Identification of Protein Transport Complexes in the Chloroplastic Envelope Membranes via Chemical Cross-Linking

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Abstract. Transport of cytoplasmically synthesized proteins into chloroplasts uses an import machinery present in the envelope membranes. To identify the components of this machinery and to begin to examine how these components interact during transport, chemical cross-linking was performed on intact chloroplasts containing precursor proteins trapped at a particular stage of transport by ATP limitation. Large cross-linked complexes were observed using three different reversible homobifunctional cross-linkers. Three outer envelope membrane proteins (OEP86, OEP75, and OEP34) and one inner envelope membrane protein (IEP110), previously reported to be involved in protein import, were identified as components of these complexes. In addition to these membrane proteins, a stromal member of the hsp100 family, ClpC, was also present in the complexes. We propose that ClpC functions as a molecular chaperone, cooperating with other components to accomplish the transport of precursor proteins into chloroplasts. We also propose that each envelope membrane contains distinct translocation complexes and that a portion of these interact to form contact sites even in the absence of precursor proteins.

I N eukaryotic cells, newly synthesized proteins must be targeted to their proper compartment to maintain proper cellular functions. Although chloroplasts have their own genome and synthesize some of their own proteins, most chloroplastic proteins are encoded in nuclear genes and synthesized in the cytoplasm. Except for certain outer envelope membrane proteins (Salomon et al., 1990; Li et al., 1991), cytoplasmically synthesized chloroplastic proteins are thought to share a common import pathway, using a translocation apparatus embedded in the two envelope membranes. Chloroplastic proteins are synthesized with NH_2-terminal extensions, called transit peptides, which direct proteins to chloroplasts (Chua and Schmidt, 1978). Transit peptides are proteolytically removed during or after import (Oblong and Lamppa, 1992). The newly imported proteins are then folded or further targeted to other destinations (for review see de Boer and Weisbeek, 1991; Theg and Scott, 1993).

Protein transport across the envelope membranes can be divided into two distinct steps based on different energy requirements. The first is irreversible binding of precursor proteins to the chloroplastic surface, a step requiring 50–100 μM ATP in the intermembrane space (Olsen et al., 1989; Olsen and Keegstra, 1992). The second is translocation of precursor proteins across both the outer and inner envelope membranes. Higher levels of ATP (1–3 mM) in the stroma are required for this step (Pain and Blobel, 1987; Theg et al., 1989). Unlike mitochondria (Pfanner and Neupert, 1986; Eilers et al., 1987), a membrane potential across the envelope membranes is not required for protein transport into chloroplasts (Theg et al., 1989). In addition, GTP may be involved in early steps, as two components of the translocation apparatus in the outer envelope membrane, outer envelope membrane proteins of 86 and 34 kD (OEP86 and OEP34),[1] have GTP-binding domains (Kessler et al., 1994), and the binding of precursor proteins is affected by nonhydrolyzable GTP analogues (Olsen and Keegstra, 1992; Kessler et al., 1994).

Recent efforts from several labs have identified some of the envelope components that constitute the import machinery (for review see Gray and Row, 1995; Schnell, 1995). Most attention to date has focused on three outer envelope membrane proteins, OEP86, OEP75, and OEP34, and one inner envelope membrane protein of 110 kD

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1. *Abbreviations used in this paper*: DSP, dithiobissuccinimidylpropionate; DST, disuccinimidyltartarate; DTSSP, dithiobissulfosuccinimidylpropionate; IEP and OEP, inner and outer envelope membrane proteins; LDS, lithiumdodecylsulfate; LS, the large subunit of ribulose 1,5-bisphosphate carboxylase; prSS, precursor to the small subunit of ribulose 1,5-bisphosphate carboxylase; Rubisco, ribulose 1,5-bisphosphate carboxylase; S78, stromal hsp70; SS, mature small subunit.

(IEP110) (Perry and Keegstra, 1994; Schnell et al., 1994; Hirsch et al., 1994; Tranel et al., 1995; Kessler and Blobel, 1996; Lübeck et al., 1996). OEP86 is considered to be a receptor, as precursor proteins first associated with OEP86 and then with OEP75 in an ATP-dependent manner (Perry and Keegstra, 1994; Ma et al., 1996). Binding of precursors was inhibited by the Fab fragment of anti-OEP86 antibodies (Hirsch et al., 1994). OEP75 is postulated to be a protein translocation channel containing multiple transmembrane β-strands (Schnell et al., 1994; Tranel et al., 1995). The function of OEP34 is unknown, but based on its ability to bind GTP, it has been postulated to have regulatory functions (Kessler et al., 1994; Seedorf et al., 1995). These three proteins form a stable complex in the outer envelope membrane (Ma et al., 1996). The involvement of IEP110 in protein import was recently demonstrated, although its function is also unknown (Kessler and Blobel, 1996; Lübeck et al., 1996). In addition to the components mentioned above, the following have also been identified as putative components: two hsp70s localized in the outer envelope membrane (Waegemann and Soll, 1991; Schnell et al., 1994; Wu et al., 1994), one outer envelope membrane protein of 44 kD (Wu et al., 1994; Ko et al., 1995), three inner envelope membrane proteins of 36 kD (Schnell et al., 1994), 44 kD (Wu et al., 1994; Ko et al., 1995), and 21 kD (Ma et al., 1996), as well as a 25-kD protein whose localization has not yet been determined (Ma et al., 1996). Finally, members of the hsp70 family of molecular chaperones have been identified as transport components in other systems (for review see Schatz and Dobberstein, 1996). In chloroplasts, hsp70 homologues are present in the stroma (Marshall et al., 1990; Marshall and Keegstra, 1992) and were reported to interact with newly imported proteins in pumpkin chloroplasts (Tsugeki and Nishimura, 1993). Although the list of putative components has grown rapidly in recent years, it seems likely that additional components still need to be identified. Moreover, the role of each of these putative components during transport remains unclear.

Another important, unresolved issue is how these components interact with each other and precursor proteins during protein translocation. Protein transport has been localized to contact sites where both the outer and inner envelope membranes are held in close proximity (Schnell and Blobel, 1993). But it is still unclear whether contact sites are permanent structures or whether they are transiently formed during protein translocation. In mitochondria, translocation components embedded in both the outer and inner membranes were coimmunoprecipitated by antibodies against each component only when precursor proteins were trapped (Horst et al., 1995). This supports a model involving the dynamic formation of contact sites.

In this paper, we have used a cross-linking strategy to investigate some of these questions. When chloroplasts containing precursor proteins trapped at an early stage of transport by ATP limitation were treated with chemical cross-linkers, large complexes containing radiolabeled precursors were observed. Immunoprecipitation of these cross-linked complexes led to the identification of four polypeptides previously identified as translocation components (OEP86, OEP75, OEP34, and IEP110). In addition, a stromal member of the hsp100 family of molecular chaperones, ClpC, was present in the complexes. Our data support the presence of two stable complexes, one each in the outer and inner envelope membranes. In addition, a portion of the complexes interact to form some contact sites in the absence of precursor proteins.

Materials and Methods

Isolation of Chloroplasts

Chloroplasts were isolated from 8- to 12-d-old pea (*Pisum sativum* var. *little marvel*) seedlings as previously described (Bruce et al., 1994). After isolation, chloroplasts were suspended to 1 mg chlorophyll/ml in import buffer (50 mM Hepes-KOH, pH 8.0, 330 mM sorbitol).

In Vitro Translation of Precursor Protein

Synthesis of precursor to the small subunit of Rubisco (prSS) was performed using a wheat germ extract (Bruce et al., 1994) with [^{35}S]methionine (DuPont/NEN, Boston, MA) for labeling.

Binding and Cross-Linking Reactions

1 vol of chloroplast suspension (1 mg chlorophyll/ml of import buffer) was mixed with in vitro–translated ^{35}S-labeled prSS ($\sim$1.67 $\times$ 10^5 dpm/μl; 5 $\times$ 10^5 dpm/1 μg chlorophyll) and 75 μM Mg-ATP, brought to 3 vol of import buffer, and incubated on ice for 20 min in the dark. Intact chloroplasts were repurified through a 40% (vol/vol) Percoll cushion and washed twice with import buffer. Chloroplasts containing bound precursors were resuspended in 0.95 vol of import buffer, and then 0.05 vol of various concentrations of cross-linkers dissolved in DMSO were added. Cross-linking reactions were incubated for 15 min on ice in the dark and then quenched by adding glycine to a final concentration of 50 mM and incubating on ice for another 15 min in the dark. Treated chloroplasts were recovered by centrifugation. Cross-linking reagents, disuccinimidyltartarate (DST), and dithiobissulfosuccinimidylpropionate (DTSSP) were purchased from Pierce (Rockford, IL); dithiobissuccinimidylpropionate (DSP) was from Sigma Chemical Co. (St. Louis, MO).

Lysis of Chloroplasts and Solubilization of Membranes

For hypertonic lysis (Keegstra and Yousif, 1986), chloroplasts treated with cross-linker were suspended to 2 mg chlorophyll/ml in TE-0.6 (10 mM Tricine-NaOH, 2 mM EDTA, pH 7.5, 0.6 M sucrose) with a cocktail of protease inhibitors (PIC; 100 μM PMSF, 2 μg/ml pepstatin, 1 μg/ml aprotinin, 1 μg/ml capric acid, 1 μg/ml leupeptin) and incubated for 10 min on ice. After two cycles of freezing at −80°C and thawing at room temperature, TE-0-PIC (10 mM Tricine-NaOH, 2 mM EDTA, pH 7.5, with PIC) was added to the chloroplast suspension to bring sucrose concentration to 0.2 M. A total membrane fraction, which contained both outer and inner envelope membranes and thylakoid membrane, was recovered by centrifugation at 100,000 *g* for 30 min at 4°C. After washing, the total membrane fraction was solubilized by 1% lithiumdodecylsulfate (LDS) as follows. The total membrane fraction was suspended to 2.5 mg chlorophyll/ml in HG-10 (25 mM Hepes-LiOH, pH 7.5, 10% glycerol) with 1% LDS at 4°C for 30 min, followed by ultracentrifugation at 100,000 *g* for 30 min at 4°C. The supernatant solution was saved and stored at −80°C until use.

Gel Filtration

Solubilized membrane proteins (1.5 ml) were loaded onto Sephacryl S-500 (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) gel filtration column (1.5 $\times$ 42 cm) and eluted by HG-10 containing 0.25% LDS; then, 1.5-ml fractions were collected. After gel filtration, three consecutive fractions were pooled.

Sources of Antibodies

All antibodies used for this study were raised in rabbits, except for the mouse monoclonal antibodies against the large subunit of Rubisco (LS). Antibodies against OEP34 and IEP35 were a gift from D. Schnell (Schnell et al., 1994). Antibodies against ClpC (immunopurified) were a gift from

J. Shanklin (Shanklin et al., 1995). Mouse monoclonal antibodies against LS were a gift from A. Portis (University of Illinois, Urbana, IL). Antibodies against hsp60 (SPA-804) were purchased from StressGen (Victoria, BC Canada). All other antibodies raised against OEP86 (Perry, 1993), OEP75 (Tranel et al., 1995), IEP110 (see below), stromal hsp70 (S78) (see below), and the small subunit of Rubisco (SS) were prepared in our own laboratory. SS was purified by gel electrophoresis on ProSieve Agarose (FMC BioProducts, Rockland, ME) and injected into rabbits without additional adjuvant. For producing antibodies against IEP110, E. coli overproduced truncated IEP110 (gift from J. Soll) (Lübeck et al., 1996), which consisted of the COOH-terminal 782 amino acid residues of IEP110, was used. It was dissolved in adjuvant (TiterMax; Vaxcel, Inc., Norcross, GA) and was injected into rabbits. For producing antibodies against S78, a portion of the S78 cDNA (Marshall and Keegstra, 1992) corresponding to the COOH-terminal 142 amino acid residues of the protein was PCR-amplified and fused in-frame to the 3′ end of the glutathione S–transferase gene in the overexpression vector pGEX-2T (Pharmacia LKB Biotechnology, Inc.). The resulting fusion protein was overexpressed in E. coli and then purified to homogeneity by the glutathione Sepharose CL-4B column (Pharmacia LKB Biotechnology, Inc.). Purified GST-S78 fusion protein was mixed with the adjuvant (TiterMax; Vaxcel, Inc.) and injected into rabbits. The specificity of these antibodies was determined by immunoblotting various chloroplastic fractions containing the different chloroplastic Hsp70 homologues. Antibodies against S78 specifically recognized a single 78-kD polypeptide in whole chloroplasts and stromal fractions but did not react with outer envelope membrane fractions, confirming that the antibodies were specific to the S78.

Purification of Antibodies

For antibodies against OEP34, IgG was purified by ImmunoPure® (A/G) IgG Purification Kit (Pierce), following the instructions provided. IgGs were finally dialyzed against PBS (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl).

Antibodies against OEP75, OEP86, and IEP110 were affinity purified. Purification was done as follows: Overproduced IEP110 in E. coli or outer envelope membrane (gift from M. Cleveland) prepared from pea chloroplasts as described by Keegstra and Yousif (1986) was purified by SDS-PAGE and then transferred to ProBlott™ membrane (Applied Biosystems, Foster City, CA). Strips from both sides of the filter were stained with Coomassie brilliant blue R-250 and the regions containing OEP75, OEP86, and IEP110 were cut out. These sections containing the antigen were blocked with 5% dry milk in TTBS (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20). They were then incubated with each antiserum diluted fourfold with 1% BSA in TTBS for 2 h at room temperature with shaking. The membrane sections were washed three times with TTBS. Bound antibodies were eluted from a membrane by incubating with 100 mM glycine, pH 2.5, at 4°C for 10 min. The eluent was immediately adjusted to neutral pH by addition of 1 M Na-phosphate, pH 8.0, followed by dialysis against PBS. Mouse monoclonal antibodies against LS were purified by ammonium sulfate precipitation (Harlow and Lane, 1988).

Immunoprecipitation

Both the total membrane fraction and gel-filtered samples were subjected to immunoprecipitation assays. Antibodies were used as crude sera, IgGs, or affinity purified (see above). Immunoprecipitation assays were done in HETN (25 mM Hepes-LiOH, 1 mM EDTA, pH 8.0, 1% Triton X-100, 150 mM NaCl, 0.1% LDS). After incubation of samples and the desired amount of antibodies for 60 min with shaking at room temperature, HETN-prewashed protein A–Sepharose CL-4B (Pharmacia LKB Biotechnology, Inc.) was added and incubated for another 60 min. Immunoprecipitates were recovered by centrifugation at 5,000 g for 3 min at room temperature. The pellets were washed with HETN several times before resuspension in Laemmli's system sample buffer either with or without 10% β-mercaptoethanol (Laemmli, 1970).

Electrophoresis and Immunoblotting

Cross-linked complexes were analyzed by SDS-PAGE, followed by fluorography. For this purpose, 1.5–16.2%T(2.7%C) gradient gels with 3.75%T, 25%C stacking gels were made. The %T and %C indicate polyacrylamide gel concentration and percentage cross-linker against total monomer, respectively. For other purposes, 6–16.2%T(2.7%C) gradient gel with 4.5%T(2.7%C) stacking gel was made. All SDS-PAGE was done with the buffer system reported by Laemmli (1970).

For immunoblotting, proteins were transferred onto Immobilon™-P membrane (Millipore Corp, Bedford, MA) in the transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) (Towbin and Gordon, 1984) with 0.05% SDS. Primary antibodies were detected with horseradish peroxidase–conjugated goat anti–rabbit or anti–mouse antibodies (Kirkegaard and Perry Labs, Inc., Gaithersburg, MD). The secondary antibodies were detected by chemiluminescent reagents (Pierce).

Results

Cross-Linking of Precursor Proteins Bound to Chloroplasts

Our strategy to further characterize the envelope-based translocation complex is shown schematically in Fig. 1. Briefly it involves: (i) binding of radiolabeled precursor proteins to chloroplasts; (ii) treatment of chloroplasts containing precursors with a cleavable cross-linker; (iii) solubilization and purification of cross-linked complexes; and (iv–a) analysis of cross-linked complexes or (iv–b) cleavage of cross-links and analysis of the components.

Most studies were conducted with prSS, although other precursors gave similar results. Several cross-linkers were examined and found to be useful to some extent, but the highest yields of cross-linked complexes were obtained with
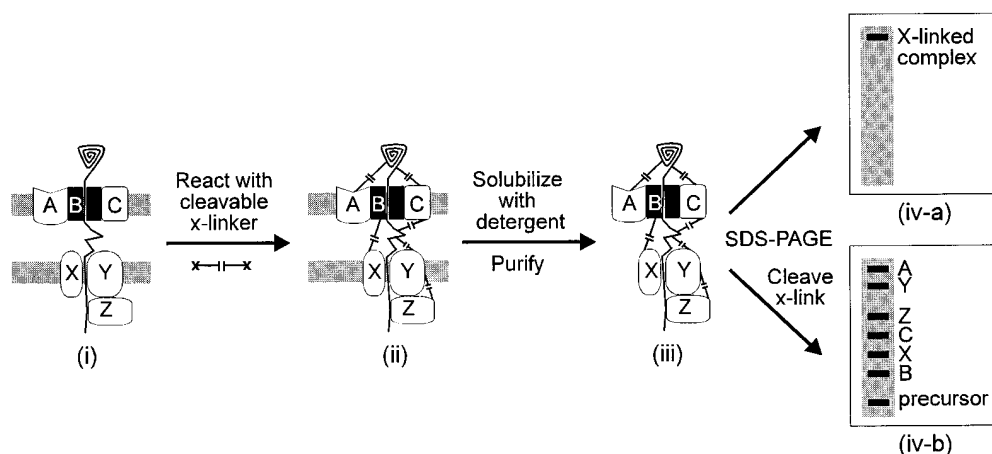
Figure 1. Cross-linking scheme. (i) Precursor proteins are bound to chloroplasts in the presence of low levels of ATP. (ii) Chloroplasts containing precursor proteins are treated with a cleavable cross-linker. (iii) Cross-linked complexes are solubilized with detergent and purified. (iv) Complexes are analyzed by SDS-PAGE either with (iv-b) or without (iv-a) cleavage of cross-links.
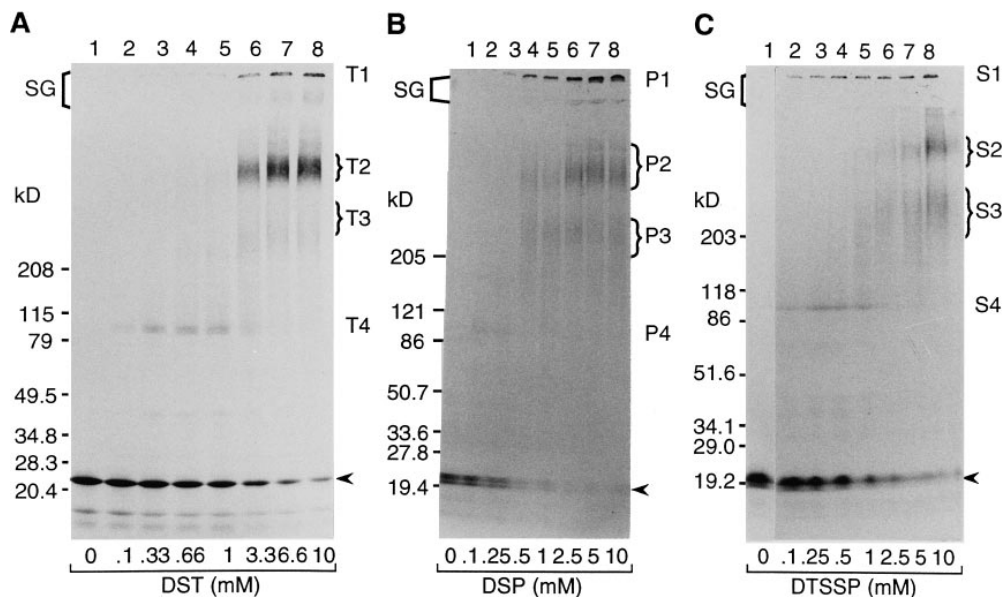
Figure 2. Chloroplast-bound precursor protein treated with various cross-linkers. In vitro translated ^{35}S-labeled prSS and chloroplasts were incubated in the dark with 75 μM ATP for 20 min at 4°C. Intact chloroplasts were re-purified through a cushion of 40% Percoll and then treated with indicated concentration of DST (*A*), DSP (*B*), or DTSSP (*C*) for 15 min at 4°C. Cross-linking reactions were quenched with 50 mM glycine. Chloroplasts were recovered by centrifugation and dissolved in electrophoresis sample buffer. Cross-linked products were analyzed by SDS-PAGE under reducing (*DST*) or non-reducing (*DSP* and *DTSSP*) conditions, followed by fluorography. Major cross-linked complexes generated by DST (*T*), DSP (*P*), and DTSSP (*S*) are shown with numbers 1 to 4 in order of decreasing size. *Arrowhead*, prSS; *SG*, stacking gel.

the following three: DST, DSP, and DTSSP. DST can be cleaved with periodate; DSP and DTSSP can be cleaved with reducing reagents. DSP and DST are membrane-permeable cross-linkers, whereas DTSSP is a water-soluble analog of DSP that does not cross biological membranes (Ji, 1983; Staros and Anjaneyulu, 1989). Because small molecules are able to cross the chloroplastic outer envelope membranes (Flügge and Benz, 1984), we expected DTSSP could react with the intermembrane space proteins and the inner envelope membrane proteins exposed on the outer surface, in addition to outer envelope membrane proteins. On the other hand, DSP and DST should gain access to both surfaces of both envelope membranes.

When chloroplasts containing bound, radiolabeled prSS were treated with DST, four radiolabeled cross-linked complexes were observed (Fig. 2 *A*; complexes are labeled *T1* to *T4* in order of decreasing size). Three of these complexes, T2, T3, and T4, entered the gel and migrated with estimated molecular masses of 600, 300, and 110 kD (see below). The largest complex, T1, did not enter the gel and remained at the origin of the stacking gel. The yield of complex T4 increased as the concentration of DST increased but then decreased and disappeared at the highest DST concentrations.

When DSP was used for cross-linking, four complexes were also observed (Fig. 2 *B*; the complexes were labeled *P1* to *P4* in order of decreasing size). The complexes generated with DSP were not as well resolved as with DST-treated samples because reducing reagents could not be added to the samples treated with DSP. In contrast to DST, where the yield of cross-linked complexes continued to increase up to cross-linker concentrations of 10 mM, with DSP the yield of cross-linked proteins was maximal at 2.5 mM (Fig. 2 *B*, lanes *6–8*). Similar results were observed when precursor to the 23-kD protein of oxygen-evolving enhancer (prOEE23) and precursor to plastocyanin (prPC) were used in place of prSS (data not shown).

When DTSSP was used as the cross-linker, four radiolabeled complexes were again observed, with the yield of larger complexes increasing as the concentration of cross-linker increased (Fig. 2 *C*; the complexes were labeled *S1* to *S4* in order of decreasing size). However, complexes S2 and S3 (Fig. 2 *C*, lanes *6–8*) were smaller and migrated more diffusely than complexes T2 and T3 observed with DST or complexes P2 and P3 observed with DSP (Fig. 3, compare lanes *2* and *7*).

With all three cross-linkers, the largest complex remained on top of the stacking gel. The amount of each complex did not change, whether or not samples were boiled just before loading onto the gel (data not shown). For the complexes that entered the separating gel, estimates of molecular weight were made using a calibration curve based on standard molecular weight markers. The calibration curve was nearly linear (data not shown). However, because the complexes contained several proteins cross-linked to each other, the complexes will likely migrate anomalously, making estimates of molecular size only approximations.

The smallest complexes, with an estimated molecular mass of 110 kD, were observed only at lower cross-linker concentrations (Fig. 2, *A*, *B*, and *C*). They were immunoprecipitated by anti-OEP86 and -OEP75 antibodies, but not by anti-OEP34 antibodies (data not shown). Based on molecular size estimates and previous studies (Perry and Keegstra, 1994), we conclude that complexes T4, P4, and S4 are a mixture of prSS-OEP86 and prSS-OEP75. Under reducing conditions (Fig. 2 *A*), complex T4 sometimes resolved into a doublet, probably representing the two cross-linked adducts.

Complexes T2 and P2 migrated with an apparent molecular mass of 600 kD (ranging from 500–700 kD), whereas complexes T3 and P3 migrated with an apparent molecular mass of 300 kD (ranging from 250–350 kD). In contrast, complexes S2 and S3, generated with DTSSP, mi-
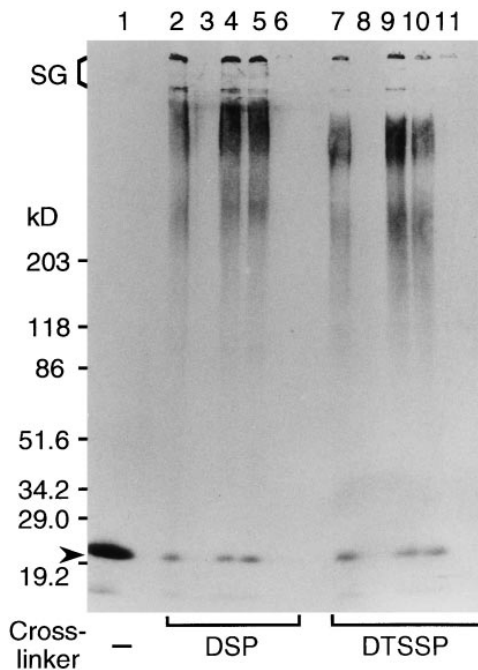
Figure 3. Fractionation of cross-linker–treated chloroplasts. After cross-linking with 2.5 mM DSP (lanes *2–6*) or 10 mM DTSSP (lanes *7–11*), intact chloroplasts containing bound prSS (lanes *2* and *7*) were hypertonically lysed, and soluble (lanes *3* and *8*) and insoluble (lanes *4* and *9*) fractions were separated by centrifugation. The pellet was resuspended in solution containing 1% LDS and subjected to another centrifugation. Solubilized (lanes *5* and *10*) and insoluble (lanes *6* and *11*) fractions were analyzed by SDS-PAGE and fluorography. Lane *1* shows a sample before cross-linking treatment. *Arrowhead*, prSS; *SG*, stacking gel.

grated with apparent molecular masses of 500 kD (ranging from 400–600 kD) and 250 kD (ranging from 200–300 kD), respectively. The smaller sizes of complexes generated with DTSSP probably reflect its membrane impermeability, resulting in the lack of cross-linking to stromal proteins and to inner membrane proteins accessible only from the stromal side.

Before continuing with solubilization and characterization of the complexes, the cleavage of cross-linkers with periodate or reducing reagents was measured. Complexes generated with DSP and DTSSP were completely cleaved by DTT or β-mercaptoethanol, whereas complexes generated with DST were not completely cleaved with periodate (data not shown). Thus, DSP (2.5 mM) and DTSSP (10 mM) were used for the studies presented below.

Solubilization and Characterization of Cross-Linked Complexes

Because translocation complexes should be located in the envelope membranes, our first efforts to characterize the complexes focused on purification of envelope membranes. Regardless of whether chloroplasts were lysed hypertonically (Keegstra and Yousif, 1986) or hypotonically (Perry and Keegstra, 1994), the large majority of the envelope membranes were recovered in the thylakoid fraction when chloroplasts treated with cross-linkers were fractionated
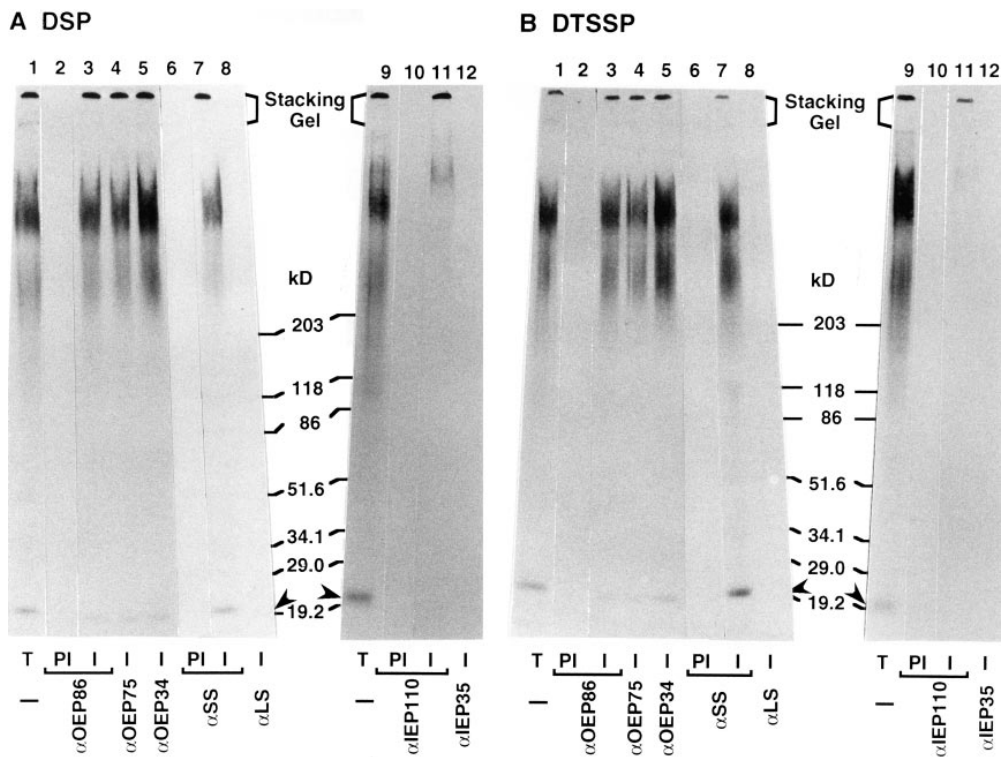
(data not shown). The reasons for this aberrant fractionation were unclear, but it caused the yields of purified envelope membranes and associated precursors to be very low. Consequently, total membranes, containing both of the envelope membranes as well as thylakoids (Fig. 3, lanes *4* and *9*), were used for most experiments. Radioactive precursors and complexes were not observed in the supernatant fraction after lysis (Fig. 3, lanes *3* and *8*). Chloroplastic membranes treated with cross-linkers could not be adequately solubilized with mild detergents (data not shown); consequently, membranes were solubilized with 1% LDS. All the cross-linked complexes that entered the resolving gel, as well as most of the large complexes that remained at the top of the stacking gel, were recovered in the supernatant after LDS solubilization (Fig. 3, compare lanes *5* with *6* and lanes *10* with *11*).

Preliminary characterization of the LDS-solubilized complexes was performed via immunoprecipitation. Antibodies against known translocation components, OEP86, OEP75, OEP34, and IEP110, were used to determine whether each component was present. Antibodies against SS, which should be in the complex as prSS, were used as a positive control. Antibodies against IEP35, an inner membrane protein not in translocation complexes (Schnell et al., 1994), and antibodies against LS were used as negative controls.

For DSP-treated samples (Fig. 4 *A*), complexes P1, P2, and P3 were immunoprecipitated by anti-OEP86, -OEP75, and -OEP34 antibodies (Fig. 4 *A*, lanes *3–5*, respectively). The proportions of these complexes in all three immunoprecipitated samples were similar to that of the sample before immunoprecipitation (Fig. 4 *A*, lane *1*), indicating that all three complexes contained all three components. With antibodies against IEP110 (Fig. 4 *A*, lane *11*), only complex P1, at the top of the stacking gel, and the upper part of complex P2 were efficiently immunoprecipitated, indicating that IEP110 was either absent or poorly exposed in the smaller complexes.

For DTSSP-treated samples (Fig. 4 *B*), similar results were obtained. Again, the three larger complexes, S1, S2, and S3, were immunoprecipitated by anti-OEP86, -OEP75, and -OEP34 antibodies (Fig. 4 *B*, lanes *3–5*) in the same proportion as found in the sample before immunoprecipitation (Fig. 4 *B*, lane *1*). Complex S1 at the top of the stacking gel was immunoprecipitated by anti-IEP110 antibodies, whereas immunoprecipitation of the 500-kD complex, S2, was very inefficient (Fig. 4 *B*, lane *11*). The observation that complexes generated with DTSSP contain some IEP110 provides evidence that either part of this molecule is present on the outer surface of the inner envelope membrane or that some DTSSP gains access to the stromal space. These two possibilities are discussed in more detail below.
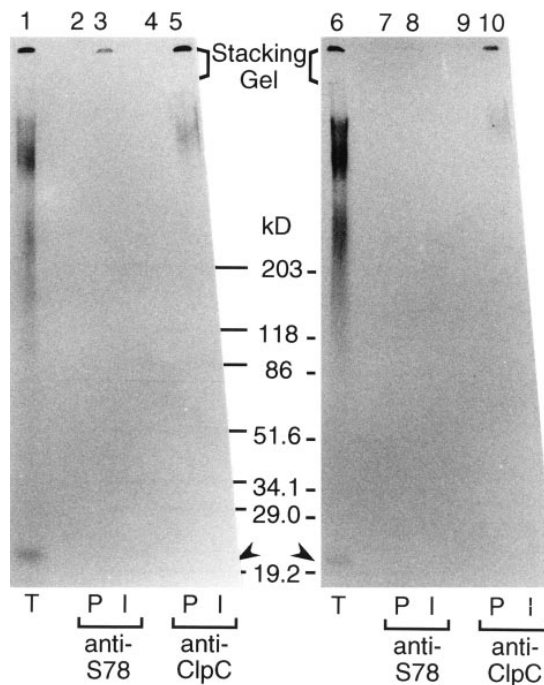
For both DSP- and DTSSP-treated samples, only trace amounts of radioactivity, probably resulting from nonspecific binding, were immunoprecipitated by preimmune sera (Fig. 4, *A* and *B*, lanes *2*, *6*, and *10*), anti-IEP35 antibodies (Fig. 4, *A* and *B*, lane *12*), or anti-LS antibodies (Fig. 4, *A* and *B*, lane *8*). As expected, all radioactive molecules, including prSS monomer, were immunoprecipitated by anti-SS antibodies (Fig. 4, *A* and *B*, lane *7*). Smaller amounts of prSS monomer were also observed in immunoprecipitates produced by anti-OEP86, -OEP75,

A DSP   1 2 3 4 5 6 7 8   9 10 11 12

Stacking Gel

kD
— 203
— 118
— 86
— 51.6
— 34.1
— 29.0
— 19.2

T │ PI │ I │ I │ PI │ I │ I   T │ PI │ I │ I
— αOEP86 αOEP75 αOEP34 αSS αLS   — αIEP110 αIEP35

B DTSSP   1 2 3 4 5 6 7 8   9 10 11 12

Stacking Gel

kD
— 203
— 118
— 86
— 51.6
— 34.1
— 29.0
— 19.2

T │ PI │ I │ I │ PI │ I │ I   T │ PI │ I │ I
— αOEP86 αOEP75 αOEP34 αSS αLS   — αIEP110 αIEP35

*Figure 4.* Immunoprecipitation of cross-linked products. Cross-linked complexes generated with DSP (*A*) and DTSSP (*B*) were solubilized with 1% LDS before immunoprecipitation by antibodies raised against OEP86 (lane *3*), OEP75 (lane *4*), OEP34 (lane *5*), SS (lane *7*), LS (lane *8*), IEP110 (lane *11*), and IEP35 (lane *12*). Controls included immunoprecipitation by preimmune sera for OEP86 (lane *2*), SS (lane *6*), and IEP110 (lane *10*). 20% of the sample subjected to immunoprecipitation is shown in lanes *1* and *9. Arrowhead*, prSS.

and -OEP34 antibodies (Fig. 4, *A* and *B*, lanes *3–5*). This monomer may be produced by cleavage of cross-links during the preparation of samples for SDS-PAGE. Taken together, the immunoprecipitation results are consistent with the hypothesis that complexes P3 and S3 consist exclusively of outer envelope membrane components, whereas the larger complexes, P1, P2, and S1, contain components from both outer and inner envelope membranes. One important question was whether the larger complexes also contained stromal molecular chaperones.

Because mitochondrial molecular chaperones, especially matrix hsp70, interact with preproteins during translocation (Rassow et al., 1994; Schneider et al., 1994), we sought to determine whether chloroplastic molecular chaperones were part of the cross-linked complexes. Immunoprecipitation was performed with antibodies specific for S78 (see Materials and Methods) and ClpC (a member of the hsp100 family) (Moore and Keegstra, 1993; Shanklin et al., 1995). Immunoprecipitation with antibodies against ClpC (Fig. 5, lanes *5* and *10*) produced results similar to those obtained with anti-IEP110 antibodies (Fig. 4, *A* and *B*, lane *11*). Specifically, only complexes P1 and S1, as well as the larger regions of complexes P2 and S2, were immunoprecipitated. The efficiency of immunoprecipitation of complexes generated with DTSSP was lower (Fig. 5, lane *10*) than complexes generated with DSP (Fig. 5, lane *5*). ClpC is localized in both the soluble and the inner envelope membrane fractions (data not shown). The portions associated with the inner envelope membrane are likely to be peripheral membrane proteins located on the stromal side only. The observation that a portion of the complexes generated by DTSSP were immunoprecipitated by anti-ClpC antibodies provides evidence that at least a small

1   2 3   4 5   6   7 8   9 10

Stacking Gel

kD
203 -
118 -
86 -
51.6 -
34.1 -
29.0 -
19.2 -

T │ P I │ P I   T │ P I │ P I
  anti- │ anti-   anti- │ anti-
  S78   ClpC   S78   ClpC

Figure 5. Immunoprecipitation of cross-linked complexes with antibodies against chloroplastic chaperones. Cross-linked complexes generated with DSP (lanes *1–5*) and DTSSP (lanes *6–10*) were solubilized with 1% LDS before immunoprecipitation by antibodies (*I*) raised against S78 (lanes *3* and *8*) and ClpC (lanes *5* and *10*). Controls included immunoprecipitation by preimmune sera (*P*) for S78 (lanes *2* and *7*) and ClpC (lanes *4* and *9*). 20% of the samples subjected to immunoprecipitation are shown (*T*) in lanes *1* and *6. Arrowhead*, prSS.
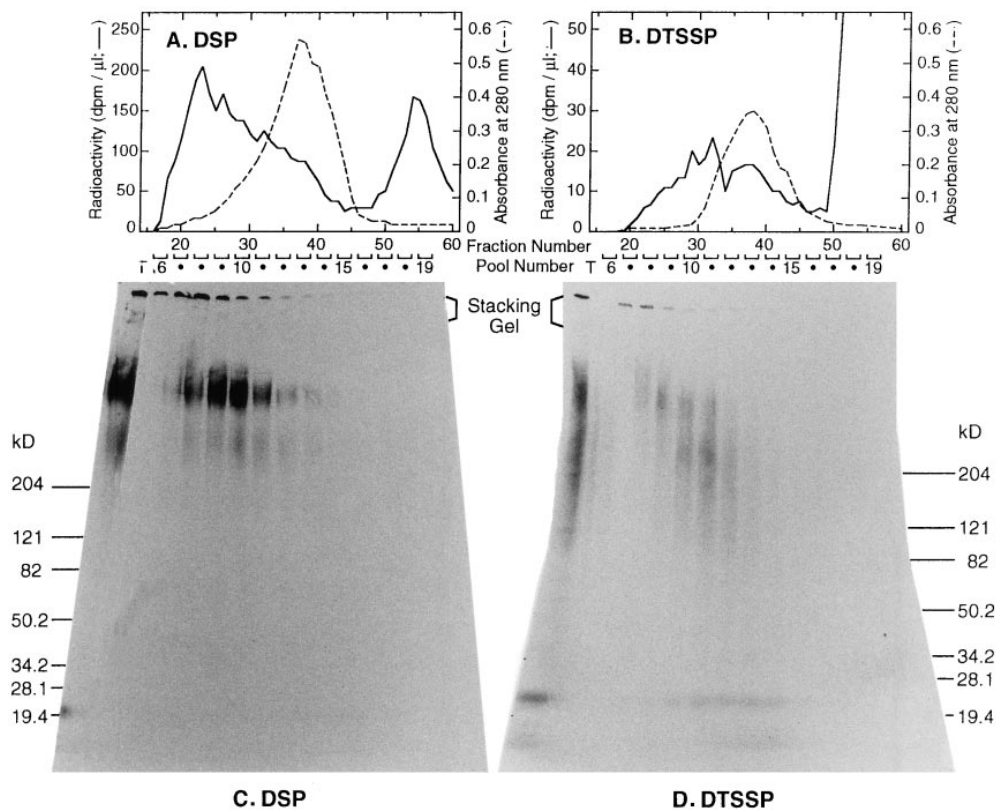
Figure 6. Sephacryl S-500 gel filtration of cross-linked complexes generated with DSP and DTSSP. Cross-linked complexes generated with DSP or DTSSP were solubilized with 1% LDS before being loaded onto a Sephacryl S-500 size exclusion column (1.5 × 42 cm). The elution patterns of radioactivity (*solid line*) and absorbance at 280 nm (*dashed line*) are shown in *A* (*DSP*) and *B* (*DTSSP*). Three consecutive fractions were combined to generate pools as shown on the bottom of *A* and *B*. Each pool was analyzed by SDS-PAGE under nonreducing conditions, followed by fluorography, as shown in *C* (*DSP*) and *D* (*DTSSP*).

amount of DTSSP gained access to the stromal space. Native complexes generated without the use of cross-linkers and solubilized with mild detergents could also be immunoprecipitated by anti-ClpC antibodies (Nielsen et al., 1997). Taking all these results together, we concluded that ClpC is part of the translocation complex involved in protein import into chloroplasts, possibly as a molecular chaperone.

Anti-S78 antibodies caused immunoprecipitation of only complex P1 at the top of the stacking gel (Fig. 5, lanes *3* and *8*). Both preimmune sera yielded only background levels of immunoprecipitation (Fig. 5, lanes *2*, *4*, *7*, and *9*). From these results, it is difficult to discern whether the presence of S78 has physiological significance. However, if S78 is involved in protein transport into chloroplasts, it appears to be present only in very large translocation complexes. Interestingly, a similar conclusion was reached using a very different strategy (Nielsen et al., 1997).

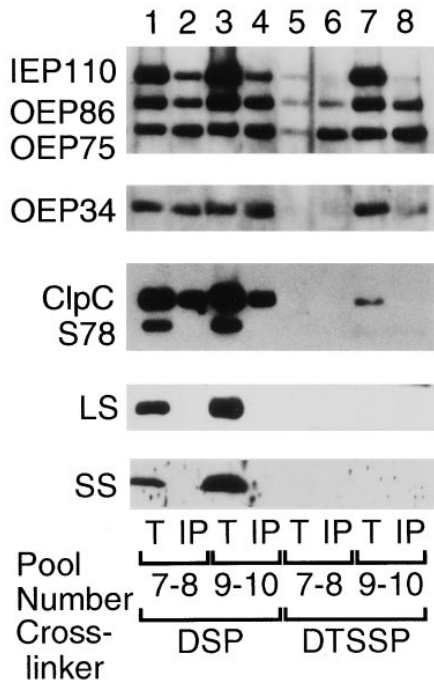### Partial Purification of Cross-Linked Complexes

Further characterization of the solubilized complexes was accomplished by purification via gel filtration. Solubilized membrane proteins, isolated from either DSP- or DTSSP-treated chloroplasts, were loaded onto a Sephacryl S-500 gel filtration column. The elution patterns of both samples were monitored by following both radioactivity and absorbance at 280 nm (A_{280}); the results obtained with DSP- and DTSSP-treated chloroplasts are shown in Fig. 6, *A* and *B*, respectively. The column fractions were combined into pools as shown in Fig. 6, and each pool was analyzed by SDS-PAGE; the resulting fluorographs are shown in Fig. 6 *C* (*DSP*) and *D* (*DTSSP*). Radiolabeled complexes

generated with DSP began to elute near the void volume (fractions 16–18, pool 6) and peaked near fractions 22–24 (pool 8). Complexes P1, P2, and P3, generated with DSP treatment, eluted differently from each other during gel filtration, but overlapped each other (Fig. 6 *C*). A similar pattern of incomplete separation was observed if solubilized membrane proteins were analyzed by sucrose gradient centrifugation (data not shown). Because the samples have been solubilized with LDS, neither gel filtration nor sucrose gradient centrifugation can be used to provide accurate estimates of the molecular weights of either the cross-linked adducts or the monomeric proteins.

Complexes generated with DTSSP began to elute a little later from the column; however, the results were similar in that the different complexes failed to separate completely (Fig. 6 *D*). Monitoring the column effluent for A_{280} showed that most of the protein eluted between fractions 31 and 42 (pools 11–14) for both cross-linker–treated samples and for mock-treated samples (data not shown). This result demonstrates that most chloroplastic proteins were not cross-linked into complexes. However more proteins were shifted into larger complexes (pools 8–10) when DSP was used as a cross-linker (Fig. 6 *A*) than when DTSSP was used (Fig. 6 *B*). This is consistent with DSP entering the stromal space and forming some cross-linked complexes with stromal proteins.

The large peak of radioactivity that appeared later (fractions 52–58; pools 18 and 19) was most likely free [^{35}S]methionine (Fig. 6, *A* and *B*). This conclusion is supported by the observation that no bands of radioactive proteins were observed in fluorographs (Fig. 6, *C* and *D*) of samples from these fractions.
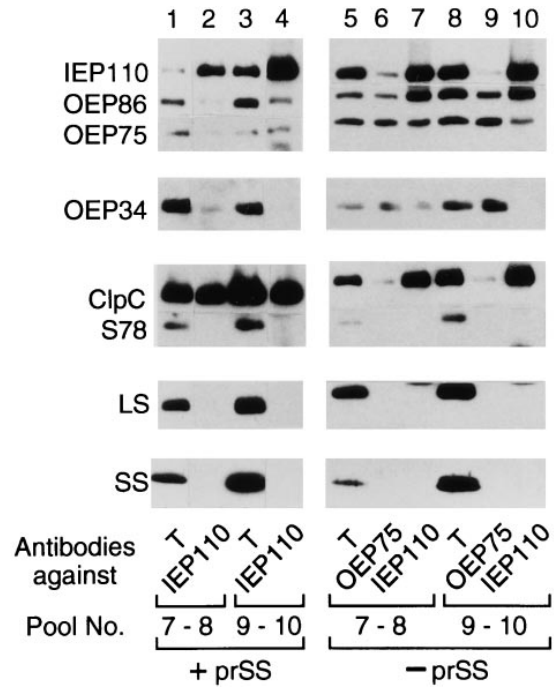
Figure 7. Analysis of complexes purified by immunoprecipitation. Cross-linked complexes generated with DSP (lanes *1–4*) or DTSSP (lanes *5–8*) and partially purified by gel filtration on Sephacryl S-500 were further purified by immunoprecipitation with anti-OEP75 antibodies. The immunoprecipitates were treated with β-mercaptoethanol to cleave the cross-links. The resulting proteins were resolved by SDS-PAGE, followed by detection via immunoblotting probed with anti-IEP110, -OEP86, -OEP75, -OEP34, -ClpC, -S78, -LS, and -SS antibodies. Two consecutive pools from the Sephacryl S-500 column were combined and used for immunoprecipitation. The immunoprecipitates from combined pools 7 and 8 (*7–8*) are shown in lanes *1, 2, 5,* and *6*; the immunoprecipitates from combined pools 9 and 10 (*9–10*) are shown in lanes *3, 4, 7,* and *8.* The samples before and after immunoprecipitation are shown (*T* and *IP*). 20% of the samples subjected to immunoprecipitation were loaded on *T* lanes.

Figure 8. Comparison of components in the complexes generated in the presence of prSS or in the absence of prSS. Cross-linked complexes were generated with DSP in the presence of prSS (lanes *1–4*) or in the absence of prSS (lanes *5–10*). The complexes were partially purified by gel filtration on Sephacryl S-500 and purified by immunoprecipitation with antibodies against OEP75 (lanes *6* and *9*) or IEP110 (lanes *2, 4, 7,* and *10*). The immunoprecipitates were treated with β-mercaptoethanol and analyzed as described in Fig. 7. Two consecutive pools from the Sephacryl S-500 column were combined and used for immunoprecipitation. The immunoprecipitates from combined pools 7 and 8 (*7–8*) are shown in lanes *1, 2, 5, 6,* and *7;* the immunoprecipitates from combined pools 9 and 10 (*9–10*) are shown in lanes *3, 4, 8, 9,* and *10.* Samples before immunoprecipitation are shown (*T*). The volume used was 20% of that subjected to immunoprecipitation.

Analysis of Components Present in Complexes

Further purification of gel-filtered complexes was accomplished by immunoprecipitation with antibodies against OEP75 and IEP110. These two antisera were chosen to investigate connections to outer and inner membrane complexes, respectively. The immunoprecipitated complexes were then cleaved by treatment with β-mercaptoethanol, and the polypeptides present in the complexes were analyzed by SDS-PAGE and immunoblotting. Two pairs of consecutive pools (7 and 8, 9 and 10) were combined and analyzed in this way (Figs. 7 and 8).

When complexes generated with DSP in the presence of prSS were immunoprecipitated with antibodies against OEP75, cleaved, and analyzed by immunoblotting, IEP110, OEP86, OEP75, OEP34, and ClpC were detected (Fig. 7, lanes *2* and *4*). When the ratios of each component were compared in the complexes immunoprecipitated by antibodies against OEP75 (Fig. 7, lanes *2* and *4*) versus the samples before immunoprecipitation (Fig. 7, lanes *1* and *3*), lesser amounts of IEP110 and ClpC were observed in the

immunoprecipitates. From this observation, along with the earlier results of immunoprecipitation (Figs. 4 and 5), we concluded that not all the complexes contained IEP110 and ClpC. The simplest explanation is that only a portion of the complexes containing OEP75 are connected to complexes containing IEP110 and ClpC. The remainder of IEP110 and ClpC are most likely present in inner membrane complexes that are not connected to outer membrane complexes. In support of this point, significant quantities of ClpC were immunoprecipitated with antibodies against IEP110 (Fig. 8, lanes *2* and *4*). It should be noted that complexes of inner membrane proteins may not be radiolabeled by association with prSS, thus we have no independent assessment of where these complexes eluted from the column.

S78 was not detected in the immunoprecipitates produced with either antibodies against OEP75 (Fig. 7, lanes *2* and *4*) or with antibodies against IEP110 (Fig. 8, lanes *2* and *4*), even though S78 was present in the samples before immunoprecipitation (Figs. 7 and 8, lanes *1* and *3*). From this result alone, we conclude that S78 is not part of these translocation complexes. This conclusion is inconsistent with

results from the immunoprecipitation experiments where S78 was found to be present in the largest complex (Fig. 5, lane 2). The resolution of this apparent inconsistency may be that S78 is a very minor component of a complex that is itself a minor proportion of the total population of complexes. Thus, S78 may be such a minor component that it could not be detected in the immunoblotting assay.

Recently, Kessler and Blobel (1996) reported an association between IEP110 and the stromal chaperonin, hsp60. Consequently, we investigated whether hsp60 was present in the complexes generated with DSP. Hsp60 could not be detected either in the samples before immunoprecipitation or in the samples immunoprecipitated with antibodies against OEP75 and IEP110 (data not shown). However, hsp60 could easily be detected in the column fractions containing the majority of the protein, e.g., pools 11 and 12 (data not shown). Thus, we conclude that hsp60 is not present in the complexes generated by DSP. This observation is not necessarily inconsistent with the observations of Kessler and Blobel (1996); it simply means that hsp60 was not cross-linked into complexes observed here. Any associations observed by Kessler and Blobel would have been disrupted by the strong detergents used to solubilize the cross-linked complexes.

LS and SS, which were both present in the total samples (Figs. 7 and 8, lanes 1 and 3), were absent from the immunoprecipitates (Figs. 7 and 8, lanes 2 and 4), confirming the specificity of the immunoprecipitation. The presence of Rubisco in the total samples was not surprising. Rubisco is a common contaminant of chloroplastic membranes. The membrane-permeable cross-linker probably adds to the proportion of Rubisco migrating with the membrane fractions. However, the fact that Rubisco was not present in the complexes purified by immunoprecipitation is important, leading us to conclude that Rubisco had not been cross-linked into translocation complexes. Because Rubisco is the most abundant protein in chloroplasts, this provides evidence that the translocation complexes do not contain extraneous proteins. It is also interesting to note that prSS was not detected in the immunoblots, although radioactivity was observed in the immunoprecipitates (data not shown). From this we conclude that prSS was not present in equimolar quantities in the complexes. One possible explanation is that some of the complexes exist in the absence of precursor proteins.

To investigate this possibility, complexes generated by cross-linker in the absence of precursor proteins were prepared and analyzed as described above. The elution patterns from the Sephacryl S-500 gel filtration column, as monitored by A_{280} and by SDS-PAGE analysis and Coomassie staining of each pool, were similar, regardless of whether the complexes were generated in the presence or the absence of precursor proteins (data not shown). The same pools (i.e., 7 and 8, 9 and 10) from the Sephacryl S-500 gel filtration column were analyzed directly (Fig. 8, lanes 5 and 8) or were subjected to immunoprecipitation with antibodies against OEP75 (Fig. 8, lanes 6 and 9) or antibodies against IEP110 (Fig. 8, lanes 7 and 10) before analysis. Similar results were obtained whether cross-linked complexes were generated in the presence of precursor proteins or in the absence of precursor proteins. For example, compare lanes 5 and 6 from Fig. 8 with lanes 1 and 2 from

Fig. 7; or in Fig. 8, compare lanes 3 and 4 with lanes 8 and 10. In making these comparisons, it is important to notice that comparisons of band intensities within a single lane are not a valid indication of the relative quantities of the various proteins. Each protein is being detected by a different antiserum, each with a different sensitivity and limit of detection. On the other hand, comparisons of band intensities for a single protein between lanes should provide a valid indication of relative quantities of that protein in the different samples. For example, in Fig. 8, lane 6 contains much less ClpC than lane 5, whereas lane 7 contains more ClpC than lane 5. From these observations we conclude that ClpC is a relatively minor component in complexes immunoprecipitated with antibodies against OEP75 but is an abundant component of complexes immunoprecipitated with antibodies against IEP110. Finally, it is important to note that variability is an inherent part of complex experiments, such as those shown in Figs. 7 and 8. For example, lane 1 in Fig. 8 is a duplicate of lane 1 in Fig. 7. Despite some minor differences, these replicates produce essentially the same results. All the experiments reported in Figs. 7 and 8 have been repeated between three and five times with essentially the same results, although some quantitative variations have been observed.

Despite the limitations described above, some important conclusions can be drawn from the experiments presented in Figs. 7 and 8. One important observation is that complexes immunoprecipitated with antibodies against OEP75, an outer membrane protein, contain at least low levels of IEP110, an inner membrane protein, and ClpC, a stromal protein (see lanes 6 and 9 in Fig. 8). Additionally, complexes immunoprecipitated with antibodies against IEP110, an inner membrane protein, contain OEP86, OEP75, and small amounts of OEP34 (see lanes 7 and 10 in Fig. 8). From these results, we concluded that some portion of the complexes present in each envelope membrane are in sufficiently close proximity that they can be cross-linked to each other. This indicates that some contact sites are present in the absence of precursor proteins.

It is also interesting to note that lesser amounts of OEP34 were recovered in the immunoprecipitates of pools 9 and 10 with antibodies against IEP110 (Fig. 8, lanes 4 and 10), whether the samples included precursors or not. Taking into consideration with the proposed function of OEP34 (Kessler et al., 1994; Seedorf et al., 1995), one possible explanation is that complexes containing OEP86, OEP75, and IEP110, but not OEP34, are formed in the absence of precursor proteins.

For complexes generated with DTSSP, we detected OEP86, OEP75, OEP34, and lesser amounts of IEP110 in samples immunoprecipitated with antibodies against OEP75 (Fig. 7, lanes 6 and 8). ClpC was not present in the precipitates, although much less ClpC was present in the total samples (Fig. 7, compare lanes 1 and 3 with 5 and 7). SS and LS were not observed in the pools (Fig. 7, lanes 5 and 7). The absence of Rubisco in the total samples suggests that if any DTSSP is gaining access to the stromal space, the quantities must be very limited. The lack of ClpC and the small quantities of IEP110 in complexes generated with DTSSP and immunoprecipitated with antibodies against OEP75 support the conclusion that this cross-linker does not effectively connect the outer envelope membrane com-

plexes to the inner envelope membrane complexes. The results presented in Figs. 7 and 8 are consistent with the earlier results of immunoprecipitation presented in Figs. 4 and 5 and provide further evidence regarding the composition of the translocation complexes.

Discussion

Several cross-linking strategies have been used to identify and characterize chloroplastic translocation components (Cornwell and Keegstra, 1987; Perry and Keegstra, 1994; Wu et al., 1994; Ma et al., 1996), each with their own advantages and disadvantages. The main advantage of label-transfer cross-linking reagents (Perry and Keegstra, 1994; Ma et al., 1996) is that individual translocation components in close proximity of a trapped precursor are specifically labeled. Sensitive detection of the putative translocation components is possible because of the radiolabel. However, a disadvantage is that the only components labeled are those in close physical proximity to the site on the trapped precursor that contains the cross-linking reagent. Thus, all translocation components may not be detected. In contrast, the strategy employed in this manuscript (Fig. 1) has the advantage that it is theoretically possible to cross-link all the components of the translocation apparatus into one large complex. Although it is possible to detect the complex using radiolabeled precursors (Figs. 2–6), detection of individual components is more difficult. We used specific antibodies against putative components, but this technique is limited to those components for which antibodies are available. Another possibility is to purify sufficient quantities of the complex so that individual components can be detected via protein staining after SDS-PAGE. This approach is currently not possible because the complexes are not sufficiently pure. The complexes recovered after gel filtration contain other chloroplastic proteins, and the complexes recovered after immunoprecipitation contain proteins from the antiserum (Akita, M., unpublished observations). However, we are continuing to pursue purification of the complexes because this strategy should reveal other translocation components.

Another potential disadvantage of the strategy presented in Fig. 1 is that abundant proteins not related to translocation might be cross-linked into the complex. Two pieces of evidence argue that this is not a significant problem in the studies reported here. First, if extraneous proteins were incorporated into these complexes, one would expect to observe a continuous distribution of radiolabeled complexes from small to large during SDS-PAGE (Fig. 2). Such distribution was not observed, with the possible exception of the large complexes that migrated at the top of the stacking gel. Second, neither LS nor SS was present in the purified cross-linked complexes (Figs. 4, 7, and 8). If extraneous proteins were cross-linked into translocation complexes, the most likely candidates would be those proteins present in highest quantities. The most abundant protein in chloroplasts is Rubisco, and its absence from the immunopurified complexes (Figs. 7 and 8) makes it unlikely that other extraneous proteins contribute significantly to the translocation complexes.

Wu et al. (1994) used a strategy nearly identical to that reported here, although with different precursors. A detailed comparison of our results with theirs is difficult for a number of reasons. First, they observed cross-linking to membrane components only when a chimeric precursor was used, but not when an authentic chloroplastic precursor was used (Wu et al., 1994). This contrasts with our studies, which were all performed with prSS. Second, they did not report some experimental details, such as the cross-linker concentrations they used. Our results indicate that the concentration of cross-linker dramatically influences the extent of cross-linking observed (Fig. 2). Finally, we used different antibodies than those used by Wu et al. (1994) to detect cross-linking to chloroplastic components. The only component examined by both groups is the large protein of the inner envelope membrane. If one assumes that our IEP110 is the same as their Cim97, then we obtained results similar to theirs, i.e., DSP caused effective cross-linking to this inner membrane protein, but DTSSP did not. Further studies will be needed to determine whether the other components examined by Wu et al. (1994) were present in the complexes we observed.

Although cross-linking generates a static "snapshot" of the interactions present at a single stage in transport, by comparing results with different cross-linkers and different cross-linking conditions, it is possible to develop a hypothesis regarding the sequence of events that occurs during transport. For example, radiolabeled precursors were found cross-linked into complexes of different sizes, which may represent different stages in transport. The smallest complex, near 110 kD, was formed by cross-linking precursors to OEP86 or OEP75. It is possible that precursors first associate with monomeric receptors before moving into larger complexes containing other components. However, it seems more likely that the 110-kD product is a consequence of incomplete cross-linking of a larger complex because at higher concentrations of cross-linker, only the larger complexes are formed (Fig. 2). Precursors were also found associated with complexes that contained only outer membrane components. Because some of these complexes were present even at the highest levels of cross-linker, especially when DSP and DTSSP were used, it seems likely that this complex represents a discrete stage in the transport process. This conclusion is further supported by the recent results of Ma et al. (1996), who used a different approach to arrive at the same conclusion. The evidence presented here and by Ma et al. (1996) indicates that OEP86, OEP75, and OEP34 are all part of this complex. It is possible that the complex contains other components. For example, the results of Wu et al. (1994) predict that Com 70 and Com 44 should also be part of this complex. We have not yet tested this prediction. Finally, precursors were found in larger complexes that contained proteins from the inner envelope membrane and stromal molecular chaperones, in addition to the components from the outer envelope membrane. The most likely explanation for these complexes is that they represent putative translocation contact sites. This interpretation receives support from other studies (Perry and Keegstra, 1994; Ma et al., 1996; Nielsen et al., 1997) that have come to similar conclusions using different approaches.

In addition to the complexes that contained radiolabeled precursors, it is likely that cross-linking, especially with DSP, generated complexes of translocation components

that did not contain precursors. Indeed, the observation that radiolabeled prSS was not present in stoichiometric amounts in the complexes that could be detected with antibodies (Figs. 7 and 8, and associated discussion) indicates that some of these complexes did not contain precursor. Furthermore, the observation that not all of the complexes containing IEP110 and ClpC could be immunoprecipitated with antibodies against OEP75 (Fig. 7) plus the observation that substantial amounts of ClpC were immunoprecipitated with antibodies against IEP110 (Fig. 8) indicated that some of the inner membrane components were present in complexes that were not associated with outer membrane components. Thus, we conclude that both the inner and outer envelope membranes contain complexes that exist independent of the formation of translocation contact sites. One important question that needs to be answered is whether precursors are needed to form translocation contact sites or whether some occur in the absence of precursors. The available evidence in mitochondrial protein transport favors a dynamic model where translocation contact sites form only in the presence of precursors (Horst et al., 1995). The data on complexes generated in the absence of precursors shown in Fig. 8 leads to the conclusion that some contact sites exist in the absence of precursors. Although this conclusion is different from that reached by Kessler and Blobel (1996), the evidence from the two studies are not mutually exclusive. Kessler and Blobel did not use cross-linkers to stabilize translocation complexes, thus the interactions that we observed here may have dissociated under their conditions. Alternatively, our results indicate that only a small proportion of the complexes present in the inner and outer membrane interact in the absence of precursor, and these few complexes may have escaped detection in their assays. It remains to be determined whether additional contact sites are formed when precursor proteins are present. It is also possible that some components join the complexes only when precursor proteins are present. Further studies will be needed to examine these and other important questions regarding the translocation process.

One final conclusion derived from our studies was that ClpC, a stromal member of the hsp100 family of molecular chaperones, was present in the translocation complexes. Additional evidence to support this conclusion was provided by Nielsen et al. (1997) using a very different strategy. They investigated translocation complexes formed in the absence of cross-linkers and solubilized with mild detergents. Consequently they were able to investigate the dynamics of ClpC interaction with precursors and found ClpC interacted in an ATP-dependent manner only with precursors engaged in the process of translocation. On the other hand, coimmunoprecipitation of ClpC with antibodies against IEP110 (Fig. 8) indicates the possible interaction between IEP110 and ClpC in chloroplasts, similar to the interaction between Tim44 and matrix hsp70 in mitochondria (Kronidou et al., 1994; Rassow et al., 1994; Schneider et al., 1994). Thus, it is tempting to speculate that during protein transport into chloroplasts, ClpC fills the role played by hsp70 during protein transport into mitochondria. Precedence for such substitution has been demonstrated in *E. coli*, where ClpA can substitute for DnaK in certain functions (Wickner et al., 1994). Consistent with this hy-

pothesis, very little S78, the stromal hsp70, was found associated with the translocation complexes. Further work will be needed to evaluate this hypothesis.

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