A Stromal Protein Factor Maintains the Solubility and Insertion Competence of an Imported Thylakoid Membrane Protein

Luis A. Payan and Kenneth Cline
Fruit Crops Department, University of Florida, Gainesville, Florida 32611

Abstract. The light-harvesting chlorophyll a/b protein (LHCP) is an ~25,000-D thylakoid membrane protein. LHCP is synthesized in the cytosol as a precursor and must translocate across the chloroplast envelope before becoming integrally associated with the thylakoid bilayer. Previous studies demonstrated that imported LHCP traverses the chloroplast stroma as a soluble intermediate before thylakoid insertion. Here, examination of this intermediate revealed that it is a stable, discrete ~120,000-D species and thus either an LHCP oligomer or a complex with another component. In vitro-synthesized LHCP can be converted to a similar form by incubation with a stromal extract. The stromal component responsible for this conversion is proteinaceous as evidenced by its inactivation by heat, protease, and NEM. Furthermore, the conversion activity coelutes from a gel filtration column with a stromal protein factor(s) previously shown to be necessary for LHCP integration into isolated thylakoids. Conversion of LHCP to the 120-kD form prevents aggregation and maintains its competence for thylakoid insertion. However, conversion to this form is apparently not sufficient for membrane insertion because the isolated 120-kD LHCP still requires stroma to complete the integration process. This suggests a need for at least one more stroma-mediated reaction. Our results explain how a hydrophobic thylakoid protein remains soluble as it traverses the aqueous stroma. Moreover, they describe in part the function of the stromal requirement for insertion into the thylakoid membrane.

Thylakoids are the photosynthetic membranes within chloroplasts. Thylakoids contain at least 50 different polypeptides of which about half are encoded on nuclear genes and synthesized in the cytosol and the remainder encoded and synthesized within the organelle (Murphy, 1986; Keegstra, 1989; Keegstra et al., 1989). Thylakoids provide a unique system for protein localization studies because they are completely enclosed within the two chloroplast envelope membranes. Thus, nuclear-encoded thylakoid proteins must translocate across the envelope and traverse the aqueous stromal matrix before becoming finally assembled into the thylakoid membrane (Keegstra, 1989; Smeekens et al., 1990). This process can be conceptually divided into two sequential operations; import into the organelle and localization within the organelle. Thylakoid proteins are imported in a manner similar to that for other plastid proteins. They are synthesized as larger precursors, bind to protein receptor(s) on the plastid surface, and translocate across the envelope by an ATP-dependent mechanism (Keegstra et al., 1989). Much of our knowledge concerning intraorganellar localization derives from studies of the light-harvesting chlorophyll a/b protein (LHCP). LHCP is the ~25,000-D apoprotein of the LHC II antenna complex; it binds chlorophyll and xanthophyll and is thought to span the thylakoid bilayer at least three times (Murphy, 1986). LHCP is synthesized as a precursor (pLHCP) with a transient ~35 residue amino-terminal "transit peptide" that is necessary for import into the chloroplast but not for sorting to thylakoids (Viitanen et al., 1988).

Recent studies indicate that intraorganellar localization of LHCP proceeds via a "soluble intermediate" pathway in which pLHCP is transported across both envelope membranes and immediately processed to mature size; a soluble form of LHCP then traverses the stroma and inserts into the thylakoids by a thylakoid-based integration system. The evidence for this pathway includes: the appearance and kinetics of stromal LHCP during in vitro chloroplast import assays (Reed et al., 1990); the enhanced accumulation of stromal LHCP when the thylakoid integration step is inhibited (Cline et al., 1989; Reed et al., 1990); the demonstration that stromal LHCP can "chase" into thylakoids (Reed et al., 1990); and the in vitro reconstitution of the thylakoid insertion step with isolated chloroplast subfractions (Cline, 1986, 1988). This latter process requires either pLHCP or LHCP, thylakoids, a stromal protein 'integration factor,' and ATP (Cline, 1986, 1988; Fulsom and Cline, 1988).

In the present study, the stromal LHCP intermediate was further characterized, the conditions of its formation deter-

1. Abbreviations used in this paper: LHCP, light-harvesting chlorophyll a/b protein; LS, large subunit; pLHCP, precursor to light-harvesting chlorophyll a/b protein; Rubisco, ribulose-1,5-bisphosphate-carboxylase.
Assays for Integration and Conversion to the Soluble LHCP Complex

Integration assays were conducted in 1.5 ml microcentrifuge tubes by incubating 200 μl stromal preparation, 50 μl 4× thylakoids, 25 μl 120 mM Mg-ATP in import buffer, and 25 μl adjusted [3H]pLHCP or LHCP for 30 min at 25°C. After incubation, thylakoids were recovered and treated for 40 min with 50 μg thermolysin (Cline, 1986). Thermolysin treatments were terminated by addition of 0.5 ml import buffer, 14 mM EDTA. Thylakoids were recovered by centrifugation and subjected to SDS-PAGE/fluorography (Cline, 1988). Integration was assessed as the amount of LHCP-DP, a protease-protected LHCP peptide that is characteristic for properly inserted LHCP (Cline, 1986).

Preparation of Complexes for Immunoprecipitation Studies

The soluble pLHCP complex was prepared by incubating 200 μl of in vitro-synthesized pLHCP with 400 μl of a 2× stromal extract (adjusted to import buffer plus 10 mM MgCl₂) at 25°C for 15 min and then centrifuging the mixture at 40,000 rpm in a rotor (model SW 50.1; Beckman Instruments, Inc., Palo Alto, CA) at 4°C for 60 min to remove the aggregated pLHCP. The material from the top half of the centrifuge tube contained very little aggregated pLHCP and was used for the immunoprecipitation experiment. The complex between radioiodiated large subunit (LS) of ribulosebisphosphate-carboxylase (Rubisco) and cpn60 was prepared by protein synthesis in isolated pea chloroplasts by a modification of the method of Mullet et al. (1986). The reaction mixture (375 μl) contained 125 μg chlorophyll of intact chloroplasts, 125 μCi of [3H]leucine, 6 mM di-thiothreitol, 2.4 mM Mg-ATP, 5 μM of each amino acid (except leucine), and 0.33 M sorbitol, 50 mM Hepes/KOH, pH 8. Reaction mixtures were incubated at 25°C for 12.5 min under light and the reaction stopped by cooling the reaction mixture on ice. Chloroplasts were diluted with 0.5 ml import buffer, pelleted at 1,200 g for 3 min, and lysed on ice with 50 μl of HKM buffer before adding 5 U of aprase. A stromal extract was obtained from the lysate and adjusted to 1.33× stroma, import buffer, 10 mM MgCl₂.

Preparation of Antibodies and Immunoprecipitation with Protein A–Sepharose Preadsorbed with Antibodies

LHC II was isolated from pea thylakoid membranes according to Steinbeck et al. (1982). LHCP was purified from LH II preparations by electroelution excised LHCP bands from 12.5% SDS-PAGE gels. Antibodies to the electroeluted LHCP in 0.1% SDS (wt/vol) were prepared in rabbits by Cocalico Biologicals, Inc., Reamstown, PA.

Protein A–Sepharose was hydrated and washed three times with HK buffer. Washed protein A–Sepharose (70 μl swollen packed gel) was incubated with 50 μl serum and HK buffer to a final volume of 375 μl for 1.5 h at 4°C with gentle shaking. The beads were recovered, washed three times with import buffer, 10 mM MgCl₂, and resuspended in 250 μl of the same buffer. Immunoprecipitation reactions were conducted by incubating 12.5 μl of the complexes described above with 75 μl of protein A–Sepharose beads preadsorbed with the respective antibodies at 4°C for 1.5 h with gentle agitation. The beads were then pelleted and washed three times with 0.5 ml import buffer, 10 mM MgCl₂. During the last wash the beads were transferred to a fresh microcentrifuge tube. Protein A–Sepharose immunoglobulin-antigen complexes were then dissociated in 25 μl SDS-PAGE sample buffer.

Immunoblotting

Proteins were separated by SDS-PAGE and transferred electrophoretically to nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH) in a Mini Trans-Blot apparatus (Bio-Rad Laboratories, Richmond, CA). The membrane was blocked with 2% BSA, 0.02% sodium azide at 4°C overnight, washed with TBS-Tween buffer (10 mM Tris-HCl [pH 7.4], 140 mM NaCl, 0.1% [vol/vol] Tween 20) for 5 min, and then incubated with antigroEL at a 1:2,000 dilution, anti-LHCP at a 1:3,000 dilution, or non-immune serum at a 1:2,000 dilution at room temperature for 60 min. All dilutions were made in HST buffer (10 mM Tris-HCl [pH 7.4], 1 M NaCl, 0.5% [vol/vol] Tween 20). After extensive washing with TBS-tween buffer, the blots were incubated with alkaline phosphatase-conjugated affinity-
purified antibodies to rabbit IgG (Kirkgaard & Perry Laboratories, Inc.,
Gaithersburg, MD) at a 1:1,000 dilution in HST buffer at room temperature
for 1 h. Membranes were washed as above and immunoreactive proteins
visualized by staining with bromochloroindolyl phosphate/nitro blue
tetrazolium (Harlow and Lane, 1988).

Miscellaneous
Glycerol gradient sedimentation was conducted by applying 200-300 µl
samples to 5 ml linear 10-30% glycerol gradients in 25 mM Hepes/KOH
(pH 8). Gradients were centrifuged at 50,000 rpm (SW50.1 rotor; Beckman
Instruments, Inc.) for 13 h at 4°C and fractionated from the bottom of the
centrifuge tubes into 0.45 ml fractions. SDS-PAGE was conducted on 0.75
mM, 12.5% gels as described (Laemmli, 1970). Nondenaturating PAGE was
conducted on 0.75 mM, 6% T/2.6% C polyacrylamide gels. Gels were pre-
pared in 0.375 M Tris-Cl pH 8.8 and run with 25 mM Tris, 192 mM glycine
at 10 mA/gel for 2 h at 7°C. Fluorography was performed as described
(Cline, 1986). Protein was determined by Bradford (1976) and chlorophyll
by Arnon (1949).

Results

The Stromal LHCP Intermediate Is a Soluble
\(~120,000\)-D Species

The stromal LHCP intermediate has previously been ob-
served during in vitro import of pLHCP into chloroplasts ei-
ther by using a rapid stopping analysis (Reed et al., 1990)
or by intentionally inhibiting the thylakoid integration step
with ionophores (Cline et al., 1989). Those investigations
demonstrated that stromal LHCP is completely soluble. This
property is notable because fully assembled LHCP requires
organic solvents or detergents for solubilization (Schmidt et
al., 1981; Steinback et al., 1982). Even freshly prepared in-
 vitro–synthesized pLHCP and LHCP are only transiently
soluble (Fulsom and Cline, 1988; Viitanen et al., 1988; and
see below).

As shown in Fig. 1, glycerol gradient sedimentation analy-
sis demonstrated that stromal LHCP exists as a discrete
species. Analysis of fractions by SDS-PAGE/fluorography
showed that LHCP sedimented in a narrow band near the
center of the gradient (Fig. 1 a). This discrete form of LHCP
could also be monitered by subjecting gradient fractions

Figure 1. The stromal LHCP inter-
mediate sediments through a
glycerol gradient as a stable,
discrete, species. Stromal LHCP
was sedimented through a gly-
cerol gradient and the fractionated
gradient analyzed by SDS-PAGE/
fluorography (a) and nonden-
aaturing PAGE/fluorography (b).
Stromal LHCP was prepared by
import of in vitro–synthesized
pLHCP into intact chloroplasts
(300 µg chlorophyll) in the pres-
ence of 0.5 µM nigericin, 10 µM
valinomycin, and 2.5 mM ATP in
darkness for 7 min as described
(Cline et al., 1989). Recovered
chloroplasts were treated with
thermolysin, repurified on Per-
coll (Cline et al., 1989), lysed
with 250 µl HK buffer plus 4 mM
MgCl2, and a stromal extract
prepared.

Figure 2. Analysis of the stromal LHCP intermediate by two-
dimensional PAGE. A stromal extract containing the stromal LHCP
intermediate was prepared as in Fig. 1 and was directly applied to
nondenaturing PAGE. The gel lane was excised and subjected to
second dimension SDS-PAGE/fluorography. The locations of sample
well, Rm \(\sim 0.4\), and dye front of the nondenaturing gel are
marked. A mixture of pLHCP and LHCP translation products was
run on the left side of the SDS-gel as reference standards.
Stroma Converts LHCP into the Soluble Complex Form

Freshly prepared in vitro-synthesized LHCP or pLHCP rapidly became aggregated when analyzed by either of the above methods. When subjected to nondenaturing PAGE after a brief incubation at 25°C, (p)LHCP remained in the sample well (Fig. 4). When subjected to glycerol gradient sedimentation, most of the (p)LHCP was found near the bottom of the gradient or adhering to the centrifuge tube walls (not shown). Consequently, LHCP must be converted to the soluble complex form during import into chloroplasts, either during translocation across the envelope or upon interaction with the stroma. The latter possibility was investigated by incubating LHCP or pLHCP with a stromal extract and resolving the mixture by nondenaturing PAGE (Fig. 4). A considerable percentage of the (p)LHCP (20–35%) was converted to soluble complex form. This value is two- to threefold the percentage of (p)LHCP that is inserted into thylakoids in an integration assay (Fulsom and Cline, 1988). The LHCP complex migrated with the same Rm and possessed the same relative molecular mass as the stromal LHCP intermediate shown in Fig. 1 (Fig. 4). The pLHCP complex migrated more slowly with an estimated Mr of ~125,000 (Figs. 3 and 4). Two-dimensional gel analysis (not shown) demonstrated the presence of the respective (p)LHCP species in the electrophoretically mobile bands and also showed that unconverted (p)LHCP accounted for the radioactivity in the sample well. This latter material is presumably a "nonconvertible" aggregate because increased amounts of stroma failed to convert it into soluble complex form. These and other results described below suggest that transiently soluble forms of (p)LHCP can be converted into the more stable soluble complex form, but that once aggregated, (p)LHCP can no longer be converted. In support of this interpretation is the observation that denaturation of (p)LHCP with urea immediately before incubation with stroma results in a substantially higher percentage of soluble complex form (not shown). Conversion of the precursor as well as the mature form of LHCP is consistent with their equal effectiveness as substrates for integration into isolated thylakoid membranes (Viitanen et al., 1988) and also with the fact that under certain in vitro conditions imported pLHCP localizes into thylakoids without processing (Chitnis et al., 1986).

Conversion of (p)LHCP was dependent upon the quantity of stroma, increasing with increasing stromal extract (Fig. 4). Comparable amounts of soluble complex were obtained when assays were conducted in low to moderate buffer concentrations (0.01–0.05 M Hepes/KOH) and low to moderate salt (<100 mM KCI). However, conversion was usually enhanced by supplementing reaction mixtures with either MgCl₂ or Mg-ATP (5–10 mM). Consistent with this result was the observation that removal of low molecular mass components from stroma and translation products with Sephadex G-25 resulted in a dramatic reduction in complex formation. This was restored in part by supplementing the assay mixture with MgCl₂ and to a greater extent with Mg-ADP, Mg-ATP, or Mg-β,γ-methylene ATP (not shown). The role of these additives in complex formation is unknown. It clearly is unrelated to the millimolar ATP requirement for thylakoid integration of LHCP because virtually no integration occurs when ATP is replaced with MgCl₂, ADP, or
Figure 4. (p)LHCP is converted to the soluble complex form by incubation with a stromal extract. In vitro-synthesized pLHCP and in vitro-synthesized LHCP were incubated with a stromal extract and then analyzed for the presence of soluble complex form. Reaction mixtures received 10 μl (p)LHCP, 10 μl 60 mM Mg-ATP, and 40 μl of HK buffer containing increasing amounts of stromal extract. Reactions were incubated and then analyzed by nondenaturing PAGE/fluorography as described in Materials and Methods. a shows a picture of the fluorogram. b displays the percentage of (p)LHCP converted to the soluble complex form. Displayed values were corrected for background radioactivity that occurs in the absence of stroma. Essentially the same results were obtained if MgCl₂ replaced Mg-ATP in the assay.

β,γ-methylene ATP (Cline, 1986). However, in order to routinely obtain maximum conversion, the assays reported here were supplemented with 10 mM Mg-ATP.

A Stromal Protein Mediates (p)LHCP Conversion

The essential macromolecular component(s) of stroma exhibited properties expected of a protein (Fig. 5). Pretreatment of the stromal extract with elevated temperatures, proteinase K, or N-ethyl maleimide (NEM) destroyed its ability to convert (p)LHCP. The inhibitory effects of proteinase K and NEM did not occur if these agents were inactivated with PMSF and DTT, respectively, before treatment. Conversion resulted from a specific protein effect because other pro-
tein preparations, including BSA, wheat germ extract, and reticulocyte lysate, were ineffective in promoting complex formation (not shown). This conclusion is further supported by the fact that converting activity eluted as a single peak when a stromal extract was fractionated on Sephacryl S-300 (Fig. 6).

We previously reported (Fulsom and Cline, 1988) that a stromal protein factor(s) is essential for integration of (p)LHCP into isolated thylakoids and that this “integration” factor is sensitive to heat, protease, and NEM (see also Fig. 5, top). Interestingly, the integration factor eluted from the S-300 column in the same fractions as converting activity.

Stromal Factor(s) Maintains LHCP Competent for Insertion into Isolated Thylakoids

The above results demonstrated that (p)LHCP is soluble when in the complex form. It seemed likely that conversion to this form has a functional significance, i.e., to maintain LHCP in a conformation that is competent for insertion into the thylakoid bilayer. To address this question, pLHCP was incubated in the presence or absence of stroma at 25°C. The ability of the incubated pLHCP to insert into isolated thylakoids was then determined in the presence of fresh stroma. pLHCP incubated in buffer alone rapidly lost the ability to insert into thylakoids. This is shown in Fig. 7 and quantified in Fig. 8. The loss of integration competence coincided with the failure to be converted into the complex form in the presence of stroma, indicative of inactivation due to heat, protease, and NEM.

Stroma was treated and then assayed for its ability to integrate [3H]LHCP into isolated thylakoids (top) and to convert [3H]LHCP into the soluble complex form (bottom). Stroma (2× in HK buffer) was pretreated independently with various temperatures (0, 25, 37 and 100°C) for 10 min (a), or with active or PMSF-deactivated proteinase K (10 μg/ml final concentration) (b), or with active or DTT-deactivated NEM (1 mM final concentration) (c) for 30 min on ice. Proteinase K and NEM were de-activated before (lanes I) or after (lanes A) stromal treatment as described (Fulsom and Cline, 1988). In the figure, integration is apparent as the amount of LHCP-DP, a protease resistant fragment of LHCP that is characteristic of proper insertion in the membrane. Similar results were obtained when in vitro-synthesized pLHCP was used. Analysis of unprocessed assay mixtures by SDS-PAGE/fluorography showed no degradation of the pLHCP or LHCP during the assays, indicating that protease inactivation was complete.

Figure 5. The stromal component that mediates soluble complex formation is sensitive to heat, proteinase K, and N-ethylmaleimide (NEM). Stroma was treated and then assayed for its ability to integrate [3H]LHCP into isolated thylakoids (top) and to convert [3H]LHCP into the soluble complex form (bottom). Stroma (2× in HK buffer) was pretreated independently with various temperatures (0, 25, 37 and 100°C) for 10 min (a), or with active or PMSF-deactivated proteinase K (10 μg/ml final concentration) (b), or with active or DTT-deactivated NEM (1 mM final concentration) (c) for 30 min on ice. Proteinase K and NEM were de-activated before (lanes I) or after (lanes A) stromal treatment as described (Fulsom and Cline, 1988). In the figure, integration is apparent as the amount of LHCP-DP, a protease resistant fragment of LHCP that is characteristic of proper insertion in the membrane. Similar results were obtained when in vitro-synthesized pLHCP was used. Analysis of unprocessed assay mixtures by SDS-PAGE/fluorography showed no degradation of the pLHCP or LHCP during the assays, indicating that protease inactivation was complete.

Figure 6. Converting activity of stroma elutes as a single peak from Sephacryl S300. Stroma was fractionated on a 1.5 × 46-cm Sephacryl S300 column and the fractions assayed for their ability to convert [3H]LHCP into the soluble complex form and to integrate [3H]LHCP into isolated thylakoids. A 10× stromal extract was adjusted to 20 mM Hepes/KOH (pH 8), 65 mM KCl, 1 mM DTT, 1 mM Mg-ATP, 1% (vol/vol) ethylene glycol. After 15 min on ice, 1.3 ml was chromatographed in the same buffer at 4.5 ml per h. The displayed values, corrected by subtracting the background values obtained in the absence of stroma, represent the percentage of activity relative to that in the peak fractions. The peak fraction converted 26% of the added LHCP to soluble complex form and integrated 7% of the added LHCP into thylakoids. Similar results were obtained in experiments in which pLHCP was used as the substrate.

The Journal of Cell Biology, Volume 112, 1991 608
Figure 7. Stroma maintains LHCP competent for integration into thylakoids. pLHCP was incubated with stroma, buffer, or BSA at 25°C and its ability to integrate into isolated thylakoids was monitored with time. In vitro-synthesized pLHCP was combined with an equal volume of a 4× stromal extract prepared in HKM buffer (a), with an equal volume of HKM buffer (b), or with HKM buffer containing BSA (2 mg/ml) (c). Aliquots were removed at 0, 1, 3, and 5 h of incubation and adjusted to comparable composition by supplementing the buffer- and BSA-incubated pLHCP aliquots with an equal volume of 25°C-incubated 2× stromal extract and supplementing stroma-incubated pLHCP with buffer. Aliquots were then immediately assayed for the ability of the incubated pLHCP to integrate into thylakoids (top). Reaction mixtures contained 200 µl adjusted aliquots, 25 µl fresh 10× stroma, 25 µl 120 mM Mg-ATP, and 50 µl 4× thylakoids. Aliquots were also assayed for the ability of the incubated pLHCP to be converted to the soluble complex (bottom). Reaction mixtures contained 40 µl adjusted aliquot, 4 µl fresh 10× stroma, 10 µl 60 mM Mg-ATP, and 6 µl import buffer plus 5 mM MgCl₂. Parallel assays showed that it was unnecessary to add fresh stroma to assays of the stroma-incubated pLHCP. During the 5-h incubation at 25°C, there was no loss of radioactive pLHCP from the incubated samples.

The Soluble Complex Form of LHCP Is Competent but Not Sufficient for Membrane Insertion

The rapid loss of competence for insertion or conversion in the absence of stroma indicated that (p)lHCP is inherently unstable in aqueous solution, but stabilized by interaction with stromal components. The presumption is that soluble (p)lHCP complex remains active and that aggregated (p)lHCP is irreversibly inactivated. To address this point more directly, a mixture of (p)lHCP and a stromal extract was incubated at 25°C and later fractionated into soluble (p)lHCP complex and aggregated (p)lHCP by glycerol gradient sedimentation (Fig. 9 a). Aggregated (p)lHCP was recovered at the bottom of the gradient (Fig. 9, Fraction 1) and soluble complex near the middle (Fractions 6 and 7). Nondenaturing PAGE/fluorography verified that most of the (p)lHCP in fractions 6 and 7 was in the soluble complex form. The aggregated (p)lHCP and soluble (p)lHCP complex were assayed for their ability to integrate into isolated thylakoids. Aggregated (p)lHCP was incapable of inserting regardless of the quantity of fresh stroma added to the reaction mixture. This did not result from inhibitory components present in the gradient because aliquots of fractions from a mock gradient did not inhibit the insertion of freshly prepared (p)lHCP (Fig. 9 b).

In contrast, the (p)lHCP soluble complex was able to insert into thylakoids (Fig. 9 c). However, it was necessary to supplement insertion reactions with fresh stroma for this to occur. We interpret this result to mean that the soluble complex, while competent for integration, requires an additional stroma-mediated reaction in order to fold into the thylakoid bilayer.
LHCP Is Not Complexed with the Chloroplast Chaperonin 60 (Cpn60)

Several studies have demonstrated that hsp60 type chaperone proteins, collectively referred to as cpn60 (Lubbon et al., 1989), form soluble complexes with newly synthesized organelar proteins before assembly (Roy, 1989, Ostermann et al., 1989). The chloroplast cpn60, which is homologous to the bacterial groEL (Hemmingsen et al., 1988), binds to Rubisco LS and presumably mediates the formation of Rubisco holoenzyme (Roy, 1990). In addition, it has recently been reported that cpn60 forms soluble complexes with several imported chloroplast proteins including LHCP (Lubben et al., 1989). Although the soluble LHCP complex described here is smaller than previously described cpn60 complexes, we chose to directly determine if cpn60 is bound to (p)LHCP by subjecting the soluble (p)LHCP complex to immunoprecipitation with protein A-Sepharose preadsorbed with antibodies to cpn60. For this experiment anti-groEL antibodies, which are highly reactive with cpn60 on Western blots (Fig. 10 a), were used. Nonimmune serum was used as a negative control and two positive controls were included. First, immunoprecipitation was conducted with anti-LHCP to demonstrate that the LHCP soluble complex could be removed from solution with appropriate antibodies. Secondly, as a control for coprecipitation of proteins bound to cpn60, LS of Rubisco was prepared by in organelle protein synthesis. LS made in this fashion was found exclusively bound to cpn60 (Fig. 10 b). As can be seen in Fig. 10 c, anti-groEL coimmunoprecipitated LS-cpn60 but not the soluble LHCP complex. This demonstrates that cpn60 is not a member of the LHCP soluble complex.

Discussion

The basic requirement for membrane protein assembly is to accurately target the protein into the correct membrane and, at the same time, to avoid aggregation before bilayer insertion. This requirement is compounded in cases where the ultimate target membrane is distal from the site of initial translocation/insertion. Such is the case for proteins of the endomembrane system, e.g., plasma membrane, Golgi, and lysosomal membranes, and for nuclear encoded thylakoid membrane proteins. The manner by which cells accomplish such assembly processes has been the subject of intense in-
Soluble LHCP is not complexed with chaperonin 60 as determined by immunoprecipitation with antibodies to groEL. Soluble (p)LHCP complex was subjected to immunoprecipitation analysis with anti-groEL antibodies. As controls, a Rubisco LS-cpn60 complex was immunoprecipitated with anti-groEL, and both complexes were subjected to immunoprecipitation with anti-LHCP antibodies and nonimmune serum. The specificity of antibodies is shown by the immunoreplica analysis of a Western blot of total chloroplast proteins. Soluble pLHCP complex (LH) was prepared by incubating [3H]pLHCP with stroma and centrifuging to remove the aggregated pLHCP (Materials and Methods). The complex between the LS of Rubisco and cpn60 (LC) was prepared by in organello protein synthesis with [3H]leucine (Materials and Methods). Analysis of complexes by SDS-PAGE/fluorography demonstrated the radiochemical purity of pLHCP and LS. Nondenaturing PAGE demonstrated that virtually all of the pLHCP was in the soluble complex form and LS in the LS-cpn60 complex. These complexes were challenged with protein A-Sepharose beads preadsorbed with the antibodies shown as described in Materials and Methods. The recovered beads were dissociated in SDS buffer and subjected to SDS-PAGE/fluorography.

Investigation. It is now widely recognized that endomembrane proteins are synthesized on ER-bound ribosomes (Walter and Lingappa, 1986) and that subsequent transport to distal membranes occurs via membrane vesicles (Lodish, 1988; Pfeffer and Rothman, 1987). Solubility problems for these proteins are circumvented by cotranslational bilayer insertion and subsequent routing in membrane vesicles. Studies of LHCP biogenesis suggest that transport and routing of thylakoid proteins differs substantially from that of endomembrane proteins. First, pLHCP is posttranslationally imported into chloroplasts. Second, LHCP does not stably insert into the chloroplast envelope, but instead translocates across both membranes into the stroma (Cline et al., 1989; Reed et al., 1990). Finally, LHCP is routed to the thylakoids through the stroma and not via membrane vesicles. The consequence of this pathway is that solubility and transport competence of LHCP species must be maintained in the cytosol before import as well as in the stroma before thylakoid integration. Recent work by Waegemann et al. (1990) indicates that import competence of pLHCP is maintained by cytosolic factors, although the manner in which such factors accomplish this is unknown. In the present work, we have examined the manner by which LHCP traverses the stroma.

Several salient characteristics of stromal LHCP have been determined. First, solubility of LHCP is achieved by conversion to a larger species, possibly a complex. Although this is apparent from several different observations, the most convincing is the fact that in vitro-synthesized (p)LHCP is not present in this form and exhibits poor and only transient solubility (Figs. 4 and 7; Fulsom and Cline, 1988; Viitanen et al., 1988). When (p)LHCP is converted to the larger complex form, this species is then soluble (Figs. 4 and 9). Second, conversion to soluble complex form maintains (p)LHCP competent for insertion into thylakoids. Only the soluble complex form of LHCP is insertionally competent as shown in Fig. 9 and it remains stable for up to 5 h at 25°C (Figs. 7 and 8). Finally, a stromal protein mediates conversion of (p)LHCP to the larger, soluble form. The evidence that implicates a stromal protein in this process includes its sensitivity to protein-inactivating agents and its behavior during gel filtration chromatography (Figs. 5 and 6).

The precise composition of soluble LHCP complex is unknown. It could be an LHCP oligomer. The LH II antenna complex in the thylakoid membranes is thought to be trimmeric (Kuhlbrandt, 1984), suggesting that LHCP monomers are capable of interacting. If the soluble LHCP complex is an oligomer, it might indicate that some folding and assembly occurs before membrane insertion. On the other hand, the soluble form of LHCP could be a complex between LHCP and a stromal protein. Adequate precedence exists for such a possibility. Soluble proteins involved in the assembly and membrane transport of newly synthesized proteins have

**Figure 10.** Soluble LHCP is not complexed with chaperonin 60 as determined by immunoprecipitation with antibodies to groEL. Soluble (p)LHCP complex was subjected to immunoprecipitation analysis with anti-groEL antibodies. As controls, a Rubisco LS-cpn60 complex was immunoprecipitated with anti-groEL, and both complexes were subjected to immunoprecipitation with anti-LHCP antibodies and nonimmune serum. The specificity of antibodies is shown by the immunoreplica analysis of a Western blot of total chloroplast proteins. Soluble pLHCP complex (LH) was prepared by incubating [3H]pLHCP with stroma and centrifuging to remove the aggregated pLHCP (Materials and Methods). The complex between the LS of Rubisco and cpn60 (LC) was prepared by in organello protein synthesis with [3H]leucine (Materials and Methods). Analysis of complexes by SDS-PAGE/fluorography demonstrated the radiochemical purity of pLHCP and LS. Nondenaturing PAGE demonstrated that virtually all of the pLHCP was in the soluble complex form and LS in the LS-cpn60 complex. These complexes were challenged with protein A-Sepharose beads preadsorbed with the antibodies shown as described in Materials and Methods. The recovered beads were dissociated in SDS buffer and subjected to SDS-PAGE/fluorography.
been described in a variety of biological systems (Chirico et al., 1988; Deshaies et al., 1988; Lecker et al., 1989; Ostermann et al., 1989; Rothman, 1989). Such proteins have been termed chaperones (Ellis and Hemmingsen, 1989). Certain chaperones, e.g., the presequence binding factor (Murakami and Mori, 1990), and the trigger factor, SecB protein, and groEL of Escherichia coli (Lecker et al., 1989), prevent aggregation and maintain membrane transport competence of precursor proteins by entering into soluble 1:1 complexes with them. The presence of chaperone proteins in organelles has been documented. Marshall et al. (1990) reported that the stroma of pea chloroplasts contains two different hsp70 proteins, one which is homologous to the E. coli dnaK protein. The potential involvement of these hsp70 proteins in chloroplast protein assembly has not been examined. We presently cannot rule out the involvement of hsp70 in formation of the stromal LHCP complex, but we note that several of our stromal subfractions are enriched in polypeptides immunoreactive with anti-hsp70 and yet are inactive in either integration or conversion assays.

Chloroplasts also contain an hsp60 type chaperone (cpn60) that is homologous to the groEL protein (Hemmingsen et al., 1988). cpn60 assists in the assembly of Rubisco and has been shown to bind a variety of imported proteins (Lubben et al., 1989). Similarly, in mitochondria, cpn60 proteins bind several different imported proteins and assist in their folding and assembly (Cheng et al., 1989; Ostermann et al., 1989). Our results indicate that stromal LHCP is not complexed with cpn60. cpn60 complexes are 800,000 D, or six to seven times as large as the LHCP complex (Lubben et al., 1989; Roy, 1989). Although it is conceivable that the 120-kD LHCP results from dissociation of a high molecular mass cpn60 complex, we think that unlikely. Dissociation of the large cpn60 complexes requires ~100 μM ATP (Bloom et al., 1983; Musgrove et al., 1987). The soluble LHCP complex can be formed in the absence of exogenous ATP (see Results) and also during import into intact chloroplasts (Figs. 1 and 2) under conditions similar to those in which the LS-cpn60 complex remains intact (Fig. 10). In both cases, the large cpn60 complex is apparent when gels are stained with Coomassie blue. The fact that anti-cpn60 antibodies failed to coprecipitate soluble LHCP under conditions that successfully coprecipitated LS bound to cpn60 demonstrates that cpn60 is not present in the 120-kD LHCP complex.

Although our experiments suggest that soluble LHCP is not complexed with cpn60, they don't rule out the involvement of cpn60 (or hsp70) during LHCP import and assembly. Lubben et al. (1989) detected a small amount of imported LHCP associated with cpn60. In addition, the recent study by Ostermann et al. (1989) demonstrated that the Rieske Fe/S protein of yeast mitochondria initially binds to the mitochondrial cpn60 upon entering mitochondria, but upon release from cpn60 is found as a 70-kD soluble species. In view of the fact that the Fe/S protein must subsequently be inserted back through the inner mitochondrial membrane, we suggest that the 70-kD species is functionally analogous to the 120-kD LHCP form. Thus it is possible that LHCP binds to cpn60 upon import and is rapidly released to the 120-kD form. We cannot rule out the potential involvement of cpn60 because all of our active preparations contain cpn60, even though frequently the amount present is very low. However, we are persuaded that cpn60 is not sufficient for either the LHCP conversion activity nor the insertion activity because many different stromal subfractions are rich in cpn60, but devoid of either activity (Yuan, J., and K. Cline, unpublished results).

Additional investigations are necessary to determine the precise nature of the soluble LHCP complex and to explore the potential role of chaperone-type proteins in the conversion process. In addition, the generality of complex formation described here for LHCP is yet to be fully explored. Preliminary results indicate that other thylakoid-destined proteins are converted into larger forms, i.e., in our standard assay, stroma converts at least four other related chlorophyll-binding apoproteins into larger forms that on nondenaturing PAGE appear analogous to the soluble LHCP complex (Cline, K., unpublished results). In contrast, we have failed to observe any stroma-induced conversion of plastocyanin to a larger form. Plastocyanin is a thylakoid lumenal protein that is thought to be imported into the stroma and processed to an intermediate size precursor before transport across the thylakoid bilayer (Smekens et al., 1990). In our hands, the intermediate size-preplastocyanin that results from incubation with stroma sediments as a monomeric soluble species. This is consistent with the apparent lack of a stromal requirement for plastocyanin transport into thylakoids (Bauerle, C., and K. Keegstra, personal communication).

The results presented here have identified one function for the stromal requirement for LHCP insertion into thylakoids, i.e., maintenance of LHCP solubility and insertion competence. But they also suggest that the involvement of stroma is more complex than maintenance of insertion competence. This is obvious from the results shown in Fig. 9. Although the complex was competent, integration did not occur unless fresh stroma was added to the reaction mixture. This conclusion is further supported by our observations on the effects of urea on the insertion reaction (Cline, K., unpublished results). Urea denaturation of pLHCP stimulated insertion two- to threefold as would be expected if disaggregation renders LHCP insertion competent. Nevertheless, integration of urea-treated LHCP was still dependent upon stroma and ATP. This is also consistent with our observation that during ion exchange chromatography, integration activity is lost when the resolution is increased, thereby confounding purification attempts. The results reported here now offer an alternative strategy for purifying at least one component of the stromal integration requirement, i.e., purification based upon interaction with LHCP.

We thank Clas Dahlin, Ken Keegstra, Jerry Marshall, Jianguo Yuan, and Eduardo Vallejos for critical reading of the manuscript, and Mark Schulle for excellent technical assistance. We also thank Paul Vittingen for providing the clone for LHCP and Thomas Lubben and George Lorimer for antibodies to groEL.

This investigation was supported in part by National Science Foundation grant DCB-8718560 to K. Cline. This paper is Florida Agricultural Station Journal Series No. R-01099.

Received for publication 6 August 1990 and in revised form 24 October 1990.

References

Arnon, D. I. 1949. Copper enzymes in isolated chloroplasts. Plant Physiol. 24:1-15.

Bloom, M., P. Milos, and H. Roy. 1983. Light dependent assembly of ribulose-
Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.

Cheng, M. Y., F.-U. Hartl, J. Martin, R. A. Pollack, F. Kalousek, W. Neuert, and A. L. Horwich. 1989. Mitochondrial heat shock protein hsp60 is essential for assembly of proteins imported into yeast mitochondria. *Nature (Lond.)*. 337:620–625.

Chitin, P. R., E. Harel, E. D. Kohorn, E. M. Tobin, and J. P. Thornher. 1986. Assembly of the precursor and processed light-harvesting chlorophyll a/b protein of *Lemna* into the light-harvesting complex II of barley etiochloroplasts. *J. Cell Biol.* 102:982–988.

Chirico, W. J., M. G. Waters, and G. Blobel. 1988. 70K heat shock related proteins stimulate protein translocation into microsomes. *Nature (Lond.)*. 332:805–810.

Cline, K. 1986. Import of proteins into chloroplasts: membrane integration of a thylakoid precursor protein reconstituted in chloroplast lysates. *J. Biol. Chem.* 261:14804–14810.

Cline, K. 1988. Light-harvesting chlorophyll a/b protein: membrane insertion, proteolytic processing, assembly into LHC II, and localization to appressed membranes occurs in chloroplast lysates. *Plant Physiol.* 86:1120–1126.

Cline, K., D. R. Fulsom, and P. V. Viitanen. 1989. An imported thylakoid protein accumulates in the stroma when insertion into thylakoids is inhibited. *J. Biol. Chem.* 264:14225–14232.

Deshaies, R. J., B. D. Koch, M. Werner-Washburne, E. A. Craig, and R. Schekman. 1988. A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. *Nature (Lond.)*. 332:800–805.

Ellis, R. J., and S. M. Hemmingsen. 1989. Molecular chaperones: proteins essential for the biogenesis of some macromolecular structures. *Trends Biochem. Sci.* 14:339–342.

Fulsom, D. R., and K. Cline. 1988. A soluble protein factor is required in vitro for membrane insertion of the thylakoid precursor protein, pLHCP. *Plant Physiol.* 88:1146–1153.

Harlow, E., and D. Lane. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 471-510.

Hedrick, J. L., and A. J. Smith. 1968. Size and charge isomer separation and estimation of molecular weights of proteins by disc gel electrophoresis. *Arch. Biochem. Biophys.* 126:155–164.

Hemmingsen, S. M., C. Woolford, S. M. van der Vies, K. Tilly, D. T. Dennis, C. P. Georgopoulos, R. W. Hendrix, and R. J. Ellis. 1988. Homologous plant and bacterial proteins chaperone oligomeric protein assembly. *Nature (Lond.)*. 333:330–334.

Keegstra, K. 1989. Transport and routing of proteins into chloroplasts. *Cell.* 56:247–253.

Keegstra, K., L. J. Olsen, and S. M. Theg. 1989. Chloroplastic precursors and their transport across the envelope membranes. *Annu. Rev. Plant Physiol. Mol. Biol.* 40:471–501.

Kahlbrandt, W. 1984. Three dimensional structure of the light-harvesting chlorophyll a/b protein complex. *Nature (Lond.)*. 307:478–480.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head bacteriophage T4. *Nature (Lond.)*. 227:680–685.

Locke, S., R. Litt, T. Ziegelhofer, C. Georgopoulos, P. J. Bassford, C. A. Kumamoto, and W. Wickner. 1989. Three pure chaperone proteins of *Escherichia coli*, SecB, trigger factor and groEL, form soluble complexes with precursor proteins in vitro. *EMBO (Eur. Mol. Biol. Organ.)*. 8:2703–2709.

Lodish, H. F. 1988. Transport of secretory and membrane glycoproteins from the rough endoplasmic reticulum to the Golgi. *J. Biol. Chem.* 263:2107–2110.

Lubben, T. H., G. K. Donaldson, P. V. Viitanen, and A. A. Gatesby. 1989. Several proteins imported into chloroplasts form stable complexes with the groEL-related chloroplast molecular chaperone. *Plant Cell.* 1:1223–1230.

Marshall, J. S., A. E. DeRoche, K. Keegstra, and E. Vierling. 1990. Identification of heat shock protein hsp70 homologues in chloroplasts. *Proc. Natl. Acad. Sci. USA.* 87:374–378.

Mullet, J. E., R. R. Klein, and A. R. Grossman. 1986. Optimization of protein synthesis in isolated higher plant chloroplasts. Identification of paused translation intermediates. *Eur. J. Biochem.* 155:331–338.

Murakami, K., and M. Mori. 1990. Purified presequence binding factor (PBF) forms an import-competent complex with a purified mitochondrial precursor protein. *EMBO (Eur. Mol. Biol. Organ.)*. 9:3201–3208.

Murphy, D. J. 1986. The molecular organization of the photosynthetic membranes of higher plants. *Biochim. Biophys. Acta.* 864:33–94.

Musgrove, J. E., R. A. Johnson, and R. J. Ellis. 1987. Dissociation of the ribulosebisphosphate large-subunit binding protein into dissimilar subunits. *Eur. J. Biochem.* 163:579–534.

Ostermann, J., A. L. Horwich, W. Neupert, and F.-U. Hartl. 1989. Protein folding in mitochondria requires complex formation with hsp60 and ATP hydrolysis. *Nature (Lond.)*. 341:125–130.

Pfeffer, S. R., and J. E. Rothman. 1987. Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. *Annu. Rev. Biochem.* 56:829–852.

Reed, J., K. Cline, L. C. Stephens, K. O. Bacot, and P. V. Viitanen. 1990. Early events in the import/assembly pathway of an integral thylakoid membrane protein. *Eur. J. Biochem.* 194:33–42.

Rothman, J. E. 1989. Polypeptide chain binding proteins: catalysis of protein folding and related processes in cells. *Cell.* 59:591–601.

Roy, H. 1989. Rabisco assembly: a model system for studying the mechanism of chaperone action. *Plant Cell.* 1:1035–1042.

Schmidt, G. W., S. G. Bartlett, A. R. Grossman, A. R. Cashmore, and N.-H. Chua. 1981. Biosynthetic pathways of two polypeptide subunits of the light-harvesting chlorophyll a/b protein complex. *J. Cell Biol.* 91:468–478.

Smeekens, S., P. Weisbeek, and C. Robinson. 1990. Protein transport into and within chloroplasts. *Trends Biochem. Sci.* 15:73–76.

Steinback, K. E., J. E. Mullet, and C. J. Artuzen. 1982. Fractionation of thylakoid membrane protein complexes by sucrose density-gradient centrifugation. *In Methods in Chloroplasts Molecular Biology.* M. Edelman, R. B. Hallick, and N.-H. Chua, editors. Elsevier/North Holland Biomedical Press, Amsterdam. 863–872.

Viitanen, P. V., E. R. Doran, and P. Dunsmuir. 1988. What is the role of the transit peptide in thylakoid integration of the light-harvesting chlorophyll a/b protein? *J. Biol. Chem.* 263:15000–15007.

Wangemann, K., H. Paulsen, and J. Soll. 1990. Translocation of proteins into isolated chloroplasts requires cytosolic factors to obtain import competence. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 261:89–92.

Walter, P., and V. Lingappa. 1986. Mechanism of protein translocation across the endoplasmic reticulum membrane. *Annu. Rev. Cell Biol.* 2:499–516.