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Stable association of chloroplastic precursors with protein translocation complexes that contain proteins from both envelope membranes and a stromal Hsp100 molecular chaperone

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Cytoplasmically synthesized precursors interact with translocation components in both the outer and inner envelope membranes during transport into chloroplasts. Using co-immunoprecipitation techniques, with antibodies specific to known translocation components, we identified stable interactions between precursor proteins and their associated membrane translocation components in detergent-solubilized chloroplastic membrane fractions. Antibodies specific to the outer envelope translocation components OEP75 and OEP34, the inner envelope translocation component IEP110 and the stromal Hsp100, ClpC, specifically co-immunoprecipitated precursor proteins under limiting ATP conditions, a stage we have called docking. A portion of these same translocation components was co-immunoprecipitated as a complex, and could also be detected by co-sedimentation through a sucrose density gradient. ClpC was observed only in complexes with those precursors utilizing the general import apparatus, and its interaction with precursor-containing translocation complexes was destabilized by ATP. Finally, ClpC was co-immunoprecipitated with a portion of the translocation components of both outer and inner envelope membranes, even in the absence of added precursors. We discuss possible roles for stromal Hsp100 in protein import and mechanisms of precursor binding in chloroplasts.

Keywords: chloroplasts/envelope membranes/hsp100/precursor proteins/protein import

Introduction

Most chloroplastic proteins are encoded by nuclear genes and are translated on cytoplasmic ribosomes. To reach their correct position, these proteins must be transported across the double membrane system surrounding chloroplasts (Chua and Schmidt, 1978; Highfield and Ellis, 1978). These proteins are synthesized as precursors containing an N-terminal transit peptide responsible for their targeting (Schmidt *et al.*, 1979). Translocation of precursors into chloroplasts can be divided into two discernible steps based on their differing energy requirements. The first is association of a precursor with the chloroplastic translocation apparatus, and the second is transport across the membranes. Stable association of precursors with the translocation apparatus requires low levels of ATP or other NTPs (Olsen *et al.*, 1989), and results in the irreversible interaction of precursors with the chloroplastic envelopes. At this stage, the precursor remains susceptible to exogenous protease and the transit peptide is not cleaved by the stromal processing peptidase, indicating that the precursor has not completely traversed the envelope membranes (Cline *et al.*, 1985). Translocation of precursors across the envelope membranes can be initiated by raising stromal ATP concentrations (Pain and Blobel, 1987; Theg *et al.*, 1989). After a precursor has traversed the envelope membranes, the transit peptide is proteolytically removed by a stromal processing peptidase, producing a mature-sized protein in the stromal compartment (Reed *et al.*, 1990).

Translocation of precursors across the two chloroplastic envelope membranes is thought to occur simultaneously at 'contact sites' (Schnell and Blobel, 1993), a term given to regions where both envelope membranes are found in close physical proximity. By analogy with mitochondria, where precursors must also cross two membranes, precursors at contact sites are thought to interact with proteinaceous complexes from both the inner and outer membranes (for review, see Schatz and Dobberstein, 1996). In mitochondria, translocation complexes from the outer and inner membranes can act independently from one another, forming contact sites only when precursors associate with both complexes simultaneously (Segui-Real *et al.*, 1993; Horst *et al.*, 1995). Whether simultaneous engagement is required in chloroplasts is presently unknown.

Recent work on the chloroplastic protein import apparatus has resulted in the identification of several components of the envelope-based translocation complex (for reviews, see Gray and Row, 1995; Schnell, 1995). Several of these translocation complex members including the outer envelope membrane proteins OEP86, OEP75 and OEP34, and the inner envelope-membrane protein IEP110, have been identified, and their corresponding cDNAs have been cloned (Hirsch *et al.*, 1994; Schnell *et al.*, 1994; Seedorf *et al.*, 1995; Tranel *et al.*, 1995; Lübeck *et al.*, 1996). All of these proteins are integral membrane proteins, and only OEP86 and OEP34 show any sequence homology with other proteins that could provide insight into their functions. Both OEP86 and OEP34 contain GTP-binding motifs and can bind GTP (Hirsch *et al.*, 1994; Kessler *et al.*, 1994; Seedorf *et al.*, 1995). The exact functions of these proteins have yet to be determined but, based on biochemical and structural features, OEP86 has been proposed to be the transit peptide receptor protein, and OEP75 may represent a translocation pore (Perry and Keegstra, 1994).

Chloroplasts also contain soluble molecular chaperones that may play a role in protein import. A cDNA encoding

the major stromal Hsp70 (S78) has been isolated and shows significant sequence similarity with both bacterial and mitochondrial Hsp70 homologs (Marshall and Keegstra, 1992). Also, a cDNA clone encoding a stromal Hsp100 (ClpC) has been isolated and shows high sequence similarity with prokaryotic Hsp100s (Moore and Keegstra, 1993). Given the central role chaperones play in protein translocation in other systems (for review, see Schatz and Dobberstein, 1996), we sought to evaluate the hypothesis that stromal chaperones were involved in protein transport. We identified interactions of both stromal ClpC and S78 with translocation complexes. However, only complexes with ClpC were effectively solubilized in non-ionic detergent. This complex formed under binding and import conditions, and was influenced by the presence of ATP. The implications of these results for the formation of translocation complexes during binding is considered.

Results

Translocation components of the outer membrane, inner membrane and stroma form a stable complex with the precursor under binding conditions

In vitro import of precursors into intact chloroplasts can be halted at the envelope membranes in the presence of low ATP concentrations (Olsen *et al.*, 1989). These bound precursors form a stable interaction with the chloroplastic protein transport machinery, but can be fully imported when internal ATP concentrations are increased to adequate levels (Cline *et al.*, 1985; Olsen and Keegstra, 1992). We sought to determine whether precursor proteins trapped at this early stage of translocation were sufficiently stably associated with translocation components to survive detergent solubilization and analysis. Three different methods of analysis were used to detect complexes. The first was co-immunoprecipitation of radiolabeled precursors with antibodies directed against individual translocation components. To accomplish this, radiolabeled precursor to the small subunit (prSS) of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) was allowed to interact with isolated, intact chloroplasts in the presence of 100 μM ATP. After re-isolation, intact chloroplasts were lysed hypotonically and separated into membrane and supernatant fractions by centrifugation. Chloroplastic membranes were solubilized with buffer containing decylmaltoside and subjected to centrifugation to remove aggregates and incompletely solubilized membranes. Using decylmaltoside, ~80–90% of precursors and translocation components remained in the supernatant after centrifugation (data not shown). The solubilized membrane proteins were subjected to immunoprecipitation using antibodies to specific components of the chloroplastic protein translocation machinery. Sufficient antiserum was added in each case to ensure that >80% of each translocation component was immunoprecipitated (data not shown). Immunoprecipitates were analyzed by SDS–PAGE and fluorography to detect co-immunoprecipitation of radiolabeled prSS (Figure 1). An aliquot of the solubilized membrane fraction was analyzed by SDS–PAGE prior to immunoprecipitation to assess the amount of prSS that was bound to chloroplasts (Figure 1, lane 1). Antibodies specific for OEP75 and OEP34, two outer membrane translocation components, were capable of co-immunoprecipitating prSS (Figure 1, lanes 3 and 4), while pre-immune sera to OEP75 (Figure 1, lane 2) and OEP34 (data not shown) were not. In addition, antibodies specific to IEP110, an inner membrane translocation component, were also capable of co-immunoprecipitating prSS (Figure 1, lane 5), whereas the corresponding pre-immune serum was not (data not shown). As the amount of protein present in the total solubilized membrane fraction is equivalent to one-fifth of that added to each immunoprecipitation, we estimated that ~10% of the prSS added to each immunoprecipitation was associated with translocation complexes (compare lane 1 with lanes 3–5). These co-immunoprecipitation efficiencies are comparable with those observed with mitochondrial transport complexes (Manning-Krieg *et al.*, 1991; Ungermann *et al.*, 1994).

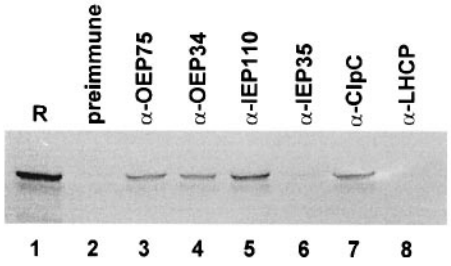

Fig. 1. Association of prSS with known translocation components. Gel-filtered, ^{35}S-labeled, prSS was incubated with isolated chloroplasts in 100 μM ATP. After incubation for 10 min at room temperature, intact chloroplasts were repurified, lysed hypotonically, and the chloroplastic membranes were resuspended in buffer containing decylmaltoside. Immunoprecipitation reactions were performed with anti-OEP75 (lane 3), anti-OEP34 (lane 4), anti-IEP110 (lane 5) or anti-ClpC (lane 7) antibodies, and OEP75 pre-immune control (lane 2). Ten per cent of each reaction was removed before immunoprecipitation (R, a representative sample is shown in lane 1); the remaining 90% was split into equal portions to which immune (I lanes) or pre-immune sera (P lanes) to OEP75, OEP34, IEP110 or ClpC were added. Only a representative pre-immune control for OEP75 is shown (lane 2); other pre-immune controls were similar. Instead of a pre-immune control, an anti-IEP35 (lane 6) immunoprecipitation was performed on the other half of the anti-LHCP (lane 8) immunoprecipitation. Samples were analyzed by SDS–PAGE and fluorography.

To evaluate the possibility that co-immunoprecipitation of prSS was due to non-specific interaction of precursors with membrane proteins, immunoprecipitation was performed using antibodies raised against two membrane proteins that are not part of the translocation apparatus. Antibodies raised against either the inner envelope membrane protein, IEP35 (Schnell *et al.*, 1994), or the thylakoid membrane light-harvesting complex protein (LHCP) family (Payan and Cline, 1991) were incapable of co-immunoprecipitating prSS (Figure 1, lanes 6 and 8), demonstrating the specificity of the co-immunoprecipitation procedure.

Because soluble molecular chaperones have been shown in other systems to interact with translocation complexes (Schatz and Dobberstein, 1996), we investigated whether any chloroplastic chaperones were present in this complex. Antibodies to ClpC, a chloroplastic molecular chaperone of the Hsp100 family, were capable of co-immunoprecipitating prSS (Figure 1, lane 7), but no association was detected with pre-immune serum (data not shown). S78 could also be detected in association with translocation complexes, but this interaction displayed characteristics

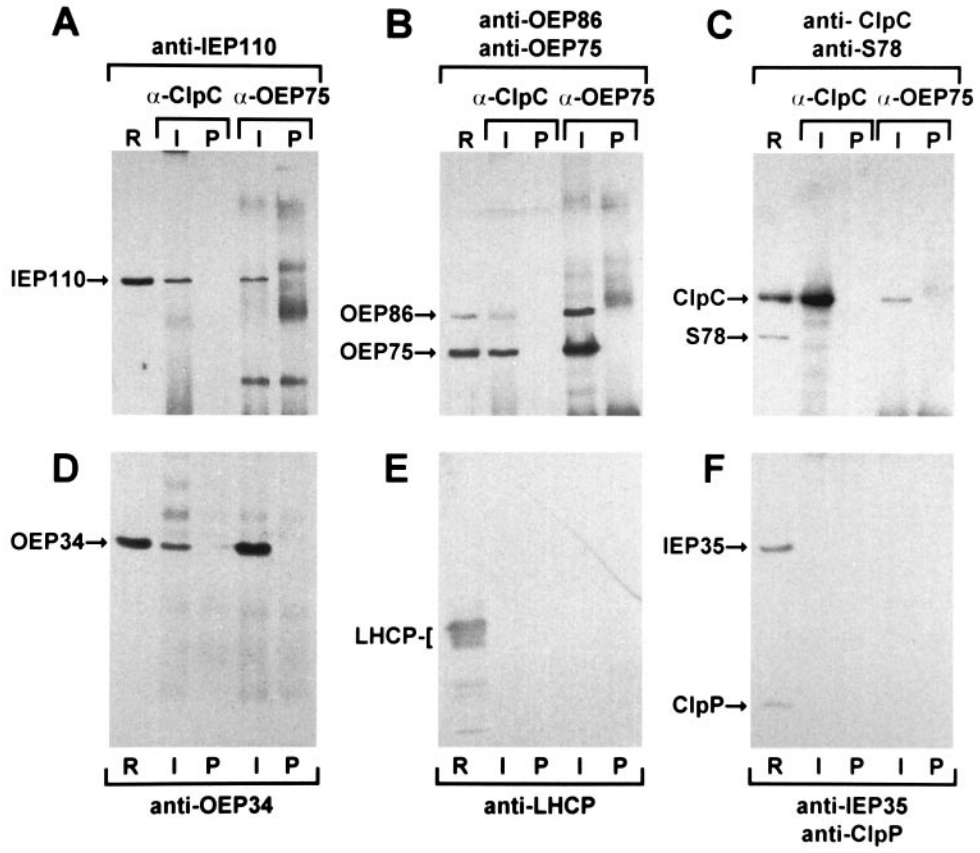

Fig. 2. Complexes immunoprecipitated by anti-OEP75 and anti-ClpC contain other translocation components. Radiolabeled prSS was incubated with isolated chloroplasts in 100 μM ATP. After a 10 min incubation, intact chloroplasts were repurified, lysed hypotonically, and isolated chloroplastic membranes were solubilized in buffer containing decylmaltoside. Immunoprecipitation reactions were performed with anti-OEP75 or anti-ClpC antibodies, or their corresponding pre-immune controls. Ten per cent of each reaction was removed for direct analysis on SDS–PAGE (R lanes); the remaining 90% was split into two equal fractions and immunoprecipitated with either anti-OEP75 serum (I lanes) or the corresponding pre-immune control (P lanes). Similar reactions were analyzed with anti-ClpC antibodies. Three replicates of the immunoprecipitations were analyzed by SDS–PAGE and transferred to Immobilon-P membrane. After immunoblotting, each membrane was divided into two parts, above (A–C) and below (D–F) the position of IgG, and probed with antibodies against IEP110 (**A**), OEP75 and OEP86 (**B**), ClpC and S78 (**C**), OEP34 (**D**), LHCP (**E**) and IEP35 and ClpP (**F**).

that required further investigation (see below). Collectively, the results described above suggested that prSS forms a stable association with known translocation components from both the outer envelope membrane and the inner envelope membrane, as well as a stromally localized chaperone.

The most likely explanation for the results described above was that all of these translocation components were associated with one another in a large complex. To evaluate this prediction, the solubilized complex was analyzed via a second strategy. The putative complexes were first immunoprecipitated with anti-OEP75 or anti-ClpC antibodies. Again, sufficient antibodies were added to ensure that >80% of these components were immunoprecipitated (data not shown). The composition of the immunoprecipitates was then analyzed by SDS–PAGE and immunoblotting (Figure 2). Antibodies against these two proteins were chosen because, based on the known location of OEP75 and ClpC within chloroplasts, they should be situated at opposite sides of a putative translocation complex. To demonstrate the relative amounts of the proteins present before immunoprecipitation, a sample of the solubilized membranes from the chloroplast-binding reaction was analyzed (Figure 2, R lanes). The complexes associated with both OEP75 and ClpC contained IEP110 (Figure 2A, I lanes), OEP34 (Figure 2D, I lanes) and OEP86 (Figure 2B, I lanes). In addition, complexes associated with ClpC contained OEP75 (Figure 2B, I lanes), and OEP75 was found in complexes containing ClpC (Figure 2C, I lanes). By comparing the amounts of the translocation components co-immunoprecipitated with amounts present in the total soluble membrane fractions, we estimated that between 10 and 20% of most translocation components remained associated with ClpC, or OEP75 after detergent solubilization and immunoprecipitation. Lower levels of OEP86 were associated with immunoprecipitated ClpC complexes, and both OEP86 and OEP34 were co-immunoprecipitated with OEP75 at higher levels.

These immunoprecipitations were specific, as the samples immunoprecipitated with the corresponding pre-immune sera did not contain any of these proteins (Figure 2, P lanes). No association of LHCP or IEP35 was found in immunoprecipitates of OEP75 or ClpC (Figure 2E and F, respectively). Several proteins of differing molecular weights were detected by the LHCP antiserum, reflecting the presence of members of an antigenically related family of proteins (Payan and Cline, 1991).

ClpC is only one class of stromal chaperones identified in chloroplasts (Gething and Sambrook, 1992; Moore and Keegstra, 1993; Shanklin *et al.*, 1995; Marshall *et al.*, 1996). Thus, we sought to determine whether molecular chaperones of the Hsp70 family were associated with the translocation complex, because they have been shown to interact with translocating precursors in other protein transport systems (Kang *et al.*, 1990; Scherer *et al.*, 1990; Vogel *et al.*, 1990). Antibodies raised against the stromal Hsp70, S78, were used to probe the immunoblot of the immunoprecipitated complexes. The presence of S78 in the solubilized membrane fraction demonstrated that some stromal S78 was present in the isolated membranes (Figure 2C, R lane). However, no association of S78 with the complexes immunoprecipitated with anti-OEP75 or anti-ClpC antibodies could be detected (Figure 2C, I lanes). We concluded that S78 was not stably associated with the solubilized translocation complex in these immunoprecipitations.

In prokaryotes, the Hsp100 chaperone family can function as subunits of the Ti protease (for review, see Squires and Squires, 1992). This protease is active as a hetero-oligomer containing the Hsp100 homolog and a separate subunit, ClpP, which contains protease activity (Hwang *et al.*, 1988; Maurizi *et al.*, 1990). Because both of these proteins have homologs in chloroplasts (Shanklin *et al.*, 1995), it was possible that the association between ClpC and translocation complex members reflected association with a protease, and did not indicate that ClpC itself was a translocation component. To evaluate this possibility, complexes immunoprecipitated by anti-OEP75 and anti-ClpC antibodies were probed with antibodies to stromal ClpP (Figure 2F). A reactive band at the correct molecular weight (Figure 2F, R lane) demonstrated the presence of ClpP in the membranes, presumably from stromal contamination. No significant association of this protein with the ClpC immunoprecipitate was detected (Figure 2F, I lanes) even when longer exposures were examined (data not shown). We concluded that the majority of the ClpC associated with translocation components was not associated with stromal ClpP and, therefore, the association was not involved in proteolysis of these complexes.

To obtain further evidence that solubilized translocation components were present in a complex, a third method of analysis was used. Solubilized chloroplastic envelope membranes containing translocation components and prSS were layered over a sucrose density gradient and fractionated to enable study of their sedimentation patterns (Figure 3). Radiolabeled prSS, analyzed by scintillation counting, appeared in two peaks, with the majority (~80% of total) found near the top of the gradient in fractions 1–9, and a smaller peak (~20% of total) migrating further into the gradient at fraction 23. Because prSS is rapidly processed and assembled into Rubisco holoenzyme after import into isolated chloroplasts (Archer and Keegstra, 1993), it was necessary to determine whether both peaks contained full-length precursor. This was measured by SDS–PAGE followed by fluorography, and all radioactivity was found in prSS (Figure 3B). Additionally, silver staining indicated that some Rubisco holoenzyme and Cpn60/10 could be detected in the gradient fractions, presumably due to stromal contamination of the isolated membranes, but these complexes sedimented primarily in fractions 11 and 15, respectively (data not shown). To determine the sedimentation patterns of the translocation components, OEP86, OEP75, OEP34 and IEP110, and of the chaperone ClpC, the gradient fractions were analyzed by SDS–PAGE, followed by immunoblotting with specific antibodies (Figure 3C). These components sedimented in two distinct peaks, similar to the sedimentation pattern of prSS (compare Figure 3B and C). However, two control proteins, IEP35 and LHCP, were observed to have different sedimentation patterns (Figure 3C). From these results, we conclude that a significant portion of the radiolabeled precursor had been incorporated into a large complex containing components of the translocation apparatus from the outer and inner membranes as well as the stromal Hsp100 chaperone ClpC. This complex sedimented far into the gradient at fraction 23, with significant amounts of the various translocation components being present in this fraction. Interestingly, OEP86 and OEP34 migrated no further into the gradient than fraction 23, while OEP75, IEP110 and ClpC were also found in fraction 25. Whether these differences represented different complexes was

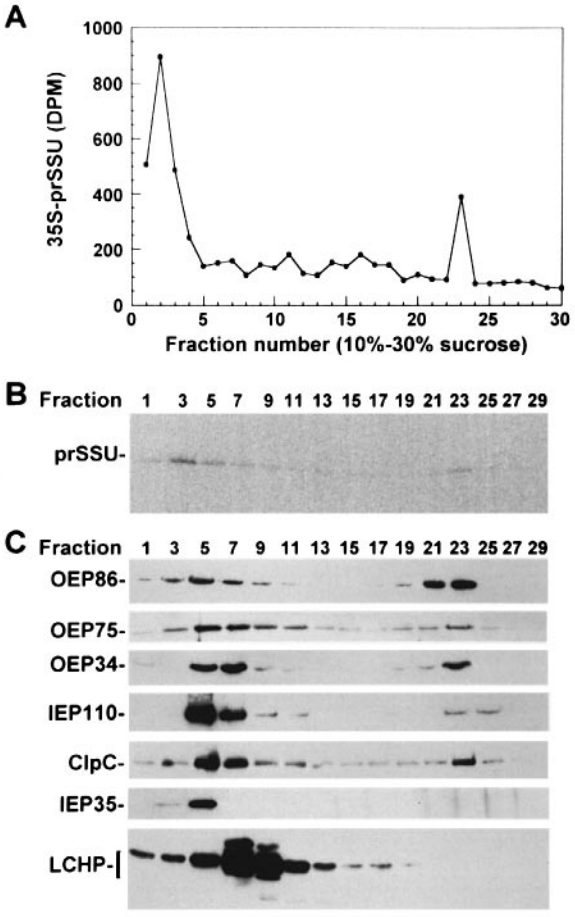

Fig. 3. Translocation components and prSS co-sediment as a complex. Radiolabeled prSS was incubated with isolated chloroplasts in 100 μM ATP. After incubating for 10 min, intact chloroplasts were repurified, lysed hypotonically, and isolated membranes were solubilized in buffer containing decylmaltoside. The solubilized membrane fraction was layered over a 10–30% linear sucrose density gradient and sedimented at 150 000 *g* for 18 h. Fractions were removed and the sedimentation patterns were analyzed by scintillation counting (**A**), SDS–PAGE and fluorography (**B**) and SDS–PAGE and transferring to Immobilon-P for immunoblotting with antibodies against the indicated proteins (**C**).

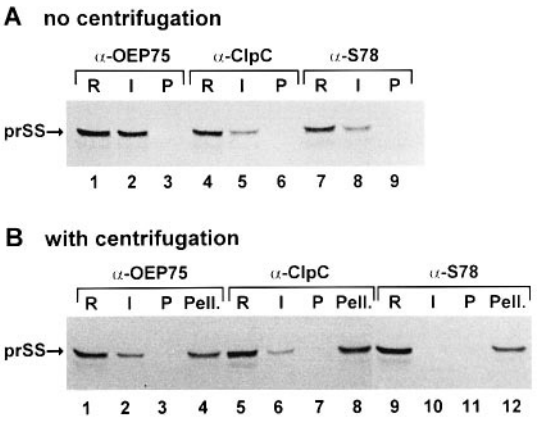

Fig. 4. Translocation complexes containing S78 are insoluble. Radiolabeled prSS was incubated with isolated chloroplasts in 100 μM ATP. After a 10 min incubation, intact chloroplasts were repurified and immediately solubilized in buffer containing decylmaltoside. (**A**) Immunoprecipitation reactions were performed directly after solubilization with anti-OEP75 (lane 2), anti-ClpC (lane 5) or anti-S78 (lane 8) antibodies, and their corresponding pre-immune controls (lanes 3, 6 and 9). Ten per cent of each reaction was removed for direct analysis by SDS–PAGE (R, lanes 1, 4 and 7); the remaining 90% was split into two equal fractions and immunoprecipitated with immune (I lanes) or their corresponding pre-immune sera (P lanes). (**B**) Solubilized chloroplasts were centrifuged at 150 000 *g* for 5 min before immunoprecipitation with anti-OEP75 (lane 2), anti-ClpC (lane 6) or anti-S78 (lane 10) antibodies, and their corresponding pre-immune controls (lanes 3, 7 and 11). Ten per cent of each reaction was removed for direct analysis by SDS–PAGE (R, lanes 1, 5 and 9); the remaining 90% was split into equal fractions and immunoprecipitated with immune (I lanes), or their corresponding pre-immune sera (P lanes). After centrifugation, the pellet was resuspended in SDS–PAGE buffer and analyzed by SDS–PAGE (Pell., lanes 4, 8 and 12). Samples were analyzed by SDS–PAGE and fluorography.

beyond the resolution of the sucrose gradient and could not be determined.

A significant portion of both the radiolabeled precursors and translocation components was observed at the top of the gradient. One possible explanation for the presence of translocation components near the top of the gradient is that they represent individual components that were not part of translocation complexes. Another, more likely, explanation is that some of the translocation complexes dissociated into individual components during experimental manipulation and could have been maintained as complexes if milder or more stabilizing conditions had been used. Further work will be needed to distinguish between these two possibilities.

ClpC and S78 both interact with translocation complexes, but only the association with ClpC is stable in solubilized complexes

ClpC was detected in a complex with other translocation components and prSS but, using similar conditions, association of the stromal Hsp70, S78, could not be detected (Figure 2). Because Hsp70 homologs have been observed to be important members of other protein translocation systems (Kang *et al.*, 1990; Scherer *et al.*, 1990; Vogel *et al.*, 1990), their possible involvement was examined in more detail. Our previous experiments had been performed with complexes prepared from isolated membranes. Because lysis and fractionation of chloroplasts during membrane isolation might disrupt interactions between chaperones and translocation complexes, whole chloroplasts were solubilized and the resulting complexes examined for the presence of S78 (Figure 4). The absence of processed mature-sized Rubisco small subunit (mSS) in aliquots of the solubilized chloroplasts indicated that import had not occurred (Figure 4A, lanes 1, 4 and 7). Antibodies to OEP75, ClpC and S78 were able to co-immunoprecipitate prSS from the solubilized chloroplasts (Figure 4A, lanes 2, 5 and 8, respectively). No prSS could be detected with the corresponding pre-immune controls (Figure 4A, lanes 3, 6 and 9), demonstrating the specificity of the immunoprecipitation reactions. Centrifugation was not performed prior to these immunoprecipitations; thus, it was possible that S78 had not been detected in earlier studies (Figure 2) either because its association with translocation complexes was disrupted during chloroplast lysis and fractionation or because S78 was in a complex which was pelleted during the centrifugation step. To examine the latter possibility, detergent-solubilized chloroplasts were subjected to centrifugation before immunoprecipitation was performed on the supernatant. In the soluble complexes, prSS could still be co-immunoprecipitated with anti-OEP75 and anti-ClpC antibodies, but not with anti-S78 antibodies (Figure 4B, compare lanes 2 and 6, with lane 10). When the pellet fraction was analyzed, significant amounts of prSS were present (Figure 4B, lanes 4, 8 and 12). Immunoblotting with antibodies to OEP75, ClpC and S78 demonstrated that these proteins were present in the pellet, but other proteins not involved in protein translocation were also present (data not shown).

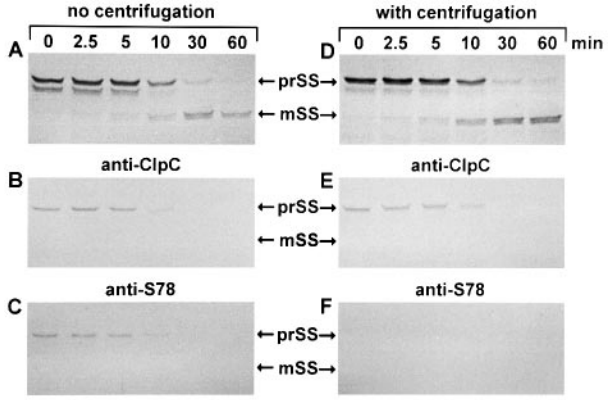

Fig. 5. Association of ClpC and S78 with prSS-containing translocation complexes during import. Radiolabeled prSS was incubated with isolated chloroplasts in 100 μM ATP. After a 10 min incubation, intact chloroplasts were repurified, import was initiated by addition of 4 mM ATP at time = 0, and aliquots were removed at the given timepoints. Intact chloroplasts were again repurified, and then solubilized in buffer containing decylmaltoside. The solubilized chloroplasts from each timepoint were either immunoprecipitated directly (A–C), or subjected to centrifugation for 5 min at 150 000 *g* (D–F). Ten per cent of each timepoint was removed for direct analysis on SDS–PAGE (**A** and **D**). The remaining 90% of each timepoint was split into equal fractions and immunoprecipitated with either anti-ClpC (**B** and **E**) or anti-S78 (**C** and **F**) antibodies. Samples were analyzed by SDS–PAGE and fluorography.

The interaction of ClpC and S78 with translocation complexes was characterized further by investigating how the interactions changed during an import timecourse (Figure 5). First, radiolabeled precursors were allowed to bind to isolated chloroplasts in the presence of low levels

of ATP. After removal of unbound precursors, chloroplasts were resuspended in the presence of high levels of ATP, thereby allowing import to occur. Analysis of the import timecourses demonstrated that mSS accumulated at significant levels only after a 2.5 min lag, but accumulation then continued for 30 min (Figure 5A and D). Precursor, but not mSS, was detected in the anti-ClpC immunoprecipitated fractions whether or not a centrifugation step was performed prior to immunoprecipitation (Figure 5B and E). Complexes immunoprecipitated by anti-ClpC antibodies were present at high levels at time 0, and decreased as the import reaction progressed (Figure 5B and E). Precursors were detected in the anti-S78-immunoprecipitated fractions if centrifugation was not performed (Figure 5C) but, after centrifugation, precursors were not observed in the anti-S78 immunoprecipitates (Figure 5F). Interestingly, no mSS was found in association with the anti-ClpC- or anti-S78-precipitated fractions. Analysis of the immunoprecipitation supernatant fractions showed that the mSS was not degraded by proteolysis (data not shown). We concluded that both ClpC and S78 interacted with precursors that were productively bound because these complexes disappeared in later timepoints, when mSS appeared. Because complexes precipitated by anti-S78 antibodies sedimented by centrifugation, we concluded that they were either very large or represented an aggregation of translocation complexes or incompletely solubilized membrane fragments. At present we are unable to distinguish between these possibilities.

To evaluate alternative explanations for the presence of molecular chaperones in the solubilized complexes, additional control reactions were performed. First, radiolabeled prSS was not associated with complexes immunoprecipitated with anti-ClpC or anti-S78 in the absence of chloroplasts (data not shown). Therefore, molecular chaperones present in the wheat germ translation system were not responsible for the association of prSS with the anti-ClpC or anti-S78 immunoprecipitates. Second, when immunoprecipitation of prSS bound to chloroplasts was performed after the proteins were denatured by boiling in 2% SDS, no prSS could be found in complexes precipitated by anti-ClpC or anti-S78 antibodies (data not shown). This experiment demonstrated that the antisera did not cross-react with prSS, causing direct immunoprecipitation. Third, chloroplasts were solubilized in immunoprecipitation buffer before radiolabeled precursor was added. When comparable amounts of precursor were present, co-immunoprecipitation of prSS was not observed (data not shown). We concluded that formation of complexes required intact chloroplasts, and did not occur as a result of mixing of the stromal compartments with the exterior of the chloroplast during solubilization. Finally, if ClpC was associated specifically with translocation complexes, then only the ClpC associated with the envelope membranes should cause co-immunoprecipitation of precursors. To test this prediction, radiolabeled prSS was allowed to associate with isolated chloroplasts, which were then lysed and separated into chloroplastic membrane and supernatant fractions. Immunoprecipitation reactions were performed with anti-ClpC antibodies using both solubilized membranes and soluble fractions. Only the solubilized membrane fractions showed co-immunoprecipitation of prSS (data not shown). These results, combined with the observation that pre-solubilized chloroplasts did not support association between ClpC and prSS, further supported an interaction between ClpC and translocation complexes during binding that is relevant to protein import.

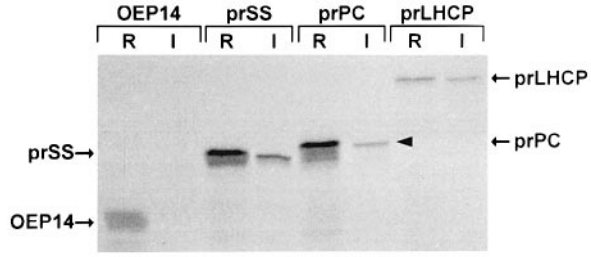

Fig. 6. ClpC interacts with translocation complexes formed by other precursors that use the general translocation apparatus. Radiolabeled OEP14, prSS, prPC and prLHCP were incubated with isolated chloroplasts in 100 μM ATP. After a 10 min incubation, intact chloroplasts were repurified and lysed hypotonically, and the isolated chloroplastic membranes were solubilized in buffer containing decylmaltoside. Twenty per cent of each reaction was removed for direct analysis by SDS–PAGE (R lanes); the remaining 80% was immunoprecipitated by anti-ClpC antibodies (I lanes). Samples were analyzed by SDS–PAGE and fluorography.

A translocation complex containing ClpC is formed with other precursors and is destabilized by ATP

If ClpC has a general role in protein import it should associate with translocation complexes formed during import of other precursors. To test this prediction, several precursors, targeted to different chloroplastic subcompartments, were allowed to associate with chloroplasts. Putative complexes were then analyzed by co-immunoprecipitation with anti-ClpC antibodies. In addition to prSS, we examined the precursor to plastocyanin (prPC), a thylakoid lumenal protein, and the precursor to LHCP (prLHCP), a thylakoid membrane protein. Association of the various precursors with chloroplasts during binding was analyzed (Figure 6, prSS, prPC, prLHCP, R lanes). All three precursors could be co-immunoprecipitated with anti-ClpC antibodies (Figure 6, prSS, prPC, prLHCP, I lanes). To demonstrate that the association of ClpC was specific to precursors associated with the chloroplastic protein import apparatus, radiolabeled OEP14 was allowed to associate with chloroplasts before solubilization and immunoprecipitation with anti-ClpC antibodies. This protein inserts into the outer membrane of chloroplasts, but does not have a cleavable N-terminal transit peptide and does not utilize the general import apparatus (Li *et al.*, 1991). This protein associated with chloroplasts in amounts comparable with the other precursors tested (Figure 6, compare OEP14 R lane with other R lanes), but no association with immunoprecipitated ClpC could be detected (Figure 6, compare OEP14 I lane with other I lanes). Thus we conclude that ClpC associated with several precursors that used the general import pathway, but not with OEP14, a protein that does not use this pathway.

The interaction of members of the Hsp100 chaperone family with substrate proteins has been shown to be regulated in an ATP-dependent manner (Wickner *et al.*, 1994; Wawryznow *et al.*, 1995). To analyze the effect of ATP on the interaction between ClpC and translocation complexes, chloroplasts were bound with precursor as described above, but were then lysed and chloroplastic

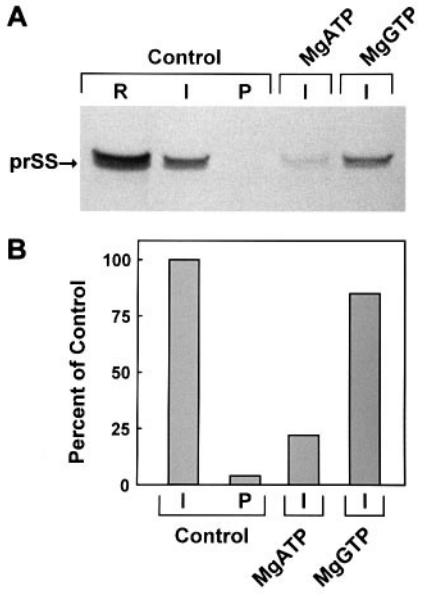

Fig. 7. ATP destabilizes the association of ClpC and translocation complexes. (**A**) Radiolabeled prSS was incubated with isolated chloroplasts in 100 μM ATP. After a 10 min incubation, intact chloroplasts were repurified, lysed hypotonically, and isolated membranes were solubilized in unsupplemented decylmaltoside buffer (Control), or decylmaltoside buffer supplemented with either 10 mM ATP (ATP) or 10 mM GTP (GTP). Ten per cent of each reaction was removed for direct analysis by SDS–PAGE (R lane); the remaining 90% was split into two equal fractions and immunoprecipitated by anti-ClpC antibodies (I lanes) or pre-immune serum (P lane). Representative reaction (R) and pre-immune (P) lanes are shown only for the control experiment. Samples were analyzed by SDS–PAGE and fluorography. (**B**) Quantitation of lanes from (A) was conducted by direct analysis on a Phosphorimager (Molecular Dynamics, Inc.).

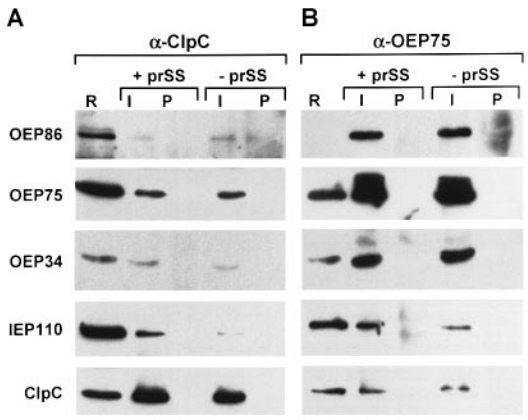

Fig. 8. Translocation complexes containing ClpC and OEP75 form in the absence of added precursor. Isolated chloroplasts were incubated in 100 μM ATP, either in the presence (+prSS) or absence (–prSS) of radiolabeled precursor. After a 10 min incubation, intact chloroplasts were repurified, lysed hypotonically, and isolated membranes were solubilized in decylmaltosode buffer. Ten per cent of each reaction was removed for direct analysis by SDS–PAGE (R lanes, –prSS not shown); the remaining 90% was split into two equal fractions and immunoprecipitated either with anti-ClpC (αClpC) or anti-OEP75 (αOEP75) antibodies (I lanes) and their corresponding pre-immune sera (P lanes). Samples were analyzed by SDS–PAGE and immunoblotting with antibodies to OEP86, OEP75, OEP34, IEP110 or ClpC. The experiment was repeated three times with similar results each time. All the panels shown were taken from a single set of anti-ClpC or anti-OEP75 immunoprecipitations and were developed for equivalent times except for the anti-ClpC-immunoprecipitated, anti-OEP86-probed panel which was from a separate experiment and was developed four times longer to visualize the OEP86 present in the total membrane fraction.

membranes were solubilized and immunoprecipitation reactions performed in the presence of Mg·ATP or Mg·GTP (Figure 7). The ClpC-containing complex was destabilized if immunoprecipitation was performed in the presence of Mg·ATP, but only slight destabilization was seen if Mg·GTP was included instead (Figure 7), indicating that this particular step was specific for ATP. The addition of Mg·ATP did not simply stimulate import in the lysed chloroplast membranes, as no mSS could be detected in the total protein control (data not shown).

A translocation complex can form in the absence of added precursors

OEP86, OEP75 and OEP34 have been shown previously to form a complex even in the absence of added precursors (Waegemann and Soll, 1991; Ma *et al.*, 1996). We have shown that in the presence of added precursors ClpC and IEP110 can also interact with these outer membrane translocation components (Figures 1–3). We therefore wanted to determine if ClpC and IEP110 could associate with outer membrane translocation components in the absence of added precursors. Chloroplasts were incubated with 100 μM ATP in the presence or absence of prSS. Intact chloroplasts were recovered, lysed and membranes isolated and solubilized as described above for the experiment shown in Figure 2. After a portion was removed for direct analysis on SDS–PAGE, the remaining samples were split into equal fractions and immunoprecipitated with anti-ClpC or anti-OEP75 antibodies and their respective pre-immune sera. Sufficient antiserum was added to ensure that at least 80% of the ClpC and OEP75 were immunoprecipitated (data not shown). The immunoprecipitates were analyzed by SDS–PAGE and immunoblotting (Figure 8). Complexes that co-immunoprecipitated with ClpC contained IEP110, OEP86, OEP75 and OEP34, regardless of whether precursor was present or not (Figure 8A, compare I lanes, + and –prSS). Conversely, complexes associated with OEP75 also contained OEP86, OEP34, IEP110 and ClpC whether precursor was present or not (Figure 8B, compare I lanes, + and –prSS). Interestingly, when immunoprecipitation was performed with anti-OEP75 antibodies, the levels of OEP34 and OEP86 present in the complexes were significantly higher than that observed in ClpC immunoprecipitates. This may indicate that the majority of OEP86, OEP34 and OEP75 can associate in a separate complex that does not contain inner membrane and stromal components, and that only a portion of these complexes associate with inner membrane and stromal components.

In the presence of precursors, ~10% of IEP110 and OEP75 proteins, and lower levels of OEP86 and OEP34, were found associated with ClpC-precipitated complexes, as measured by comparing with the amount of the proteins present in the total solubilized membrane fractions (Figure 8A, compare R and I lanes). However, it should be noted that only ~20% of the prSS associated with chloroplasts could be found in high molecular weight complexes after separation on a sucrose density gradient (Figure 3). The remaining 80% of the prSS was found in fractions consistent with partially or completely destabilized complexes,

and might explain the low levels of co-immunoprecipitation of complexes containing both outer and inner membrane components. While the proportion of OEP86, OEP34 and OEP75 associated with ClpC-precipitated complexes remained unchanged in the absence of added precursors, the amount of IEP110 present in ClpC-precipitated complexes was reduced. However, we are uncertain whether this is significant because the level of reduction varied in different experiments (data not shown), and the amounts of IEP110 associated with OEP75 remained about the same in the presence or absence of added precursors (Figure 8B).

Discussion

Recent identification of several members of the chloroplastic protein translocation machinery has allowed for further refinement of our understanding of the mechanism by which precursors are transported into chloroplasts. We have attempted to define the composition of complexes that form during translocation using co-immunoprecipitation techniques with antibodies specific to translocation components. We have observed that precursors could be found in stable association with translocation complexes after solubilization with a mild detergent, decylmaltoside. Characterization of these complexes has led to two conclusions: (i) that under limiting ATP conditions, precursors associated with translocation complexes containing components of the outer and inner envelope membranes; and (ii) that the chaperone ClpC, a stromal Hsp100 homolog, was associated with precursor-containing complexes under these limiting ATP conditions.

The data presented here suggest a new role for the stromal Hsp100 homolog ClpC, i.e. as a component of the translocation complex. Three experimental results provided evidence that the association of ClpC with the chloroplastic protein translocation apparatus was physiologically significant. First, the amount of precursor associated with translocation complexes containing ClpC decreased in a time-dependent manner during an import reaction (Figure 5). Second, multiple precursors could be co-immunoprecipitated with ClpC under binding conditions, indicating that ClpC interacted as a component of the general import apparatus and, through this interaction, associated with various precursors. This association with precursors during binding depended upon their utilization of the general import apparatus (Figure 6). Finally, the ability of ATP to destabilize the association of ClpC from precursor-containing complexes (Figure 7) is consistent with the previous observations that ClpC interacts with substrate proteins in an ATP-dependent manner (Wickner *et al.*, 1994; Wawryznow *et al.*, 1995). This observation could be interpreted in two ways. First, ClpC could be released from the complex, leaving precursor associated with other components. Second, precursor could be released from a complex that still contains ClpC. Because complexes containing ClpC can be detected in the absence of added precursors (Figure 8), we favor this second explanation. This implies that it is a precursor–ClpC interaction that stabilizes the complexes observed here. Further work is needed to confirm this conclusion and to explore the implications of this hypothesis for the mechanism of protein transport into chloroplasts.

Is the stromal Hsp70 homolog S78 also involved in protein import into chloroplasts? Precursor could be co-immunoprecipitated by anti-S78 antibodies under binding and import conditions (Figures 4 and 5). This co-immunoprecipitation was not simply due to an insoluble aggregation or association of S78 with residual membranes, as no precursor was detected in the pre-immune control. Additionally, precursor co-immunoprecipitated by anti-S78 antibodies appeared to be a true import intermediate (Figure 5), and was dependent on intact chloroplasts (data not shown). For these reasons, we cannot at this point eliminate the possibility of an interaction between S78 and translocating precursor. However, while ClpC associated with detergent-soluble translocation complexes, the complexes containing S78 and precursor were sedimented after a centrifugation step. The relevance of this S78–precursor interaction to protein import was called into question by the observation that non-translocation components were also found in this pellet. Additionally, S78 did not co-sediment with prSS and other translocation components on a sucrose density gradient (data not shown).

Previously, outer membrane hsp70 homologs have been identified as members of translocation complexes (Waegemann and Soll, 1991; Ko *et al.*, 1992; Schnell *et al.*, 1994). It was proposed that these proteins might play a role in insertion of precursors through the outer membrane translocation complex. Because the S78 antiserum was specific to the stromal Hsp70 and did not cross-react with the outer membrane Hsp70 (M.Akita, E.Nielsen and K.Keegstra, submitted), we were unable to confirm or refute these findings in this study.

Even though the role of stromal Hsp70 homologs in chloroplastic protein import remains equivocal, our proposal that the stromal Hsp100 homolog ClpC acts as a translocation component in a manner relevant to protein import is consistent with findings in other laboratories. Clp homologs were identified originally as members of the ATP-dependent Ti protease in *Escherichia coli* (Hwang *et al.*, 1988; Maurizi *et al.*, 1990). Recent experiments have demonstrated that these proteins can act as chaperones in the absence of the proteolytic subunit ClpP (Wickner *et al.*, 1994). Because no stromal ClpP was detected in our immunoprecipitations, we concluded that ClpC interacted with precursor as a chaperone and not as part of a protease (Figure 2). This raised the possibility that ClpC interacts with precursors in a manner analogous to that observed for Hsp70 homologs in the mitochondrial and endoplasmic reticulum (ER) import systems (for review, see Schatz and Dobberstein, 1996). In mitochondria, a matrix Hsp100 homolog can interact with translocation intermediates, though only under specific conditions which limited levels of functional Hsp70 (Schmitt *et al.*, 1995). Based on our results, interaction of precursors with Hsp100 homologs would appear to be the dominant association during chloroplastic protein import.

The ATP-dependent association of precursor with contact sites would allow for interaction of ClpC with the translocation apparatus in a membrane-associated complex. This interaction could involve direct association of ClpC with the bound precursor, or interaction of ClpC with a protein in the membrane translocation apparatus. Molecular chaperones have been observed to interact directly with translocating precursors when in association

with membrane translocation components in both the mitochondria and ER. In mitochondria, TIM44, an inner membrane translocation component, has been observed to interact with the matrix Hsp70 during import of precursors (Blom *et al.*, 1993; Kronidou *et al.*, 1994; Rassow *et al.*, 1994; Schneider *et al.*, 1994). In the ER, the lumenal Hsp70 has been found to form a complex with Sec63p, a membrane protein component of the translocation machinery (Sanders *et al.*, 1992). In both systems, these interactions can occur with or without associated precursor and depend on the ATP-bound state of the Hsp70 homolog (Brodsky and Schekman, 1995; von Ahsen *et al.*, 1995). We have also demonstrated an ATP sensitivity of the interaction between ClpC and translocation complexes that contain precursors (Figure 7). In addition, a fraction of ClpC interacts tightly with the chloroplastic envelopes in the absence of precursor, despite the fact that the majority of ClpC is a soluble protein (data not shown). Some of these membrane-bound ClpC proteins interacted with IEP110 and OEP75, and to a lesser degree with OEP86 and OEP34 (Figure 8). Perhaps an interaction similar to that observed for Hsp70 homologs in mitochondria and ER is occurring with the Hsp100 homolog ClpC in chloroplasts.

The observation that a portion of the protein translocation components of the outer and inner membranes associated in complexes even in the absence of added precursors (Figure 8) presents an intriguing difference from protein import into mitochondria, in which outer and inner membrane translocation complexes can act independently, and are only connected by translocating precursors (Segui-Real *et al.*, 1993; Horst *et al.*, 1994). Kessler and Blobel (1996) reported that in the absence of precursors IEP110 was not found in association with outer membrane translocation components either by detection with Coomassie Blue or immunoblotting techniques. We clearly observe IEP110 in association with OEP75-precipitated complexes (Figure 8). At present, the reasons for these discrepancies are not clear. Detergents and salt concentrations used in our experiments differed from those used by Kessler and Blobel. Additionally, we incubated chloroplasts in 100 μM ATP prior to lysis and solubilization, which may have affected the association of inner and outer membrane complexes.

On the basis of data presented here and previous work, we offer a revised hypothesis for the topology of the precursor during ATP-dependent association with chloroplasts (Figure 9). Earlier views depicted precursors as associated simply with outer membrane components in the presence of low ATP concentrations (for reviews, see Gray and Row, 1995; Schnell, 1995). The data presented here argue that these precursors also engage inner membrane and stromal components of the transport apparatus either directly (Figure 9A) or indirectly (Figure 9B). Although other models cannot be excluded, the two possibilities presented are simplest and are consistent with our current observations.

Previous models, showing bound precursors associated only with outer envelope membranes, were based on observations that, in limiting ATP conditions, precursors were not inserted sufficiently into the translocation machinery to be protected from exogenous protease, or to allow cleavage of the transit peptide by stromal processing

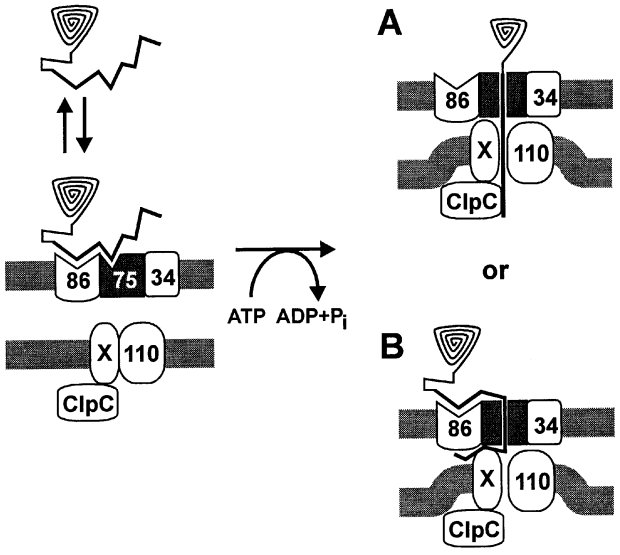

Fig. 9. A model for formation of translocation complexes during ATP-dependent docking to chloroplasts. In this scheme, the initial interaction of cytoplasmically synthesized precursors with receptor proteins occurs in the absence of added energy. At this stage, the association of precursors with receptor proteins remains reversible, and is not necessarily confined to contact sites. Upon the addition of ATP, the precursor transit peptide is inserted across both envelope membranes (**A**), or across the outer membrane only (**B**), forming a contact site, and allowing interaction of the transit peptide with inner envelope membrane and stromal translocation components.

peptidase (for reviews, see Archer and Keegstra, 1990; Soll and Alefsen, 1993; Tian *et al.*, 1995). This ATP-dependent stage of import, where precursors associate stably with chloroplastic membranes, has been termed binding. While binding traditionally has referred to a reversible process, the ATP-dependent association of precursors with chloroplastic membranes is not reversible, and these precursors are no longer in equilibrium with free precursor proteins (Olsen *et al.*, 1989; Schnell and Blobel, 1993). Indeed, these precursors are even partially resistant to extraction by high salt and carbonate (Waegemann and Soll, 1991). Additionally, these models do not explain why bound precursors associate with chloroplastic membranes in 'patches,' correlated with contact sites, nor do they explain why, upon lysis of intact chloroplasts and separation of chloroplastic membranes, bound precursors migrate with mixed membrane fractions containing contact sites rather than with purified outer membranes (Schnell and Blobel, 1993; Perry and Keegstra, 1994; J.Ostrom and K.Keegstra, unpublished results). In light of these observations, as well as the work presented here and by others (Wu *et al.*, 1994; M.Akita, E.Nielsen and K.Keegstra, submitted), we propose that a more appropriate term for this stage in chloroplastic protein translocation is 'docking.' We define docked precursors as having progressed to a discrete step following initial recognition that would occur during binding, but halted prior to full translocation of the precursor into the stroma. At this stage in import, docked precursor would associate at contact sites in an ATP-dependent fashion forming complexes containing translocation components of both outer and inner envelope membranes. The association of precursors with these complexes would be stable, as

opposed to the reversible association of precursors with receptor proteins that would occur during binding.

The hypothesis presented in Figure 9 shows the formation of a single translocation complex consisting of components of several compartments of the chloroplast. Using both co-immunoprecipitation (Figures 1 and 2) and differential centrifugation techniques (Figure 3), we detected translocation complexes containing precursors and translocation components of the outer envelope membrane, inner envelope membrane and stromal compartments. Similar complexes have been identified when cross-linked complexes containing docked precursors are analyzed (Wu *et al.*, 1994; M.Akita, E.Nielsen and K.Keegstra, submitted). In addition, ATP-dependent associations of precursors with chloroplasts localize in a punctate pattern in intact chloroplasts correlating with regions of close association of the inner and outer chloroplast membranes (Schnell and Blobel, 1993). Furthermore, these precursors migrate with chloroplastic membrane fractions containing contact sites (Schnell and Blobel, 1993; Perry and Keegstra, 1994; J.Ostrom and K.Keegstra, unpublished results). All these results are consistent with formation of a translocation complex containing components of both outer and inner envelope membranes.

Because the complex we characterized does not represent reversible binding, the question arises of whether there is an earlier binding step in chloroplastic protein import. Several lines of evidence support the formation of a different binding complex earlier than the docking step. While stable association of precursor with intact chloroplasts requires ATP, OEP86 and, to a lesser degree, OEP75 can be cross-linked specifically to prSS in the absence of nucleotide (Perry and Keegstra, 1994; Ma *et al.*, 1996). This complex migrates with purified outer envelopes rather than contact sites and, upon addition of ATP, can be chased into the contact sites, where it interacts with OEP75 and an inner membrane protein (Ma *et al.*, 1996). Since, the docking complex includes inner envelope membrane and stromal components, it is unlikely it would migrate with purified outer envelopes.

Outer envelope membrane translocation complexes may not always be associated with inner envelope membrane translocation components during import of precursors into chloroplasts. A slowly translocating precursor can be found in a translocation complex that includes only outer envelope membrane translocation components (Schnell *et al.*, 1995). A membrane protein complex isolated from purified outer membranes has been shown to interact with precursors in an ATP-dependent and transit peptide-specific manner (Soll and Waegemann, 1992). Also, when chloroplasts are subjected to hypertonic conditions to separate the outer and inner envelope membranes, precursors can accumulate in the inter-membrane space during an import reaction (Scott and Theg, 1996). These results suggest that an outer membrane complex is capable of recognizing and translocating precursors independently of inner membrane components. We detected IEP110 and ClpC in OEP75-precipitated complexes, but OEP86 and OEP34 were present at significantly higher levels (Figure 8). These results suggest that, although some OEP75 can interact with inner membrane and stromal components, even in the absence of added precursors, much more is found associated only with OEP34 and OEP86. This result is consistent with the work of Ma *et al.* (1996), who showed that OEP75, OEP34 and OEP86 were associated with one another even in the absence of added precursors. While the authors observed no association of inner membrane components under these conditions, antibodies to inner membrane components were not tested, and Coomassie Blue staining detection methods employed may have not have been sufficiently sensitive to detect lower amounts of inner membrane components.

In conclusion, we have used co-immunoprecipitation techniques to analyze formation of chloroplastic protein translocation complexes. Under docking conditions, a translocation complex containing outer and inner membrane components as well as the stromal chaperone ClpC could be detected. We posit that the interaction of the stromal chaperone ClpC is relevant to the protein import process because association was seen only under conditions that could support docking or import. Further evidence of involvement by other approaches will be required to prove a functional role for this Hsp100 in the import process. We are currently attempting to address the extent to which the precursor has been inserted into the translocation complex using cross-linking techniques.

Materials and methods

Isolation of chloroplasts

Chloroplasts were isolated from 8- to 12-day-old pea seedlings (*Pisum sativum* var. *little marvel*) as previously described (Bruce *et al.*, 1994), and suspended in import buffer (50 mM HEPES–KOH pH 8.0, 300 mM sorbitol) at a concentration of 1 mg chlorophyll/ml.

In vitro translation of precursor proteins

Transcription of mRNA was performed as previously described (Bruce *et al.*, 1994). Plasmids containing cDNA clones of precursors to Rubisco small subunit (pRBCS, *Pst*I-cut; Olsen and Keegstra, 1992), plastocyanin (pPPC, *Eco*RI-cut; Baurle *et al.*, 1991), light-harvesting complex protein (pAB80, *Eco*RI-cut; Payan and Cline, 1991) and 14 kDa outer envelope protein (p14kom, *Eco*RI-cut; Li *et al.*, 1991) were linearized using the appropriate restriction enzymes, transcribed into mRNA. mRNAs were translated and labeled with [^{35}S]methionine (NEN-DuPont) as previously described (Bruce *et al.*, 1994). After translation, residual nucleotides were removed by gel filtration as previously described (Olsen *et al.*, 1989).

Chloroplastic binding and import reactions

Fifty μl of isolated, intact chloroplasts (1 mg chlorophyll/ml) that had been pre-treated with 5 μM nigericin to inhibit photophosphorylation were mixed with *in vitro* translated precursors (1×10^6 d.p.m.) in 100 μl of import buffer supplemented with 100 μM ATP. Reactions were incubated at room temperature in the dark for 10 min. The reactions were terminated by re-isolation of intact chloroplasts with associated precursors by sedimentation through a 40% Percoll cushion. If import was carried out, the above steps were performed as a batch reaction corresponding to the number of timepoints in the import reaction (i.e. six timepoints = 6×150 μl reaction volume). After re-isolation of intact chloroplasts, import was initiated by resuspending chloroplasts in import buffer (50 μl/timepoint) supplemented with 4 mM ATP. The batch import reaction was carried out at room temperature, 50 μl aliquots were removed at given timepoints and import was halted by re-isolation of intact chloroplasts by sedimentation through a 40% Percoll cushion.

Immunoprecipitation

Except where specified, the repurified, intact chloroplasts were lysed hypotonically in 200 μl of lysis buffer (25 mM HEPES–KOH pH 8.0, 4 mM $MgCl_2$). The lysis reaction was incubated on ice for 5 min in the dark, and the supernatant and membrane fractions were separated by centrifugation (5 min, 100 000 *g*, Sorvall RP100-AT2). Isolated chloroplastic membranes and supernatant fractions were suspended in 1 ml of IPES-DM [25 mM HEPES–NaOH pH 7.5, 50 mM NaCl, 2 mM EDTA pH 8.0, 2 mM EGTA pH 8.0, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% (w/v) decylmaltoside] and incubated for 5 min on

ice in the dark. Except where specified, insoluble material was removed from the detergent-solubilized fractions by a centrifugation step (5 min, 100 000 *g*, Sorvall RP100-AT2). The resulting supernatant was immunoprecipitated with the appropriate antisera (10 µl of OEP75, OEP34, IEP110, IEP35, S78 or LHCP), or affinity-purified IgGs (15 µg of ClpC), and 10 mg (dry weight) of IPES-DM pre-washed protein A–Sepharose CL-4B (Pharmacia, Inc.). Immunoprecipitation was carried out for 2 h at 4°C, in the dark. Immunoprecipitated pellets were washed three times with 1 ml of IPES-DM, and once with 1 ml of IPES (without decylmaltoside), and resuspended in SDS–PAGE sample buffer (Laemmli, 1970).

Preparation of antibodies

All antibodies were polyclonal and raised in rabbits. Antisera and preimmune sera to OEP34, OEP86 and IEP35 (Schnell *et al.*, 1994) were a gift from D.Schnell. Affinity-purified, anti-ClpC IgG (Shanklin *et al.*, 1995) was a gift from J.Shanklin. Antiserum to LHCP (Payan and Cline, 1991) was a gift from K.Cline. Antiserum to OEP75 was raised against the mature region of the OEP75 protein overexpressed in *E.coli* using the pET expression system (Novagen, Inc.) (our unpublished results). Antisera to IEP110 were raised as discussed in M.Akita, E.Nielsen and K.Keegstra (submitted). To make antiserum that would recognize specifically S78, a portion of the S78 cDNA (Marshall and Keegstra, 1992) corresponding to the C-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, Inc.). The resulting fusion protein was overexpressed and then purified to homogeneity from *E.coli* using a glutathione–Sepharose CL-4B column (Pharmacia, Inc.). Purified GST–S78 fusion protein was mixed with Titre-Max adjuvant (Vaxcel, Inc.) and injected into rabbits. The specificity of the antiserum was determined as described in M.Akita, E.Nielsen and K.Keegstra (submitted).

Electrophoresis and immunoblotting

All electrophoresis was performed as previously described (Laemmli, 1970) using the Hoefer gel electrophoresis system (Bio-Rad, Inc.). Immunoblotting consisted of transferring proteins onto Immobilon-P membrane (Millipore, Inc.), in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.05% SDS) as described by Towbin and Gordon (1984). Detection of immunoblotted proteins was performed with horseradish peroxidase conjugated to goat anti-rabbit Fabs (Kirkegaard and Perry, Inc.), and were visualized by Western-ECL chemiluminescence (Amersham, Inc.) exposed to X-ray film (Eastman-Kodak, Inc.).

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