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Envelope Membrane Proteins That Interact with Chloroplastic Precursor Proteins

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The post-translational transport of cytoplasmically synthesized precursor proteins into chloroplasts requires proteins in the envelope membranes. To identify some of these proteins, label transfer cross-linking was performed using precursor to the small subunit of ribulose-1,5-bisphosphate carboxylase (prSSU) that was blocked at an early stage of the transport process. Two envelope proteins were identified: an 86-kD protein and a 75-kD protein, both present in the outer membrane. Labeling of both proteins required prSSU and could not be accomplished with SSU lacking a transit peptide. Labeling of the 75-kD protein occurred only when low levels of ATP were present, whereas labeling of the 86-kD protein occurred in the absence of exogenous ATP. Although both labeled proteins were identified as proteins of the outer envelope membrane, the labeled form of the 75-kD protein could only be detected in fractions containing mixed envelope membranes. Based on these observations, we propose that prSSU first binds in an ATP-independent fashion to the 86-kD protein. The energy-requiring step is association with the 75-kD protein and assembly of a translocation contact site between the inner and outer membrane of the chloroplastic envelope.

INTRODUCTION

Most chloroplastic proteins are encoded by genes in the nucleus and synthesized on free polysomes in the cytoplasm as larger precursor proteins that must be post-translationally transported into chloroplasts (Chua and Schmidt, 1978; Highfield and Ellis, 1978). The additional size is due to an amino-terminal extension called a transit peptide (Schmidt et al., 1979). Transit peptides are necessary for transport into chloroplasts; a protein without a transit peptide does not associate with chloroplasts (Mishkind et al., 1985; Anderson and Smith, 1986). Transit peptides are also sufficient for transport in that they can direct foreign proteins into chloroplasts (Lubben et al., 1988).

The steps in the transport process include binding of precursors to the surface of chloroplasts and translocation across the membranes of the envelope. The transit peptide is proteolytically removed during or shortly after transport, yielding a mature-sized protein (Schmidt and Mishkind, 1986). Sorting and assembly of the protein may then occur.

Although binding traditionally refers to a reversible process, binding of precursor proteins to chloroplasts is not reversible. Precursors bound in a nucleoside triphosphate–dependent manner are no longer in equilibrium with free precursor proteins (Olsen et al., 1989). When chloroplasts with bound precursor are fractionated, the bound precursor does not localize with outer membranes in a sucrose gradient (J. Ostrom and K. Keegstra, unpublished results). Instead, bound precursor fractionates with a region of the sucrose gradient believed to contain contact sites between the inner and outer membrane (Cline et al., 1985b). Contact sites have been proposed to be the location of precursor translocation across the envelope. In view of these characteristics, it may be more appropriate to consider these "bound" precursor proteins as "early translocation intermediates."

It is widely accepted that the transport process requires a translocation apparatus present in the chloroplastic envelope and that envelope membrane proteins are essential components of the translocation apparatus. However, the identity and function of envelope proteins involved in precursor binding and translocation remain unclear despite several attempts to identify such proteins. Cornwell and Keegstra (1987) used a heterobifunctional cross-linker to identify an 86-kD adduct of ^{3}H-small subunit of ribulose-1,5-bisphosphate carboxylase precursor (prSSU) and a putative receptor protein. By subtracting the 20-kD molecular mass of prSSU, the putative receptor's molecular mass was estimated to be 66 kD. However, no 66-kD protein was visible on stained gels of envelope proteins. This may be because the 66-kD protein is too low in abundance. Alternatively, the assumptions that the ratio of prSSU to receptor is one to one and that the adduct runs as the sum of the molecular masses of individual components could be wrong.

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Chloroplastic proteins of 30 and 52 kD were identified by two methods. Kaderbhai et al. (1988) performed cross-linking to isolated inner and outer membranes using a synthetic peptide corresponding to a portion of the transit peptide of prSSU. Pain et al. (1988) raised antiidiotypic antibodies against a synthetic peptide corresponding to a portion of the transit peptide of prSSU. These antiidiotypic antibodies should mimic the transit peptide in binding to receptor proteins. Immunoblotting demonstrated that the antiidiotypic antibodies reacted with a 52-kD protein and a 30-kD protein. The 52-kD protein was found to be in the soluble fraction and was shown to be the large subunit of ribulose bisphosphate carboxylase. The 30-kD protein, after isolation of a cDNA clone, was found to be the same protein identified previously as the phosphate translocator (Flügge et al., 1989, 1991; Schnell et al., 1990; Willey et al., 1991). This protein's role as the phosphate translocator has recently been confirmed by transforming yeast with the cDNA clone and assaying for phosphate translocator activity (Loddenkötter et al., 1993). The possibility still remains that this protein also functions in protein transport. If so, it must be part of the transport machinery in the inner membrane, because that is the location of the phosphate translocator.

Because transport requires ATP as an energy source, Hinz and Flügge (1988) used ^{32}P-ATP to examine differential phosphorylation of envelope proteins upon association with precursor. This approach assumes that the ATP requirement is involved in phosphorylation of a transport component. They found a correlation between inhibition of protein transport and stimulation of phosphorylation of a 51-kD protein. Binding of a chimeric precursor led to an increase in phosphorylation of the 51-kD protein.

Another approach for the identification of putative transport components was to bind precursors to isolated outer membranes and then solubilize the membranes with detergent to identify a complex that remains associated with the bound precursor. Ten outer envelope proteins cosedimented in a sucrose gradient with bound prSSU, including an 86-kD protein, a heat shock cognate protein 70, and a 34-kD protein (Waegemann and Soll, 1991; Soll and Alefsen, 1993). In a subsequent paper, the authors report that this complex isolated from the outer membrane can interact with precursor proteins (Soll and Waegemann, 1992).

In this paper, we report on studies using a label transfer cross-linking reagent to identify putative proteinaceous components of the transport apparatus. This approach has been used in studies of nuclear transport (Li and Thomas, 1989) and bacterial secretion (Joly and Wickner, 1993). The advantage of this cross-linking strategy is that the cross-linker is cleavable, leaving the putative transport component modified with the radiolabeled portion of the cross-linker. Thus, this technique can be considered as an affinity-labeling procedure. When the reaction products are analyzed by SDS-PAGE, the molecular mass of the radiolabeled protein(s) corresponds to the molecular mass of the putative transport component(s). There is no need to estimate the molecular mass by subtracting the molecular mass of the ligand from that of the adduct. Outer membrane proteins of 86 and 75 kD were labeled using this approach. Low levels of ATP stimulated labeling of the 75-kD protein, whereas labeling of the 86-kD protein was ATP independent. The cross-linking data were interpreted in terms of a working model in which the ATP-dependent step of binding is formation of a translocation contact site.

RESULTS

Identification of Envelope Proteins by Label Transfer Experiments

We used a label transfer cross-linking technique to identify putative components of the chloroplastic protein transport apparatus. This technique requires use of a chemically pure precursor protein. Large quantities of prSSU were acquired by expression in *Escherichia coli*. The protein was easily purified because it forms inclusion bodies within the bacterial cells. The bacterial cells were ruptured, insoluble inclusion bodies were collected by centrifugation and washed, and the inclusion proteins were solubilized in guanidine-HCl. Precursor prepared by this method was greater than 90% pure when analyzed by SDS-PAGE and silver staining. PrSSU produced in this manner has been shown to be import competent when diluted from denaturant and incubated with chloroplasts under transport conditions (B. Bruce, M. Hurley, and K. Keegstra, manuscript submitted). Import of *E. coli*–produced precursors is similar to import of in vitro–synthesized prSSU in that both require ATP and thermolysin-sensitive chloroplastic components (B. Bruce, M. Hurley, and K. Keegstra, manuscript submitted).

The cross-linker used in the label transfer experiments was APDP (Pierce Chemical Co., Rockford, IL). This cross-linker is heterobifunctional: one end is sulfhydryl reactive, the other is activated by illumination with UV light causing reaction with nearby molecules. APDP can be labeled with iodine, and the cross-linked adduct can be cleaved by reducing agents, such as DTT. The first step in the cross-linking procedure was to iodinate APDP. The radiolabeled cross-linker was then reacted with sulfhydryl groups of cysteine residues in prSSU to generate the labeled azidophenylthio- (APT-) adduct of prSSU. When analyzed by SDS-PAGE and autoradiography, labeled prSSU was observed as a band near 20 kD (data not shown). Diffuse bands of radiolabel near 40 kD and 60 kD were also observed (data not shown) and most likely represent dimers and trimers of ^{125}I-APT-prSSU. These dimers and trimers of modified prSSU also associated with the envelope membranes and can be seen in the lanes labeled −DTT in Figure 1.

The next step in the label transfer experiment was to bind ^{125}I-APT-prSSU to chloroplasts. Low levels of ATP (75 μM) were added to support binding, and the reaction was incubated on ice in the dark for 15 min. Intact chloroplasts with bound ^{125}I-APT-prSSU were repurified over a 40% Percoll cushion, washed with import buffer, and exposed to UV light, thereby

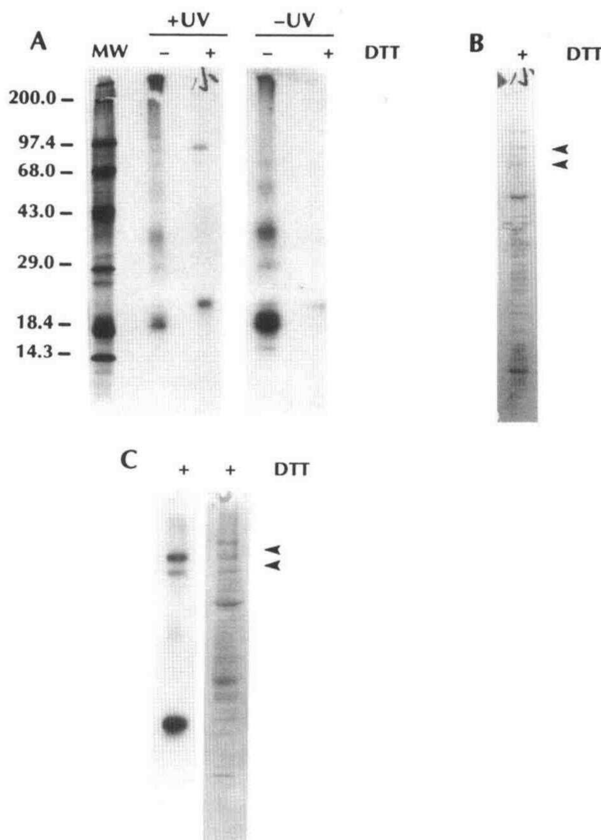

Figure 1. Photolysis of Bound ^{125}I-APT-prSSU Labels Envelope Proteins.

(A) Products of a cross-linking reaction in envelope fraction I. ^{125}I-APT-prSSU was bound to chloroplasts on ice in the dark with 75 μM of ATP. Intact chloroplasts were reisolated over 40% Percoll in import buffer and resuspended in 1 mL of import buffer. The azido group on the cross-linker was photolyzed by irradiation of the samples with UV light for 10 min on ice (+UV). The control (−UV) was not exposed to UV light but was otherwise treated in the same manner. Chloroplasts were fractionated to obtain envelopes that were resuspended in sample buffer with and without 50 mM DTT (+ and −, respectively). The samples were boiled for 2 to 3 min. A photograph of the autoradiogram obtained from analyzing envelope fraction I on a 10 to 15% gradient gel is shown. Molecular mass markers in kilodaltons are indicated at left.
(B) Protein profile of envelope fraction I. A photograph of the Coomassie blue–stained gel corresponding to the lane in **(A)** labeled +UV and +DTT is shown. Arrowheads mark the stained proteins corresponding to the labeled protein bands.
(C) Products of a label transfer reaction in envelope fraction II. Envelope fraction II from a label transfer reaction resuspended in sample buffer with 50 mM DTT was analyzed on a 10 to 15% SDS-PAGE. The left lane shows a photograph of the autoradiogram of a sample solubilized in sample buffer with 50 mM DTT (+). The right lane shows a photograph of the Coomassie blue–stained gel from which the autoradiogram in the left lane was exposed. Arrowheads mark the stained proteins corresponding to the labeled protein bands.

activating the azido group on the cross-linker and causing covalent reaction with nearby molecules, presumably components of the transport apparatus. Chloroplasts were lysed and separated into soluble, thylakoid, and envelope fractions, as described previously (Perry et al., 1991). The envelope membranes were collected in two fractions: membranes that sedimented at a 0.46/1.0 M sucrose interface in a sucrose step gradient (envelope fraction I) and membranes that sedimented at a 1.0/1.2 M sucrose interface (envelope fraction II). Envelope fraction II was slightly green in color, indicating some contamination with thylakoids, but when analyzed by SDS-PAGE, the protein profile, which is shown in Figure 1C, demonstrated that this fraction contained primarily envelope membrane proteins. When envelope fraction I was boiled in sample buffer that did not contain DTT and then analyzed by SDS-PAGE and autoradiography, the most prominent band of radiolabeled protein observed was at ~100 kD (Figure 1A, +UV and −DTT). Labeled prSSU bound to the envelope was also observed at 20 kD.

When DTT was present in the sample buffer, the disulfide in the envelope protein-^{125}I-APT-prSSU adduct was cleaved, leaving the envelope protein covalently modified with the radioiodinated portion of the cross-linker and causing a shift in the molecular mass of the labeled material. Figure 1A shows a band of labeled protein at 86 kD and a fainter band of labeled protein at 75 kD in the +UV, +DTT lane. These proteins were also labeled in envelope fraction II, but the 75-kD protein was generally more strongly labeled than in envelope fraction I (Figure 1C). PrSSU was also labeled when DTT was present because the precursor cross-links to itself upon illumination with UV light. The labeled prSSU ran slower if DTT was present because intramolecular disulfide bonds were reduced, yielding a more extended protein conformation that migrated more slowly through the gel.

Because the radiolabeled portion of the cross-linker remained with the envelope proteins when the cross-linker was cleaved, thereby removing prSSU from the adduct, it was not necessary to estimate the molecular mass of the envelope proteins by subtracting the molecular mass of prSSU from the molecular mass of the adduct. The label transfer reaction generates only a small increase in molecular mass and no change in charge of the labeled protein (Li and Thomas, 1989). Labeled protein in the lanes containing DTT runs at the same position as the unlabeled envelope protein, thereby allowing the identity of the protein on SDS-PAGE to be determined by aligning the autoradiogram with the stained gel. Figures 1B and 1C show photographs of the Coomassie blue–stained pattern of the same lanes that produced the autoradiograms shown in Figures 1A and 1C, respectively (lane marked +UV, +DTT in Figure 1A). The arrowheads mark the stained protein bands corresponding to the labeled proteins.

Photolysis of the bound ^{125}I-APT-prSSU was required for label to be incorporated into the 86- and 75-kD proteins. If the UV treatment was not performed, no label was observed in the 86- and 75-kD proteins (Figure 1A, −UV). This demonstrates that incorporation of label into the 86- and 75-kD proteins

was caused by the label transfer reaction. In the absence of UV treatment, a significant amount of ^{125}I-APT-prSSU, including dimers and trimers of modified prSSU, remained bound to the envelope, as can be seen in the lane without DTT (Figure 1A).

Identification of Labeled Proteins on a Two-Dimensional Gel

Because envelope membranes have more than one protein of ~86 kD (Werner-Washburne et al., 1983) and of 75 kD (Marshall et al., 1990), two-dimensional gels were run to determine precisely which proteins were labeled. Previous work demonstrated that the inner and the outer membranes of the chloroplastic envelope each contain an 86-kD protein (Werner-Washburne et al., 1983). The outer membrane 86-kD protein remains at the basic end of the isoelectric focusing gel, while the inner membrane 86-kD protein migrates into the isoelectric focusing gel (Werner-Washburne et al., 1983). Figure 2 shows the results of two-dimensional gel analysis of envelope fraction II from a label transfer experiment. The labeled 86-kD protein remained at the basic end of the isoelectric focusing gel and therefore was the outer membrane 86-kD protein (Figure 2A). The labeled 75-kD protein corresponded to the major 75-kD protein in the envelope, which is also an outer membrane protein (Figure 2A; Werner-Washburne et al., 1983). The diffuse spot in the lower right in the photograph of the autoradiogram corresponds to a pI of ~8.5 and a molecular mass of 20 kD and represents modified prSSU that was bound to the envelope (Figure 2A). Figure 2B shows a photograph of the Coomassie blue–stained gel from which the autoradiogram was exposed. Arrowheads mark the positions of labeled 86- and 75-kD proteins on the autoradiogram and the stained proteins on the Coomassie blue–stained gel that correspond to the labeled proteins. The same proteins were labeled in envelope fraction I (data not shown).

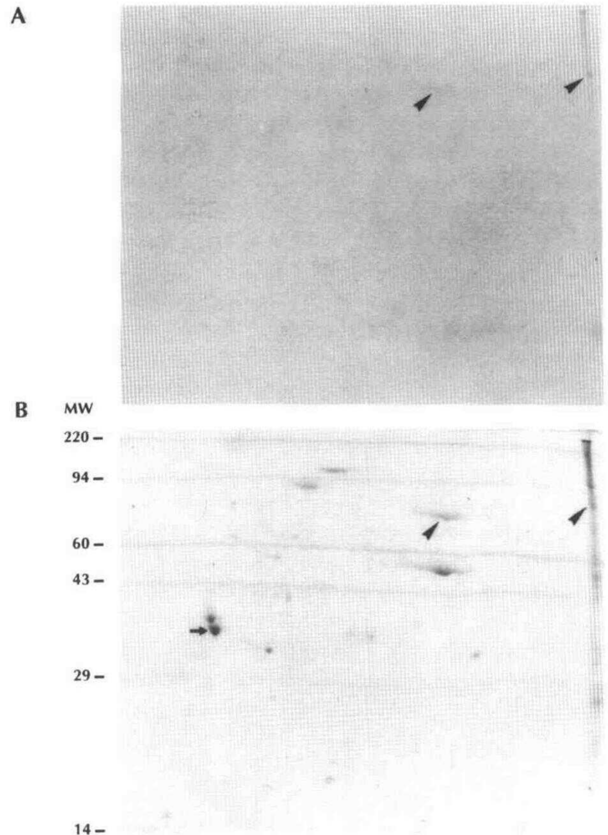

Figure 2. Two-Dimensional Analysis of a Cross-Linking Experiment.

(A) Autoradiogram of the two-dimensional gel. The cross-linking experiment was performed as described in Figure 1. Envelope fraction II was mixed with nonlabeled membranes of envelope fraction II isolated separately so that a total of 30 μg of protein was loaded onto the isoelectric focusing gel. The isoelectric focusing gel had a pH gradient of 4 to 8. Isoelectric focusing was followed by analysis on a 10% SDS–polyacrylamide slab gel. A photograph of the autoradiogram is shown. Arrowheads mark the labeled 86- and 75-kD proteins.
(B) Coomassie blue–stained two-dimensional gel. A photograph of the Coomassie blue–stained gel from which the autoradiogram in **(A)** was exposed is shown. The arrow marks a standard added to the sample when isoelectric focusing was performed. The arrowheads mark the proteins labeled in the cross-linking experiment. The horizontal lines represent molecular weight markers added to the polyacrylamide gel. The molecular mass of these markers is indicated at left in kilodaltons.

Association with the 86- and 75-kD Proteins Represents a Productive Step in the Transport Process

Precursors that are specifically bound to the chloroplastic surface should be competent for subsequent translocation across the envelope. Precursor to small subunit that is synthesized by in vitro translation is 60 to 85% translocation competent from the bound stage upon addition of sufficient amounts of ATP and warming the reaction to room temperature (Cline et al., 1985a; Friedman and Keegstra, 1989). To evaluate whether ^{125}I-APT-prSSU was bound specifically, we attempted to complete import of modified prSSU before exposure to UV light. As shown in Figure 3A, when extra ATP was added to chloroplasts containing bound ^{125}I-APT-prSSU, less radiolabel was found in the 86- and 75-kD proteins (compare lanes labeled Bound and Chased). Concomitantly, a radiolabeled protein with a molecular mass of 14 kD appeared in the soluble fraction (Figure 3B, compare lanes B and C). This was mature-sized SSU that derived from translocation and processing of ^{125}I-APT-prSSU. These results show that precursor associated with the 86- and 75-kD proteins was on a productive pathway for transport into the stroma.

ATP Dependence of Labeling of Envelope Proteins

Because tight binding of precursors to chloroplasts is dependent on low levels of nucleotide triphosphate (Olsen et al., 1989), we investigated the ATP dependence of labeling of the 86- and 75-kD proteins. Figure 4 shows that labeling of both the 86-kD and 75-kD proteins was stimulated by the presence of 75 μM of ATP. Labeling of the 75-kD protein required ATP, with almost no label incorporated into this protein in the absence of ATP. A binding reaction without exogenously added ATP, but with apyrase present to consume ATP from chloroplasts, resulted in the 86-kD protein still being labeled upon cross-linking. This was true in both envelope fractions. We conclude that ATP was required for association of precursor with the 75-kD protein but association with the 86-kD protein can still occur in the absence of ATP.

Interestingly, labeled 75-kD protein was present predominantly in envelope fraction II. One interpretation is that once precursors associate with the 75-kD protein, the complex is found only at contact sites, which are isolated predominantly in fraction II. To evaluate this idea, we investigated the location of precursors first bound in the absence of ATP and then chased with low levels of ATP. ATP was added to a final concentration of 75 μM to one-third of the reaction without ATP before illumination with UV light (Figure 4). This sample was incubated on ice in the dark for 15 min, followed by photolysis

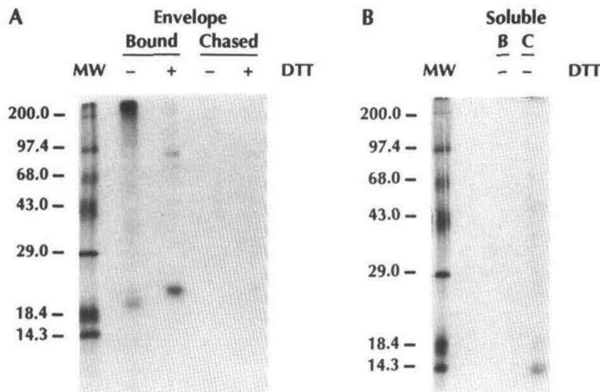

Figure 3. Association of ^{125}I-APT-prSSU with the 86- and 75-kD Envelope Proteins as a Productive Step in the Transport Pathway.

(A) Products of a label transfer reaction in the envelope fraction. ^{125}I-APT-prSSU was bound to the chloroplasts as in Figure 1. Intact chloroplasts were recovered over 40% Percoll and resuspended in import buffer. One-half of the reaction was exposed to UV light and fractionated (Bound) as described in Methods. ATP was added to a final concentration of 3 mM to the other half, and the reaction incubated at 25°C for 15 min, followed by exposure to UV light and fractionation (Chased). Samples were boiled in sample buffer with and without 50 mM DTT (+ and −, respectively) and analyzed on a 10 to 15% gradient gel. A photograph of the autoradiogram obtained from analyzing envelope fraction I is shown. Molecular mass markers (MW) in kilodaltons are indicated at left.

(B) Products of a label transfer reaction in the soluble fraction. The soluble fraction of the fractionated chloroplasts from the label transfer experiments described in **(A)** was boiled in sample buffer without DTT (−) and analyzed by SDS-PAGE on a 10 to 15% gradient gel. The lane labeled B corresponds to the label transfer reaction in which photolysis was performed after binding. The lane labeled C corresponds to the label transfer reaction in which the bound ^{125}I-APT-prSSU was chased by addition of ATP to a final concentration of 3 mM and incubation at 25°C before photolysis. A photograph of the autoradiogram is shown. Molecular mass markers (MW) in kilodaltons are indicated at left.

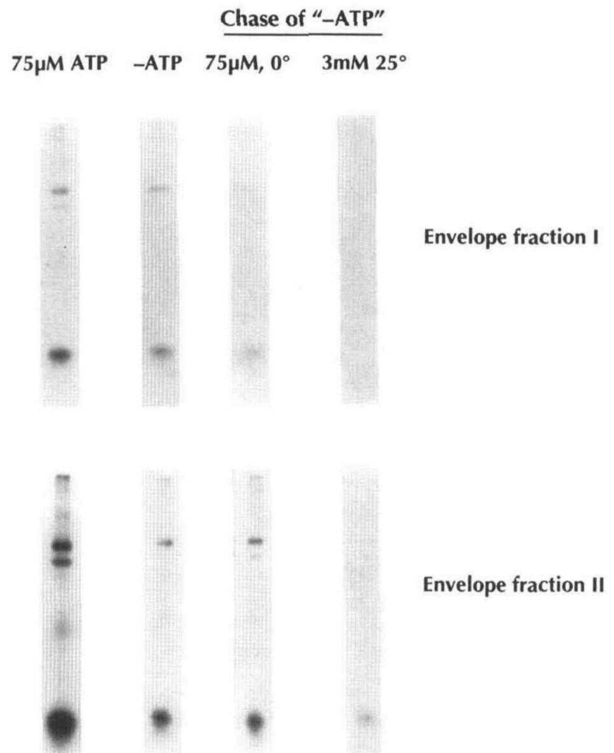

Figure 4. ATP Dependence of Labeling of the 86- and 75-kD Envelope Proteins.

The control reaction contained 75 μM ATP in the binding reaction as described in Figure 1 (75 μM ATP). A binding reaction without exogenously added ATP and with apyrase present was also incubated on ice for 15 min in the dark (−ATP). The intact chloroplasts were repurified over 40% Percoll and washed with import buffer. The 75-μM ATP and one-third of the −ATP reactions were exposed to UV light, the chloroplasts fractionated, and envelope fractions I and II analyzed by SDS-PAGE. To another third of the −ATP reaction, ATP was added to a final concentration of 75 μM and was incubated on ice (Chase of −ATP, 75 μM, 0°C). The last third had ATP added to a final concentration of 3 mM and was incubated at 25°C (Chase of −ATP, 3 mM, 25°C). After incubation for 15 min, these reactions were exposed to UV light, fractionated, and analyzed by SDS-PAGE. A photograph of the autoradiogram is shown.

of the cross-linker. Label was incorporated into the 75-kD protein in envelope fraction II. Less label was incorporated into the 86-kD protein, and no label appeared in the 75-kD protein in envelope fraction I. These results suggest that micromolar concentrations of ATP caused ^{125}I-APT-prSSU associated in an ATP-independent manner with the 86-kD protein to associate with the 75-kD protein, and this complex sedimented preferentially with envelope fraction II.

ATP was added to a final concentration of 3 mM to one-third of the reaction without ATP, and this aliquot was incubated at 25°C for 15 min (conditions that support translocation) before illumination with UV light. Radiolabel disappeared from both proteins in both envelope fractions I and II (Figure 4), with a concomitant increase in a labeled 14-kD protein in the soluble fraction (data not shown), demonstrating that ^{125}I-APT-prSSU bound to the 86-kD protein in an ATP-independent manner was on a productive pathway for transport and processing to the mature-sized protein in the stroma.

Previous work has demonstrated that ATP dependently bound precursor fractionates not with the outer membrane but with mixed membranes that have been shown to contain contact sites, sites that have been proposed to be the location of translocation of precursor proteins (J. Ostrom and K. Keegstra, unpublished results; Schnell and Blobel, 1993). Envelope fraction II sediments in the sucrose gradient at a density expected for mixed membranes containing contact sites. To confirm these results, chloroplasts were lysed hypertonically after cross-linking. Hypertonic lysis allows separation of purified outer membrane from mixed membranes that include contact sites. Figure 5 shows that the only protein labeled in the outer membrane was the 86-kD protein, even though two-dimensional gels have confirmed that the labeled 75-kD protein was an outer membrane protein. In mixed membranes, in the reaction incubated without ATP, mainly the 86-kD protein was labeled. Fractionation of labeled 86-kD protein with mixed membranes was most likely because separation of the outer membrane from other fractions was not complete (Cline et al., 1985b). The 75-kD protein was more strongly labeled in the mixed membrane fraction if 75 μM of ATP was present in the binding reaction (Figure 5, M.M., +ATP). Upon chasing precursor bound in the absence of ATP to an ATP-dependent stage, less label was observed in the 86-kD protein in the outer membrane and more label was incorporated into the 75-kD protein, which fractionates with mixed membranes (data not shown). The small amount of label incorporated into the 75-kD protein in the reaction without ATP may be explained by some ATP derived from chloroplasts supporting a low level of ATP-dependent binding. Although apyrase was included in the binding reaction to consume any ATP, apyrase cannot cross the outer membrane. ATP to support binding is utilized in the intermembrane space (Olsen and Keegstra, 1992). Thus, apyrase would not be able to block binding caused by ATP from the chloroplast stroma exported to the intermembrane space.

Together, these results suggest that the precursor first associated in an ATP-independent manner with the outer membrane 86-kD protein and fractionated with the outer membrane at this stage. The ATP-dependent step in binding was association with the outer membrane 75-kD protein. This complex no longer fractionated with the outer membrane but instead sedimented with mixed membranes, presumably with contact sites between the outer and inner membrane.

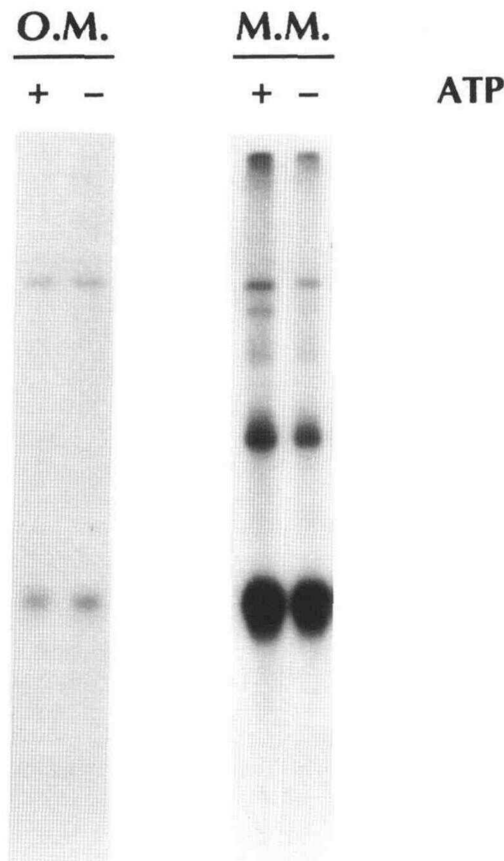

Figure 5. Resolution of Outer Membrane from Mixed Membranes.

The cross-linking experiment with and without ATP (+ and −ATP, respectively) was performed as described in Figure 4. After exposure to UV light, the chloroplasts were lysed under hypertonic conditions in 0.6 M sucrose in 25 mM Hepes, pH 8.0, and the outer membrane (O.M.) was separated from mixed inner and outer membranes (M.M., mixed membranes). Each was analyzed by SDS-PAGE. A photograph of the autoradiogram is shown.

Specificity of the Label Transfer Experiment

A transit peptide is necessary for transport into chloroplasts (Mishkind et al., 1985; Anderson and Smith, 1986). Thus, a critical test of the specificity of the labeling reaction was to determine whether labeling of the 86- and 75-kD proteins required the precursor form of SSU. Mature-sized small

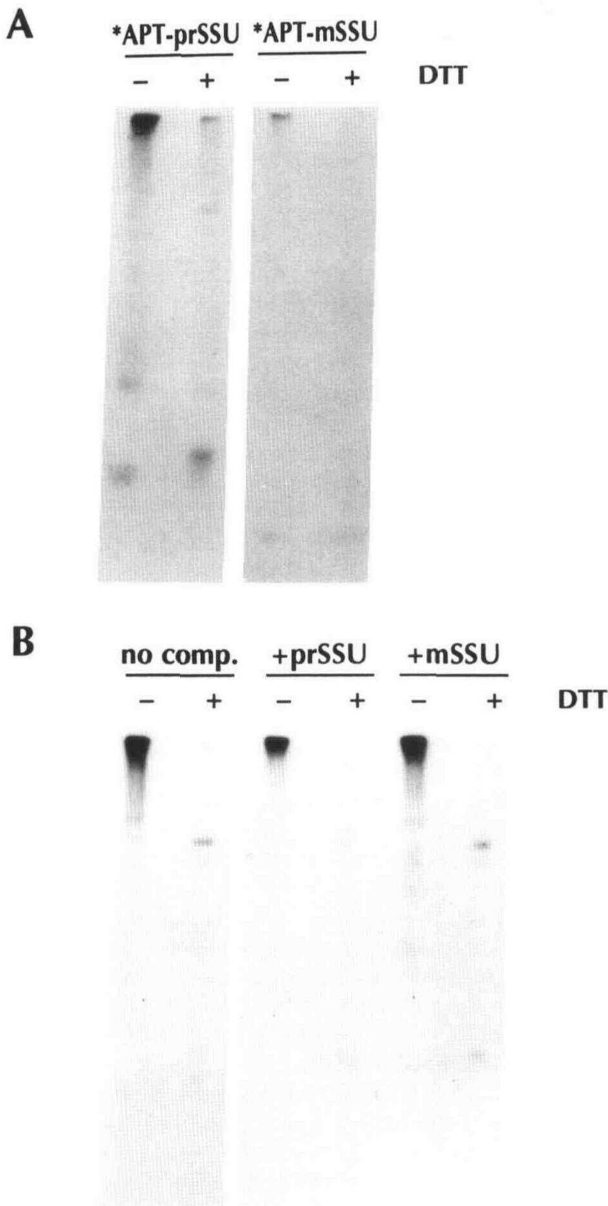

Figure 6. Labeling of Envelope Proteins Is Ligand Specific.

(A) Cross-linking using ^{125}I-APT-mSSU. Either prSSU or mSSU modified with ^{125}I-APDP was incubated in a binding reaction with chloroplasts, as described in Figure 1. Intact chloroplasts were recovered and washed, and the samples were exposed to UV light to activate the azido group on the cross-linker. Fractionation of chloroplasts was performed, and envelope fraction I was analyzed by SDS-PAGE in the presence and absence DTT (+ and −DTT, respectively). A photograph of the autoradiogram is shown.

(B) Competition of binding by unmodified prSSU or mSSU. The binding reaction of ^{125}I-APT-prSSU to chloroplasts was performed as described in Figure 1 except that a 10-fold molar excess of either unmodified prSSU or mSSU (+prSSU and +mSSU, respectively) was added to the binding reaction. A control without competing ligand was also performed (no comp.). Samples were resuspended in sample buffer with and without 50 mM of DTT (+ and −DTT, respectively). A photograph of the autoradiogram obtained by analysis of envelope fraction I is shown.

subunit (mSSU) from expression in *E. coli* was modified with ^{125}I-APDP. ^{125}I-APT-mSSU was incubated in a binding reaction with chloroplasts, and cross-linking was performed as for ^{125}I-APT-prSSU. Figure 6A shows no label incorporated into either the 86- or the 75-kD proteins when ^{125}I-APT-mSSU was used as the ligand in the label transfer reaction. A small amount of label can be seen at approximately 14 kD, which represents a low level of nonspecific binding of ^{125}I-APT-mSSU to chloroplasts.

A further test of ligand specificity was performed by competing binding of ^{125}I-APT-prSSU with a 10-fold molar excess of either unmodified prSSU or mSSU (Figure 6B). Labeling of both envelope proteins was decreased when prSSU was used as the competing ligand. When mSSU was the competitor, no reduction of labeling of the 86- or 75-kD proteins was observed. Thus, association of small subunit with the 86- and 75-kD envelope proteins required a transit peptide, demonstrating ligand specificity of the label transfer reaction.

Protease Sensitivity of the Labeled Proteins

One piece of evidence for a proteinaceous receptor is the protease sensitivity of binding (Cline et al., 1985a; Friedman and Keegstra, 1989). Thus, we investigated whether labeling of the 75- and 86-kD proteins was dependent upon protease-sensitive components. When ^{125}I-APT-prSSU was bound and cross-linked to thermolysin-pretreated chloroplasts, no label was observed in the 86- or 75-kD proteins, and no bound precursor was associated with the envelope, as shown in Figure 7 in the lane marked "pre-." Thus, a thermolysin-sensitive surface component was required for association of modified precursor with the 86- and 75-kD proteins.

Bound precursor is exposed externally on chloroplasts and can be digested by thermolysin treatment after binding (Cline et al., 1985a). When chloroplasts were treated with thermolysin after cross-linking, the bound precursor was removed (compare the radiolabeled protein band at 20 kD in Figure 7, control in the left lane versus "post-"), and the 86-kD protein was cleaved yielding a smaller fragment of ~50 kD. The 75-kD protein remained uncleaved by thermolysin, arguing that it was not a thermolysin-sensitive protein exposed at the surface of chloroplasts.

Another possibility was that both the 86- and the 75-kD proteins were thermolysin sensitive. The 86-kD protein may have been cleaved to a 75-kD fragment and the 75-kD protein cleaved to a 50-kD fragment. Envelopes from chloroplasts that were treated with thermolysin after the label transfer experiment were analyzed by two-dimensional gel electrophoresis.

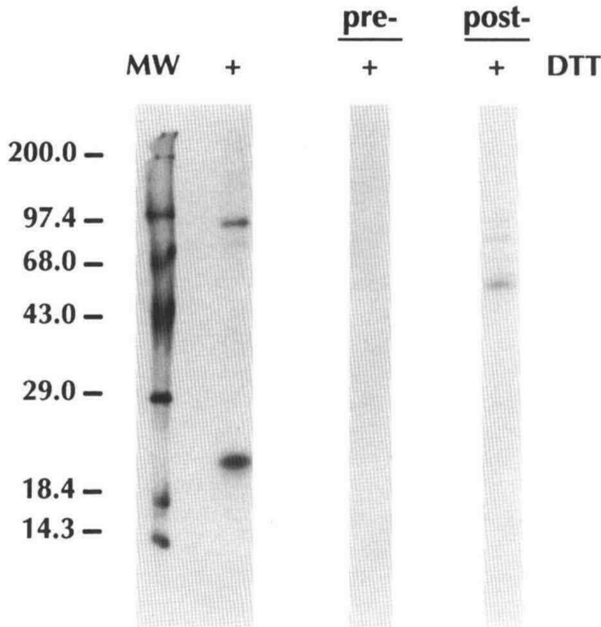

Figure 7. Thermolysin Sensitivity of Proteins Labeled in the Cross-Linking Reaction.

The control cross-linking reaction was performed as given in Figure 1 (left lane). After exposure of the sample to UV light, half of the reaction was treated with thermolysin, as described in Methods (post−). ^{125}I-APT-prSSU was also incubated in a binding reaction with chloroplasts that had been pretreated with thermolysin (pre−). Photolysis and fractionation were performed as in Figure 1. The results from envelope fraction I resuspended in sample buffer containing 50 mM DTT (+DTT) are shown as a photograph of the autoradiogram. Molecular mass markers (MW) are given in kilodaltons at left.

The labeled 75-kD protein of pI 7.5 remained undigested by thermolysin treatment, whereas the labeled 86-kD protein disappeared (data not shown). Thus, the 86-kD protein may represent the thermolysin-sensitive component of the transport apparatus required for binding of precursor proteins. The 75-kD protein was not itself thermolysin sensitive but was not labeled when thermolysin-pretreated chloroplasts were used in cross-linking reactions. One likely explanation is that the precursor must first bind to the thermolysin-sensitive 86-kD protein before association with the 75-kD protein.

DISCUSSION

Despite considerable progress in describing the process of protein transport into chloroplasts, relatively little is known about the identity of the components that mediate transport. To address this latter problem, we used prSSU as the ligand in label transfer cross-linking reactions. This procedure led to the identification of two proteins of the outer envelope membrane as putative components of the transport apparatus. A working model incorporating these observations is shown in Figure 8. Although other models cannot be excluded, this simple model is consistent with the current evidence.

Several experimental results provided evidence that the labeled 86- and 75-kD proteins were components of the transport apparatus. First, labeling of the two proteins was specific for the precursor form of SSU and did not occur when labeling was attempted with mature SSU (Figure 6). Second, bound precursors modified with the iodinated cross-linker were on a productive pathway for transport as demonstrated by their translocation into chloroplasts when extra ATP was added, thereby preventing the labeling of the two envelope components. Finally, labeling of the 86- and 75-kD proteins required a thermolysin-sensitive chloroplastic protein (Figure 7, "pre-"). If thermolysin treatment was performed after the label transfer reaction, the 86-kD protein was digested, but the 75-kD protein was thermolysin resistant (Figure 7, "post-"). This is consistent with results reported in Cline et al. (1984), who demonstrated that the 75-kD protein of the outer membrane is largely thermolysin resistant, whereas the outer membrane 86-kD protein is thermolysin sensitive.

The protease results presented in Figure 7 also support the hypothesis that the 86-kD protein represents the thermolysin-sensitive protein required for binding of precursor proteins to chloroplasts. Although the 75-kD protein was thermolysin resistant, it was not labeled when thermolysin-pretreated chloroplasts were used in the binding reaction. This is consistent with the hypothesis that precursors first bind to the thermolysin-sensitive 86-kD protein, as shown in Figure 8.

The sequential association of precursors, first with the 86-kD protein, and later as a larger complex including the 75-kD protein, as shown in Figure 8, is further supported by the experiments presented in Figure 4. If low levels of ATP were added to an aliquot of the binding reaction without ATP, and the reaction incubated on ice for 15 min before exposure to UV light, upon photolysis of the cross-linker and hypotonic lysis, less label was associated with the 86-kD protein in envelope fraction I and more label appeared in the 75-kD protein in envelope fraction II. Therefore, precursor associated in an ATP-independent manner with the 86-kD protein could be subsequently chased to an ATP-dependent complex including both the 86- and the 75-kD proteins. This later complex preferentially fractionated with envelope fraction II.

Although it was expected that transport components labeled by association with bound precursors would be present in the outer envelope membrane, it was important to provide experimental verification of this point. The labeled 86-kD protein was demonstrated to be an outer membrane protein by two-dimensional gel electrophoresis (Figure 2) and by fractionation with the outer membrane (Figure 5). The 75-kD protein has been used as a marker for the outer membrane, but there is evidence that more than one 75-kD protein exists in this membrane, one of which is a heat shock protein 70 (Hsp70) (Marshall

et al., 1990). Soluble Hsp70 proteins have been demonstrated to be involved in transport across membranes in mitochondria (Deshaies et al., 1988; Murakami et al., 1988; Kang et al., 1990; Scherer et al., 1990) and in secretion (Chirico et al., 1988; Zimmermann et al., 1988). Furthermore, Waegemann and Soll (1991) reported that a heat shock cognate protein cosediments with a complex associated with bound precursor protein. Two-dimensional gel analysis has demonstrated that the labeled 75-kD protein corresponded to the major 75 kD that has a pI of ~7.5. Hsp70 proteins tend to have slightly acidic pIs (Craig, 1985). Also, the envelope Hsp70 is not the major 75-kD protein of the envelope (Marshall et al., 1990). Therefore, we consider it unlikely that the labeled 75-kD protein is an Hsp70.

Friedman and Keegstra (1989) calculated that putative receptors should be low-abundance proteins in the chloroplastic envelope based on their observations of 1500 to 3000 binding sites per chloroplast. In contrast, the 75-kD protein is the most abundant outer membrane protein, whereas the 86-kD protein is easily visible on a Coomassie blue–stained two-dimensional gel. Why are relatively abundant proteins being labeled? One possibility is that some other unidentified protein is the limiting component of the transport apparatus, and the 86- and 75-kD proteins are present in excess. Some of the label transfer experiments showed labeling of a relatively minor envelope protein of approximate molecular mass of 200 kD (for example, Figure 3A, lane marked Bound, +DTT). Another possible explanation is that the earlier estimates of the number of binding sites, which were made using in vitro–synthesized precursor, were not accurate. In support of this, the number of molecules of overexpressed ^{35}S-prSSU bound per chloroplast was approximately 10-fold higher than found for in vitro–synthesized prSSU (S.E. Perry, unpublished observation).

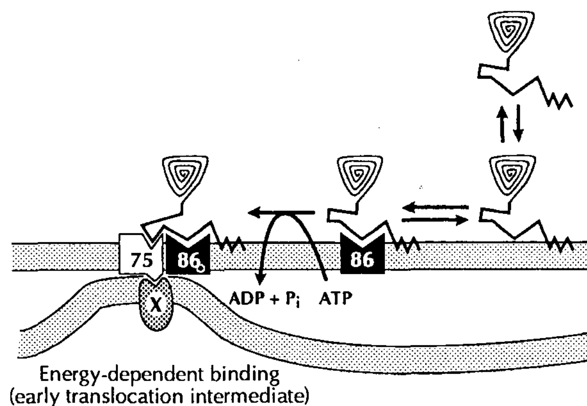

Figure 8. Model of Binding of Precursor to Chloroplasts and Formation of a Translocation Contact Site.

In this model, precursors may interact first with the lipid bilayer. The lipid bound precursor could diffuse in the plane of the membrane to associate with the 86-kD protein, a putative receptor protein. The ATP-dependent step is association with the 75-kD protein and formation of a translocation contact site. Unidentified inner membrane protein(s) (X) may be involved in formation of a translocation contact site. The transit peptide may be inserted into the translocation site at this point.

It is noteworthy that the intensity of labeling of envelope proteins is different from the relative abundance of the proteins in the membranes (Figure 1). Therefore, envelope proteins are not nonspecifically labeled according to their abundance. Furthermore, the phosphate translocator, which is the most abundant envelope protein, was not labeled in these experiments. This was true even when aggregation of this protein, which occurs when samples are heated in preparation for electrophoresis, was prevented by preparation of samples without heating (data not shown).

The working model presented in Figure 8 shows that precursors accumulate at contact sites in the presence of low levels of ATP. Again, this aspect of the model is consistent with the available evidence. Previous work has demonstrated that precursors bound in the presence of low levels of ATP do not fractionate with the outer membrane, although they are still exposed at the chloroplastic surface based on their sensitivity to thermolysin (Schnell and Blobel, 1993; J. Ostrom and K. Keegstra, unpublished observations). Precursors at this stage of transport fractionated with membranes previously shown to contain contact sites (Cline et al., 1985b). The concept of a translocation contact site has been proposed both in chloroplasts and in mitochondria as a means for the precursor to traverse two lipid bilayers in one step. Regions where the inner and outer envelope membranes are compressed have been described by electron microscopy in both mitochondria and chloroplasts (Hackenbrock, 1968; Cline et al., 1985b). Translocation intermediates in which the targeting sequence is cleaved by the processing protease inside the organelle, but part of the precursor remains exposed on the exterior of the organelle as shown by sensitivity to exogenously added protease, have been described in both organelles as well (Schleyer and Neupert, 1985; Schnell and Blobel, 1993). These protease-sensitive, mature-sized proteins must span both lipid bilayers of the envelope simultaneously to be exposed both on the interior and exterior of the organelle. We believe that envelope fraction II represents a fraction of the envelopes enriched in contact sites, and the ATP-dependent step in binding is formation of a translocation contact site. To corroborate this, hypertonic lysis of chloroplasts after label transfer experiments was performed.

Hypertonic lysis allows separation of purified outer membrane from mixed inner and outer membrane. The only labeled protein in the outer membrane fraction was the 86-kD protein. It is interesting that no labeled 75-kD protein fractionated with purified outer membranes under hypertonic lysis conditions because the 75-kD protein is an outer membrane protein. Therefore, we propose that after association with the 75-kD protein, the complex of 86-kD, 75-kD, and precursor protein fractionated with membranes containing contact sites. Because the 75-kD protein has been used as a marker for outer membrane in fractionation protocols, it must not exist permanently

in stable contact sites. Formation of a translocation contact site must be induced upon association with the 75-kD protein as in the dynamic model of translocation contact sites proposed in mitochondria (Pfanner et al., 1992).

The disposition of the transit peptide in this early translocation complex is not known. The model in Figure 8 shows the transit peptide still exposed at the surface of chloroplasts. In this case, the formation of a translocation contact site is mediated by interaction of transport components in the outer and inner membranes. The unidentified inner membrane component is labeled as X in Figure 8. Alternatively, the transit peptide may be inserted into the translocation complex, causing the precursor protein to engage simultaneously the transport apparatus present in the inner and outer membrane, thereby forming a translocation contact site. In mitochondria, these translocation contact sites are distinct from membrane adhesion sites where the two membranes are held stably near one another (Pfanner et al., 1992). It remains to be established whether such a distinction can be made in chloroplasts.

In the current model describing protein transport into mitochondria, the precursor first binds to a protease-sensitive receptor in the outer membrane such as MOM19 and MOM72 (Pfanner et al., 1992). The precursor is then inserted into the outer membrane by general insertion site proteins, including MOM38. If a similar system operates in chloroplasts, then the 86-kD protein shown in Figure 8 may act as a surface receptor, whereas the 75-kD protein may have a role analogous to a general insertion site protein. Work is currently underway to evaluate the validity of the working model shown in Figure 8.

METHODS

Overexpression of prSSU

BL21(DE3) competent cells (Novagen, Madison, WI) were transformed with the plasmid pet11D-prSSU or pet11C-mSSU (Klein and Salvucci, 1992) provided by Dr. R. Klein (University of Kentucky, Lexington). Single colonies were used to inoculate 5 mL of Luria-Bertani broth that contained 50 μg/mL ampicillin. The synthesis of the precursor or mature form of the small subunit of ribulose-1,5-bisphosphate carboxylase (prSSU and mSSU, respectively) was induced by addition of isopropyl β-D-thiogalactopyranoside to 1 mM when the OD of the culture was between 0.7 and 1.0. Induced cultures were grown for 2 to 3 hr, and cells were harvested by centrifugation for 5 min at 12,000*g*. Bacterial pellets were washed once in 50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.6 (buffer A), and resuspended in 1 mL of buffer A. Bacterial suspensions were ruptured via sonication for two 15-sec pulses using a probe sonicator (setting 6; Heat Systems-Ultrasonics, Plainview, NY). The insoluble fraction containing the inclusion bodies was pelleted by centrifugation for 10 min at 12,000*g*. The pellet was washed three times with 5 mL of buffer A containing 1% Triton X-100. The final pellet was resuspended in 6 M guanidine-HCl and 10 mM β-mercaptoethanol in buffer A and incubated at room temperature for 5 min to solubilize proteins present in the inclusion bodies. Insoluble debris was removed by centrifugation in a microcentrifuge for 10 min at 14,000 rpm. The supernatant containing solubilized prSSU or mSSU was divided into aliquots and stored at −80°C. A sample of the purified protein was analyzed by SDS-PAGE and silver stained to assess the purity of the preparation. Overexpressed prSSU and mSSU were typically greater than 90% pure.

Generation of ^{125}I-APT-prSSU

Eppendorf tubes were coated with 40 μg of IODO-GEN iodination reagent (Pierce Chemical Co., Rockford, IL) by dissolving the IODO-GEN in chloroform at a concentration of 1 mg/mL and adding 40 μL to each tube. The tubes were dried in a Speedvac (Savant, Farmingdale, NY) without heat and stored with desiccant at −20°C. An IODO-GEN-coated tube was washed once with 0.1 M sodium bicarbonate, pH 9.0, to remove any microscopic flakes of IODO-GEN. The cross-linker APDP (Pierce Chemical Co.) was dissolved in DMSO and diluted 20-fold with 0.1 M sodium bicarbonate, pH 9.0, immediately before use. Carrier-free Na-^{125}I (2 mCi) and 44.8 nmol of APDP were added to the IODO-GEN-coated tube and incubated at room temperature for 5 min in the dark.

Precursor protein was reduced by addition of 1% β-mercaptoethanol and incubation at 37°C for 15 min. The β-mercaptoethanol was removed by passing the reaction over a Sephadex G-25 (Pharmacia) column equilibrated with 6 M guanidine-HCl in 0.1 M sodium bicarbonate, pH 9.

The iodination reaction was terminated by removing the reaction from the IODO-GEN tube and adding it to the tube with reduced prSSU (18 nmol) and unlabeled NaI (1 μmol) in 6 M guanidine-HCl, 0.1 M sodium bicarbonate, pH 9. The conjugation reaction of cross-linker and prSSU was incubated on ice for 2 to 3 hr in the dark. The free iodine, cross-linker, and guanidine were removed by passing the reaction over a Bio-gel P2 (Bio-Rad) column equilibrated with import buffer with 50 mM of KCl (import buffer is 0.33 M sorbitol, 50 mM Hepes, pH 8.0) and collecting ^{125}I-APT-prSSU in the void volume. This was added immediately to a binding reaction containing intact chloroplasts.

Cross-Linking Experiments

Intact chloroplasts were purified from 10- to 13-day-old pea seedlings (*Pisum sativum* var Green Arrow) using differential centrifugation followed by sedimentation through a Percoll gradient (Cline, 1986). Chloroplasts were washed twice with import buffer and resuspended to 5 mg chlorophyll per mL. Chloroplasts were kept on ice in the dark until use.

^{125}I-APT-prSSU (25 μL) was added to chloroplasts containing 1.5 mg of chlorophyll, and ATP was added at a final concentration of 75 μM in a 600-μL reaction. For binding without ATP, no ATP was added, but 6 units of apyrase were included in the reaction mixture. For competition experiments, a 10-fold molar excess of unlabeled, unmodified prSSU or mSSU was added. Binding was for 15 min on ice in the dark. Intact chloroplasts were separated from broken chloroplasts and free ^{125}I-APT-prSSU by centrifuging through a cushion of 40% Percoll in import buffer. Samples to be chased to different steps in transport had ATP added to 75 μM or 3 mM and were incubated on ice or at 25°C, respectively, for 15 min before exposure to UV light. The intact chloroplast pellet was resuspended in 1 mL of cold import buffer. Samples were spread on Pyrex dishes on ice and exposed to UV light for 10 min using a UV cross-linker 1800 (Stratagene) with 254-nm bulbs. The samples were mixed by gentle swirling halfway through illumination. After exposure to UV light, chloroplasts were pelleted, resuspended

in lysis buffer (25 mM Hepes, pH 8.0), and incubated on ice for 10 min. An equal volume of 0.6 M sucrose, 4 mM $MgCl_2$ in 25 mM Hepes, pH 8.0, was added to the lysis reaction, and the sample was layered on top of a sucrose step gradient (1.2 mL of 1.2 M sucrose, 1.5 mL of 1.0 M sucrose, 1.5 mL of 0.46 M sucrose; all sucrose solutions were in 25 mM Hepes, pH 8.0). The gradient was centrifuged for 1 hr at 47,000 rpm in a SW50.1 rotor (Beckman).

Two aliquots of the soluble fraction were collected from the top region of the gradient, and ice cold acetone was added to 80% to precipitate the soluble proteins. After precipitation at −20°C for at least an hour, insoluble proteins were collected by centrifuging for 15 min at top speed in a microcentrifuge. The pellet was dried in a Speedvac and resuspended in SDS-PAGE sample buffer with or without 50 mM of DTT.

Envelope fraction I was collected from the 0.46/1.0 M sucrose interface. Envelope fraction II was collected from the 1.0/1.2 M sucrose interface. Both were diluted with two volumes of lysis buffer, split into two aliquots, and the membranes collected by centrifugation for 45 min at 48,000*g*. Membrane pellets were resuspended in sample buffer with and without 50 mM of DTT.

Thylakoids present in the pellet at the bottom of the tube were resuspended in 1-mL import buffer, divided into two aliquots, and centrifuged for 5 min at 7500 rpm in a microcentrifuge. Pellets were resuspended in sample buffer with or without DTT.

All samples were boiled 3 min, analyzed on a 10 to 15% gradient SDS-PAGE, stained with Coomassie Brilliant Blue R250, dried, and exposed to Kodak XAR5 x-ray film with a Cronex Lightening Plus intensifying screen (Du Pont, Wilmington, DE).

Resolution of Outer Membrane from Mixed Membranes

To separate the outer membrane from mixed membranes, chloroplasts were lysed under hypertonic conditions before fractionation. After illumination with UV light, the chloroplasts were pelleted and then resuspended in 0.6 M of sucrose in 25 mM Hepes, pH 8.0, to a concentration of 2 mg of chlorophyll per mL. The suspension was incubated on ice for 10 min and then frozen at −20°C for at least an hour. After thawing at room temperature, the lysed chloroplasts were centrifuged at 4000*g* for 5 min. The supernatant was removed to a new tube. Hypotonic lysis buffer was added to the pellet (1 mg of chlorophyll per mL), the suspension was incubated on ice for 10 min and centrifuged at 4000*g* for 5 min, and the supernatant was pooled with the first supernatant. The supernatant was diluted with 3 volumes of 25 mM Hepes, pH 8.0, and the membranes pelleted at 48,000*g* for 1 hr. The pellet was resuspended in 0.3 M of sucrose/Hepes and homogenized to break up any clumps. This suspension was loaded onto a sucrose gradient of steps of 1 mL each 1.2, 1.0, 0.8, and 0.6 M sucrose in 25 mM Hepes, pH 8.0, and centrifuged 14 to 19 hr in a SW50.1 rotor (Beckman) at 28,500 rpm. Fractions of 0.5 mL were collected starting from the top of the gradient, diluted with 1 mL of Hepes, and centrifuged at 48,000*g* for 1 hr. Membrane pellets were resuspended in sample buffer with 50 mM of DTT and analyzed by SDS-PAGE.

Thermolysin Treatment

Some samples were either pretreated with thermolysin (treatment of chloroplasts before binding of ^{125}I-APT-prSSU) or post-treated (treatment of chloroplasts after photolysis of the cross-linker). In both cases, the chloroplasts were pelleted and resuspended in import buffer containing 200 µg per mL thermolysin and 1 mM $CaCl_2$. The protease digestion reaction was incubated on ice for 30 min. Protease treatment was stopped by adding EDTA to a final concentration of 10 mM. For pretreatment of chloroplasts, the intact chloroplasts were reisolated over 40% Percoll and washed with import buffer. A sample was counted on a hemocytometer, and the chloroplasts were resuspended to the same number of chloroplasts per milliliter as chloroplasts not thermolysin pretreated.

Two-Dimensional Gel Electrophoresis

Two-dimensional electrophoresis was performed according to the method of O'Farrell (1975) by Kendrick Labs, Inc. (Madison, WI) as follows: isoelectric focusing was conducted in glass tubes of inner diameter 1.0 mm, using 2.0%, pH 4 to 8, ampholines for 9600 volt hr. One microgram of an isoelectric focusing internal standard, tropomyosin protein, with a molecular mass of 33 kD and a pI of 5.2 was added to the samples.

After equilibration for 10 min in 10% glycerol, 50 mM DTT, 2.3% SDS, and 0.0625 M Tris, pH 6.8, the tube gel was sealed to the top of a stacking gel that is on top of a 10% polyacrylamide slab gel and SDS slab gel electrophoresis conducted for ~4 hr at 12.5 mA per gel. Molecular weight standards were added to the agarose that sealed the tube gel to the slab gel and appeared as horizontal lines on the Coomassie blue–stained gel.

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