.

Schnell and Blobel (1993) were the first to report experiments supporting a contact site model of protein import for chloroplasts. They used a chimeric protein to identify a membrane-spanning transport intermediate. This intermediate had been processed to its mature size, but continued to cofractionate with bound precursor proteins in the envelope membranes. These data led them to propose that chloroplasts import proteins at regions of contact between the two membranes. Other labs have now also reported detection of such contact sites in chloroplasts, and have used them to identify components of the plastid envelope translocation machinery (Alefsen et al., 1994; Kessler et al., 1994; Schnell et al., 1994; Wu et al., 1994; for reviews see Gray and Row, 1995; Tranel et al., 1995). Perry and Keegstra (1994) provided additional functional support for contact site transport when they found that a photoactivatable chloroplast precursor could be cross-linked to an 86-kD outer membrane protein, now thought to be the major precursor receptor in these membranes (Hirsch et

al., 1994; Kessler et al., 1994). On addition of ATP, this "precursor-receptor" complex moved into a more dense membrane fraction, and became associated with a 75-kD protein thought to be present in the translocation apparatus (cf. Gray and Row, 1995). They suggested that the presence of the precursor protein and an energy source causes the formation of a "translocation" contact site through which the polypeptide passes into the stroma.

In this paper we present data indicating that sequential transport across the two chloroplast envelope membranes can also occur, demonstrating that functionally independent transport machineries exist in each membrane. We have further characterized the energy requirements of the independent transport events. Our experiments suggest that contact sites are not a functional requirement of the mechanism of chloroplast protein import, and that the import process may be mediated by at least three discrete ATPases.

Materials and Methods

Chloroplast Isolation and Preparation

Intact chloroplasts were isolated from 12–14-day-old pea seedlings (*Pisum sativum*) (Theg and Geske, 1992) and resuspended in import buffer (IB) containing 330 mM sorbitol and 50 mM K-Tricine, pH 8.0 (1X IB). Osmotically shrunken chloroplasts were prepared 1 h before the initiation of import reactions by diluting them to a concentration of 1 mg chlorophyll/ml in a buffer containing 50 mM K-Tricine, pH 8.0, and 660 mM sorbitol (2X IB). ATP-depleted chloroplasts were kept in the dark after dilution. Nigericin/valinomycin (nig/val)–treated chloroplasts were preincubated with 6 μM of each ionophore, and the final concentration of nig/val during the import reactions was 2 μM. Where indicated chloroplasts were preincubated with either 10 mM glycerate or 3 mM o-ATP.

Preparation of Radiolabeled Precursors

Cloned genes for the small subunit of ribulose bisphosphate carboxylase/oxygenase (prSS), the phosphate translocator (prP36) and the 17 and 23 subunits of the oxygen evolving complex (prOE17, and prOE23) (Cashmore, 1983; Schnell et al., 1990; Ettinger and Theg, 1992; Wales et al., 1989a; Wales et al., 1989b) were transcribed and translated in vitro (Olsen et al., 1989) to obtain ^{3}H-labeled precursors. In experiments where <1 mM ATP was added, precursors were desalted to remove ATP remaining after the translation reaction by centrifugation through Sephadex G-25 columns as described by Olsen et al. (1989). Apyrase treated precursor was incubated at 30°C for 1 h with 0.01 U of apyrase/ml translation reaction.

Protein Import Reaction Conditions

Reactions with osmotically shrunken chloroplasts were performed in 2X IB; isotonic reactions contained 1X IB. All reactions contained 5 mM $MgCl_2$, 500,000 DPM of radiolabeled precursor protein, and 3 mM ATP, unless indicated otherwise in the figure legend. The reactions were started by the addition of 20 μl of chloroplasts from a 1-mg chlorophyll/ml stock. The final reaction volume was 60 μl. All reactions were terminated exactly at the indicated time points by the protease or mock protease treatments described below. After protease treatment, chloroplasts were reisolated and prepared for SDS-PAGE (Theg et al., 1989). The resulting gels were fluorographed and quantitated using a Bio Image analysis system (Ettinger and Theg, 1991). All quantitated data represent the average of two or more experiments.

Protease Treatment

As consistent protease activity was essential to the outcome of these experiments, particular care was exercised in preparation and use of the added protease. Thermolysin stock solutions were made fresh immediately before use at a concentration of 4 mg/ml in 2X IB supplemented with 5 mM $CaCl_2$. Import reactions were terminated by the addition of 3.3 mM $HgCl_2$ (except in the case of chase reactions and the reactions in Fig. 9;

Reed et al., 1990) and 5 mM CaCl₂. Protease-treated (+ protease) samples received thermolysin at a final concentration of 200 μg thermolysin/ml and were incubated on ice for 15 min. Digestions were stopped by the addition of 60 μl of 25 mM EDTA in 1× IB. After protease or mock protease treatment, chloroplasts were repurified by centrifugation through silicon oil into perchloric acid as described (Theg et al., 1989). Mock protease–treated (−protease) samples were treated as above except that thermolysin was omitted. Protease efficiency was assessed for each experiment by digesting precursor that was bound to chloroplasts in the presence of HgCl₂. As no transport can take place under these conditions, this bound polypeptide should be completely digested by thermolysin.

Fractionation of Envelope Membranes

At the time points indicated in the legend for Fig. 9, 300 μl protein import reactions were stopped by the addition of 1 ml of ice cold 2× IB (All buffers used in the fractionation procedure contained a cocktail of protease inhibitors which included aprotinin, leupeptin, pepstatin, and PMSF.). The reactions were immediately pelleted and resuspended in 2× IB. The chloroplasts were lysed by one freeze/thaw cycle. Import reactions containing imported p36, bound prSS and OE17 were then pooled, diluted to 0.3 M sorbitol, loaded onto a 0.46–1 M linear sucrose gradient, and centrifuged at 45,000 rpm in a TLS-55 rotor (Beckman Instrs., Fullerton, CA). 80-μl fractions were collected and analyzed. Both acid precipitation and heating were avoided as p36 aggregates under these conditions.

Kinetic Analysis of Protein Import

The kinetic analysis shown in Fig. 3 *B* was performed on the quantitated data of Fig. 3 *A* (points). The solid lines show fits to these data analyzed according to the sequence

$$P_f \xrightarrow{k_1} P_b \xrightarrow{k_2} PPP \xrightarrow{k_3} I \xrightarrow{k_4} M$$
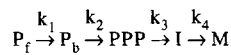
with P_f, P_b, and PPP representing the amount of precursor free in solution, bound to the external chloroplast surface and present in the protease-protected form after crossing the outer envelope membrane, respectively; I and M represent the amount of stromal intermediate and mature protein present, respectively. The equations for the instantaneous concentrations of each species were derived from the general solution for a series of *n* consecutive irreversible first-order reactions as given by Capellos and Bielski (1980). The fits were generated using these equations and data points with the SIMPLEX algorithm (Caceci and Cacheris, 1984). A solution was sought in which one set of rate constants ($k_1 - k_4$) was used to simultaneously fit all four data sets representing $[P_b]$, $[PPP]$, $[I]$, and $[M]$; data representing $[P_f]$ cannot be determined in these experiments, and $[P_f]_0$ was sought as a fifth adjustable parameter. The best-fit parameters are given in the legend to Fig. 3.

Results

Detection of Protease-protected, Precursor-sized Polypeptides

We sought to develop an assay to assess whether the two chloroplast envelope membranes are competent to transport proteins independently of one another. To this end we placed isolated chloroplasts in a hypertonic medium, a procedure which pulls the inner membrane away from the outer membrane without disrupting all structural contact sites (Cline et al., 1985a). These osmotically shrunken chloroplasts were then used in import experiments with radiolabeled chloroplast precursor proteins. The import reactions were stopped at specific time points by adding HgCl₂, a chemical poison of the chloroplast transport machinery (Reed et al., 1990). Finally, the chloroplasts were treated with the protease thermolysin to digest those precursors remaining in solution or bound to the outside of the plastid; precursors which survive the protease treatment are considered to have crossed the outer envelope membrane (Cline et al., 1984).

Fig. 1 *A* shows the results of such an experiment performed with the precursor (pr) to a thylakoid protein, OE17, a 17-kD subunit of the photosynthetic oxygen-evolving enzyme complex. The activity of the protease was evaluated in this (and every) experiment by performing mock import reactions in the presence of HgCl₂. This poi-
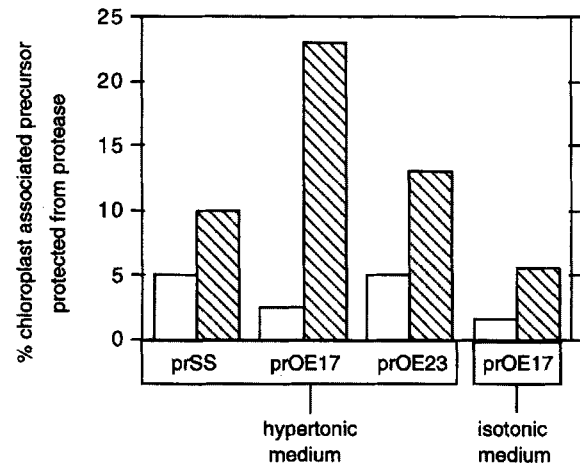
A
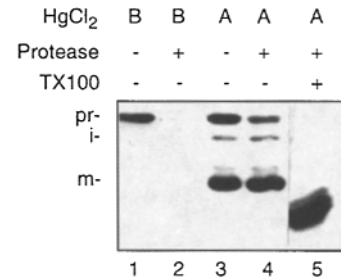
B

Figure 1. Formation of the protease-protected precursor. Precursor proteins were incubated with chloroplasts for 2 min. 3.3 mM HgCl₂ was either added before the reaction was initiated (*B*) or was added at 2 min after the import reaction started (*A*). + protease indicates treatment with 200 μg/ml thermolysin for 15 min on ice; − protease indicates that the sample was subjected to the same incubations and additions except that no protease was added. Where indicated, 1% Triton X-100 was added before protease treatment. (*A*) prOE17 was imported. *pr*, precursor; *i*, stromal intermediate; *m*, mature. (*B*) Chloroplast precursors prSS, prOE17, and prOE23 were imported as above. Empty bars represent the percent of the precursor bound to chloroplasts in the presence of HgCl₂ which was left undigested after protease treatment (a measure of protease efficiency). Striped bars represent the percent of chloroplast-associated precursor-sized protein resistant to protease digestion after a 2-min import reaction stopped with HgCl₂. The last two bars represent the same experiment performed with prOE17 in isotonic buffer containing 330 mM sorbitol. All quantitated data represents the average of two experiments. The difference between the amount of protease-protected precursor detected after 2 min of import (*striped bars*), and the amount of precursor left undigested after import in the presence of HgCl₂ (*empty bars*) was shown to be statistically relevant by *t* test analysis.

son prevented any membrane transport from taking place, and led to a population of externally bound precursor proteins (Fig. 1 A, lane 1). When this sample was treated with thermolysin, the precursor was essentially completely digested (Fig. 1 A, lane 2 and Fig. 1 B), indicating that both were outside the chloroplast, and that the protease efficiently degraded the precursor under these reaction conditions. However, when the transport reaction was allowed to proceed for 2 min before being arrested with $HgCl_2$ and treated with thermolysin, a large portion of the precursor polypeptide was resistant to the added protease (Fig. 1 A, compare lanes 3 and 4). The presence of the intermediate and mature-sized bands indicates that some transport into the stroma and the thylakoid lumen, respectively, had occurred during the incubation period. As the chloroplast envelope membranes have been shown to possess strong precursor unfolding activity (Guera et al., 1993; Endo et al., 1994; Reinbothe et al., 1995; Clark, S. A., and S. M. Theg, unpublished observations), it is unlikely that this precursor species represents a newly folded, compact form which is still bound to the outside of the plastid. When the envelope membranes were solubilized with the detergent Triton X-100 before the protease treatment, the previously protected precursor polypeptide became susceptible to digestion by thermolysin again. These results suggest that a population of the precursor protein had crossed the outer membrane during the 2-min import reaction and became protected from exogenously added protease. At the same time, this protein had not reached the stromal compartment where it would be immediately processed to a lower molecular weight form by the stromal processing peptidase (Reed et al., 1990). This polypeptide species, which we have termed the protease-protected precursor, apparently represents a partially imported form of prOE17.

Fig. 1 B shows quantitative results for the experiment described above performed with several different chloroplast precursors (small subunit of ribulose bisphosphate carboxylase/oxygenase [SS] and oxygen-evolving complex subunits [OE] OE17, and OE23). The empty bars report the efficiency with which the protease degraded the externally bound species of each precursor, and indicate that thermolysin left between 3 and 6% of these polypeptides undigested. The striped bars represent the amount of precursor which was protected from the exogenous protease after a 2-min import reaction stopped with $HgCl_2$. As these values range from 11 to 47%, the protease-protected precursor population was significantly higher than can be explained by inefficiency of the protease, and therefore must have arisen because the precursors had crossed the outer envelope membrane. The final set of bars represent the same experiment performed with chloroplasts kept in isotonic buffer and demonstrate that a low but statistically significant level of the protease-protected precursor can be produced under these conditions. Subsequent modification of the assay allowed us to accumulate a much larger fraction of protease-protected precursor in chloroplasts in isotonic buffer (see Fig. 6 B). The precursors used in the experiment of Fig. 1 B represent both stromal and thylakoid-resident proteins. Our ability to detect the protease-protected precursor with each indicates that this phenomenon is general and is likely related to the mechanism of chloroplast protein import. We consistently observed that the protease-protected precursor species was made most efficiently with prOE17. Therefore, we concentrated on this protein for the remainder of the experiments described.
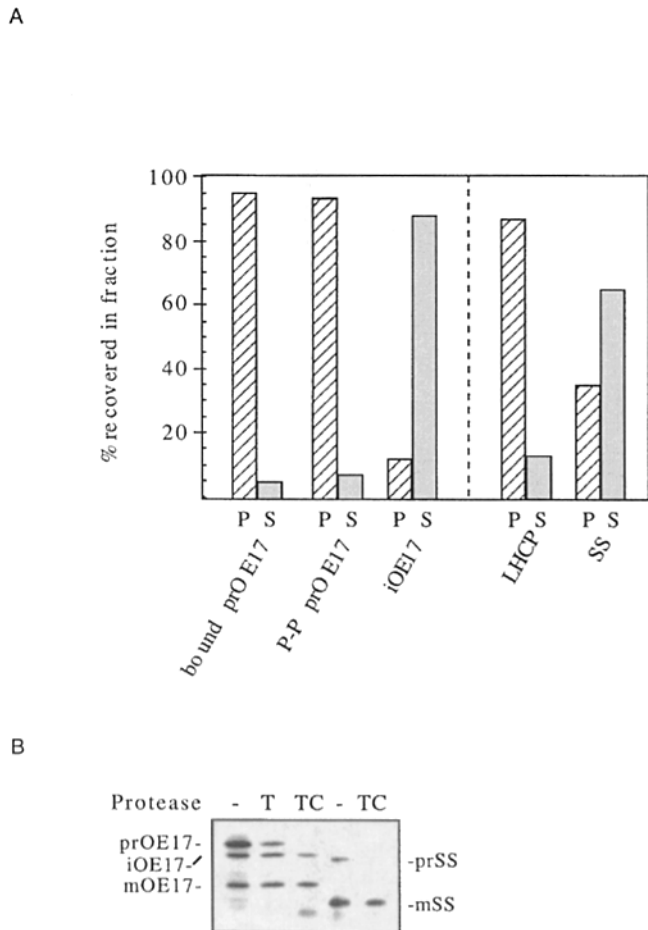
The Protease-protected Precursor Is Associated with the Chloroplast Membranes

Although the stromal processing peptidase acts quickly to remove the transit peptide from precursors (Reed et al., 1990), the possibility that the protease-protected precursor had in fact already crossed into the stroma and escaped processing was investigated. To this end, chloroplasts containing the protease-protected prOE17 were fractionated into the membrane and soluble components. The protease-protected precursor was recovered exclusively in the pellet along with other envelope and thylakoid membrane markers (Fig. 2 A), indicating that it is associated with the envelope membranes and is not soluble in the stroma or in the intermembrane space.

The combination of trypsin and chymotrypsin has been used as a probe of the intermembrane space (Cline et al., 1984; Marshall et al., 1990; Schnell et al., 1994; Wu et al., 1994). At relatively high concentrations these proteases penetrate the outer envelope membrane and degrade proteins accessible from the intermembrane space. To demonstrate that protease-protected prOE17 is protected by virtue of having crossed the outer membrane but not the inner membrane, import reactions were treated with this combination of proteases. Although prOE17 can survive thermolysin treatment (indicating its passage across the outer membrane), no protease-protected prOE17 was observed after digestion with trypsin and chymotrypsin (Fig. 2 B, compare the second and third lanes). Under the same conditions, neither mature SS nor i-OE17 present in the stroma was digested, indicating that the stroma was not accessed by the trypsin/chymotrypsin combination. Since trypsin/chymotrypsin degraded the protease-protected precursor without destroying stromally located proteins (SS and i-OE17), the protease-protected precursor must not be in the stroma. While this probe for the inner membrane space has not been studied extensively, the results of this experiment are consistent with the fractionation in Fig. 2 A. Together these data serve to localize the protease-protected precursor to a surface of one of the envelope membranes facing the intermembrane space.
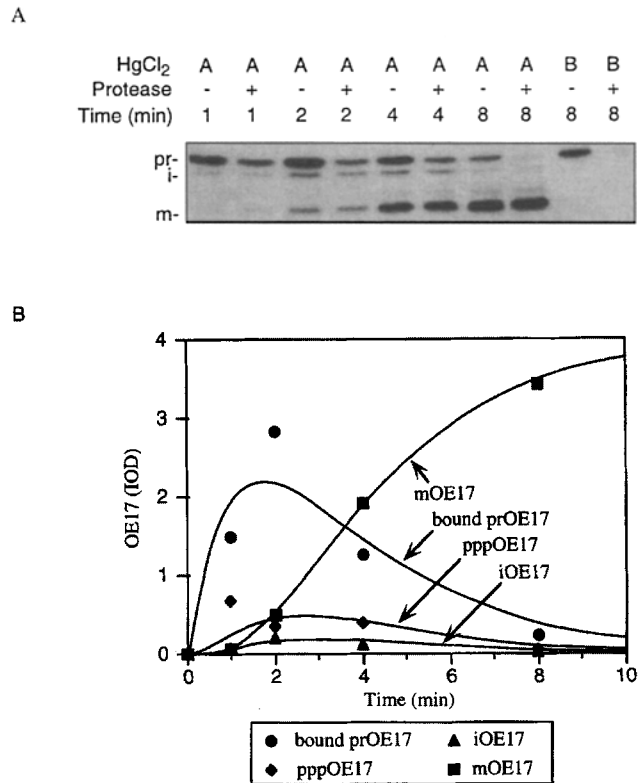
Protease-protected prOE17 Is a Productive Import Pathway Intermediate

We next examined whether the protease-protected precursor is an intermediate on the protein import pathway. If so, we would expect this species to appear at early times after the initiation of the import reaction, and then to disappear at later times as it moved across the inner envelope into the chloroplasts. Fig. 3 A shows that the protease-protected precursor was abundant at early time points but declined in later points and was absent by 8 min. Time course experiments performed with chloroplasts kept under iso-osmotic conditions displayed similar protein import kinetics, although the overall quantity of the protease-protected precursor was much lower than was seen in osmotically shrunken chloroplasts (data not shown).

Figure 2. Protease-protected prOE17 is associated with the chloroplast membranes. (A) 2 min import reactions were performed as in Fig. 1. After thermolysin (+) or mock thermolysin (−) treatment, the samples were pelleted and lysed in 10 mM Tricine (pH 8.0), 1 mM EDTA. The reactions were centrifuged at 100,000 *g* for 30 min and the pellet (P) and supernatant (S) collected. The amount of polypeptide recovered in each fraction was quantitated. (B) PrOE17 and prSS were imported for 2 and 8 min, respectively. Reactions were either not treated with protease (−), treated with thermolysin (T), or treated with 300 mg/ml trypsin and 300 μg/ml chymotrypsin (TC). Lanes *1–3*, prOE17; *4* and *5*, prSS.

A more rigorous analysis of the data in Fig. 3 *A* established the plausibility of the postulate that the protease-protected precursor is a pathway intermediate in osmotically shrunken chloroplasts. In this type of experiment, the import reaction plateaus because the pool of import-competent precursor is rapidly exhausted; addition of fresh precursor after the reaction slows leads to another round of protein import (Hashimoto, A., and S. M. Theg, unpublished data). This allows us to treat the different protein species appearing in Fig. 3 *A* as originating from a pulse of precursor. Fig. 3 *B* shows the best-fit solution to the integrated rate equations describing the progression of an unbound precursor to a mature size protein in the thylakoid lumen on a pathway passing through externally bound, protease-protected precursor and stromal-intermediate stages. As described in Materials and Methods, the data

Figure 3. Protease-protected prOE17 is an intermediate on the protein import pathway. (A) The experiment was performed exactly as in Fig. 1 except that the reactions were allowed to run for the indicated time before being quenched by $HgCl_2$. All samples were immediately transferred to ice and either protease or mock protease treated as indicated. (B) Data from A were quantitated and subjected to kinetic analysis as described in Materials and Methods. The relative intensities of the bands are reported as units of integrated optical density. ●, bound precursor ('bound prOE17'); ◆, protease-protected precursor ('pppOE17'); ▲, stromal intermediate ('iOE17'); ■, mature OE17 ('mOE17'). From top to bottom at *t* = 6 min, the lines correspond to computer-generated fits to the mature OE17, bound precursor, protease-protected precursor, and stromal intermediate; the adjustable parameters determined by computer fit were $[P_f]_0 = 4.08$ IOD units, $k_1 = 0.86$ min^{-1}, $k_2 = 0.35$ min^{-1}, $k_3 = 1.48$ min^{-1} and $k_4 = 3.61$ min^{-1}.

fitting procedure required that one set of rate constants was used to calculate the instantaneous concentrations of the four species for which data were available. While some of the calculated lines fit more closely than others, each line falls reasonably near their respective data points and thus adequately describes the trends of the observed kinetic data. Although fitting data to a model is not sufficient to prove the model, these calculations demonstrate that the protease-protected precursor behaves in a manner consistent with its placement on the productive import pathway.

To confirm that the protease-protected precursor is a productive intermediate, we designed a protocol in which the protease-protected precursor could be arrested between the two envelope membranes, and subsequently chased across the inner membrane in a later step. To accomplish this it was necessary to stop the import reaction without adding the irreversible chemical poison $HgCl_2$.

Therefore, reactions were stopped by placing the sample on ice before the protease treatment. Although this method is less effective in arresting the import reaction (Leheny and Theg, 1994), we were still able to detect a significant population of protease-protected precursor representing 9% of the total bound protein (compared with 23% in the HgCl$_2$-treated reaction; see Fig. 4 A, lanes 4 and 5). When the reaction containing the protease-protected precursor arrested in the absence of HgCl$_2$ was warmed to room temperature after protease treatment, the protected precursor species chased to the mature-sized polypeptide (Fig. 4 A, lanes 5 and 6). Quantitation of the bands shown in Fig. 4 A, lanes 5 and 6 indicated that both the protease-protected precursor and the stromal intermediate species were necessary to account for the increase in mature-sized OE17 detected after the chase reaction (Fig. 4 B). These data indicate that the protease-protected precursor is a productive import pathway intermediate and is competent to complete the membrane translocation step. (This point is also demonstrated in Fig. 8.)
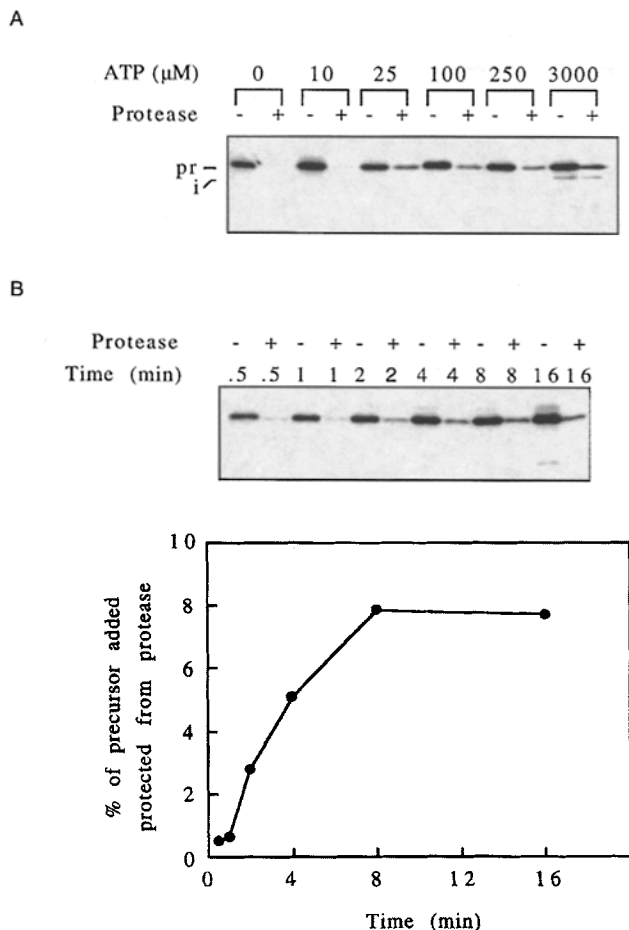
A

B
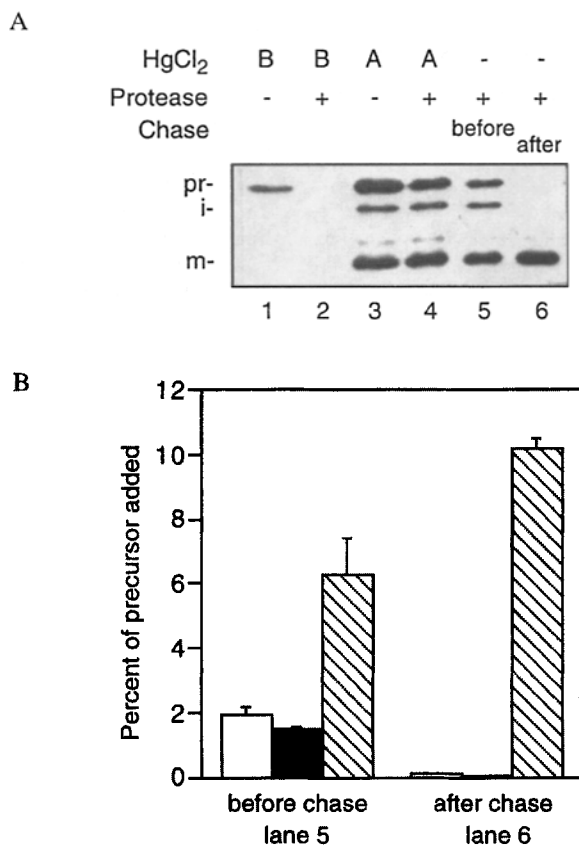
Figure 4. Chase of the arrested protease-protected precursor across the inner envelope membrane. (*A*) The experiment was performed as in Fig. 1 except that in lanes 5 and 6 the reactions were halted by being placed on ice without HgCl$_2$ treatment. Both samples were protease treated and then the sample in lane 6 was incubated for eight more minutes at room temperature. (*B*) Quantitation is from duplicate experiments such as those represented by lanes 5 and 6 in *A*. Empty bars represent prOE17; *solid bars*, the stromal intermediate; and *striped bars*, the mature-sized protein.

Intermediate Levels of ATP Support Production of Protease-protected Precursor

Because the protease-protected precursor species had crossed only the outer envelope membrane, it could be used to investigate the energetic requirements for this membrane transport step. Osmotically shrunken chloroplasts were incubated in the dark on ice to allow normal plastid metabolism to deplete endogenous ATP. The chloroplasts were also treated with the potent uncoupler combination nigericin and valinomycin (nig/val) to prevent any ATP production by incidental exposure to light during the import reaction. A side effect of this treatment is that thylakoid-directed polypeptides which require a membrane electrochemical potential for translocation into the thylakoid lumen (such as OE17) accumulate as stromal intermediates (Cline et al., 1992). However, the nig/val combination has no effect on the targeting of these polypeptides across the envelope membranes (Grossman et al., 1980; Cline et al., 1985b; Flugge and Hinz, 1986; Schindler et al., 1987; Theg et al., 1989). ATP-depleted prOE17 was added to ATP-depleted chloroplasts with increasing concentrations of exogenously added ATP (Fig. 5 A). After 4 min without added ATP, some precursor was reisolated with chloroplasts. This precursor was completely degraded by thermolysin, indicating that it had not crossed the outer envelope membrane. When 10 μM ATP was included in the reaction, the amount of precursor associated with chloroplasts increased threefold, a consequence of the ATP-dependent binding studied previously (Olsen et al., 1989). This bound species remained protease accessible. When concentrations of ATP between 25 and 250 μM were added, a significant population of the associated precursor was found to have crossed the outer envelope as evidenced by its resistance to the added protease. At 3 mM ATP the stromal intermediate-sized form of OE17 was also detected, indicating that the polypeptide had reached the stroma. This experiment suggests that there are three different ATP-requiring steps in the chloroplast protein import process. The first, ATP-dependent binding (Olsen et al., 1989), occurs in the presence of 10 μM ATP. The second step, transport across the outer membrane, requires an intermediate concentration of ATP (25–100 μM). Finally, more than 250 μM ATP is required for a precursor to cross completely into the stroma, where it is processed to the stromal-intermediate form.

In previous experiments we were forced to trap the protease-protected precursor kinetically as an early time point in the import reaction. Our observation that this species can be formed at ATP concentrations that are too low to support complete envelope transport suggested that it should accumulate at low ATP concentrations. Accordingly, we examined the time course of the appearance of the protease-protected precursor in the presence of 50 μM ATP. As predicted, the protease-protected precursor accumulated in a linear fashion until the 8-min point and then leveled off (Fig. 5 B). It is noteworthy that Fig. 5 A shows that the protease-protected precursor becomes stranded between the envelope membranes after 4 min in the presence of 250 μM ATP. This rules out the possibility that the reaction documented in Fig. 5 B plateaued after 8 min because formation of the protease-protected precursor depleted the 50 μM ATP added to this experiment.

A

ATP (µM)   0   10   25   100   250   3000
Protease   - + - + - + - + - + - +

pr —
i ⁄

B

Protease   - + - + - + - + - + - +
Time (min) .5 .5 1 1 2 2 4 4 8 8 16 16

Figure 5. Outer membrane transport requires an intermediate concentration of ATP. (A) Desalted prOE17 was imported into ATP-depleted chloroplasts containing nig/val in 2X IB (660 mM sorbitol, 50 mM Tricine; see also Materials and Methods). Import reactions were terminated after 4 min by the addition of 3.3 mM HgCl₂. ATP and protease (thermolysin) were added to the reactions as indicated. (B) Import reactions were performed exactly as in Fig. 4 A except that 50 µM ATP was added to each reaction. Time points are indicated. (C) Quantitation of "+protease" samples from B. Points represent the average of two experiments and are expressed as the percent of total radiolabeled precursor added to the import reaction which was protease resistant.

Nucleotide Specificity of Outer Membrane Transport

Because precursor binding to the outer envelope membrane of chloroplasts can be supported by NTPs other than ATP (Olsen et al., 1989), we reasoned that the ATPases that support binding and that drive outer membrane transport might be distinguishable on the basis of NTP specificity. Fig. 6 A shows protein import experiments that were performed in ATP-depleted chloroplasts supplemented with different NTPs. Only ATP was able to support outer envelope transport as indicated by the appearance of the protease-protected precursor species. The nonhydrolyzable ATP analog adenylyl-imidodiphosphate (AMP-PNP) could not substitute for ATP. Neither ADP nor other NTPs were able to drive the production of the protease-protected precursor, although all of the nucleotide triphosphates can support binding (Olsen et al., 1989). Olsen and Keegstra (1992) further demonstrated

that the ability of these NTPs to support binding was not the result of their low level conversion to ATP. As precursor binding and outer membrane transport have different nucleotide specificities, our results suggest that these two processes are driven by different ATPases.

If transport across the outer membrane is a genuine step on the import pathway, then chloroplasts in isotonic buffer should also accumulate protease-protected precursors when the ATP concentration is too low to support import into the stroma. Import experiments using ATP-depleted chloroplasts kept in isotonic buffer were performed either in the presence of 100 µM ATP or 100 µM GTP (Fig. 6 B). While the ATP supported transport across the outer membrane, GTP, did not. These data reiterate the nucleotide specificity shown in Fig. 6 A and further demonstrate that the protease-protected precursor species can also be observed in normal, isotonic chloroplasts.

Outer Membrane Transport Requires ATP in the Intermembrane Space

Next, the location of the ATP requirement for transport across the outer membrane was assessed. Previous studies have shown that when protein import into ATP-depleted chloroplasts is performed in the dark in the presence of exogenously added ATP, there is a lag of ~2 min in the kinetics of protein uptake (Theg et al., 1989). This lag has been attributed to the fact that ATP is required in the stroma to drive protein import. Consequently, externally added ATP must be transported across the inner membrane via the adenylate translocator to achieve a stromal ATP concentration that can support protein import. Consistent with this idea, the half-time for ATP transport via the adenylate translocator has been measured at ~3.5 min at room temperature (Leheny and Theg, 1994). If ATP is required outside the chloroplast or in the intermembrane space (the porous outer membrane is not an impediment to ATP diffusion [Flugge and Benz, 1984]), then ATP added to an import reaction should be immediately available for outer membrane transport. On the other hand, if ATP is required in the stroma, a lag in the kinetics of outer membrane transport of ~2 min would be expected. In control experiments, we found that when ATP-depleted chloroplasts were added to an import reaction containing 1 mM ATP and placed in the light so that ATP could also be synthesized in the stroma, considerable accumulation of intermediate- and mature-sized polypeptides (stromal and thylakoid lumenal forms, respectively) were detected after 2 min (Fig. 7 A, group 1). When the chloroplasts were treated with nig/val so that light-driven ATP synthesis could not take place, only the protease-protected precursor was formed in 2 min (Fig. 7 A, group 2). However, if the same import reaction containing nig/val and 1 mM ATP was incubated for 4 min, protein import into the stroma did take place as evidenced by the appearance of the intermediate form of OE17 (Fig. 7 A, group 3). These data are consistent with the idea that externally added ATP is immediately available for protein transport across the outer membrane, but must be translocated into the stromal compartment before transport can be completed. These results suggest that ATP either outside the chloroplast or in the inner membrane space drives protein trans-

A   B
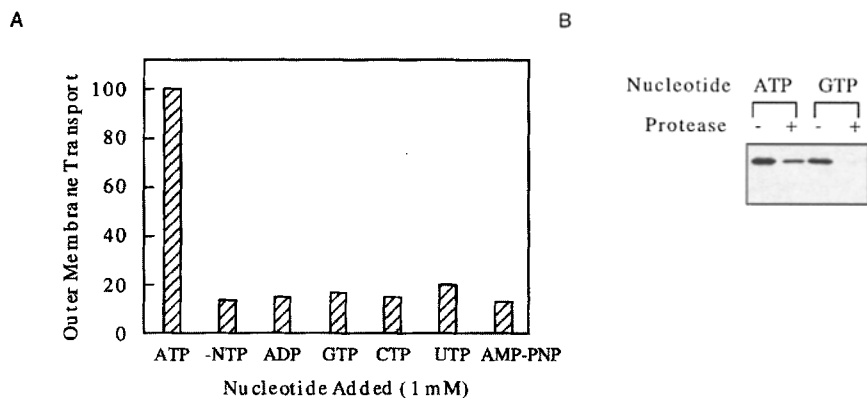
Figure 6. NTPs other than ATP cannot support outer membrane transport. (*A*) Experiments were performed exactly as in Fig. 4 except that 1 mM of the nucleotide indicated was added to each reaction. Import reactions were terminated after 2 min. The amount of protease-protected precursor formed in the presence of ATP was normalized to 100%. The data shown represent the average of two experiments. (*B*) Chloroplasts in isotonic buffer display the same energy requirements for protein transport as in hypertonic buffer. Import reactions were performed as in Fig. 1 except that chloroplasts were kept in 1× 1B (330 mM sorbitol, 50 mM Tricine). Import reactions were for 8 min in the presence of 100 μM of the indicated NTP.

location across the outer membrane, while ATP in the stroma is required for inner membrane transport.

In a second approach to address the location of the ATP requirement for protein transport across the outer membrane, we prevented ATP from accumulating in the stroma during protein import experiments. To keep the reaction conditions as consistent as possible, all import reactions were performed in the light in the presence of exogenous ATP. Additionally, samples received one or more of the following: (*1*) nig/val to prevent photophosphorylation, (*2*) periodate-oxidized ATP (o-ATP), an imperfect inhibitor of the adenylate translocator (Flugge and Hinz, 1986), and (*3*) glycerate, which causes depletion of stromal ATP through the action of the stromal enzyme glycerate kinase (cf. Olsen et al., 1989). In a control reaction (Fig. 7 *A*, group 4), o-ATP in the light had no adverse effect on protein import, as ATP should be present both inside through photophosphorylation and outside (exogenously added) the chloroplast under these conditions. Similarly, glycerate alone had no detectable effect on the levels of protein import (Fig. 7 *A*, group 5), indicating that glycerate kinase activity was not high enough to keep up with the rate of ATP synthesis via photophosphorylation. Finally, when nig/val, o-ATP, and glycerate were added together, protein import into the stroma was inhibited as indicated by the lack of intermediate-sized polypeptide, while the production of the protease-protected precursor was not (Fig. 7 *A*, group 6). The fact that no OE17 was transported into the stroma after 4 min indicates that stromal ATP levels were too low to support inner membrane transport. However, protease-protected precursor could still be formed under these conditions, again suggesting that external ATP can drive outer membrane transport.

Previous studies have shown that ATP outside the chloroplast is not required for protein import, and that the ATP required for binding is probably hydrolyzed in the inner membrane space (Olsen and Keegstra, 1992). In light of this, it was likely that the ATP required to support outer membrane transport is also used in the inner membrane space. To confirm this hypothesis, experiments were performed in the presence of glucose and hexokinase. Hexokinase is too large to permeate the outer membrane,

and has been shown to act as an efficient external ATP trap in this system (Theg et al., 1989; Olsen and Keegstra, 1992). The efficacy of glucose and hexokinase in removing ATP was tested by adding them to import reactions performed with ATP-depleted chloroplasts in the presence of 1 mM ATP in the dark (Fig. 7 *B*). Under these conditions, no ATP-dependent binding was detected for either prSS (Fig. 7 *B*) or prOE17 (data not shown), indicating that hexokinase was indeed active. However, when the ATP source for protein import was continuous light-driven ATP synthesis in the stroma, hexokinase did not prevent either complete import or production of the protease-protected precursor species. These results suggest that ATP outside the outer envelope membrane is not the driving force for outer membrane transport. Together with the data from Fig. 7 *A* which indicated that the energy for outer membrane transport is not used in the stromal compartment, this experiment implicates ATP in the inner membrane space as that which drives outer envelope transport.

## Inner Membrane Transport Requires High Levels of Stromal ATP

To examine the energetics of inner membrane protein transport independently of outer membrane transport, protease-protected prOE17 was accumulated with 100 μM ATP, and then allowed to chase across the inner membrane after protease treatment (Fig. 8). As expected, in the absence of additional nucleotide little of the protease-protected precursor chased across the inner membrane to the intermediate-sized form. The fraction of protease-protected precursor that appeared in the stroma after the chase period increased in proportion to the concentration of added ATP. Neither GTP, ADP, nor AMP-PNP could support inner membrane transport. When dihydroxycetone phosphate (DHAP) and oxaloacetic acid (OAA), a reagent combination which enables substrate-level ATP synthesis to occur in the stroma (cf. Olsen et al., 1989), were added to the chase reaction, 91% of the protease-protected precursor chased to the intermediate form, as would be predicted if inner membrane protein transport

A
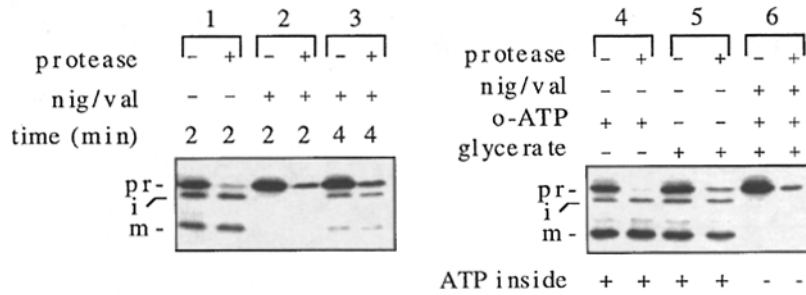
B
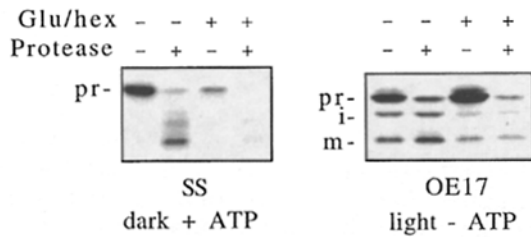
requires stromal ATP. Consistent with this idea, glucose and hexokinase added as an external ATP trap had no significant effect on the chase reaction driven by DHAP and OAA.

Since the only source of OE17 available during the chase period in this experiment was the protease-protected precursor (Fig. 8, −chase lane), the ATP-dependent appearance of iOE17 in these reactions must have resulted from a chase of this species across the inner membrane. This experiment therefore confirms our earlier conclusion (from Figs. 3 and 4) that the protease-protected precursor is a productive intermediate on the import pathway. It also in-
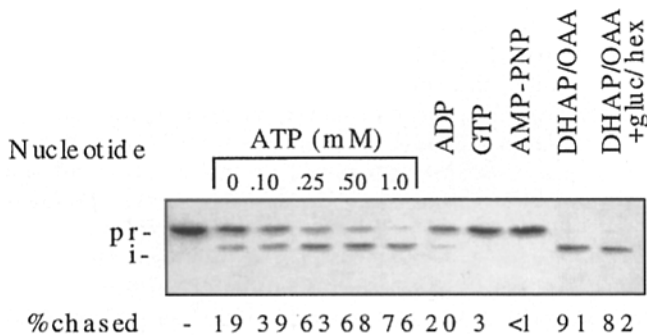
Figure 8. Inner membrane transport requires stromal ATP. prOE17 was imported into ATP- depleted chloroplasts with 100 μM ATP in the presence of nig/val for 4 min. Reactions were terminated by placing the samples on ice. All reactions were subjected to a 15-min thermolysin digestion which was stopped by the addition of 25 mM EDTA. NTPs were then added as indicated. Dihydroxycetone phosphate (DHAP) and oxaloacetic acid (OAA) were added at 1 mM each, glucose and hexokinase were added as in Fig. 6 B. Finally, the reactions were returned to room temperature and allowed to proceed for 8 more min. Quantitations are expressed as the ratio of intermediate-sized polypeptide to the total OE17 in each lane and represent the average of two experiments.

dicates that the protease-protected precursor intermediate is stable during our experiments since it survived the 27-min experiment under conditions that did not allow its import into the stroma, i.e., low ATP concentrations, ADP, GTP, and AMP-PNP.

Protease-protected prOE17 Is Associated with the Inner Envelope Membrane

When chloroplasts are lysed in hyperosmotic medium, the two envelope membranes are pulled apart and vesicles containing inner and outer membrane fragments are produced (Cline et al., 1985a). The inner and outer membranes can then be separated in a sucrose gradient by virtue of their different densities (Cline, 1985). To determine where the protease-protected precursor is located within the envelope membrane system (Fig. 2 *A*), membranes recovered from protein import reactions containing this species were fractionated using this method (Fig. 9). We excluded HgCl₂ during these separations because it has been reported to cause membrane aggregation (Reed et al., 1990). Fractionation experiments performed after protease treatment were similarly unsuccessful. Accordingly, we analyzed samples that were neither stopped with HgCl₂ nor protease treated. When samples from a 2-min import reaction that had been halted by ice treatment were examined, prOE17 was recovered across the entire sucrose gradient. Since this fractionation was performed in the absence of protease, the samples contained a large amount of prOE17 bound to the outer surface of the outer membrane; hence the high proportion of prOE17 fractionating at the top of the gradient with the outer membrane marker, prSS (Fig. 9 *B*). However, 17% of the total prOE17 recovered cofractionated with the inner membrane marker protein p36 (Schnell et al., 1990; Fig. 9 *C*). We note that this is in the correct range of protease-protected precursor detectable in import reaction halted by

A

C

B

placement on ice rather than by addition of HgCl₂ (i.e., Fig. 4). Accordingly, we suggest that the protease-protected precursor observed in Fig. 4 is the same species that fractionated with the inner envelope membrane in Fig. 9. We therefore conclude that the protease-protected precursor, observed in Fig. 2 to be membrane bound and facing the intermembrane space, is in fact bound to the outer surface of the inner membrane.

It has been reported that when prSS is bound to chloroplasts, it cofractionates with an intermediate density fraction thought to contain contact sites (Schnell and Blobel, 1993; Perry and Keegstra, 1994). In our experiments, prSS was bound to chloroplasts which were already osmotically shrunken, possibly preventing insertion into contact sites. Both prSS and prP36 (data not shown) fractionated near the top of the gradient where the outer membrane proteins would be expected. Regardless of whether the bound form of prSS fractionated with the outer membrane or with a contact site fraction, the behavior of prOE17 was clearly different. Under conditions where a protease-protected species is observed, a significant portion of prOE17 migrated to the bottom of the gradient and cofractionated with the inner membrane marker, distinct from the expected location for either outer membrane or contact site proteins.

Discussion

### Appearance and Location of the Protease-protected Precursor

In this study we report the characterization of a new intermediate on the pathway of protein import across the two chloroplast envelope membranes. The identifying features of this intermediate, which we have designated protease-protected precursor, are (*1*) resistance to rigorous protease treatment and (*2*) retention of its transit peptide. These characteristics distinguish this polypeptide species from the contact site intermediates described in both chloroplasts (Schnell and Blobel, 1993; Hirsch et al., 1994; Kessler et al., 1994; Schnell et al., 1994; Wu et al., 1994) and mitochondria (Schleyer and Neupert, 1985) which are protease-sensitive and mature-sized. In a recent paper, Wu et al. (1994) mentioned that they also observed a protease-protected precursor in their import experiments performed with low ATP concentrations. However, they did not examine this species further, nor did they discuss its implications for the mechanism of protein import. Our fractionation studies (Figs. 2 and 9) suggested that the protease-protected precursor was in the inner membrane space, and was associated with the inner envelope membrane. This intermediate was most readily observed when prOE17 was imported into osmotically shrunken plastids, but could be detected with two other precursors (Fig. 1), and with chloroplasts in isotonic buffer (Figs. 1 *B* and 6 *B*).

We performed three independent experiments to address whether the protease-protected precursor is a productive intermediate on the protein import pathway. First, under conditions in which precursors are translocated into the stroma of osmotically shrunken chloroplasts, the protease-protected prOE17 behaves kinetically as a pathway intermediate, rising early in the reaction time course and declining as the stromal intermediate-sized species accumulates (Fig. 3). Second, the protease-protected precursor could be trapped by placing samples from a 2-min incubation period on ice. Upon rewarming the samples after protease treatment, the precursor chased into the mature thylakoid-resident form (Fig. 4). Third, our evaluation of the

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energetics of envelope protein transport revealed that the protease-protected precursor could be accumulated at intermediate concentrations of ATP (Fig. 5, see below). Subsequent ATP addition resulted in the chase of the protease-protected prOE17 into the stroma (Fig. 8). Each of these experiments suggests that the protease-protected precursor is a productive intermediate capable of further transport across the inner envelope membrane under appropriate conditions. The existence of such an import intermediate shows that the envelope membranes contain functionally independent import machineries (Fig. 10).

These experiments demonstrate that chloroplasts can import proteins sequentially across the two envelope membranes under either low ATP or high osmotic strength conditions. The mechanism used when ATP is not limiting during the import of proteins into plastids held in isotonic medium is less clear. Under these conditions, the amount of protease-protected precursor observed was quite low (Fig. 1 *B*), making further analysis impossible. Furthermore, the kinetic treatment of the data obtained under hypertonic conditions (Fig. 3) examined only one possible pathway. Other models, particularly those in which proteins can enter the stroma via two pathways, only one of which passes through the protease-protected precursor intermediate, would presumably also permit reasonable fits to the data. Therefore, our experiments do not address the question of whether the protease-protected precursor, fully contained between the two envelope membranes, is an obligatory intermediate of chloroplast protein import. Our experiments do, however, indicate that this intermediate exists under certain conditions, and this discovery has important implications for the mechanistic description of the envelope protein transport reactions.
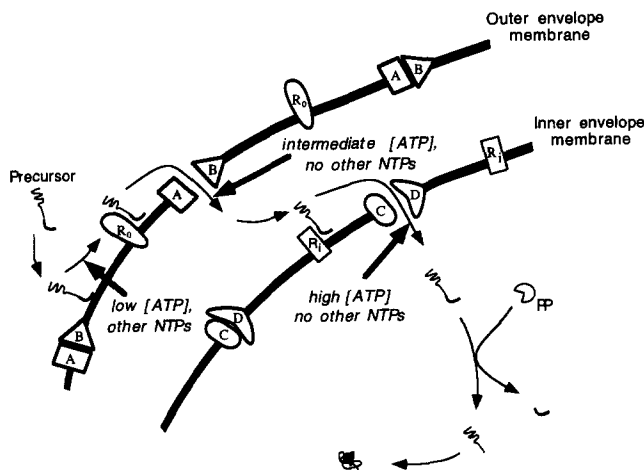
Figure 10. Schematic representation of sequential transport of proteins across the two chloroplast envelope membranes. The two envelope membranes are depicted with independent import machineries. A partially translocated polypeptide representing the protease-protected precursor species is shown bound to a receptor on the inner envelope membrane. The receptors and translocators in the outer and inner membranes were purposely left unidentified to allow for the addition of new components of the transport machinery as they become known while preserving the general nature of the model shown.

The Utilization of ATP during Envelope Protein Transport

The availability of this new protein import intermediate allowed us to identify distinct energy requirements for polypeptide transport across the outer and inner membranes. We found that outer membrane transport requires a moderate concentration of ATP outside the stroma, probably in the inner membrane space (Figs. 6 and 7). This requirement is distinguished from the energy used for precursor binding by a more restrictive nucleotide specificity and a higher demand for ATP. The amount of ATP required for outer membrane transport is significantly lower than the previously described total envelope transport ATP requirement. This, along with the requirement for high concentrations of stromal ATP for chasing trapped precursors across the inner envelope membrane, indicates that inner membrane transport is the energy expensive step of the chloroplast protein import process (Figs. 6 and 8). These conclusions are included in the scheme of Fig. 10.

The Energetics of Chloroplast Protein Import

If protein transport across cellular membranes has one unifying theme, it is the requirement for energy. Whether this energy is necessary for chaperone-mediated unfolding of translocating polypeptides, or is a more fundamental requirement of the actual process by which the polypeptide is driven across the hydrophobic membrane barrier is a central issue. Hsp70 and its homologues have been found to be associated with translocating polypeptides in a number of organelles, including mitochondria (Gambill et al., 1993), ER (Vogel et al., 1990), and chloroplasts (Waegemann and Soll, 1991; Tsugeki and Nishimura, 1993; Schnell et al., 1994; Wu et al., 1994; for review see Gray and Row, 1995). In addition to stromal hsp70s (Marshall et al., 1990; Marshall and Keegstra, 1992), chloroplasts contain one or possibly two envelope hsp70s which have been localized to the outer chloroplast membrane (Marshall et al., 1990; Ko et al., 1992; Schnell et al., 1994). It has been suggested that hsp70s provide the driving force for membrane transport by binding to the translocating polypeptide as it emerges from the membrane, thereby preventing retrograde movement (Neupert et al., 1990; Simon et al., 1992). Recent support for this idea came from work on the mitochondrial hsp70 which suggested that this matrix hsp70 homologue may play an essential role in the translocation process, probably in concert with the inner membrane protein MIM44/Isp45p (Kronidou et al., 1994; Ungermann et al., 1994), in a manner which is independent of the folding state of the translocating polypeptide (Gambill et al., 1993). It is possible that both the chloroplast outer envelope hsp70s and the stromal hsp70 function in a similar capacity in the plastid outer and inner envelope transport reactions, respectively. Our data regarding the location of the sites of ATP hydrolysis supporting these two reactions is consistent with these ideas.

The Role of Contact Sites in Chloroplast Protein Import

A number of reports have suggested that proteins can traverse the two plastid envelope membranes at contact sites (Schnell and Blobel, 1993; Perry and Keegstra, 1994;

Alefsen et al., 1994; Schnell et al., 1994; Wu et al., 1994). As elaborated above, our data do not demonstrate that all imported proteins proceed through an intermembrane space intermediate. Nevertheless, we do not believe that the import intermediate characterized herein used a contact site to cross the outer envelope membrane. While the protease-protected precursor species can be formed in normal isolated chloroplasts, at low efficiencies at high ATP concentrations (Fig. 1 *B*) or at high efficiencies at low ATP concentrations (Fig. 5 *B*), it is more readily observed in osmotically shrunken chloroplasts. Because this treatment is known to leave "structural" contact sites intact (Cline et al., 1985a), it is not obvious why osmotic shrinking would enhance the quantity of protease-protected precursor observed if the precursor were forced to enter the translocation pathway at one of these contact sites. Further, if proteins start their transport across the envelope membranes at contact sites, it would not be expected that the protease-protected precursor would act kinetically as a pathway intermediate. Yet the protease-protected precursor was among the earliest species detected in a chloroplast protein import reaction (Fig. 3). This is in contrast to the situation in mitochondria where precursors are postulated to initially engage the import machinery in contact sites, and, if further translocation across the inner membrane is blocked, diffuse with the inner membrane receptor out of the contact sites (Hwang et al., 1991; Segui-Real et al., 1993). Recent evidence suggests that the inner mitochondrial membrane receptor MIM44/Isp45p acts as an anchor for the incoming polypeptide and prevents diffusion out of the translocation pore (Kronidou et al., 1994; Schneider et al., 1994; Ungermann et al., 1995; Mayer et al., 1995). This could lead to the coupling of outer and inner membrane transport usually observed in mitochondrial protein import. The chloroplast outer envelope membrane hsp70 may use ATP in the intermembrane space to provide the same tethering of the incoming polypeptide without using the transport machinery of the inner membrane, resulting in the uncoupled transport reactions observed here.

It has been suggested that envelope membrane vesicles of intermediate density, which appear to consist of a mixture of inner membranes and outer membranes, represent a contact site fraction (Cline et al., 1985a; Schnell and Blobel, 1993; Perry and Keegstra, 1994). By this criteria, some of the protease-protected prOE17 may also cofractionate with contact sites. However, it is difficult to explain why this precursor would be resistant to exogenous protease digestion. Furthermore, it is noteworthy that, unlike the protease-sensitive mature protein import intermediates which defined contact site transport in mitochondria (Schleyer and Neupert, 1985) and chloroplasts (Schnell and Blobel, 1993; Alefsen et al., 1994; Schnell et al., 1994; Wu et al., 1994), the species we have described retains its transit peptide. If this import pathway intermediate extends into the stromal compartment, it must do so in such a way that the cleavage site of the transit peptide remains shielded from the processing peptidase.

A number of authors have raised the possibility that proteins crossing the plastid envelope membranes could engage the inner membrane translocation machinery before being released from the transport apparatus of the outer membrane, thereby creating dynamic "translocation" contact sites (Alefsen et al., 1994; Perry and Keegstra, 1994; Schnell et al., 1994; cf. Horst et al., 1995). Our experiments are consistent with this model. When the envelope membranes are pulled apart, such as in high osmotic strength buffer, this type of coupled transport would be less likely to occur, resulting in the sequential transport across the envelope membranes that we have observed. Our data add detail to this model by showing that the chloroplast envelope membranes each contain functional import machineries, and that these machineries may act either in concert in a coupled transport reaction or independently in a sequential transport reaction. Since the envelope protein import machineries can function in either coupled or uncoupled modes, contact sites might best be viewed as a consequence of coupled transport rather than an obligatory ingredient of the mechanism through which proteins are translocated across the chloroplast envelope membranes.

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