Identification of Intermediates in the Pathway of Protein Import into Chloroplasts and Their Localization to Envelope Contact Sites

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Abstract. We have used a hybrid precursor protein to study the pathway of protein import into chloroplasts. This hybrid (pS/protA) consists of the precursor to the small subunit of Rubisco (pS) fused to the IgG binding domains of staphylococcal protein A. The pS/protA is efficiently imported into isolated chloroplasts and is processed to its mature form (S/protA). In addition to the mature stromal form, two intermediates in the pathway of pS/protA import were identified at early time points in the import reaction. The first intermediate represents unprocessed pS/protA bound to the outer surface of the chloroplast envelope and is analogous to a previously characterized form of pS that is specifically bound to the chloroplast surface and can be subsequently translocated in the stroma (Cline, K., M. Werner-Washburne, T. H. Lubben, and K. Keegstra. 1985. *J. Biol. Chem.* 260:3691-3696.) The second intermediate represents a partially translo-cated form of the percursor that remains associated with the envelope membrane. This form is processed to mature S/protA, but remains susceptible to exogenously added protease in intact chloroplasts. We conclude that the envelope associated S/protA is spanning both the outer and inner chloroplast membranes en route to the stroma. Biochemical and immunochemical localization of the two translocation intermediates indicates that both forms are exposed at the surface of the outer membrane at sites where the outer and inner membrane are closely apposed. These contact zones appear to be organized in a reticular network on the outer envelope. We propose a model for protein import into chloroplasts that has as its central features two distinct protein conducting channels in the outer and inner envelope membranes, each gated open by a distinct subdomain of the pS signal sequence.

A majority of the protein components of the chloroplast are encoded in the nucleus and synthesized as higher molecular weight precursor proteins on cytoplasmic ribosomes. The distinction between the site of chloroplast protein synthesis and their sites of function requires that a system exist for the import of these proteins into the organelle en route to their proper assembly into one of the six suborganellar compartments. The initial committal step in the process of targeting and assembly of proteins destined for the internal compartments of the chloroplast is their translocation across the double membrane of the chloroplast envelope (for review see Keegstra and Olsen, 1989; de Boer and Weisbeek, 1991).

The import process can be divided into two general stages. The first stage involves the specific association of the precursor with the surface of the chloroplast (Cline et al., 1985*b*; Friedman and Keegstra, 1989). The specificity of this binding is conferred on the precursor by its intrinsic amino-terminal signal sequence and requires proteinaceous receptor(s) at the chloroplast surface (Cline et al., 1985*b*). The binding reaction also requires a low concentration of ATP which is apparently hydrolyzed in the intermembrane space (Olsen et al., 1989; Olsen and Keegstra, 1992). The second stage of import involves the translocation of the bound precursor across the double membrane of the envelope into the stroma. This process is also ATP dependent (Grossman et al., 1980), but the concentration of ATP required for import is five- to tenfold greater than that required for precursor binding (Olsen et al., 1989). In addition, the hydrolysis of this ATP occurs within the chloroplast stromal compartment (Pain and Blobel, 1987; Theg et al., 1989). Upon translocation, the signal sequence is removed by a specific endoprotease (Robinson and Ellis, 1984) and the polypeptide is free to undergo subsequent targeting or assembly reactions.

One approach to obtaining a greater understanding of the chloroplast import process is to identify and characterize intermediates in the translocation reaction. This approach has been used successfully in the case of mitochondria (for review see Pfanner et al., 1992) and the RER (Gilmore and Blobel, 1985; Sanders et al., 1992) to delineate individual steps in the import process.

In this paper, we have used a hybrid precursor protein to identify at least two intermediates in the pathway of precursor import into chloroplasts. These intermediates include an envelope bound precursor with characteristics similar to those previously described for bound small subunit of Rubisco precursor (pS), and a second mature form of the hy-

brid which appears simultaneously to span both the outer and inner membranes en route to the stroma. In addition, we have used the hybrid precursor to localize the sites of precursor import, and thereby assess the role of envelope contact sites in the chloroplast import process.

Materials and Methods

Materials

Pea seeds (Progress No. 9) were obtained from W. Altee Burpee Co. (Warminster, PA). [^{35}S]methionine and [^{35}S]EXPRE^{35}S^{35}S (cysteine/methionine) were obtained from Du Pont New England Nuclear (Wilmington, DE). Percoll silica gel was from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Nigericin was from Calbiochem-Behring Corp. (San Diego, CA). IPTG was obtained from BRL/Gibco Inc. (Gaithersburg, MD). Fluorescein-conjugated goat anti-rabbit IgG was obtained from Jackson Immunoresearch Laboratories Inc. (West Grove, PA) and gold-conjugated goat anti-rabbit IgG was obtained from Zymed Laboratories Inc. (San Francisco, CA). Plasmid pET8c and *Escherichia coli* strain BL21 (DE3) were a kind gift of Dr. F. W. Studier (Biology Department, Brookhaven National Laboratory, Upton, NY).

Isolation and Cryopreservation of Chloroplasts

Intact chloroplasts were isolated from 10-14-d-old pea seedlings (*Pisum sativum*, Progress No. 9) by homogenization and Percoll silica gel gradient centrifugation as previously described (Pain and Blobel, 1987). If chloroplasts were to be used directly in import experiments, they were resuspended in 50 mM Hepes-KOH, pH 7.7, 0.33 M sorbitol (HS buffer) to a concentration of 2 mg of chlorophyll/ml. If chloroplasts were to be used in future experiments, they were resuspended directly in HS buffer containing 20% vol/vol DMSO to a concentration of 2-3 mg chlorophyll/ml, immediately frozen in liquid nitrogen and stored under liquid nitrogen according to the procedure of Yuan et al. (1991). The cryopreserved chloroplasts were thawed at room temperature, diluted 1:1 with HS buffer, and reisolated over 40% Percoll in HS buffer at 4°C prior to use in precursor import assays. The average recovery of intact chloroplasts was 50-60% of the original frozen material.

Production of Overexpressed pS/protA in E. coli and Cell-free Transcription and Translation of pS and pS/protA

A diagram of the pET8c-pS/protA construct is shown in Fig. 1. Nucleotides 76-618 of the pS cDNA (Pain and Blobel, 1987) encoding the entire pS protein sequence were inserted into the NcoI/BamHI sites of pET8c (Rosenberg et al., 1987) after the introduction of NcoI and BamHI sites onto the ends of the pS sequence using the polymerase chain reaction and pT7-pS (Pain and Blobel, 1987) as a template. This construct was named pET8c-pS. To construct the pET8c-pS/protA plasmid, a segment of the staphylococcal protein A gene (Uhlen et al., 1984) from nucleotides 272-1104 was amplified by the polymerase chain reaction using primers that incorporated an in frame NsiI site at the 5' terminus and a stop codon immediately followed by a BamHI site at the 3' terminus with the pRIT5 plasmid (Pharmacia Fine Chemicals, Piscataway, NJ) serving as a template. This DNA fragment encodes residues −10 to 271 of the protein A polypeptide that includes the five potential IgG binding domains, but excludes the entire membrane-spanning domain and most of the signal sequence of protein A. The NsiI/BamHI fragment of protein A was inserted into the NsiI/BamHI sites of the pET8c-pS plasmid to yield pET8c-pS/protA.

The pET8c-pS and pET8c-pS/protA plasmids were introduced into *E. coli* BL21 (DE3) (Rosenberg et al., 1987). The strains were cultured in L-broth or M9 medium (Maniatis et al., 1982) containing 100 μg/ml ampicillin at 37°C. When cultures had reached OD$_{600}$ = 0.6, expression of pS or the pS/protA fusion protein was induced by the addition of IPTG to 1 mM final concentration. The induction was carried out for 2-4 h at 37°C. If the over expressed proteins were to be [^{35}S] labeled the strains were grown at 37°C in M9 medium containing MgSO$_4$ to OD$_{600}$=0.6. The cells were recovered by centrifugation at 8,000 g for 10 min at room temperature, and resuspended in M9 medium with MgCl replacing MgSO$_4$. IPTG was added to 1 mM final concentration and the culture was incubated at 37°C for 30 min. After the incubation, a mixture of [^{35}S]methionine and [^{35}S]cys-

teine (EXPRE^{35}S^{35}S; Du Pont/New England Nuclear, Wilmington, DE) was added to a final concentration of 0.1 mCi/ml and the culture was incubated at 37°C for 4 h.

The over expressed pS/protA protein was found to be sequestered in insoluble inclusion bodies in IPTG induced cultures of the appropriate strain. The inclusion body fraction was purified from the *E. coli* cells using the lysis and TX-100 washing method of Marston (1987). The inclusion bodies were concentrated by centrifugation and dissolved in 8 M urea, 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, and 2 mM DTT to a concentration of 100 μg/ml. The dissolved inclusion bodies were rapidly diluted into 60 to 100 vol of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl (dilution buffer) and incubated at room temperature for 2 h to allow refolding of the denatured pS/protA. After the incubation, the diluted solution was centrifuged for 10 min at 20,000 g to remove any aggregates. The supernatant was applied directly to a rabbit IgG-Sepharose column, and the column was subsequently washed with 5 vol of dilution buffer. The bound pS/protA was eluted with 0.2 M glycine, pH 2.2. The purified pS/protA was dialyzed into dilution buffer containing 5 mM DTT and was stored at −80°C. Before addition to the chloroplast binding or import reactions, the purified pS/protA was concentrated and dissolved in dilution buffer containing 8 M urea and 2 mM DTT to a final concentration of 100 μg protein/ml.

In vitro transcription of pT7-pS and pET8c-pS/protA was carried out as previously described (Pain and Blobel, 1987). The pS and pS/protA transcripts were translated in a wheat germ cell-free translation system in the presence of [^{35}S]methionine for 1 h at 25°C as previously described (Pain and Blobel, 1987). The postribosomal supernatant (PRS)[1] of the translation mixtures containing the newly synthesized pS or pS/protA was depleted of ATP by chromatography through Sephadex G-25 as described by Olsen et al. (1989) and used directly in the chloroplast import assays or stored at −80°C.

Chloroplast Import and Binding Assays

The import of urea denatured pS and pS/protA into intact chloroplasts was assayed essentially by a standard method (Pain and Blobel, 1987). Unless otherwise indicated, each assay contained isolated intact chloroplasts equivalent to 25-100 μg of chlorophyll in 300 μl of HS buffer containing 2.0 mM Mg(OAc)$_2$, 40 mM KOAc, 0.025 mM DTT, 100 μg/ml BSA (import buffer) and 5 mM MgATP. Purified pS/protA was added directly to each assay in 5 μl of 8 M urea at 26°C. After import, the chloroplasts were reisolated over 40% Percoll silica gel and subjected to further analysis or treated with 50 μg/ml thermolysin on ice for 30 min in the presence of 0.1 mM CaCl$_2$ (Cline et al., 1984).

The binding of urea denatured pS/protA to intact chloroplasts was assayed by a modification of previously described methods (Schnell et al., 1991). Unless otherwise indicated, each assay contained chloroplasts equivalent to 25-50 μg of chlorophyll in 300 μl of import buffer containing 50 μM ATP. After binding, the chloroplasts were reisolated and analyzed as given for the import reactions.

The import or binding of pS/protA was quantitated by direct radioanalytic imaging of SDS-PAGE gels using an AMBIS Radioanalytic Imaging System (San Diego, CA). Data plotting and analysis were carried out using the KaleidoGraph data analysis system (Synergy Software, Inc., Reading, PA). Binding or import competence was determined by calculating the fraction of pS/protA that could be bound or imported by isolated chloroplasts under conditions in which the concentration of ^{35}S-labeled pS/protA was limiting and the amount of chloroplasts were in excess in the assay reaction mixture.

Preparation of Chloroplast Envelope Membranes

Chloroplast envelope membranes were prepared by a modification of the method of Keegstra and Yousif (1986). After reisolation through 40% Percoll silica gel, chloroplasts were resuspended directly in 10 mM Tricine-HCl, pH 7.5, 2.0 mM EDTA (TE buffer) containing 0.6 M sucrose to a final concentration of no more than 0.5 mg chlorophyll/ml. After 10 min on ice, the chloroplasts were lysed by freezing at −20°C for 1 h. The samples were thawed on ice, diluted to 0.2 M sucrose with TE buffer, and homogenized by 20 strokes with piston A in a Dounce tissue homogenizer (Kontes Glass Co., Vineland, NJ). The membrane fraction was recovered by centrifugation at 30,000 rpm (114,000 g) in a rotor (SW 40 Ti; Beckman Instruments, Inc., Palo Alto, CA) for 30 min at 4°C. The supernatant containing the

stromal contents was removed for subsequent analysis, and the membrane pellet containing total chloroplast membranes was resuspended in 10 ml of 42% wt/vol sucrose in TE buffer by 20 strokes with piston A in a Dounce tissue homogenizer (Kontes Glass Co.). The resuspended membranes were transferred to a tube (SW 28; Beckman Instruments, Inc.) and overlaid with a 28-ml linear gradient of 20–38% wt/vol sucrose in TE buffer. The samples were centrifuged at 96,000 g for 17 h in a rotor (SW 28; Beckman Instruments, Inc.). After centrifugation, the gradients were separated into three major fractions: (a) the 20–22% sucrose fraction containing outer membrane vesicles; (b) the 25–32% sucrose fraction containing inner membrane and mixed inner and outer membrane vesicles; and (c) the 42% sucrose cushion containing the thylakoid membrane vesicles and residual stromal contents. The membranes in each fraction were recovered by diluting the fraction with 2 vol of TE buffer and centrifuging the samples at 200,000 g for 90 min.

For import or binding reactions containing chloroplasts equivalent to <1 mg chlorophyll, the sucrose density gradient separation of the total chloroplast envelope membranes was modified to maximize membrane yield. All procedures before the sucrose density gradient separation were identical to those described above. Instead of flotation into a linear sucrose gradient, the membranes were resuspended in 0.5 ml of 42% wt/vol sucrose in TE buffer and overlaid with a step gradient composed of 0.75 ml 38% wt/vol sucrose, 0.5 ml 25% wt/vol sucrose and 0.25 ml 20% wt/vol sucrose all in TE buffer. The samples were centrifuged in a swinging bucket rotor (TLS-55; Beckman Instruments, Inc.) for 2 h at 200,000 g in an ultracentrifuge (TL-100; Beckman Instruments, Inc.) at 4°C. The step gradients were fractionated into three major components: (a) the 20% sucrose layer containing outer membrane vesicles; (b) the 38%/25% sucrose interface containing inner and mixed inner and outer membrane vesicles; and (c) the 42% sucrose cushion containing thylakoid membranes. The membrane fractions were diluted and recovered by centrifugation as given above.

Immunofluorescence Microscopy

Chloroplasts (equivalent to 25 μg of chlorophyll) which had been incubated with the pS/protA construct under binding or import conditions were reisolated over 40% Percoll in HS buffer and prefixed in 100 μl ice-cold HS buffer containing 0.05% glutaraldehyde and 0.5% paraformaldehyde for 30 min. After the prefixation, the chloroplasts were centrifuged for 30 s at 3,500 g at 4°C and washed with 100 μl of HS buffer containing 10 mM NH₄Cl. The chloroplasts were resuspended in 100 μl of HS buffer containing 50 μg/ml of rabbit IgG and incubated for 15 min at room temperature in the dark or 1 h at 4°C in the dark. The chloroplasts were reisolated over 40% Percoll in HS buffer and incubated with 20 μg/ml of fluorescein-conjugated goat anti–rabbit IgG. After incubation, the chloroplasts were reisolated over 40% Percoll silica gel, resuspended in HS buffer, and allowed to settle onto polylysine-coated coverslips for 30 min at 4°C. The chloroplasts were fixed onto the coverslips in HS buffer containing 3% paraformaldehyde for 30 min at 4°C. The slides were viewed with a Zeiss Axiophot fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Antibodies against a 37-kD polypeptide of the chloroplast outer envelope membrane were generated in rabbits as previously described (Schnell et al., 1990). Immunofluorescence microscopic detection of the 37-kD polypeptide was carried out as for detection of the pS/protA hybrid, except chloroplasts were not incubated with pS/protA before incubation with the anti-37-kD serum.

Immunoelectron Microscopy

For the preparation of thin sections, chloroplasts (equivalent to 12.5 μg of chlorophyll) which had been incubated with the pS/protA construct under binding or import conditions were reisolated over 40% Percoll in HS buffer and prefixed in 100 μl ice-cold HS buffer containing 0.05% glutaraldehyde, 0.5% paraformaldehyde for 30 min. After the prefixation, the chloroplasts were centrifuged for 30 s at 3,500 g at 4°C and washed with 100 μl of HS buffer containing 10 mM NH₄Cl. The chloroplasts were resuspended in 100 μl of HS buffer containing 50 μg/ml of rabbit IgG and incubated for 1 h at 4°C in the dark. The chloroplasts were reisolated over 40% Percoll in HS buffer and incubated with a 1:10 dilution of goat anti–rabbit IgG conjugated to 10-nm colloidal gold (Zymed Inc., San Francisco, CA) in HS buffer. After incubation, the chloroplasts were reisolated over 40% Percoll in HS buffer, resuspended in HS buffer containing 2.5% glutaraldehyde for 30 min on ice. The chloroplasts were embedded, sectioned, and viewed as previously described (Pain et al., 1988).

The negative staining of immunolabeled chloroplasts was carried out as follows. All procedures were carried out at 0–4°C. Chloroplasts (equivalent to 12.5 μg of chlorophyll) that had been incubated with the pS/protA construct under binding conditions at 26°C were reisolated over 40% Percoll in HS buffer, resuspended in 100 μl of HS buffer containing 50 μg/ml rabbit IgG and incubated for 30 min on ice. After incubation with rabbit IgG, the chloroplasts were reisolated through 40% Percoll in HS buffer and resuspended to 2 × 10⁷ chloroplasts/ml in HS buffer. A small aliquot (3–5 μl) of the suspension was deposited onto polylysine or alcian blue treated-carbon-Formvar-coated nickel grids. The grid was placed drop-side up on a slide that had been covered with parafilm and the chloroplasts were allowed to adhere to the grid for 1 h in a moist chamber. Any nonadhering chloroplasts were removed by floating the grid, chloroplast side down, on a drop of HS buffer. The grid was transferred to a drop of HS buffer containing a 1:10 dilution of goat anti–rabbit IgG conjugated to 10 nm gold (Amersham Corp., Arlington Heights, IL) and incubated for 1–2 h. After the incubation, the grids were rinsed three to six times with HS buffer for a total of 1–2 h, and negatively stained with 2% aqueous phosphotungstic acid (PTA), pH 7.4, by a standard method (Hayat, 1972). The grids were photographed on a JEOL 100 CX electron microscope (JEOL USA, Peabody, MA) operated at 80 kV.

Results

Construction and Overexpression of pS/protA

As an import substrate for our studies, we constructed a hybrid protein between the precursor of the small subunit of Rubisco (pS) and the five potential IgG binding domains of staphylococcal protein A (protA) (Fig. 1 A). This hybrid was chosen as it embodied both the well-defined import characteristics of pS and the high affinity IgG binding sites of protein A. This second feature provided a simple means of purifying the precursor using IgG-Sepharose. Furthermore, it offered the potential to specifically localize the precursor using IgG molecules that do not react with endogenous chloroplast components.

The pS/protA fusion was overexpressed in *E. coli* using the T7 promoter based pET8c vector system (Rosenberg et al., 1987) in the presence of [³⁵S]methionine/cysteine. The overexpressed pS/protA was sequestered in insoluble inclusion bodies, thereby greatly simplifying the initial stages of its purification. The inclusion body fraction containing the ³⁵S-labeled pS/protA was prepared from induced *E. coli* cells by the method of Marston (1987). To further purify pS/protA, the inclusion bodies were dissolved in 8 M urea and the solution was rapidly diluted 100-fold at room temperature to allow refolding of the urea denatured pS/protA. The pS/protA was purified from the diluted mixture by affinity chromatography on rabbit IgG-Sepharose. A typical SDS-PAGE profile of a crude *E. coli* extract (Fig. 1, lane 1) and the IgG-Sepharose purified pS/protA (Fig. 1, lane 2) is shown in Fig. 1 B. After induction, pS/protA constitutes ~0.5% of the total *E. coli* protein with typical yields of 200 μg per liter of culture. The pS/protA was ~85% pure after enrichment by IgG-Sepharose (Fig. 1 B, lane 2) and represented 90% of the radiolabeled material in this fraction (Fig. 1 C). The pS/protA fusion had a typical specific activity of 10⁶ cpm/μg.

Binding and Import Characteristics of pS/protA

As an initial step in characterizing the import of pS/protA into chloroplasts, we compared the requirements for the import of the urea denatured substrate to those that have been determined for authentic pS synthesized in a cell free system. First, we determined the energy requirements of pS/protA
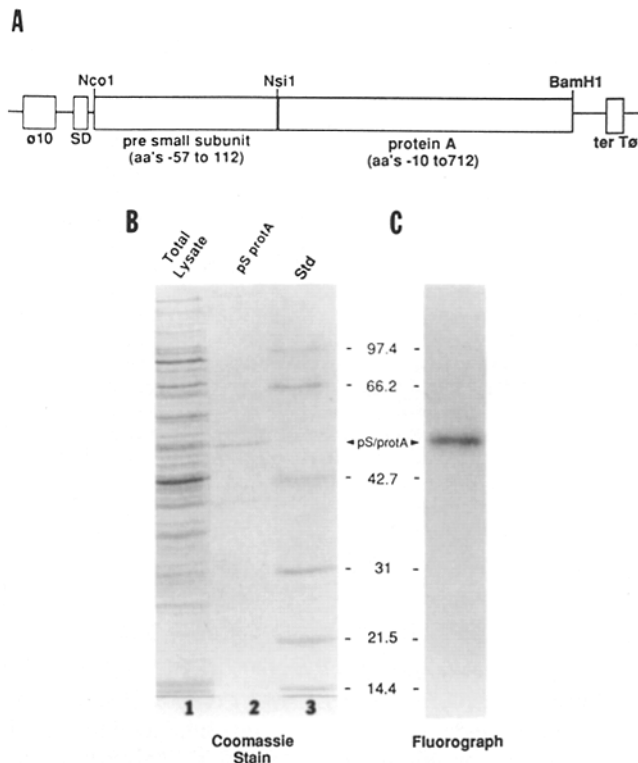
A


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φ10  SD   pre small subunit        protein A            ter Tø
          (aa's -57 to 112)        (aa's -10 to712)
     Nco1           Nsi1                    BamH1
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B   **C**


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- 97.4 -
- 66.2 -
◄pS/protA►
- 42.7 -
- 31 -
- 21.5 -
- 14.4 -
  1    2    3
Coomassie          Fluorograph
Stain
```

Figure 1. Physical map of the pET8c-pS/protA insert and overexpression of pS/protA in *E. coli.* (*A*) The 1.4-kb BglIII-EcoRV fragment of pET8c-pS/protA containing all of the sequences relevant for expression is shown. The pS/protA fragment was cloned into the NcoI and BamHI sites of pET8c under the control of the inducible φ10 promoter at a position 8-bp downstream from the translation initiation signal for the gene *10* protein of phage T7. The Tø transcription terminator is present downstream of the pS/protA insert. (*B*) Coomassie blue–stained SDS-PAGE gel of a total cell extract (lane *1*) and a pS/protA-enriched fraction (lane *2*) from *E. coli* cultures induced with IPTG for the expression of pS/protA. (*C*) Fluorograph of a pS/protA-enriched fraction from *E. coli* cultures induced for pS/protA expression in the presence of [^{35}S]methionine/cysteine.

import into isolated chloroplasts. It has been shown previously for pS synthesized in a wheat germ extract that both the specific binding of the precursor to the envelope and its subsequent import into the stroma of isolated chloroplasts requires the hydrolysis of ATP (Olsen et al., 1989). To assay the requirement of ATP in our system, the purified ^{35}S-labeled pS/protA was dissolved in 8 M urea and directly diluted into a chloroplast suspension containing a standard import buffer and various concentration of MgATP at room temperature in the dark. The final concentration of pS/protA in the reaction was 30 nM. The final concentration of urea in the import reaction did not exceed 0.2 M. Concentrations of urea up to 0.8 M had no effect on chloroplast import (data not shown). After a 30-min import reaction, the intact chloroplasts were reisolated through a 40% Percoll silica gel cushion, fractionated into crude envelope and stromal components, and both fractions were directly analyzed by SDS-PAGE and fluorography.

The effects of varying MgATP concentrations on pS/protA import into isolated chloroplasts are shown in Fig. 2. In the absence of exogenously added MgATP, no detectable pS/
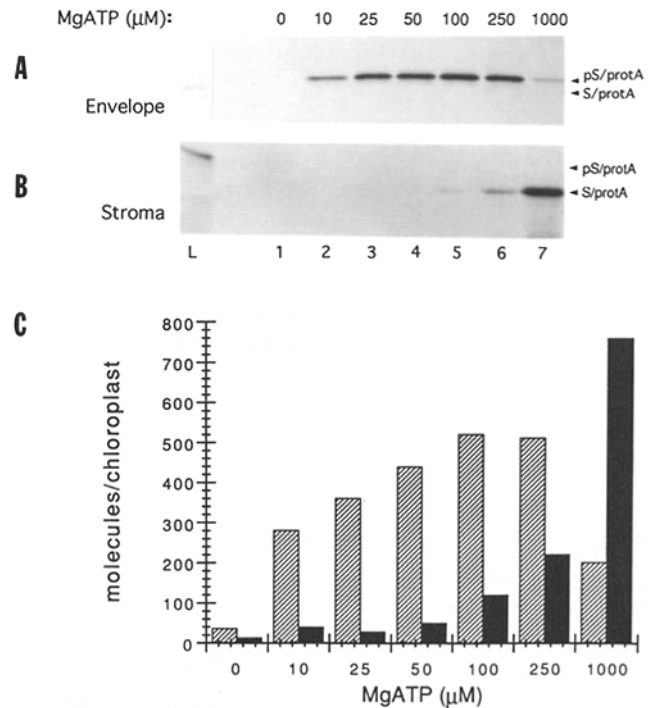
MgATP (μM): 0 10 25 50 100 250 1000

A Envelope

B Stroma

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L   1   2   3   4   5   6   7
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C

Figure 2. ATP dependence of pS/protA import into isolated chloroplasts. Aliquots of ATP-depleted chloroplasts equivalent to 25 μg chlorophyll were incubated with 30 nM urea denatured [^{35}S]pS/protA in the presence of increasing amounts of MgATP at 26°C for 30 min. The chloroplasts were reisolated over 40% Percoll silica gel and separated into crude envelope (*A*) and stroma (*B*) fractions. The fractions were analyzed by SDS-PAGE and fluorography. The concentrations of MgATP included in each assay are indicated by the numbers above *A*. The position of precursor (*pS/protA*) and mature (*S/protA*) forms of the pS/protA fusion are indicated by the arrowheads. Lane *L* contains an aliquot of the [^{35}S]pS/protA added to each reaction. (*C*) Quantitation of the data in *A* and *B*. ▨, bound; ■, imported.

protA is found associated with either the envelope or stroma of the chloroplasts (Fig. 2, *A* and *B*, lane *1*). As the MgATP concentration is increased, increasing amounts of pS/protA are specifically bound to the envelope fraction with maximum binding occurring at 50–100 μM MgATP (Fig. 2 *A*, lanes *2–5*, and Fig. 2 *C*). At concentrations of MgATP exceeding 100 μM, the amount of pS/protA bound at the envelope decreases (Fig. 2 *A*, lanes *5–7*, and *C*) with a concomitant increase in a stromal form of the pS/protA fusion (Fig. 2 *B*, lanes *5–7*, and *C*). The imported fusion protein comigrates on SDS-PAGE with mature S/protA expressed in the pET8c system (data not shown) and most likely represents mature S/protA. A small amount of mature S/protA is found associated with the envelope fraction at 1,000 μM ATP (Fig. 2 *A*, lane *7*). This processed form of the precursor is a membrane-associated form of the hybrid rather than contamination from the imported stromal S/protA (see below).

Second, we measured the affinity of pS/protA binding to the chloroplast surface to determine whether the urea denatured substrate used a recognition system of similar characteristics to that used by a wheat germ–synthesized substrate. Isolated chloroplasts that had been depleted of endogenous ATP were incubated with various concentrations of urea denatured pS/protA in the dark for 30 min at 26°C in the
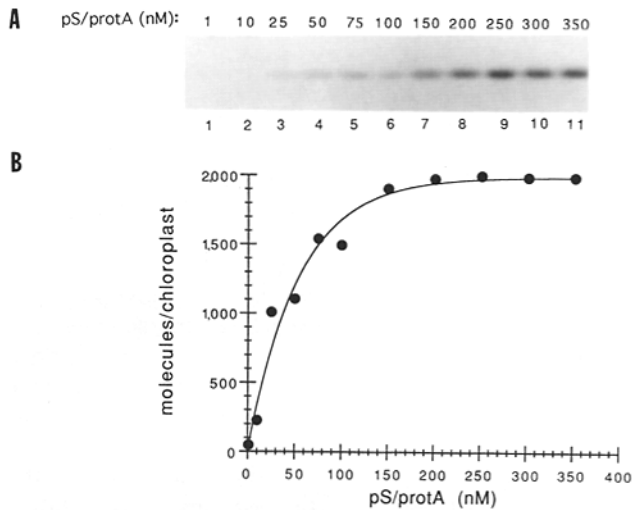
A pS/protA (nM): 1 10 25 50 75 100 150 200 250 300 350

B

Figure 3. Titration of pS/protA binding to intact chloroplasts. Aliquots of ATP-depleted chloroplasts equivalent to 25 μg chlorophyll were incubated with increasing concentrations of urea denatured [^{35}S]pS/protA in the presence of 50 μM MgATP at 26°C in the dark for 10 min. The chloroplasts were reisolated over 40% Percoll silica gel and analyzed directly by SDS-PAGE and fluorography. (*A*) Fluorograph of chloroplasts incubated with increasing concentrations of [^{35}S]pS/protA. (*B*) Quantitation of the data in *A*. The concentration of [^{35}S]pS/protA included in each reaction is given at the top of *A* in nanomolar.

presence of 50 μM MgATP. After the binding reaction, the chloroplasts were reisolated over 40% Percoll silica gel and directly analyzed by SDS-PAGE and fluorography (Fig. 3 *A*). Fig. 3 *B* shows the plot of the pS/protA binding data fit to a hyperbolic curve. The chloroplast binding sites were saturated at 1,600–2,200 molecules/chloroplast with a binding K_d of ~40 nM. This value most likely represents a minimum affinity estimate as only 65–70% of the urea denatured pS/protA added to each assay was competent for binding or import in a given experiment (data not shown).

Finally, to confirm that the import of pS/protA was using the same import pathway into chloroplasts as wheat germ synthesized pS, we tested the ability of these two precursors to compete for import. Isolated chloroplasts were incubated with various concentrations of unlabeled urea denatured pS/protA for 5 min on ice in import buffer. Following the preincubation with pS/protA, an aliquot of wheat germ PRS containing ^{35}S-labeled pS was added to the chloroplast suspension and the mixture was incubated for 15 min at 26°C. The chloroplasts were reisolated and analyzed by SDS-PAGE and fluorography. Fig. 4 shows that unlabeled pS/protA is able to compete for the import of ^{35}S-labeled pS into isolated chloroplasts (Fig. 4 *A*, compare lanes *1* through *6*, and *B*). The import of radiolabeled pS synthesized in a wheat germ extract was inhibited by pS/protA in a dose-dependent manner with >90% inhibition observed at the highest concentrations of pS/protA included in the assay (Fig. 4 *A*, lane *6*, and *B*). These data confirm that pS/protA is imported into isolated chloroplasts by a mechanism similar to that used by wheat germ synthesized pS.

Time Course of pS/protA Import

The identification of membrane bound forms of pS/protA

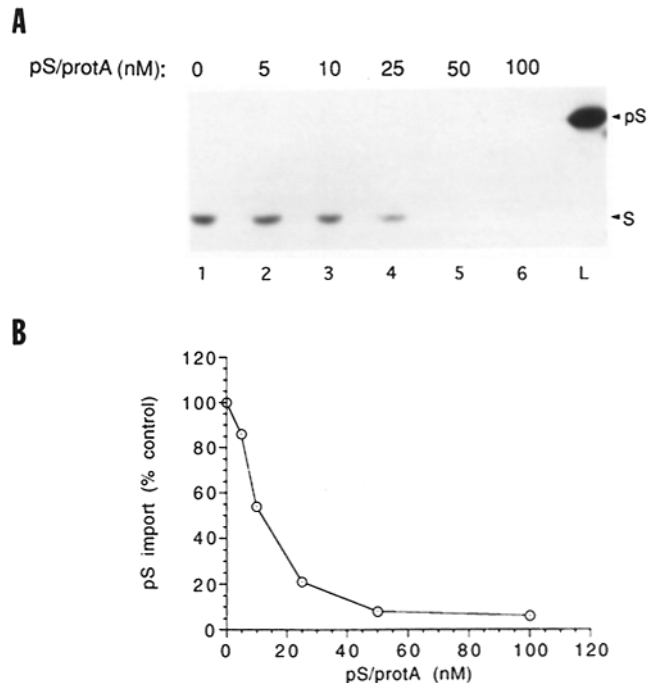**A** pS/protA (nM): 0 5 10 25 50 100

B

Figure 4. Competition of pS import into isolated chloroplasts with urea denatured pS/protA. Aliquots of chloroplasts equivalent to 25 μg chlorophyll were preincubated with increasing concentrations of unlabeled urea denatured pS/protA for 5 min on ice before the addition of 30 μl of wheat germ PRS containing [^{35}S]pS. The chloroplasts were incubated for an additional 15 min at 26°C under import conditions, reisolated over 40% Percoll silica gel, and analyzed directly by SDS-PAGE and fluorography. (*A*) Fluorograph of chloroplasts from the competition experiment. (*B*) Quantitation of data in *A*. The concentration of pS/protA in each reaction is indicated at the top of *A*. The positions of precursor (*pS*) and mature (*S*) forms of the precursor are indicated by arrowheads. Lane *L* contains an aliquot of the wheat germ PRS containing [^{35}S]pS.

and S/protA under precursor import conditions (Fig. 2) suggested that these polypeptides might represent intermediates in the pathway of precursor import into the chloroplast. Therefore, the appearance of envelope-associated pS/protA and S/protA should precede appearance of stromal S/protA, and their abundance relative to the stromal S/protA should be greater at early time points in the import reaction. To test this possibility, we carried out a time course of pS/protA import. Isolated chloroplasts were incubated with 200 nM ^{35}S-labeled pS/protA in the dark at 26°C for 5 min in the presence of 50 μM MgATP to support specific binding of the precursor but preclude precursor import. The chloroplasts were reisolated over 40% Percoll silica gel and resuspended in import buffer excluding MgATP. Import of the bound precursor was initiated by the addition of 5 mM MgATP, and at various times, an aliquot of chloroplasts was removed, chilled on ice, and reisolated through 40% Percoll silica gel. The chloroplasts were lysed by freeze thaw under hypertonic conditions and fractionated into stromal, thylakoid, and envelope fractions by flotation into sucrose step gradients (see Materials and Methods). The analysis of the stroma and envelope fractions is shown in Fig. 5. The thylakoid fraction is not shown, as none of the hybrid was detected in this fraction. Mature S/protA accumulated in the stroma up to 10 min after addition of precursor (Fig. 5 *A*, lane *6*, and *C*) with no

Time (min): 0 1 2.5 5 7.5 10 30

A Stroma — pS/protA — S/protA

B Envelope — pS/protA — S/protA

1 2 3 4 5 6 7

C

stroma S/protA

envelope S/protA

envelope pS/protA

molecules/chloroplast — time (min)

D Protease: − + − +
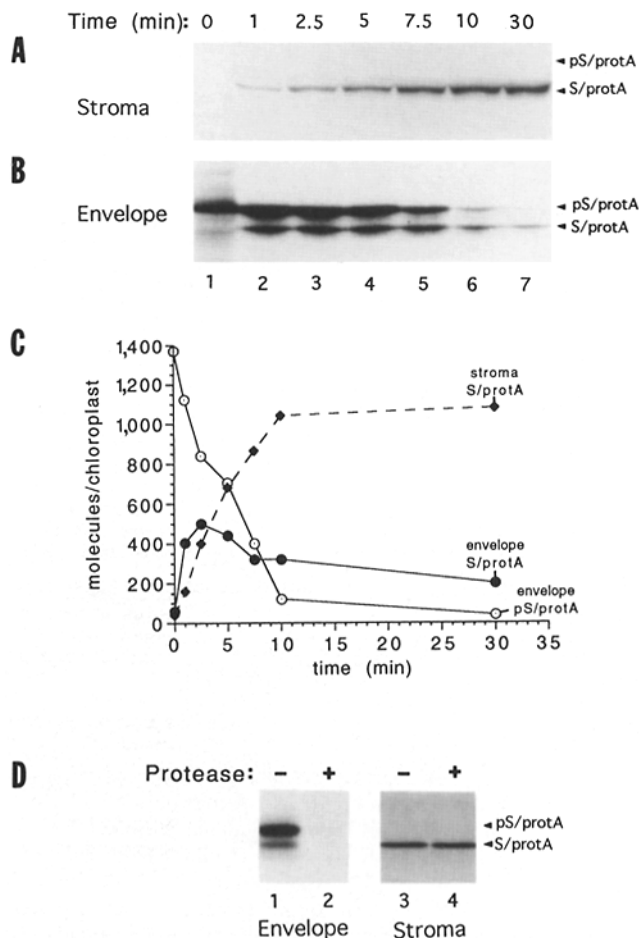
— pS/protA — S/protA

1 2 3 4

Envelope Stroma

Figure 5. Time course of pS/protA import into isolated chloroplasts. Chloroplasts equivalent to 1 mg chlorophyll were incubated with 200 nM urea denatured [^{35}S]pS/protA in the presence of 50 μM MgATP at 26°C in the dark to maximize precursor binding but preclude import. After the binding reaction, the chloroplasts were reisolated and resuspended in import buffer excluding ATP. After the addition of 5 mM MgATP, aliquots were removed from the import reaction and chilled on ice at the time points indicated. Each sample was reisolated over 40% Percoll silica gel and separated into stroma (*A*) and envelope (*B*) fractions. The fractions were analyzed by SDS-PAGE and fluorography. The time after addition of 5 mM MgATP is indicated at the top of *A* in minutes. (*C*) Quantitation of data in *A* and *B*. (♦---♦) stromal S/protA. (●—●) envelope S/protA, (○—○) envelope pS/protA. (*D*) Chloroplasts equivalent to the samples in lane *4* of *A* and *B* were incubated in the absence (−) or presence (+) of 50 μg/ml thermolysin (*Protease*) on ice for 30 min before separation into envelope (*Envelope*) and stroma (*Stroma*) fractions. The positions of precursor (*pS/protA*) and mature (*S/protA*) forms of the pS/protA fusion are indicated by arrowheads.

more additional accumulation up to 30 min (Fig. 5 *A*, lane *7*, and *C*).

The amounts of the membrane-associated forms of pS/protA over the time course of import are shown in Fig. 5, *B* and *C*. The envelope bound unprocessed pS/protA is most abundant at early time points in the import reaction with a sharp decrease in its abundance occurring at 10 min when the import reaction has plateaued (Fig. 5, *A* and *B*, compare lanes *2* and *6*, and *C*). The decrease in pS/protA corresponds to the increase in stromal S/protA strongly sug-

gesting a precursor product relationship between these forms, respectively. Approximately 70% of the envelope associated pS/protA is chased into stromal S/protA at the 30-min time point (Fig. 5 *C*). The amount of the membrane-associated S/protA form peaks at a midpoint in the import reaction and decreases at the later time points as stromal S/protA accumulates (Fig. 5, *A* and *B*, compare lanes *3* and *7*, and *5C*). The behavior of the membrane S/protA is consistent with it being an intermediate between membrane bound pS/protA and the stromal S/protA.

To determine whether the envelope-associated pS/protA and S/protA (Fig. 5 *B*) were associated with the outer surface of the chloroplast, chloroplasts from the 5 min time point of the pS/protA import time course were treated with 50 μg/ml of thermolysin prior to being separated into stroma and envelope fractions. The protease thermolysin used in the protease protection experiments has been shown previously to access only components of the outer membrane but not the inner membrane in intact chloroplasts (Cline et al., 1984). At the 5 min time point, both forms of the import intermediates in addition to the stromal form are present (Fig. 5 *A* and *B*, lane *4*). The stroma S/protA is fully protease protected (Fig. 5 *D*, compare lanes *3* and *4*) indicating that it represents a fully imported polypeptide. Interestingly, both the envelope associated pS/protA and S/protA forms at the 5 min time point are accessible to externally added protease (Fig. 5 *D*, compare lanes *1* and *2*) indicating that they are at least partially exposed at the outer surface of intact chloroplasts.

The extent to which the envelope bound pS/protA has been translocated across the envelope membranes remains to be determined. As its signal sequence is uncleaved, translocation must not have proceeded far enough to expose the signal peptide cleavage site. The membrane-associated form of mature S/protA (Fig. 5 *B*, lanes *2–7*) is of particular interest. This form is protease sensitive in intact chloroplasts (Fig. 5 *D*, lane *2*) indicating that the polypeptide is at least partially exposed at the chloroplast surface. The protease sensitivity of the membrane associated S/protA eliminates the possibility that it is contaminating S/protA from the stromal fraction (see above). Although it is clearly accessible at the chloroplast surface, the fact that this form is processed indicates that its amino-terminal signal sequence is accessible to the stromal signal peptidase. The membrane-associated S/protA represents a partially translocated intermediate in the import process with its amino terminus exposed to the stroma and its carboxy terminus exposed at the outer surface of the chloroplast.

Membrane Localization of pS/protA Import

To sublocalize the envelope bound import intermediates, we reisolated chloroplasts from 0 and 5 min time points in the pS/protA import reaction (see Fig. 5 *B*, lanes *1* and *4*), lysed the chloroplasts under hypertonic conditions and separated the membrane fraction by flotation into linear 20–38% sucrose gradients. Figure 6, panel A shows a representative profile of SDS-PAGE resolved and Coomassie blue-stained membrane polypeptides of the sucrose gradient fractions. Three major membrane subfractions are apparent. Fractions *1–3*, labeled *OM*, contain the least dense portion of the gradient (20–22% sucrose) and represent vesicles derived from outer membrane regions that are not associated with the in-
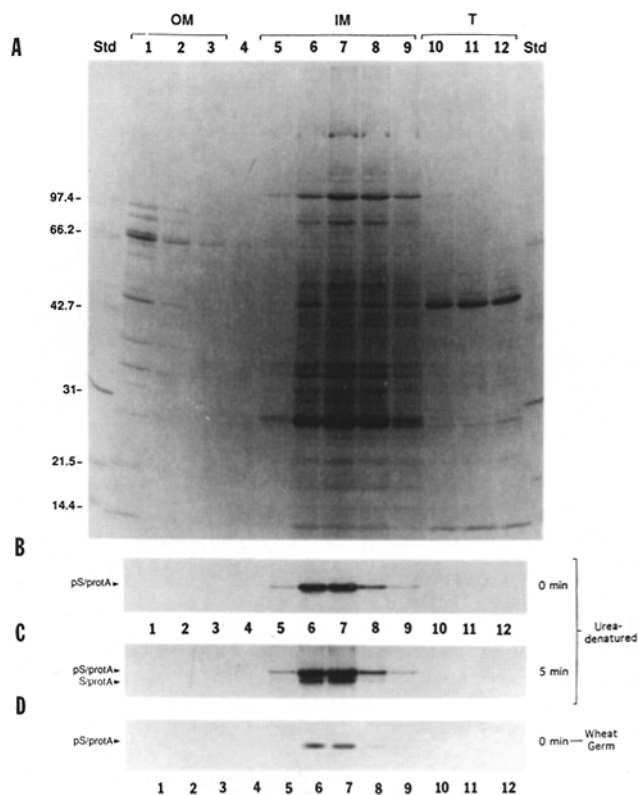
Figure 6. Separation of membranes from chloroplasts isolated from different time points in the pS/protA import reaction. Chloroplasts from 0 min and 5 min time points in the pS/protA import reaction (see Fig. 5) were reisolated over 40% Percoll silica gel, lysed under hypertonic conditions, and separated into stroma and total membrane fractions. The total chloroplast membranes were separated into outer membrane (*OM*), intermediate density membrane (*IM*), and thylakoid membrane (*T*) fractions by flotation in a 20–38% wt/vol sucrose gradient (see Materials and Methods). 1.5 ml fractions were collected and aliquots were analyzed by SDS-PAGE in 10–15% polyacrylamide gradient gels. Lanes *1–9* contained the entire 1.5-ml fraction. Lanes *10–12* contained 300 μl of the corresponding fraction to avoid overloading of the gel. (*A*) Coomassie blue stain of fractions derived from a typical sucrose gradient separation of total chloroplast membranes. (*B* and *C*) Fluorograph of gels from sucrose gradient separation of membranes derived from 0 min (*B*) and 5 min (*C*) time points from Fig. 5. (*D*) The same as *B*, but using an ATP-depleted wheat germ PRS containing [35S]pS/protA as the import substrate. The time after addition of 5 mM MgATP to the import reaction and the origin of the pS/protA substrate used in each reaction is given to the right of *B–D*. The positions of the mature (*S/protA*) and the precursor (*pS/protA*) form of pS/protA are indicated by the arrowheads. The molecular masses of standard proteins (*Std*) in kilodaltons are indicated at the left of *A*.

ner membrane. The intermediate density fraction from 25–35% sucrose (fraction *5–9*, labeled *IM*) represents the bulk of the chloroplast envelope membranes. This fraction has previously been shown to contain primarily inner membrane vesicles, but also contains mixed vesicles derived from both inner and outer membranes (Cline et al., 1985a). Fractions *10–12* represent the 42% sucrose load zone and contain predominantly the thylakoid membranes and any unbroken chloroplasts.

Fig. 6 *B* shows that pS/protA bound to chloroplasts under low ATP concentrations at 26°C (corresponding to the 0 min

time point in Fig. 5 *B*) fractionates with the intermediate density membrane fraction of the chloroplast envelope (Fig. 6 *B*, lanes *5–9*). No detectable amounts of pS/protA were found associated with free outer membrane vesicles under the conditions tested (Fig. 6, *B*, lanes *1–3*). The association of pS/protA with the intermediate density envelope membrane vesicles was not unique to the urea denatured substrate, as pS/protA that had been synthesized in a wheat germ cell-free system was also found associated with these vesicles following a precursor binding assay (Fig. 6 *D*). The localization of pS/protA to the intermediate density vesicles was also independent of temperature, as precursor that had been bound to the chloroplasts at 0°C rather than 26°C gave a similar localization pattern (data not shown). Typically, 60–65% of the total membrane associated pS/protA fractionated with the intermediate density membrane vesicles (Fig. 6 *B*, lanes *5–9*). The remaining 35–40% of the precursor remained in the 42% load zone. Immunoblot analysis using an anti-chloroplast envelope serum indicated that these results are consistent with the yield of envelope membranes from total chloroplast membranes fractionated by flotation into sucrose gradients (data not shown).

Fig. 6 *C* shows the fluorograph of chloroplast membranes fractionated from chloroplasts at the 5 min time point in the pS/protA import reaction (see Fig. 5 *B*, lane *4*). As seen with bound pS/protA, no detectable levels of either translocation intermediate were found associated with the outer membrane fraction (Fig. 6 *C*, lanes *1–3*). In fact, at each time point during the import reaction tested in Fig. 5, pS/protA was not detectable in the outer membrane fraction (data not shown). The bulk of the membrane-associated forms of both pS/protA and S/protA fractionated with the intermediate density envelope membrane vesicles (Fig. 6, *B* and *C*). At the 5 min time point, 62–66% of the membrane-associated pS/protA fractionated with the intermediate density membrane vesicles (Fig. 6 *C*, lanes *5–9*). Reliable quantitation of the percentage of membrane-associated S/protA fractioning with the intermediate density membrane vesicles was not possible as a significant amount of stromal S/protA consistently contaminated the 42% load zone. The presence of translocation intermediates in the intermediate density vesicle population is most likely due to their localization in mixed membrane vesicles. These vesicles have been shown to contain both outer and inner membranes linked by virtue of the presence of membrane contact sites (Cline et al., 1985a). The contact sites would allow the S/protA intermediate to be exposed at the chloroplast outer surface while providing the polypeptide access to the stromal signal peptidase through linked channels in the outer and inner membranes (Schnell et al., 1991).

Localization of pS/protA Import Sites at the Chloroplast Envelope

The protein A domain of the pS/protA molecule provided potential high affinity binding sites on the hybrid precursor for microscopic localization of the polypeptide at the chloroplast envelope. To test the efficiency of IgG binding by the *E. coli* expressed pS/protA molecule, we carried out a set of chloroplast import assays using urea denatured pS/protA with the inclusion of various concentrations of rabbit IgG. Presumably, formation of a bulky protein A–IgG complex would prevent the translocation of the protein A moiety of
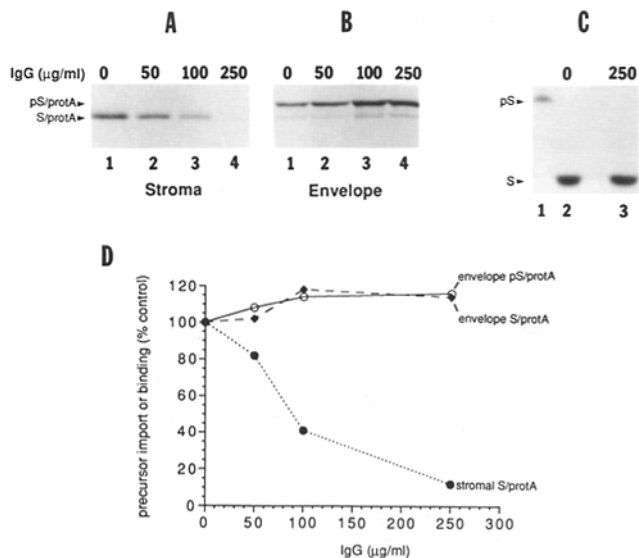
Figure 7. Inhibition of pS/protA import into isolated chloroplasts by IgG. Isolated chloroplasts (equivalent to 25 μg/ml chlorophyll) were incubated with urea denatured [^{35}S]pS/protA or a wheat germ PRS containing [^{35}S]pS in the presence of varying amounts of rabbit IgG in a standard import reaction. After the import reaction, the chloroplasts were reisolated, and analyzed directly or separated into stroma and envelope fractions prior to analysis by SDS-PAGE and fluorography. (*A* and *B*) Fluorographs of SDS-PAGE resolved stroma (*A*) and envelope (*B*) fractions from chloroplasts incubated with [^{35}S]pS/protA. (*C*) Fluorograph of SDS-PAGE resolved total chloroplast polypeptides from chloroplasts incubated with in vitro synthesized [^{35}S]pS. The numbers refer to the concentration of IgG included in the import reaction in μg/ml. The positions of the precursor (*pS/protA* or *pS*) or mature (*S/protA* or *S*) forms of the precursors are indicated by the arrows. (*C*) Quantitation of data given in panel A. (●····●) stromal S/protA, (○—○) envelope-associated pS/protA, (♦- - -♦) envelope-associated S/protA.

Figure 8. Localization of translocation intermediates by immunofluorescence microscopy. Chloroplasts from the 0 min (*A* and *D*), 5 min (*B* and *E*), or 30 min (*C* and *F*) time points in the pS/protA import reaction in Fig. 5 were reisolated over 40% Percoll silica gel, fixed, and surface bound precursor was detected by sequential incubation of the chloroplasts with rabbit IgG and fluorescein-conjugated goat anti–rabbit IgG (see Materials and Methods). Protease (+ or −) indicates that samples were treated with 50 μg/ml thermolysin following the import or binding reaction, but before incubation with the antibodies. The time point in the import reaction from which the chloroplasts were taken is given at the top of the figure in minutes. (*G*) Chloroplasts incubated with pS/protA in the absence of ATP before incubation with antibodies. (*H*) Chloroplasts incubated in the absence of pS/protA before incubation with antibodies. (*I*) Chloroplasts incubated with a rabbit antiserum raised against a 37-kD protein of the chloroplast outer membrane before incubation with fluorescein conjugated goat anti–rabbit IgG. Bar, 7 μm.

pS/protA into chloroplasts. Fig. 7 *A*, shows that the presence of IgG in the import reaction is able to effectively inhibit the import of pS/protA into the stroma in a dose-dependent manner. The inhibition of pS/protA import by IgG is specific for the hybrid precursor, as the import of urea denatured pS is not inhibited at the highest concentration of IgG included in the assay (Fig. 7 *C*, compare lanes 2 and 3).

Interestingly, the pS/protA-IgG complex is still targeted to the chloroplast surface and remains associated with the envelope membrane (Fig. 7 *B*, lanes 1-4). In addition to pS/protA, the membrane-associated mature S/protA is also generated in the presence of IgG. This observation indicates that the pS moiety of pS/protA is translocated across the envelope membranes resulting in signal peptide processing, whereas translocation of the protein A moiety of the hybrid is inhibited by the complexed IgG molecule. The amounts of the precursor and mature forms of translocation intermediates associated with the envelope remain nearly constant with increasing inhibition of import by IgG (compare Fig. 7, *A* and *B*, and *D*), suggesting that both the precursor binding and translocation machinery of the chloroplast are saturated.

The ability of the urea denatured pS/protA molecule to rapidly regain IgG binding capability after dilution into import buffer indicated that the protein A-IgG interaction could
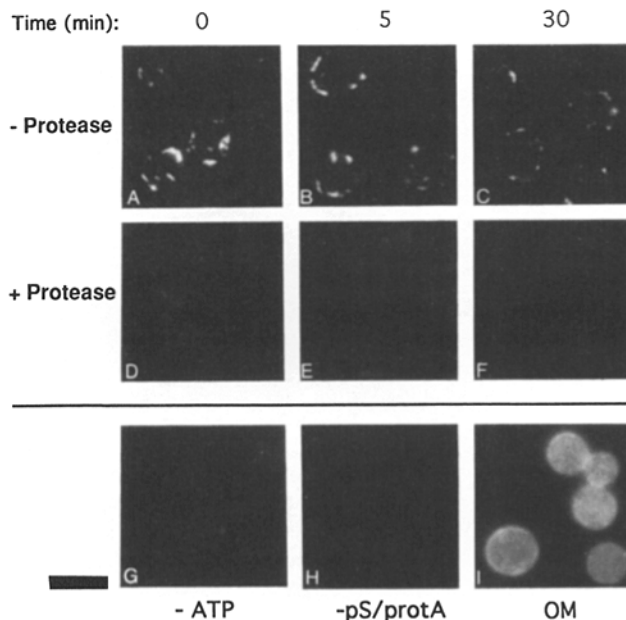
be used to visualize the translocation intermediates at the chloroplast envelope. Therefore, we used labeled IgG molecules to localize the translocation intermediates in immunofluorescence experiments with intact chloroplasts. Chloroplasts from 0, 5, and 30 min time points in the pS/protA import time course shown in Fig. 5 were reisolated, fixed under mild conditions, and incubated sequentially with rabbit IgG and fluorescein-conjugated goat anti–rabbit IgG. Chloroplasts from the 0 min pS/protA import time point gave a distinct patched pattern of immunofluorescence (Fig. 8 *A*). The immunofluorescence pattern indicated that the precursor receptor(s) at the chloroplast surface are not evenly distributed over the outer membrane but reside in distinct regions of the chloroplast envelope. A similar patched immunofluorescence pattern was observed with chloroplasts incubated with pS/protA for 5 minutes under precursor import conditions (Fig. 8 *B*). At the 30 min time point (Fig. 8 *C*), the patched pattern of immunofluorescence remained but the intensity of the immunofluorescence signal was noticeably decreased. At 30 min, most of the envelope-associated pS/protA has been chased into the S/protA form (see Fig. 5, *A* and *B*, lane 7). Although we cannot distinguish
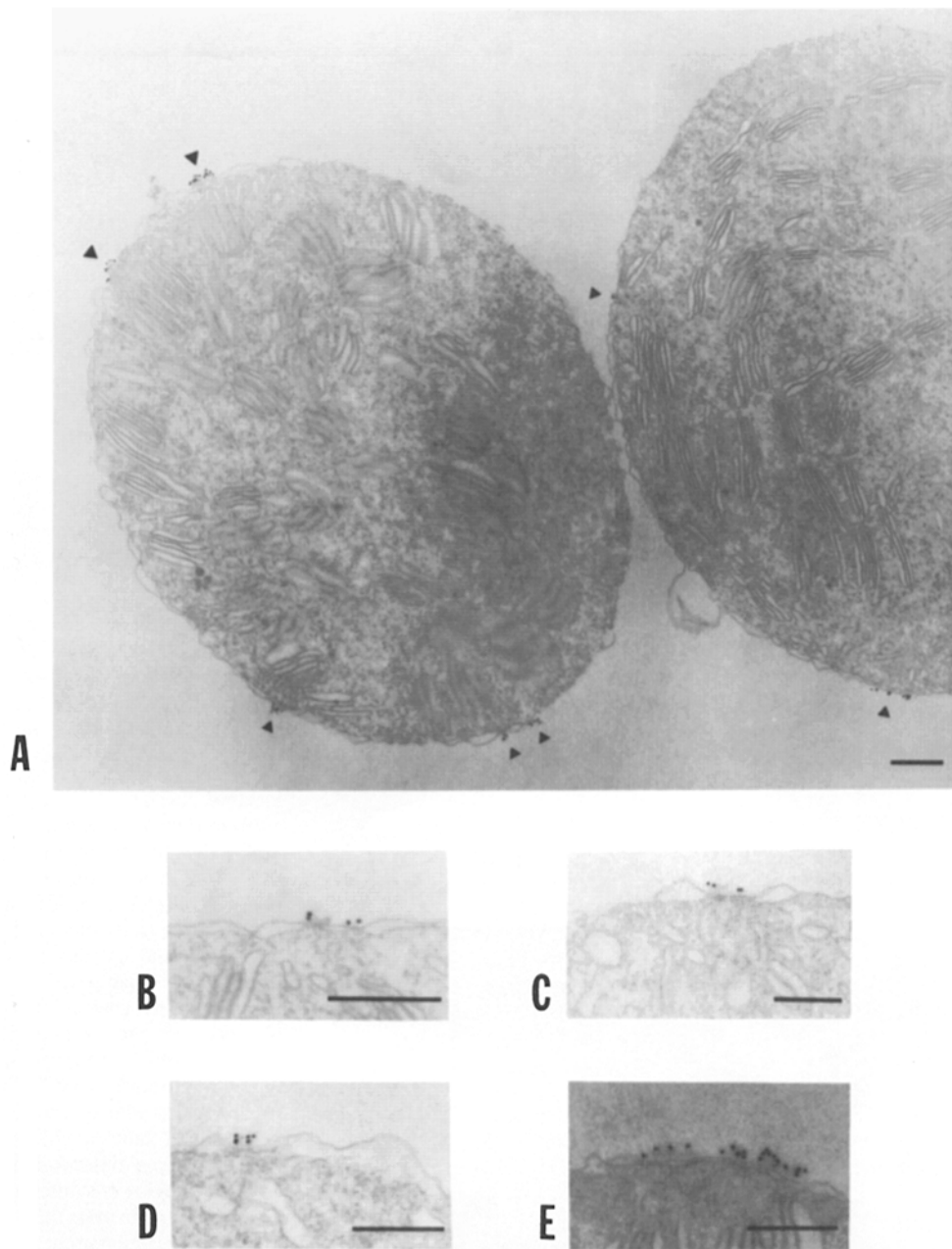
Figure 9. Localization of translocation intermediates by immunoelectron microscopy. Chloroplasts from the 0 min time point in the pS/protA import reaction in Fig. 5 were reisolated, fixed, incubated sequentially with rabbit IgG, and gold-conjugated goat anti-rabbit IgG and processed for microscopy as given in Materials and Methods. The panels give different examples of immunogold labeling at the chloroplast surface at high and low magnifications. The arrows in A indicate the positions of immunogold label on the chloroplasts. Bars: (A) 0.5 μm; (B-D) 0.25 μm.

between IgG binding to the envelope-associated pS/protA or S/protA forms, the similarity in immunofluorescence pattern exhibited at 0, 5, and 30 min time points suggests that the recognition and import machinery do not undergo a dramatic reorganization at the chloroplast surface during the import reaction. These data extend our previous observations that signal peptide analogs bound to the chloroplast surface localize to specific regions of the chloroplast outer membrane and are not evenly distributed across the membrane surface (Schnell et al., 1991).

To insure that the patched immunofluorescence pattern exhibited by the translocation intermediates was not due to artificial capping induced by the divalent IgG molecules, we carried similar immunofluorescence experiments using a rabbit anti–serum directed against a 37-kD polypeptide of the chloroplast outer membrane (OM37). As shown in Fig. 8 I, OM37 exhibits an even distribution throughout the outer

membrane. These data argue against the possibility of antigen clustering in the outer membrane induced by incubation with divalent IgG molecules.

Chloroplasts incubated in the absence of pS/protA (Fig. 8 H) or in the presence of pS/protA in the absence of ATP (Fig. 8 G) before incubation with antibodies gave no fluorescence signal. In addition, chloroplasts treated with protease immediately following the pS/protA binding or import reaction gave a dramatically reduced immunofluorescence signal (Fig. 8, D-F). These data indicate that the immunofluorescence signal was dependent upon the productive binding of pS/protA to the chloroplasts and was not due to nonspecific binding of the precursor or IgG to the chloroplast surface.

Immunoelectron microscopic localization of the pS/protA construct from the 0 min time point in Fig. 5 confirmed the patched distribution of the bound precursor at the chloroplast surface (Fig. 9 A). At higher magnification, the immunogold
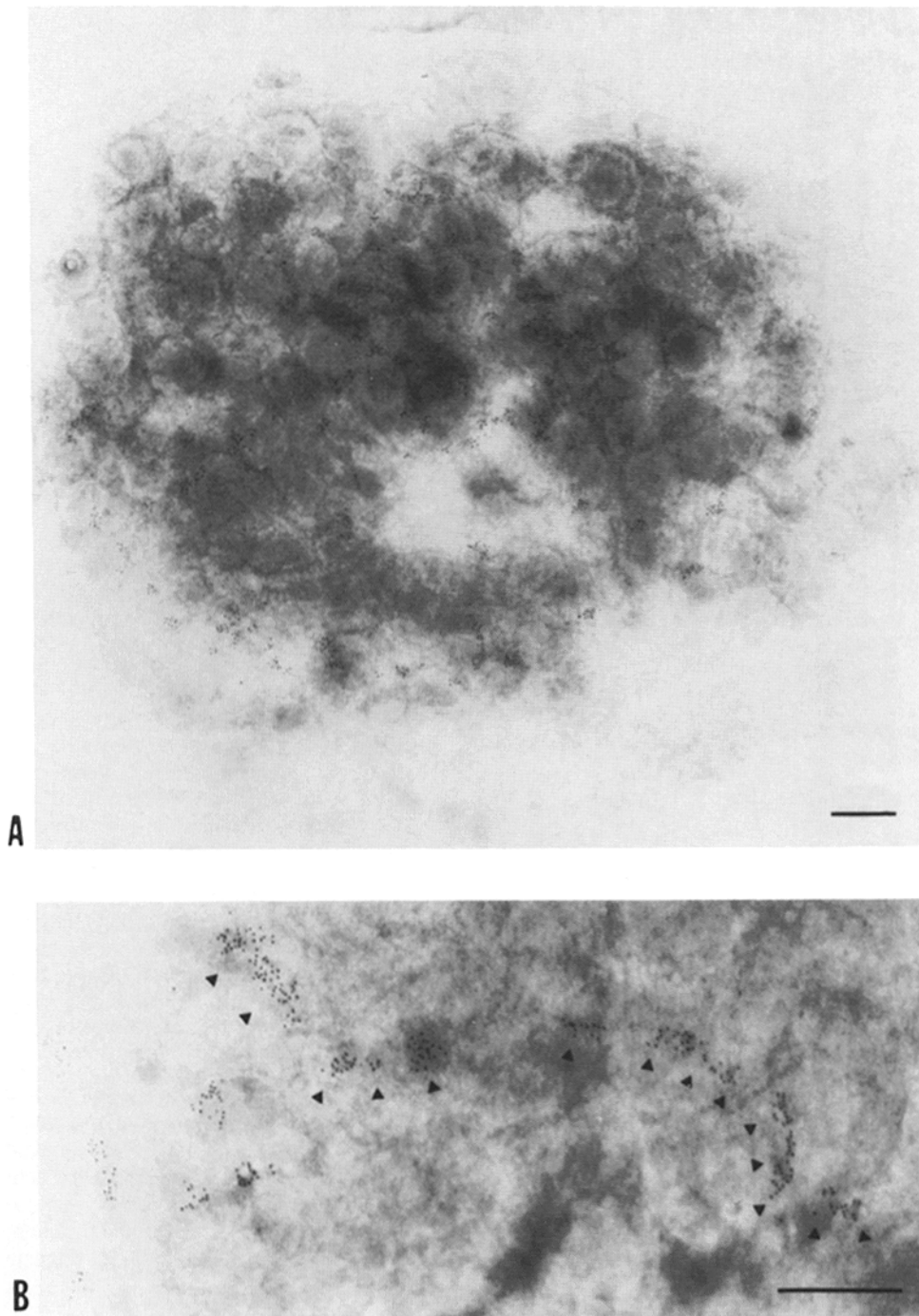
Figure 10. Localization of translocation intermediates on the surface of whole-mount chloroplasts. Chloroplasts from the 0 min time point in the pS/protA import reaction in Fig. 5 were reisolated, immobilized, and incubated sequentially with rabbit IgG and gold-conjugated goat anti-rabbit IgG. The samples were negatively stained and viewed directly by transmission EM. *A* gives an example of a single immunogold labeled chloroplast. *B* gives a high magnification of a region of a different chloroplast to illustrate the reticular organization of the immunogold-labeled precursor binding sites at the chloroplast surface (*arrowheads*). Bar, 0.5 μm.

labeling appeared to be localized to the outer surface of the outer membrane at sites in close contact with the inner envelope membrane (Fig. 9, *B–E*). The labeling pattern was indistinguishable in samples from 0, 5, or 30 min time points in the import reaction, (data not shown). The majority of the labeling was found in small clusters at contact sites (Fig. 9, *A–D*), but a small number of chloroplasts in each section exhibited labeling over an extended region of the outer membrane (Fig. 9 *E*). The extended labeling pattern invariably corresponded to extended regions of envelope contact sites. Immunogold labeling was not observed on chloroplasts that had been incubated with the gold conjugate without prior in-

cubation with pS/protA (data not shown). The localization of the translocation intermediates is consistent with the location of chloroplast import sites at contact zones where the inner and outer envelope membranes are closely apposed.

Comparison of the immunogold labeling pattern in chloroplast thin sections (Fig. 9) with the pattern observed in the immunofluorescence experiments (Fig. 8) suggested that the chloroplast import sites were organized in extended patches at the envelope surface. To directly visualize the organization of import sites at the envelope surface, immunogold labeled chloroplasts that had been mounted onto grids were negatively stained and observed directly in the transmission elec-

tron microscope. Fig. 10 shows the electron micrographs of a chloroplast from the 0 min time point in the import reaction (see Fig. 5) that had been immunogold labeled and negatively stained. The regular array of disclike thylakoid grana underlying the envelope membrane are clearly visible (Fig. 10 *A*). As suggested by the immunofluorescence photographs, the bound pS/protA molecules are not evenly distributed over the outer membrane surface, but are organized in a patchwork in specific regions of the outer envelope surface with no label apparent in large regions of the outer membrane (Fig. 10 *A*). In most cases, the patchwork appears to have an extended structure that is suggestive of grooves or ridges in the envelope (Fig. 10, *A* and *B*). These patterns of labeling were consistent with those observed in thin sections of immunogold-labeled chloroplasts (Fig. 9). In thin sections, the majority of labeling was visible in small clusters at the chloroplast surface (Fig. 9, *A–D*), but occasionally, extended patches of labeling along the chloroplast surface were observed (Fig. 9 *E*). These two patterns were apparently due to lateral or longitudinal sections through the reticular pattern, respectively. The immunogold labeling patterns observed in negatively stained chloroplasts from the 5 min time point (see Fig. 5) were indistinguishable from those shown in Fig. 10 (data not shown).

Discussion

To facilitate the discussion of these results, we propose a model for protein import into chloroplasts (Fig. 11). This model has as its central features two distinct protein conducting channels, one in the outer and one in the inner envelope membrane, each gated open by distinct subdomains of the chloroplast signal sequence. We suggest (Fig. 11) that a subdomain of the signal sequence of pS gates open a protein conducting channel in the outer chloroplast membrane after interacting with a signal sequence receptor that is a subunit of the channel. After translocation of the signal sequence across the outer chloroplast channel, another distinct subdomain of the signal sequence opens a protein conducting channel in the inner chloroplast membrane via interaction with the signal sequence receptor subunit of this channel. Protein translocation proceeds simultaneously through both channels at contact sites where the outer and inner membranes are closely apposed. Both membrane channels close successively following successive completion of protein translocation. This model also would extend to the translocation of proteins across the double membranes of the mitochondria. The existence of protein conducting channels for protein translocation across cellular membranes has been suggested some time ago (Dobberstein et al., 1977; Blobel, 1980; Pain et al., 1988; Schnell et al., 1991). Recently, protein conducting channels have been detected in the mammalian RER (Simon and Blobel, 1991) and in the *E. coli* plasma membrane (Simon and Blobel, 1992) by electrophysiological methods. The latter channel can be gated open by a signal peptide of an *E. coli* precursor protein (Simon and Blobel, 1992).

Our suggestion here that distinct subdomains of the pS signal sequence interact with cognate receptor subunits of the two protein conducting channels in the chloroplast envelope is consistent with the observation that different signal sequence mutations effect different aspects of precursor import (Reiss et al., 1989; Smeekens et al., 1989; Wasmann et al.,
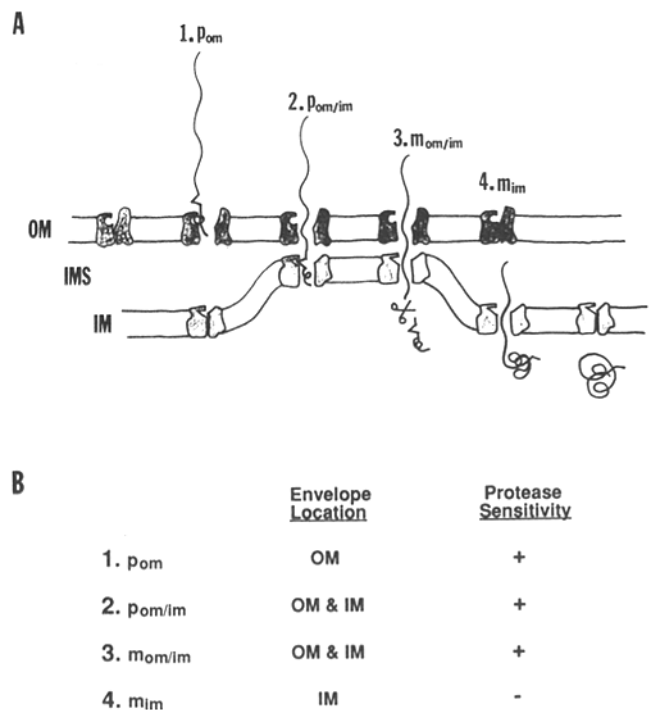

Figure 11. Model for the translocation of proteins across the chloroplast envelope. *IM*, inner membrane; *IMS*, intermediate space; *OM*, outer membrane.

1988). In addition, synthetic signal sequence analogs derived from different regions of the same signal sequence exhibit radically different effects on the binding or translocation steps when included in a chloroplast import assay (Buvinger et al., 1989; Perry et al., 1991; Schnell et al., 1991).

Not indicated in the model in Fig. 11 are other features of protein import into chloroplasts that have been shown to be important in other protein translocation systems. For example, by analogy to protein translocation across the RER (Walter et al., 1981) or the prokaryotic plasma membrane (Watanabe and Blobel, 1989), we have suggested previously that a specific signal recognition factor (SRF) will bind to the signal sequence of pS either during or shortly after synthesis (Schnell et al., 1991). Binding to SRF will prevent inactivation of the signal sequence due to protein folding. Equally important, SRF, by virtue of its binding to a cognate receptor that is restricted in its localization to the outer chloroplast membrane, will target the precursor to the chloroplast surface. Interaction of SRF with its cognate receptor would dissociate the signal sequence from SRF and allow it to open the protein conducting channel in the outer chloroplast membrane. Also not indicated in Fig. 11 is the interaction of molecular chaperones with portions of pS other than the signal sequence. The binding of chaperones on the *cis* side of the membrane would maintain the precursor in an unfolded configuration that is competent for translocation (Chirico et al., 1988; Murakami et al., 1988; Deshaies et al., 1988). In addition, chaperone binding to the emerging polypeptide on the *trans* side of the membrane could provide a thermodynamic driving force to catalyze membrane translocation (Kang et al., 1990; Simon et al., 1992). The ATP requirements for chloroplast import, therefore, would reflect

ATP hydrolysis by molecular chaperones. Evidence has been presented supporting the involvement of both an SRF and chaperones in the import of at least one precursor into chloroplasts (Waegemann et al., 1990).

By using a urea-denatured precursor in our experiments, we have bypassed the need for SRF and cytosolic chaperones. The binding of urea-denatured precursor to chloroplasts was shown here to require ATP with optimal binding at 100 μM ATP (Fig. 2), and saturation of binding was obtained at ~2,000 precursors per chloroplast. In close agreement with these results, it has previously been shown that optimal binding of wheat germ–synthesized pS also requires 100 μM ATP (Olsen et al., 1989) with binding saturation occurring at 1,500–3,500 precursors per chloroplast (Friedman and Keegstra, 1989). However, the dissociation constant for the binding of wheat germ–synthesized pS to isolated chloroplasts was determined to be 6–10 nM, a value somewhat lower than the 40 nM k_D determined here for urea-denatured pS/protA. Similar to the results obtained here, Pilon et al. (1992a) have recently shown that the binding and import of urea-denatured preferredoxin exhibit similar ATP requirements as those exhibited by wheat germ–synthesized pS and those demonstrated here for urea-denatured pS/protA. Pilon et al. (1992b) have determined the K_m for the import of purified urea–denatured preferredoxin into isolated chloroplasts to be ~100 nM. Although the value measured for urea denatured preferredoxin represents a Michaelis-Menton constant for the import reaction rather than a binding affinity constant, it is reflective of the relative affinity of the substrate for the import machinery. The higher binding affinity measured for wheat germ synthesized pS versus the urea-denatured precursors may reflect an increase in binding efficiency due to the actions of SRF and chaperones that are present in the wheat germ extract.

We envision that the high affinity bound form of the pS/protA (Fig. 5 B, lane 1) represents $p_{om/im}$ (Fig. 11 A, stage 2). This assignment is in agreement with the localization of this intermediate at membrane contact sites (Fig. 9) and its enrichment in intermediate density vesicles composed of mixed inner and outer membranes (Fig. 6, B and D). If the outer membrane channel is part of contact zones of closely apposed outer and inner membrane, it is possible that the bound precursor also includes p_{om}. This would provide one explanation for our inability to find detectable amounts of bound pS/protA associated with free outer membrane vesicles upon fractionation of chloroplast membranes (Fig. 6), or to localize pS/protA to regions of the outer membrane not engaged in contact sites with the inner membrane (Fig. 9). Alternatively, p_{om} may be a very short lived and not a rate limiting intermediate and, therefore, difficult to detect in the binding or import assays. This interpretation is compatible with a recent report demonstrating that wheat germ–synthesized pS can specifically bind to isolated outer envelope membrane vesicles in an ATP stimulated manner (Waegemann et al., 1991).

The envelope associated S/protA observed in the time course of pS/protA import (Fig. 5 B) corresponds to $m_{om/im}$ (Fig. 11 A). The localization (Figs. 6, C, and 8, B and C) and protease susceptibility (Fig. 5 D) of this intermediate are indistinguishable from bound pS/protA, but its conversion to the mature form indicates that it has accessed the stromal compartment (Fig. 5 B). We often observe a small amount

of S/protA that remains undigested following the treatment of chloroplasts with thermolysin (data not shown). This protease insensitive S/protA may represent m_{im} (Fig. 11). However, the inability to isolate preparations of inner membrane vesicles that are not contaminated with outer membrane (Cline et al., 1985a) has made it impossible to definitively localize the protease insensitive form to the inner membrane.

With the localization of the chloroplast import apparatus at envelope contact sites, definition of the composition and structural nature of these sites becomes central to an understanding of the mechanism of protein import into chloroplasts. One possibility is that contact sites are formed by structures distinct from the translocation apparatus. These structures would serve to maintain the two envelope membranes in close proximity to facilitate translocation. In this case, the two channels in the inner and outer membranes would not be stably linked, but may be restricted to specific regions of these membranes by the contact site adhesion zones. This interpretation is consistent with several observations. First, the binding of pS/protA occurs at membrane contact sites under conditions that restrict membrane fluidity (i.e., 0°C) (Figs. 6 and 9). This observation argues against a model in which protein conducting channels are randomly distributed throughout the plane of the membrane bilayer and must migrate laterally to contact sites. Second, isolated outer membrane vesicles bind pS in an ATP-dependent manner (Waegemann and Soll, 1991). The authors suggest that this binding represents insertion of pS into the protein conducting channel in the outer membrane. This interpretation would exclude the possibility that the channels in the inner and outer membranes are stably linked, and would require that freely diffusing protein conducting channels exist in both envelope membranes. It has yet to be determined whether the inner membrane of chloroplasts has the ability to bind or import precursor proteins, as does the inner membrane of mitochondria (Hwang et al., 1989). Third, the immunogold labeling pattern of the import intermediates (Fig. 10), and morphological studies of envelopes of intact chloroplasts both indicate that contact zones are organized into regular lines or grooves (Cremers et al., 1988). Finally, two groups have noted that contact zones survive the forced separation of the outer and inner membranes induced by hypotonic (Cremers et al., 1988) or hypertonic treatment of the chloroplasts (Cline et al., 1985a). Both observations suggest that contact sites are relatively stable structures that can survive physically disruptive procedures such as osmotic shock.

In addition to the insights provided concerning the overall pathway of protein import, the pS/protA intermediates provide tools by which to assess the function of putative components of the import apparatus. Using an antiidiotypic antibody approach, we have previously described the identification of a 36-kD integral membrane protein (p36) as a candidate for a signal sequence binding subunit of a protein conducting channel in chloroplast envelope (Pain et al., 1988). Antibodies to p36 were able to inhibit the import of pS by blocking the binding of the precursor to the chloroplast envelope (Schnell et al., 1990). In addition, p36 was shown to bind specifically to precursor proteins in a soluble precursor binding assay (Schnell et al., 1990). Consistent with the localization of translocation intermediates characterized in this study, p36 was localized at envelope contact sites

(Schnell et al., 1990). Recently, it has been suggested that p36 functions as the triose phosphate-3-phosphoglycerate-phosphate translocator of the envelope inner membrane and not an import receptor (Flügge et al., 1991). It is fair to state, however, that definitive proof for or against either of these assignments is still lacking. Our hope is that the pom/im and mom/im intermediates will provide a useful means to purify the protein conducting channels of the chloroplast envelope and, thereby, provide independent evidence for or against the functional assignment of p36.

Note Added in Proof. In Fig. 1 *A*, the size of the protein A domain of the pS/protA hybrid construct should read (aa's −10 to 271) instead of (aa's −10 to 712).

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