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

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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).[1] 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, ribulosebiphosphate-carboxylase.

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mined, and its functional significance investigated. We report that stromal LHCP is a stable, discrete ~120,000-D species. This contrasts with in vitro-synthesized LHCP which rapidly becomes aggregated and insertionally inactive. In vitro-synthesized LHCP can be converted into the soluble form by incubation with a stromal protein factor. Conversion is necessary to maintain LHCP in an insertionally competent state, suggesting that the stromal protein factor functions similarly to molecular chaperones. However, LHCP is not complexed with the chloroplastic hsp60 type chaperone. In addition to conversion, a further stroma-mediated reaction appears to be necessary for inserting LHCP into the thylakoid membrane because the isolated 120-kD form of LHCP requires fresh stroma to complete the integration process. These studies provide the first characterization of an intermediate of the assembly pathway for a nuclear-encoded thylakoid membrane protein and describe in part a function for the stromal requirement for insertion into the thylakoid bilayer.

Materials and Methods

Materials

Tritium-labeled leucine was from New England Nuclear (Boston, MA). Nigericin, valinomycin, and Miracloth were from Calbiochem (San Diego, CA). Mg-ATP, protein A-Sepharose 4B, β,γ-methylene ATP, and thermolysin were from Sigma Chemical Co. (St. Louis, MO). SP6 polymerase and RNasin were from Promega Biotec (Madison, WI). Percoll, G(5')ppp(5')G, and protein molecular weight standards were from Pharmacia Inc. (Piscataway, NJ). All other chemicals were reagent grade. The in vitro expression plasmids used in this study have been described. Plasmid psAB80XD/4 (Cline et al., 1989) harbors the AB 80 gene, which codes for the entire precursor to LHCP from pea. P2HPLC is a pUC18 plasmid that harbors the coding sequence for the mature form of LHCP from petunia, previously designated as Δ-TP (Viitanen et al., 1988), and was the generous gift of Dr. Paul Viitanen (DuPont, Wilmington, DE). Antibody to groEL was kindly provided by Drs. Thomas Lubben and George Lorimer (DuPont, Wilmington, DE).

Preparation of Radiolabeled pLHCP and LHCP

RNA for pLHCP and mature LHCP was transcribed with SP6 polymerase in vitro from Eco RI-linearized psAB80XD/4 and Xba I-linearized p2HPLC, respectively (Cline, 1988; Cline et al., 1989) and translated in the presence of [^{3}H]leucine in a wheat germ system (Cline et al., 1989). Translations were diluted two- to sixfold and adjusted to import buffer (0.33 M sorbitol, 0.05 M Hepes/KOH, pH 8) containing 30 mM leucine before use.

Preparation of Chloroplasts, Lysates, Thylakoids and Stroma

Intact chloroplasts were isolated from 9- to 13-d-old pea (*Pisum sativum* L. cv. Laxton's Progress 9) seedlings as previously described (Cline et al., 1989). Chloroplasts were osmotically lysed with HK buffer (10 mM Hepes/KOH, pH 8) or in HKM buffer (HK buffer plus 10 mM $MgCl_2$) (Cline, 1988). Thylakoids were prepared from lysates by centrifugation at 3,200 *g* for 8 min at 4°C and were washed once before use. Stroma was prepared from the resulting supernatant by further centrifugation at 42,000 *g* for 30 min at 4°C. Stromal extracts prepared from 0.5 mg chlorophyll/ml lysates are arbitrarily referred to as 1× stroma. Thylakoids adjusted to 0.5 mg chlorophyll/ml are referred to as 1× thylakoids.

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 [^{3}H]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).

Assays for the conversion to the soluble LHCP complex were performed by incubating 40 μl stromal preparation, 10 μl of 60 mM Mg-ATP, and 10 μl of adjusted [^{3}H]pLHCP or LHCP for 15 min at 25°C. Assays were then cooled on ice, supplemented with 10 μl of 50% glycerol, containing bromophenol blue, and subjected to nondenaturing PAGE/fluorography. Quantitation of integration and complex formation assays was accomplished by scintillation counting of radiolabeled proteins extracted from excised gel bands (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 $MgCl_2$) 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 radiolabeled 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 [^{3}H]leucine, 6 mM dithiothreitol, 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 apyrase. A stromal extract was obtained from the lysate and adjusted to 1.33× stroma, import buffer, 10 mM $MgCl_2$.

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

LHC II was isolated from pea thylakoid membranes according to Steinback et al. (1982). LHCP was purified from LHC II preparations by electroeluting 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 $MgCl_2$, 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 $MgCl_2$. 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 anti-groEL at a 1:2,000 dilution, anti-LHCP at a 1:3,000 dilution, or nonimmune 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-

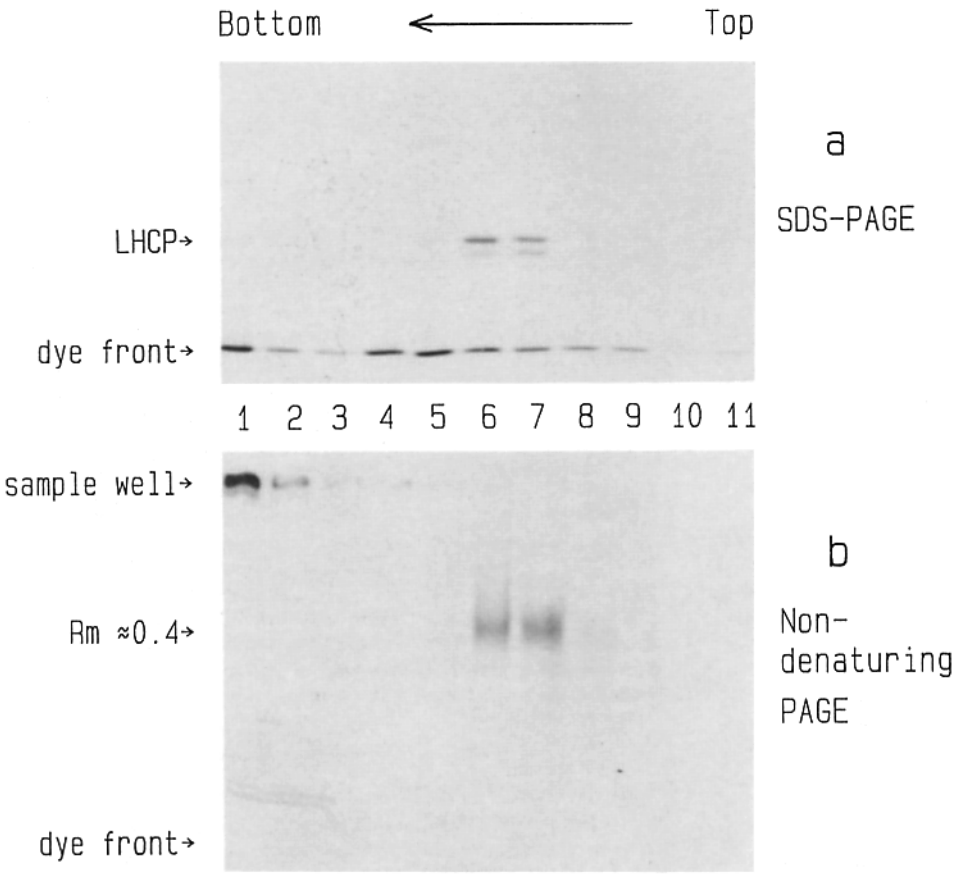


Figure 1. The stromal LHCP intermediate sediments through a glycerol gradient as a stable, discrete, species. Stromal LHCP was sedimented through a glycerol gradient and the fractionated gradient analyzed by SDS-PAGE/fluorography (*a*) and nondenaturing PAGE/fluorography (*b*). Stromal LHCP was prepared by import of in vitro-synthesized pLHCP into intact chloroplasts (300 μg chlorophyll) in the presence of 0.5 μM nigericin, 1.0 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 Percoll (Cline et al., 1989), lysed with 250 μl HK buffer plus 4 mM $MgCl_2$, and a stromal extract prepared.

purified antibodies to rabbit IgG (Kirkegaard & 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 wtih 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). Nondenaturing PAGE was conducted on 0.75 mm, 6% T/2.6% C polyacrylamide gels. Gels were prepared 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 observed during in vitro import of pLHCP into chloroplasts either 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 analysis 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 monitored by subjecting gradient fractions

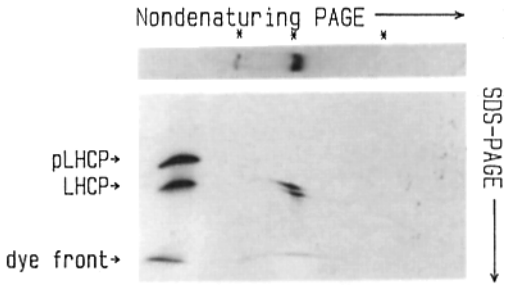


Figure 2. Analysis of the stromal LHCP intermediate by two-dimensional PAGE. A stromal extract containing the stromal LHCP intermediate was prepared as in Fig. 1 and was directly applied to nondenaturing PAGE. The gel lane was excised and subjected to second dimension SDS-PAGE/fluorography. The locations of sample well, Rm ~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.

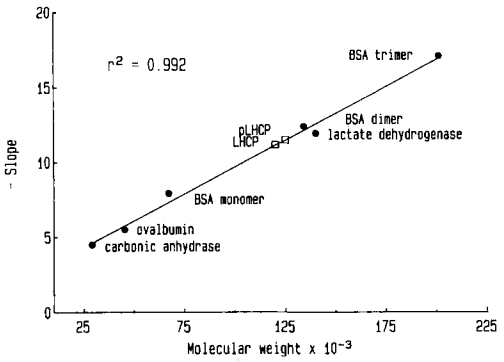


Figure 3. The apparent native molecular mass of the stromal LHCP intermediate is ~120,000 D as determined by Ferguson plot analysis. Stromal LHCP was prepared as described in Fig. 1. Soluble complex forms of in vitro-synthesized pHLCP or LHCP were prepared by incubation with stroma as described in Fig. 4. The native molecular weight of the soluble complex species was determined by electrophoresis on a series of nondenaturing gels polymerized from 6, 7, 8, 9, 10, and 11% acrylamide, with 2.6% cross-linking, at 10 mA/gel for 2 h at 7°C. Molecular weight standards were applied to the left half of each gel, which was subsequently stained with Coomassie blue R250. Radiolabeled (p)LHCP samples were applied to the right half of each gel, which was prepared for fluorography. The relative mobilities of the proteins were used to construct Ferguson plots and the slopes of these plots used to obtain the calibration line shown (Hedrick and Smith, 1968).

to nondenaturing PAGE/fluorography on 6% gels. A band with an Rm ~0.4 was observed in LHCP-containing fractions (Fig. 1 *b*).

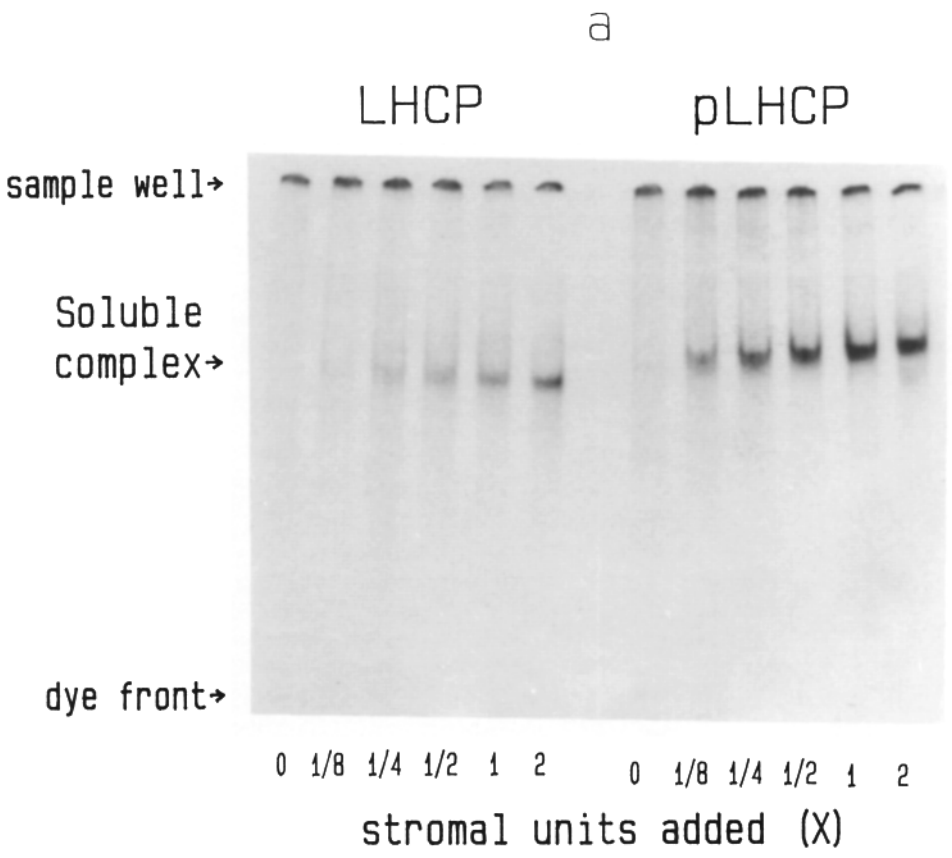
The radiolabel at the dye front of SDS-PAGE of most fractions (Fig. 1 *a*) is due to degradation products of LHCP, which are characteristic of import assays in which the thylakoid insertion step is inhibited (Cline et al., 1989). They appear to result from the action of an uncharacterized ATP-dependent protease in the stroma. Aggregated degradation products presumably give rise to the radiolabel in the sample wells of the nondenaturing PAGE (Fig. 1 *b*). However, in order to unambiguously identify the labeled component of the Rm ~0.4 band, an extract containing stromal LHCP was immediately subjected to two-dimensional gel analysis in which the first dimension was nondenaturing PAGE and the second dimension was SDS-PAGE (Fig. 2). Inspection of the two-dimensional gel revealed that all of the LHCP in this extract was present in the Rm ~0.4 band, thereby indicating that virtually all of the stromal LHCP exists in this form. These results were the first indication that stromal LHCP could readily be monitored as a discrete species by nondenaturing PAGE.

Stromal LHCP sedimented between the 4.7S BSA standard and the 7.3S lactate dehydrogenase standard (not shown). A better estimate of the native molecular mass of stromal LHCP was obtained from its migration behavior on a series of nondenaturing gels of different polyacrylamide concentrations according to Hedrick and Smith (1968). An M_r of ~120,000 D was obtained for stromal LHCP with this analysis (Fig. 3). Because LHCP has a monomeric M_r of 25,000, the stromal form of LHCP is due either to oligomerization of LHCP or to its association with another component. For convenience, we refer to this 120,000-D soluble LHCP as the soluble complex form of LHCP.

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 radiolabel 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 KCl). However, conversion was usually enhanced by supplementing reaction mixtures with either $MgCl_2$ 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 $MgCl_2$ 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 $MgCl_2$, ADP, or



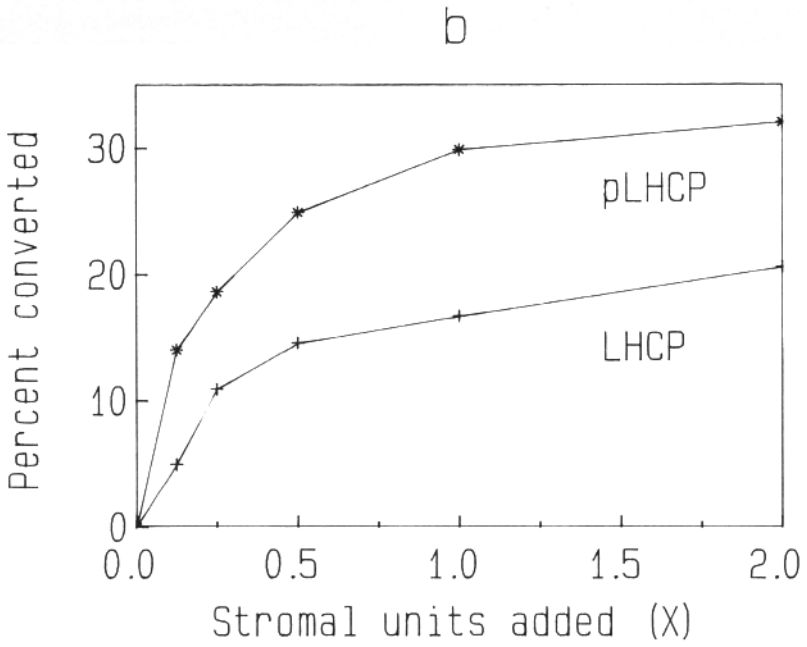


Figure 4. (p)LHCP is converted to the soluble complex form by incubation with a stromal extract. In vitro–synthesized pLHCP and in vitro–synthesized LHCP were incubated with a stromal extract and then analyzed for the presence of soluble complex form. Reaction mixtures received 10 μl (p)LHCP, 10 μl 60 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 $MgCl_2$ 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-

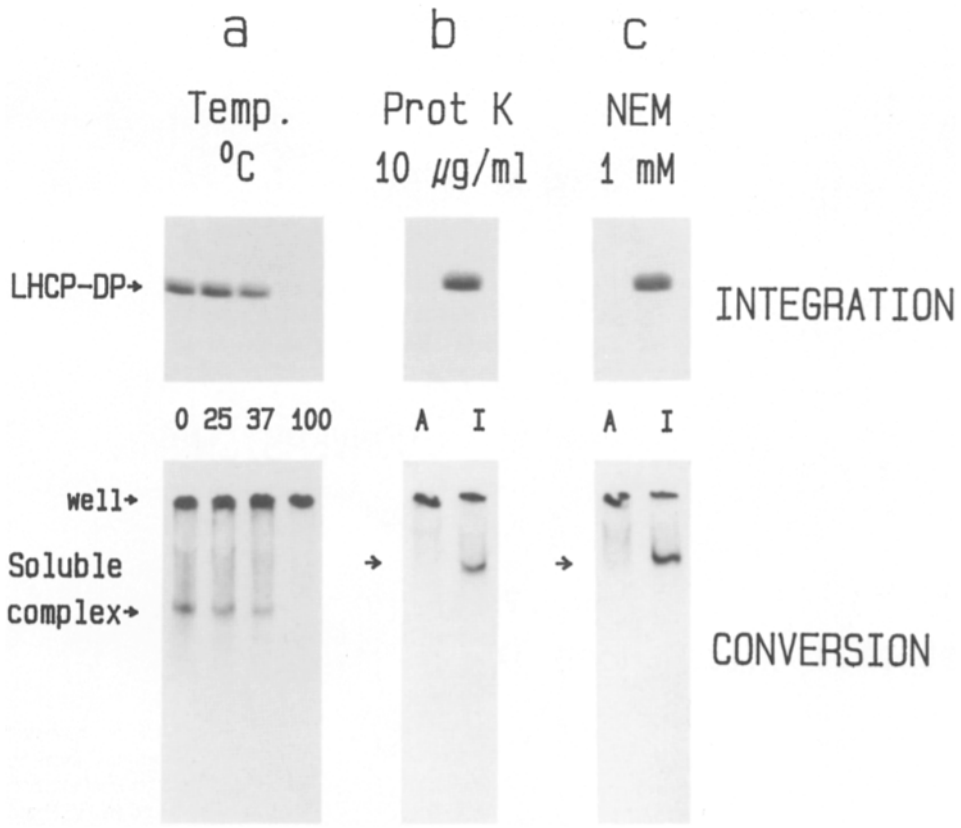


Figure 5. The stromal component that mediates soluble complex formation is sensitive to heat, proteinase K, and *N*-ethylmaleimide (*NEM*). Stroma was treated and then assayed for its ability to integrate [^{3}H]LHCP into isolated thylakoids (*top*) and to convert [^{3}H]LHCP into the soluble complex 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 deactivated 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.

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 pres-

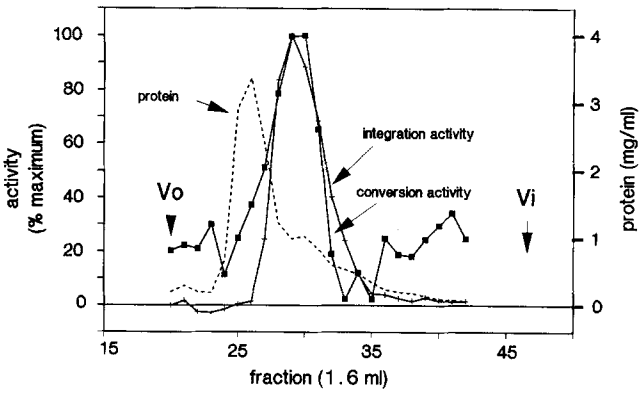


Figure 6. Converting activity of stroma elutes as a single peak from Sephacryl S300. Stroma was fractionated on a 1.5 × 46-cm Sephacryl S300 column and the fractions assayed for their ability to convert [^{3}H]LHCP into the soluble complex form and to integrate [^{3}H]LHCP into isolated thylakoids. A 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.

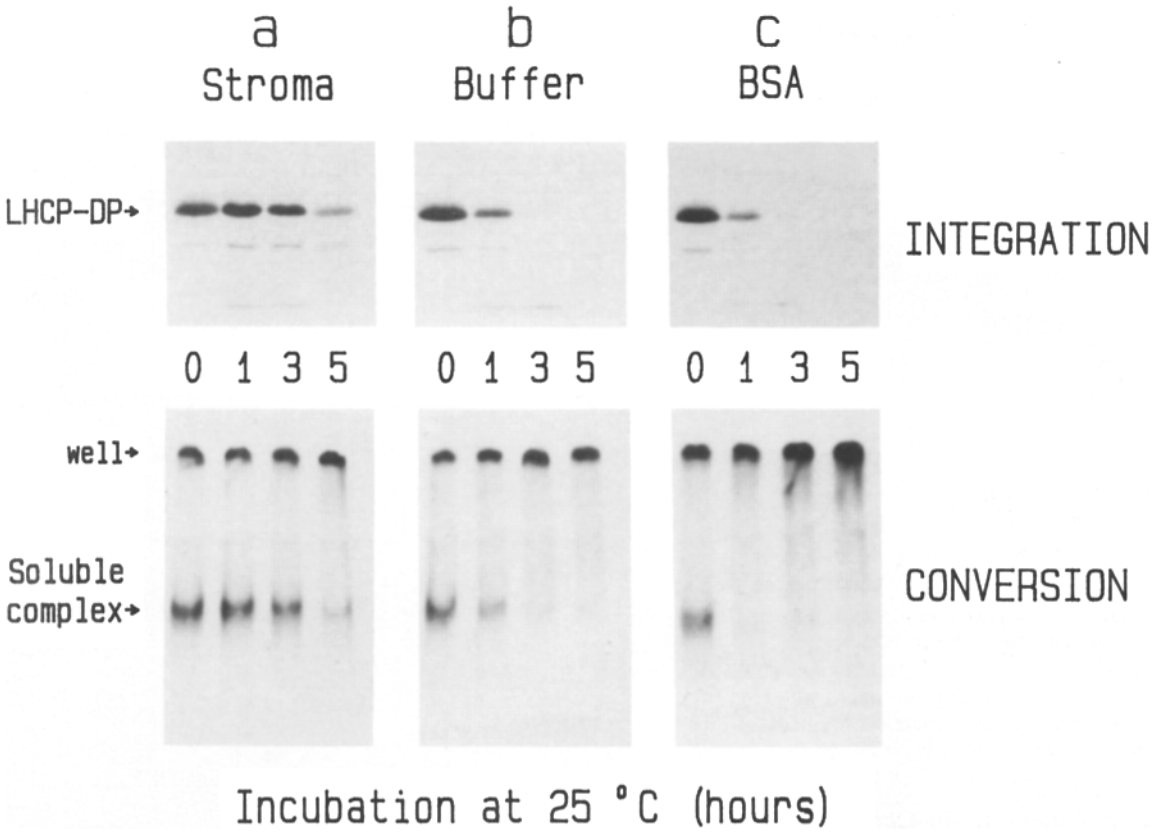


Figure 7. Stroma maintains LHCP competent for integration into thylakoids. pLHCP was incubated with stroma, buffer, or BSA at 25°C and its ability to integrate into isolated thylakoids was monitored with time. In vitro–synthesized pLHCP was combined with an equal volume of a 4× stromal extract prepared 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 $MgCl_2$. 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.

ence of fresh stroma (Fig. 7). In contrast, pLHCP incubated in the presence of stroma retained its ability to integrate for up to 5 h (Figs. 7 and 8). As expected, insertional capability correlated with the presence of the soluble complex (Fig. 7). The stabilizing effect of the stroma on pLHCP was a specific effect because BSA provided no stabilization over the "buffer alone" incubation. Further, the inability of fresh stroma to convert buffer- or BSA-incubated pLHCP into the complex form indicates that the stromal component cannot act as an "unfoldase" as was previously suggested (Smeekens et al., 1990).

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 pLHCP and a stromal extract was incubated at 25°C and later fractionated into soluble pLHCP complex and aggregated pLHCP by glycerol gradient sedimentation (Fig. 9 *a*). Aggregated pLHCP 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 pLHCP in fractions 6 and 7 was in the soluble complex form. The aggregated pLHCP and soluble pLHCP complex were assayed for their ability to integrate into isolated thylakoids. Aggregated pLHCP 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 pLHCP (Fig. 9 *b*).

In contrast, the pLHCP 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.

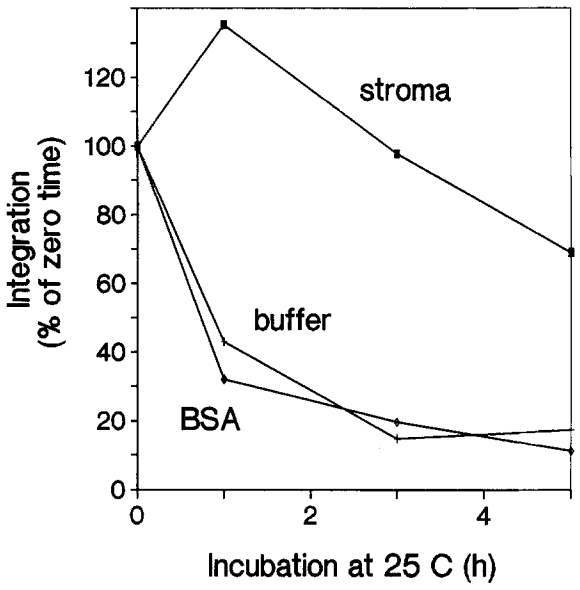


Figure 8. Quantification of integration competence of pLHCP incubated with and without stroma at 25°C. The ability of in vitro–synthesized pLHCP to integrate into isolated thylakoids after incubation at 25°C with stroma, buffer, or buffer containing BSA as shown in Fig. 7 was quantified (Materials and Methods) and displayed as a percentage of zero time integration for each sample.

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 organellar 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 LCHP 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 organello 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-

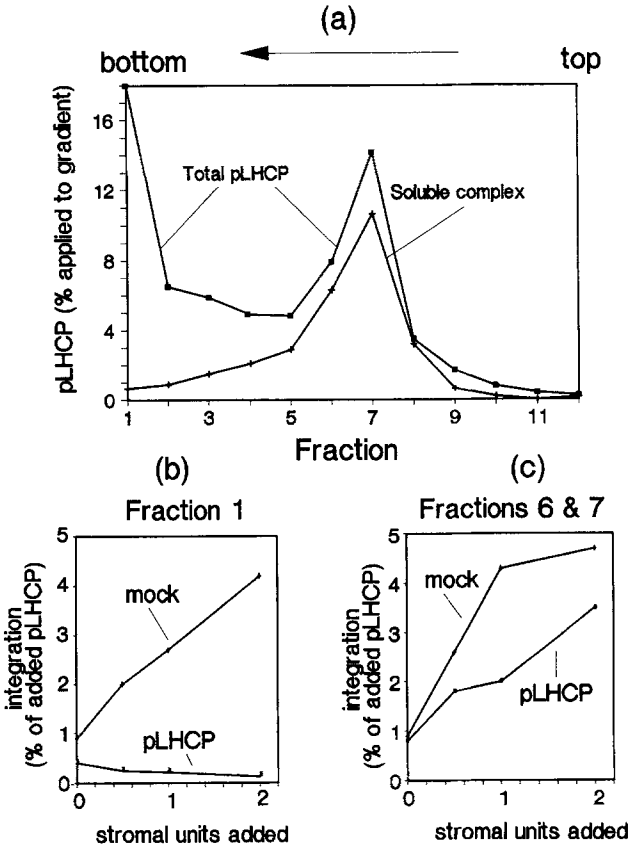


Figure 9. Only the soluble complex form of LHCP is competent for thylakoid integration. Aggregated and soluble pLHCP complex were independently assayed for their ability to integrate into thylakoids in the presence of increasing amounts of fresh stroma. A mixture of aggregated and soluble complex was prepared by incubating 100 μl [^{3}H]pLHCP with 400 μl of 2× stroma and 100 μl of 60 mM ATP for 15 min at 25°C. The mixture was fractionated by glycerol gradient sedimentation and assayed for the presence of pLHCP and soluble complex by quantitative SDS- and nondenaturing PAGE/fluorography, respectively. The aggregated pLHCP (*Fraction 1*) was diluted with 1 vol of buffer and the soluble pLHCP complex (*Fractions 6* and *7*) was pooled. As a control for the possible presence of inhibitory substances, stroma was incubated with a mock translation mixture (lacking pLHCP RNA), and this mixture was sedimented through a parallel gradient. Comparable fractions were collected and tested for their effects on integration of freshly prepared pLHCP. Integration assays included 140 μl fraction, stroma (from a 10× stock to final concentrations of 0.5, 1, and 2×) 25 μl 120 mM Mg-ATP, 50 μl thylakoids (4×), 25 μl of either freshly prepared pLHCP for the mock gradient fractions or mock translation mixture for the pLHCP gradient fractions, and HK buffer to a final volume of 300 μl per assay. (*a*) Amounts of pLHCP and soluble complex in each of the fractions from the pLHCP gradient. (*c* and *d*) Integration into isolated thylakoids using fraction 1 and fractions 6+7 from pLHCP and mock gradients, respectively.

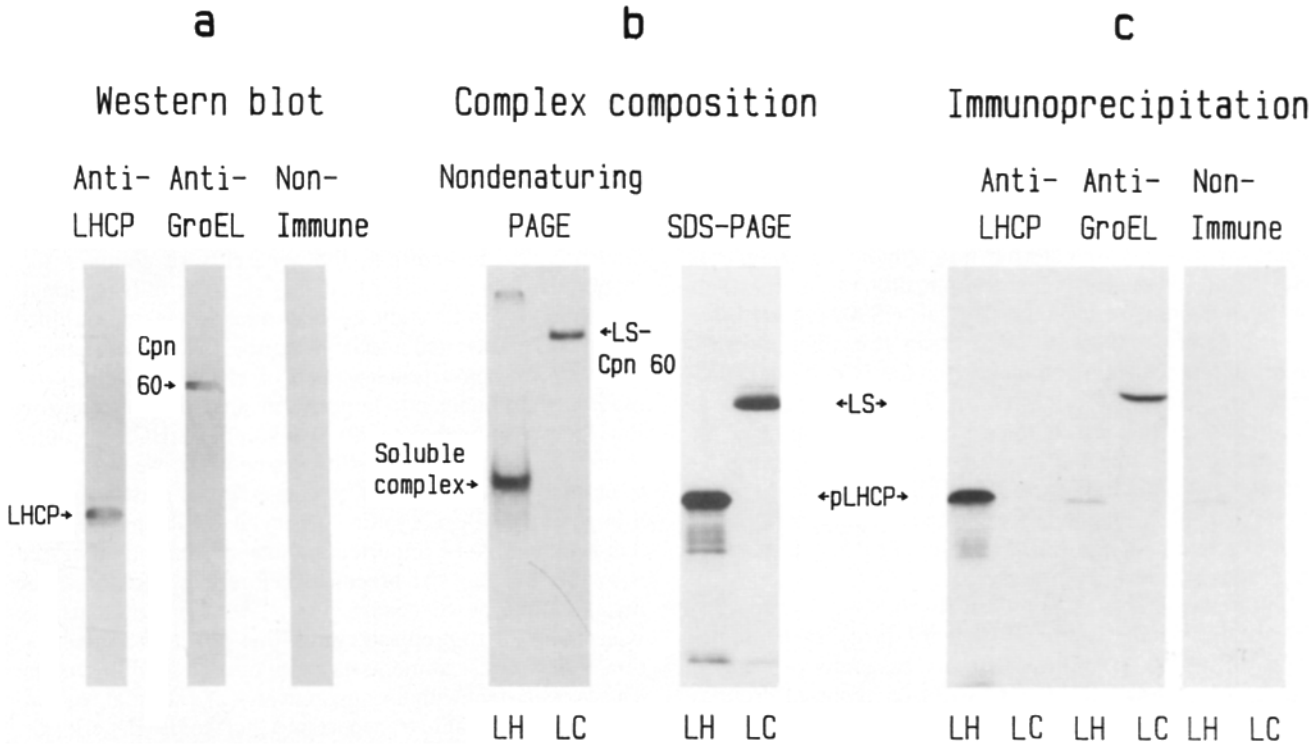


Figure 10. Soluble LHCP is not complexed with chaperonin 60 as determined by immunoprecipitation with antibodies to groEL. Soluble (p)LHCP complex was subjected to immunoprecipitation analysis with anti-groEL antibodies. As controls, a Rubisco LS-cpn60 complex was immunoprecipitated with anti-groEL, 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 (*a*). Soluble pLHCP complex (*LH*) was prepared by incubating [^{3}H]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 [^{3}H]leucine (Materials and Methods). Analysis of complexes by SDS-PAGE/fluorography (*b*) demonstrated the radiochemical purity of pLHCP and LS. Nondenaturing PAGE (*b*) 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 (*c*).

vestigation. 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 LHC II antenna complex in the thylakoid membranes is thought to be trimeric (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

been described in a variety of biological systems (Chirico et al., 1988; Deshaies et al., 1988; Lecker et al., 1989; Osterman 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 chloroplast (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 (Smeekens 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.

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