

Functional and Mutational Analysis of the Light-harvesting Chlorophyll *a/b* Protein of Thylakoid Membranes

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Abstract. The precursor for a *Lemna* light-harvesting chlorophyll *a/b* protein (pLHCP) has been synthesized in vitro from a single member of the nuclear LHCP multigene family. We report the sequence of this gene. When incubated with *Lemna* chloroplasts, the pLHCP is imported and processed into several polypeptides, and the mature form is assembled into the light-harvesting complex of photosystem II (LHC II). The accumulation of the processed LHCP is enhanced by the addition to the chloroplasts of a precursor and a co-factor for chlorophyll biosynthesis. Using a model for the arrangement of the mature polypeptide in the thylakoid membrane as a guide, we have created mutations that lie within the mature coding

region. We have studied the processing, the integration into thylakoid membranes, and the assembly into light-harvesting complexes of six of these deletions. Four different mutant LHCPs are found as processed proteins in the thylakoid membrane, but only one appears to have an orientation in the membrane that is similar to that of the wild type. No mutant LHCP appears in LHC II. The other two mutant LHCPs cannot be detected within the chloroplasts. We conclude that stable complex formation is not required for the processing and insertion of altered LHCPs into the thylakoid membrane. We discuss the results in light of our model.

IN higher plants light-harvesting complexes (LHCs)¹ located in the chloroplast thylakoid membrane transfer absorbed light energy to photochemical reaction centers (19, 46). The major protein component of the LHC of photosystem II (LHC II) of green plants is encoded by a nuclear gene family (7, 14, 15, 25, 29, 42, 47). This polypeptide, the light-harvesting chlorophyll *a/b* apoprotein (LHCP), is translated in the cytoplasm on membrane-free polyribosomes as a precursor polypeptide. It is subsequently imported into the chloroplast (3, 13, 20, 42) and then complexed with chlorophyll and carotenoid molecules to yield a processed mature thylakoid membrane protein (10, 16).

Recently, much attention has been given to the transport of nuclear coded proteins into chloroplasts (13, 34, 39, 40, 43, 50). It is now clear that amino-terminal transit peptides direct the precursors to the correct organelle (43, 50) and that the chloroplast outer envelope plays a role in the recognition of the precursors (13, 22). It has been shown for one protein, the small subunit of ribulose biphosphate carboxylase/oxygenase (SSU), that the transit peptide is cleaved from the

mature protein by a stromal specific protease in a two-step process (39, 40). SSU is subsequently assembled with the large subunit of the enzyme to yield a functional unit (9).

Less, however, is known of the events that follow the uptake of thylakoid membrane proteins into the chloroplast. For example, it remains to be determined how LHCP integrates specifically into thylakoid membranes, becomes a member of a holocomplex, and binds chlorophyll and carotenoid molecules. Moreover, depending on the species, mature LHCP is found in at least two forms that differ slightly in electrophoretic mobility on denaturing gels (7, 15, 42, 44). It has been suggested that the source of the multiple forms lies with the members of the gene families that encode LHCP (7, 14, 15, 25, 42, 47), but the situation remains unresolved.

To follow the maturation and assembly of LHCP into chlorophyll-protein complexes, we have studied the uptake and processing of LHCP precursor (pLHCP) by isolated chloroplasts. We have developed an assay for LHC assembly that uses LHCP expressed in vitro from a *Lemna gibba* genomic clone and chloroplasts isolated from *Lemna* tissue. We have previously isolated several LHCP genomic coding sequences from *Lemna* (47), and have reported the sequence of one that contains an intron (25). Here we present the sequence of a second *Lemna* genomic clone that does not have an interrupted coding region. Using this gene as template for in vitro RNA and protein synthesis, we show that a single member of the LHCP gene family can give rise to multiple mature forms

¹ Abbreviations used in this paper: ALA, 5-aminolevulinic acid; LHC, light-harvesting complex; LHC II, LHC of photosystem II; LHCP, light-harvesting chlorophyll *a/b* protein; pLHCP, LHCP precursor; RB, resuspension buffer (100 mM Tricine, pH 7.9, 300 mM glycerol, 1 mM MgCl₂, 1 mM dithiothreitol); 100 mM Tricine, pH 7.9, 300 mM glycerol, 1 mM MgCl₂, 1 mM dithiothreitol; RBE, RB containing 5 mM Na₂EDTA; SAM, S-adenosyl methionine; TE, 100 mM Tricine, pH 7.9, 5 mM Na₂EDTA, 1 mM phenylmethylsulfonfyl fluoride.

in the LHC II of the thylakoid membranes of isolated chloroplasts.

From both the deduced amino acid sequence of the *Lemna* clones and some experimental observations, we have previously predicted the conformation of the mature polypeptide in the thylakoid membrane (25). Our model is in accord with electron microscope studies (26, 31). Three α -helical portions in LHCP are predicted to form membrane-spanning segments, with the amino terminus exposed to the stroma, and the carboxy terminus within the lumen. Using this model as a guide, we have created deletions of the LHCP mature coding sequence, and have studied the assembly of the corresponding mutant proteins into thylakoid membranes of isolated chloroplasts.

Materials and Methods

Molecular Cloning and Sequencing

An Eco RI/Bam HI 1.6-kb DNA fragment homologous to a sequenced LHCP genomic clone pAB19 (25) was isolated from a 9-kb *Lemna* genomic segment, AB30 (47). This 1.6-kb piece was cloned into pUC8 (thereby creating pAB30) and sequenced using the chemical method of Maxam and Gilbert (33). The Eco RI, Bam HI, Bgl III (position 803), and Xho I sites (positions 334 and 1112) were labeled with either polynucleotide kinase (PL Biochemicals, Piscataway, NJ) using γ -³²P-ATP (ICN, Irvine, CA), or the Klenow fragment of *Escherichia coli* DNA polymerase (Bethesda Research Laboratories, Gaithersburg, MD) using the appropriate α -³²P-dNTP, and the fragments isolated by electrophoresis through low temperature gelling agarose (LGT, Marine Colloids, Rockland, ME). Both strands of the fragment were sequenced, except for the ~50 base pairs at either end of the clone.

In Vitro Transcription and Translation

We determined by S1 nuclease mapping (25) that the site of in vivo transcription initiation is within a few nucleotides of a unique SstI site at position 351 of pAB30. The 3' terminus of the message lies ~300 bases upstream of the Bam HI site. The SstI/Bam HI fragment was cloned into the SstI/Bam HI sites of pSP65 to create psp65ab30, in which the AB30 transcription unit is downstream of the SP6 RNA polymerase promoter (18). psp65ab30 was linearized with Hind III, which cleaves downstream of the plasmid insert, to provide a template for transcription. The transcription reaction included 100 μ g/ml DNA, 40 mM Tris (pH 7.5), 6 mM MgCl₂, 2 mM spermidine, 0.5 mM each of ATP, GTP, CTP, UTP, 10 mM dithiothreitol, 3 U/ml RNasin (Promega Biotec, Madison, WI), and 150 U/ml SP6 RNA polymerase (New England Nuclear, Boston, MA). The reaction was terminated after 1 h at 37°C by the addition of DNase to 0.2 mg/ml and incubation for 10 more min at 37°C. The reaction was then phenol extracted twice, ethanol precipitated, the RNA resuspended at 1 mg/ml in water, and stored at -70°C. Typically we recovered 10 μ g of RNA per μ g of linear template.

Translation of the uncapped AB30 RNA was performed in a wheat germ extract (38) treated with micrococcal nuclease (36a) in the presence of [³⁵S]-methionine (800 Ci/mmol). 1–2 μ g of RNA was added for each 50- μ l translation reaction, and under these conditions we recovered 20,000–50,000 cpm/ μ l of translation mix. Reactions were terminated by freezing and storage in liquid nitrogen.

Chloroplast Isolation

L. gibba plants were grown in darkness with intermittent red illumination (2 min/8 h) for 6 wk on E medium with sucrose (48). The plants were transferred to E medium lacking sucrose and placed in continuous white light for 24 h before harvesting. We refer to these plants as greening tissue. Chloroplast preparations from plants depleted of sucrose and exposed to white light for shorter periods of time were less efficient in incorporating LHCP. All isolation steps were performed at 4°C. Chloroplasts from ~10 g of greening tissue were used for each uptake reaction. Plants were harvested after 24 h of illumination and ground for 20 s in a Waring blender equipped with razor blades (24) in a buffer (23) containing 1.25% Ficoll-400 (Sigma Chemical Co., St. Louis, MO), 2.5% Dextran-40 (Sigma Chemical Co.), 1% bovine serum albumin (BSA, fraction V) (Sigma Chemical Co.), 12.5 mM Tris (pH 7.6), 5 mM MgCl₂, 440 mM sucrose, and 10 mM β -mercaptoethanol. The solution was filtered through 60- μ m nylon mesh (Nitetex) and centrifuged at 3,000 rpm in a Sorvall GSA rotor for 1 min. The pellet was resuspended in resuspension buffer (RB) (100 mM

Tricine, pH 7.9, 300 mM glycerol, 1 mM MgCl₂, 1 mM dithiothreitol), aliquoted into glass 15-ml Corex tubes, and centrifuged at 3,500 rpm in a Sorvall SS34 rotor for 1 min. Each test tube received ~50 μ g of protein and 100 μ g of chlorophyll as determined by the methods described in references 6 and 2, respectively.

pLHCP Import

The chloroplast pellet was resuspended in 100 μ l of RB and 65 μ l of a solution containing 1 M glycerol, 0.3 M Tricine, pH 7.9, 40 mM methionine, 4 mM ATP, 4.5 mM S-adenosyl methionine (SAM), and 4.5 mM 5-aminolevulinic acid (ALA). 125 μ l of translation products were thawed and added to the chloroplast suspension and the mixture was incubated at 25°C for 60 min with constant but gentle agitation. The test tubes were illuminated at ~30 μ Einstein/m² per s (cf. reference 8). The samples were also rotated every 15 min to keep the chloroplasts suspended. The reaction was terminated by the addition of 2 ml of RB, centrifugation for 1 min at 5,500 rpm, and resuspension of the pellet in 500 μ l of RB. Thermolysin was added to 200 μ g/ml, CaCl₂ to 5 mM, and the reaction incubated at 4°C for 30 min. The suspension was then underlaid with 2 ml of RBE (RB containing 5 mM Na₂EDTA) with 25% Percoll (Sigma Chemical Co., St. Louis, MO), 0.25% BSA (fraction V, Sigma Chemical Co.), Ficoll-400 (Sigma Chemical Co.), and 0.75% polyethylene glycol 8000 (Sigma Chemical Co.) and centrifuged for 3 min at 6,000 rpm in a Sorvall SS34 rotor. Broken chloroplasts remained above the Percoll layer, while intact organelles pelleted (20). This observation was confirmed by the fact that breakage of all the chloroplasts, obtained by dilution with water and vigorous vortexing before centrifugation through Percoll, resulted in no green pellet and a large green interface above the Percoll. The pelleted, intact organelles were then washed with 2 ml of RBE containing 1 mM phenylmethylsulfonyl fluoride (α -toluenesulfonyl fluoride), recentrifuged at 6,000 rpm, and resuspended and vortexed in TE (100 mM Tricine, pH 7.9, 5 mM Na₂EDTA, 1 mM phenylmethylsulfonyl fluoride). The suspension was centrifuged at top speed in a Fisher microfuge (15,000 g), and the soluble proteins (stroma fraction) precipitated with 10% trichloroacetic acid. The pellet of thylakoid membranes was resuspended in a denaturing buffer (4% SDS, 20% glycerol, 0.2 M dithiothreitol, 5 mM Na₂EDTA, 0.70 mM Tris, pH 6.8, 0.01% bromophenol blue) and incubated at 55°C for 60 min before electrophoresis. This method of denaturation was necessary because boiling causes starch to become viscous, and the sample could not otherwise be completely denatured. The stromal fraction was pelleted in the microfuge, and washed with 80% acetone, dried under vacuum, and dissolved by boiling for 2 min in denaturing buffer. SDS PAGE (28) and fluorography (30) were as previously described.

Chlorophyll-Protein Complex Analysis

Isolated thylakoid membranes (see above) were resolved into complexes according to the method of Peter, G. and J. P. Thornber (manuscript in preparation). Thylakoids were gently resuspended in 25 μ l of a solution containing 6 mM Tris-base, 50 mM glycine, 10% glycerol, and incubated for 15 min at 4°C. 2.5 μ l of a solution containing 3% SDS, 7% octylglucoside was then added, and the suspension was incubated for an additional 10 min at 4°C. Samples were then applied to a 6% polyacrylamide gel containing 25 mM Tris-base, 200 mM glycine, and 0.4% Deriphat-160 (McKerson's Chemical Laboratories, Minneapolis, MN). The reservoir buffer contained 12 mM Tris-base, 100 mM glycine, 0.2% Deriphat-160, and 0.02% SDS. For electrophoresis in a second, denaturing dimension, gel strips were placed horizontally above a 12.5% Laemmli SDS acrylamide gel (28), and run at 40 mA at 42°C.

Trypsin Treatment of Thylakoid Membranes

After gentle resuspension of the thylakoid pellet in 100 μ l of TE (without phenylmethylsulfonyl fluoride), trypsin (TPCK-treated) (Sigma Chemical Co.) was added to 0.25 mg/ml. The reaction was incubated for 15 min at 25°C. The digestion was then diluted with 1 ml of TE, phenylmethylsulfonyl fluoride was added to 1 mM, and the mixture was centrifuged for 10 min at 15,000 g. The pellet was resuspended in denaturing buffer, incubated for 1 h at 55°C, and subjected to electrophoresis on denaturing gels.

Western Blotting

Electrophoresis (28), electrophoretic transfer to nitrocellulose (5), and immunodetection (49) were performed as described. The LHCP monoclonal antibody was raised against nondenatured protein of tobacco LHC II and will be described in detail elsewhere (46a).

Deletion Construction

The Bam HI site of psp65ab30 was eliminated by digestion of the cloned DNA

with Bam HI, creating flush ends with the Klenow fragment of *E. coli* DNA polymerase and four dNTPs, followed by religation. The resulting clone, psp65ab30B-, was digested with either Eco RI (5' terminus of insert), or in a separate reaction, with Hind III (3' terminus). The linearized DNA was treated with exonuclease Bal 31 (Bethesda Research Laboratories) for varying amounts of time, and Bam HI linkers were ligated to the deletion termini. The Eco RI sample was digested with Hind III, and the Hind III sample with Eco RI; then the plasmid inserts of various lengths were isolated by electrophoresis in LGT agarose. The Eco RI/Bam HI or Hind III/Bam HI fragments were then cloned into pSP65, and analyzed by gel electrophoresis. Inserts of varying size were sequenced (33) from the Bam HI site to determine the extent of the deletion. Inframe LHCP coding sequences for mutants D, E, F, K, and I were recreated by combining the Eco RI/Bam HI and Bam HI/Hind III fragments at the Bam HI sites and then cloning into the Eco RI and Hind III sites of pSP65. Mutant J was created in a similar fashion except that the Bam HI ends were made flush with Klenow polymerase and four dNTPs to allow for an in-frame coding sequence. All recreated LHCP coding regions therefore have a Bam HI poly-linker within the coding sequence which contributes additional amino acids. The sequence end points (minus Bam HI linker) for each deletion are as follows, with the amino-proximal break point first, the carboxy-proximal last. The amino acids that are contributed by the Bam HI linker are listed in the single-letter code after the numbers (D:741-821, GS; E:803 (BgIII of pab30)-932, IR; F:687-821, GS; I:895-1008, RIR; J:1053-1098, GSIR; K:1109-1168, DP). The positions of the deletions in relation to the rest of the mature protein are also illustrated in Fig. 5.

Results

LHCP Sequence

We have isolated several LHCP coding sequences from *L. gibba* (47) and have studied the expression of two, AB19 and AB30 in detail. Previously, we reported the nucleotide sequence of AB19 which contains a short intron having the

characteristics of a transposable element (25). Here we present the sequence of AB30, which does not have an interrupted coding region. A probe homologous to the 3' untranslated region of the predicted mRNA for AB30 hybridizes to an RNA species of 1,200 bases that is present at a high concentration in light-grown plants and at much reduced levels in plants which have been placed in darkness for a week (data not shown). Fig. 1 shows the nucleotide and predicted amino acid sequence of AB30, and compares this genomic fragment with AB19. We have found a short region of nucleotide sequence upstream from the TATA box which is identical in AB19 and AB30 (asterisks in Fig. 1) and another that is homologous in both *Lemna* and *petunia* Cab genes (boxed region Fig. 1; cf. reference 15). We do not know the significance, if any, of these observations.

Within the mature LHCP coding sequence, 85% of the amino acids are conserved between AB19 and AB30. However, little homology is found in the first 20 residues of the amino-terminal portion of the mature polypeptides. Both AB19 and AB30 contain a proposed site of phosphorylation, a threonine (position 504), that is thought to be involved in thylakoid membrane stacking (1, 4, 45). The two transit peptides differ in their predicted hydrophobicities (27) yet do exhibit the three blocks of homology common to all known chloroplast transit sequences (25a). We do not yet know whether the presence of the two distinct transit sequences and amino termini of the mature polypeptides is of any functional significance.

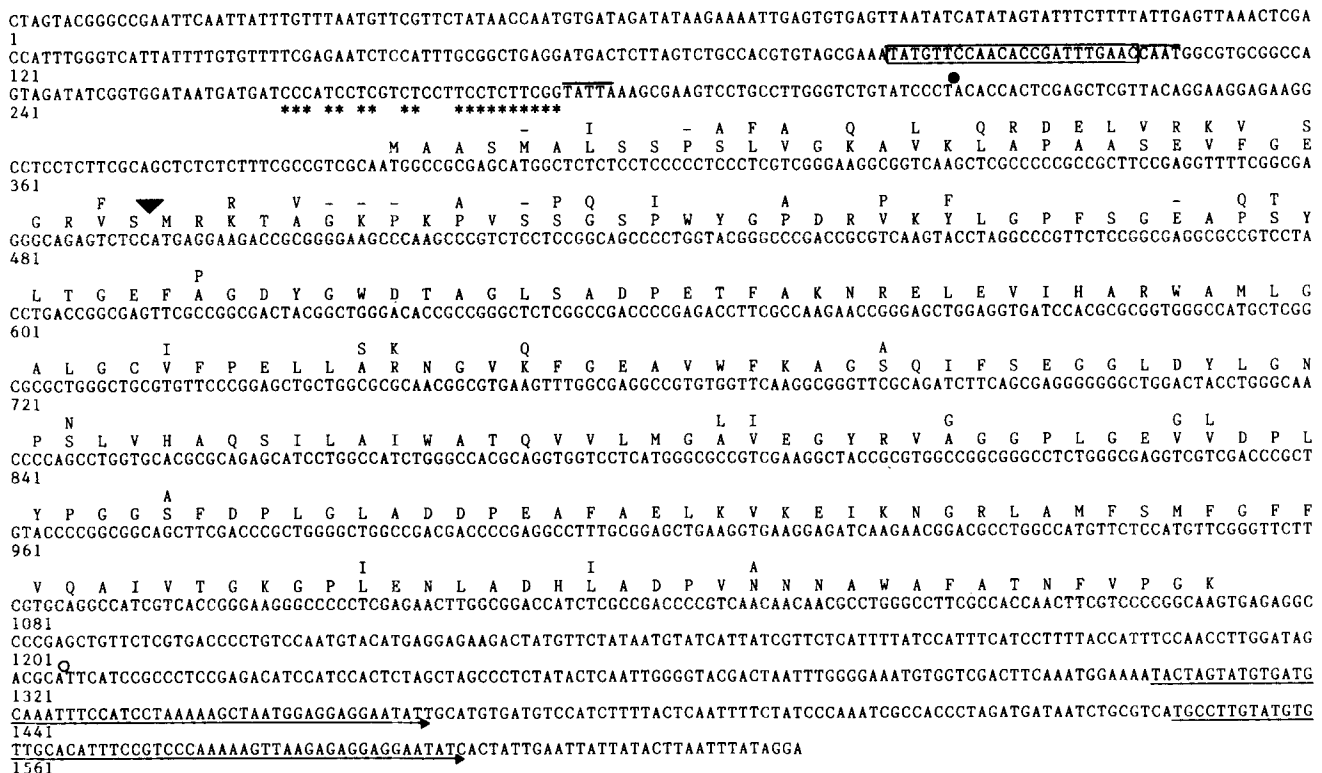


Figure 1. The nucleotide and derived amino acid sequence of AB30, and a comparison with AB19 (25). Above the noncoding DNA strand is the complete amino acid sequence for AB30. Amino acids that are different in AB19 are indicated above those for AB30, and a (-) indicates a deletion at that position. ▼ indicates the proposed site of processing (25). An asterisk (*) indicates identical bases in AB30 and AB19 that are upstream of the site of transcription initiation. The box encloses a region of homology found between the *Lemna* clones and Cab genes of *petunia* (15). The putative TATTA and CAAT boxes are overlined. A solid ball (●) and an open circle (○) denote the approximate sites of transcription initiation, and termination, respectively. Arrows appearing at the 3' end of the sequence show an imperfect direct repeat.

In Vitro Expression of pLHCP

We chose AB30 as template for *in vitro* pLHCP RNA production because this clone represents an uninterrupted coding sequence for a pLHCP. We isolated a genomic fragment, whose 5' terminus coincides with the *in vivo* transcription start site (Sst I site at 338), that contains a complete transcription unit and that extends ~300 bp beyond the *in vivo* poly (A) addition site to a Bam H1 site. This fragment was inserted into the Sst I/Bam H1 sites of pSP65 to create the plasmid psp65ab30. The *in vitro* expression of this clone is shown in Fig. 2. Transcription of Hind III-digested psp65ab30 with SP6 RNA polymerase (18) produced a ~1,300 base uncapped RNA that contains an LHCP transcription unit and an additional 300 bases at the 3' terminus (Fig. 2a). This AB30 RNA was then translated in a wheat germ extract to incorporate [³⁵S]methionine into a pLHCP that migrated at the same position (32 kD) observed for the pLHCP in *Lemna* poly (A) RNA translation products (48). The predicted molecular weight of pLHCP from the nucleotide sequence is 28,735. This size is lower than that predicted from its mobility in denaturing gels. The AB30 translation product can be immunoprecipitated with the LHCP monoclonal antibody (data not shown).

Uptake and Processing of pLHCP by Chloroplasts

We next sought to incorporate the pLHCP synthesized *in vitro* into the LHC of *Lemna* thylakoids. In an attempt to mimic the *in vivo* process as well as possible, we used chloroplasts from the same organism, and therefore conditions were established for the isolation of intact active *L. gibba* chloroplasts. Several common isolation buffers were tested

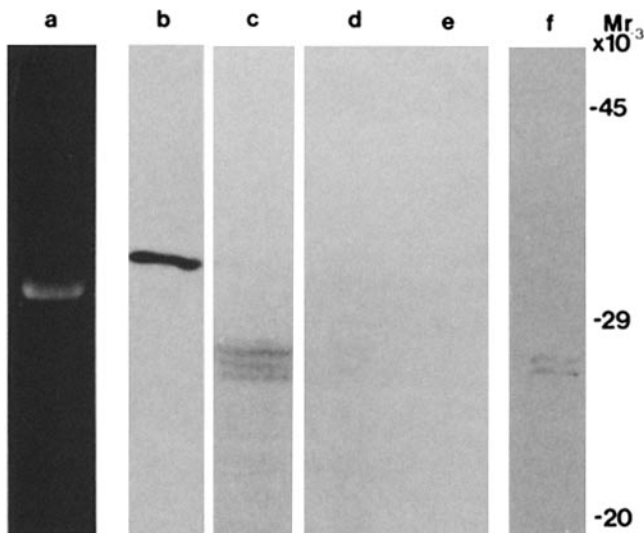


Figure 2. *In vitro* transcription, translation, and incorporation of pLHCP into thylakoid membranes. (a) Ethidium bromide stain of a denaturing agarose gel (37) showing 1 μ g of pLHCP RNA synthesized with SP6 RNA polymerase. b–e show a fluorograph of a denaturing 12.5% polyacrylamide gel containing: (b) the products of the translation of this pLHCP RNA in a wheat germ extract; (c) the thylakoid fraction of chloroplasts incubated with pLHCP; (d) same as in lane c, except the uptake reaction lacked ALA and SAM; (e) the stroma fraction of the same incubation shown in lane c; and (f) immunological staining of thylakoids from mature plastids with LHCP monoclonal antibody.

(20), but the use of a nuclear isolation buffer (23) provided the highest yield of intact, functional chloroplasts. Labeled pLHCP was incubated with a crude chloroplast preparation isolated from greening plants (see Materials and Methods). After 60 min, the reaction mixture was treated with the protease thermolysin to digest any protein that was not located within the chloroplasts. Protein within intact organelles remains undigested as thermolysin does not cross the envelope (12). We and others (8, 13) have chosen thermolysin because this protease causes less damage to the envelopes and therefore less plastid breakage compared with the more commonly used trypsin (20). After separation of intact chloroplasts from those broken during isolation and incubation, thylakoid and stromal fractions were prepared from them. The results of the uptake of pLHCP by *Lemna* chloroplasts are shown in Fig. 2. Fluorography of gels displaying the chloroplast compartments showed that labeled protein was associated only with the thylakoid fraction and migrated with a mobility that was identical to that of the mature LHCP (Fig. 2c). We conclude that pLHCP can be incorporated and processed by *Lemna* chloroplasts.

A striking observation is the detection of three radioactive polypeptides that differ slightly in electrophoretic mobility (Fig. 2c). LHCP monoclonal antibody reactions (Fig. 2f) and Coomassie Blue staining of denatured thylakoids from greening chloroplasts reveal only two prominent bands, and these correspond in size to the two faster migrating radioactive LHCPs. The slowest, third radioactive band that is of equivalent intensity to the other two bands on the fluorograph is not detected by the monoclonal antibody or Coomassie Blue staining of thylakoids from greening plants. A band which corresponds in size to the slowest migrating radioactive LHCP (Fig. 2c) could only be detected with the monoclonal antibody or Coomassie Blue stain in thylakoids prepared from mature chloroplasts (data not shown). Such plastids, isolated from *Lemna* grown on sucrose in continuous white light, contain higher levels of LHCP (48). In most *in vitro* uptake experiments using chloroplasts isolated from greening tissue, we observe three radioactive bands of approximately equal intensity, but in some cases the number and intensities of the bands vary.

We also considered the possibility that the various forms arise through the degradation of a single species during the isolation procedure. We therefore fractionated and fluorographed a sample of the uptake reaction mixture taken before the preparation of thylakoid and stroma fractions. These results are shown in Fig. 3b. Three radioactive bands were isolated from this early stage of the procedure, indicating that the multiple forms arise before the fractionation of the chloroplast. We have also added translation products to the pellet of intact chloroplasts, and we noted that the pLHCP did not undergo degradation during subsequent fractionation (Fig. 3a). Thus the three bands are not an artifact of the isolation, and we are left to conclude that the multiple LHCPs arise during the uptake and processing steps.

We wished to determine whether one LHCP band accumulated before any of the others to see whether the higher molecular forms gave rise to the lower forms. We therefore incubated the chloroplasts with pLHCP for varying amounts of time. The results of this analysis are shown in Fig. 3, c–f. The intensity of each of the LHCP bands increases over 1 h of incubation and each variant accumulates at a similar rate.

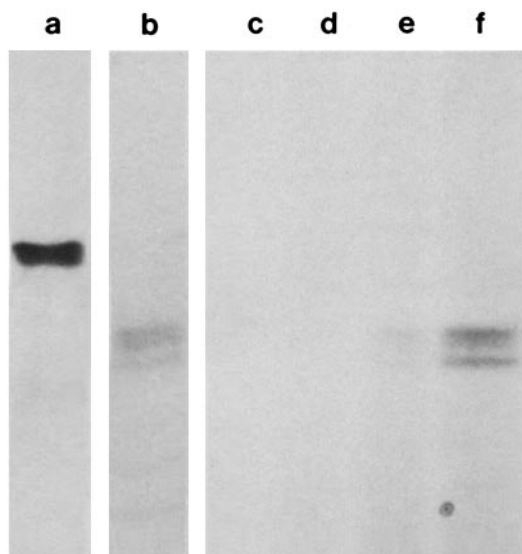


Figure 3. Evidence that the multiple LHCP bands arise during pLHCP import and processing by chloroplasts. Fluorograph of a gel containing: (a) pLHCP incubated with pelleted intact chloroplasts and fractionated with the thylakoid membranes; (b) a sample of the uptake reaction prior to chloroplast disruption and fractionation; (c–f) thylakoid preparations from intact chloroplasts incubated with pLHCP for 0, 5, 20, or 40 min, respectively.

Therefore, no precursor–product relationship is evident among the three bands.

Chlorophyll Synthesis Factors Affect pLHCP Incorporation

LHCP does not accumulate in the absence of chlorophyll, and we wished to determine whether the import of LHCP into *L. gibba* thylakoid membranes was affected by chlorophyll synthesis. We found that the addition to the reaction mixture of ALA, a precursor, and SAM, a co-factor, needed for chlorophyll synthesis (17, 21) greatly enhanced the amount of mature LHCP detected in thylakoid membranes (Fig. 2, c and d).

LHCP Is Incorporated into Chlorophyll–Protein Complexes

To determine whether the processed LHCP is assembled into LHCs and whether it might bind pigment molecules, we fractionated isolated thylakoids after an uptake reaction in a nondissociating gel system (Fig. 4, horizontal arrow; cf. Peter, G., and J. P. Thornber, manuscript in preparation). In this electrophoresis system all detectable chlorophyll remains bound to protein. When this gel is run in a second dimension (Fig. 4a, vertical arrow) under denaturing conditions (28), green band II is shown to contain only mature LHCP and two other minor proteins. A thylakoid preparation from an uptake experiment was run concurrently in the denaturing dimension to serve as a marker (Fig. 4, asterisk). The slowest migrating green bands of the first dimension (band I in Fig. 4A) are composed of polypeptides of photosystem I preparations (36).

Fluorography of such two-dimensional gels after uptake of radioactive pLHCP demonstrates that most of the labeled LHCP in the thylakoid is present in LHC II (Fig. 4B, asterisk). Although Coomassie Blue stain had revealed that all of the

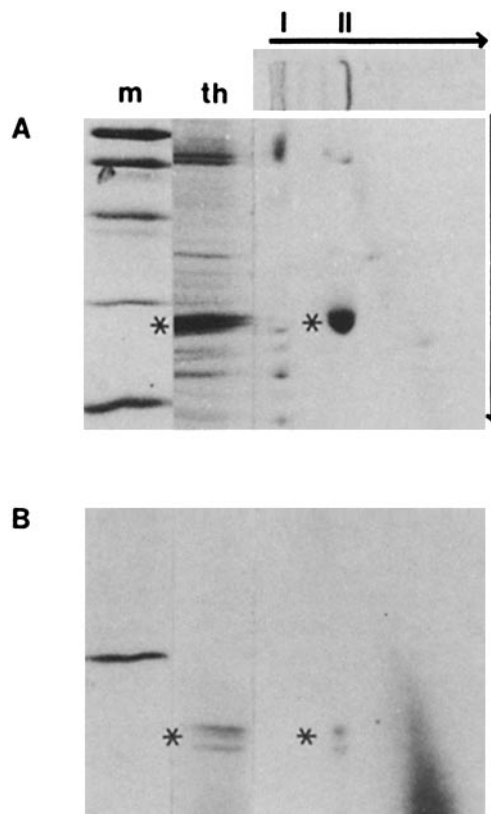


Figure 4. Assembly into LHC II of LHCP synthesized in vitro. The horizontal arrow lies above, and indicates the direction of migration in nondenaturing gel electrophoresis that displays visible amounts of chlorophyll (bands I and II). This first dimension was placed on top of a Laemmli gel (28) and subjected to electrophoresis in the direction of the vertical arrow. This second gel is shown stained with Coomassie Blue (A), and then fluorographed (B). Also shown in the second dimension are lanes *m* (molecular weight markers [$M_r \times 10^{-3}$ are 66, 58, 45, 29, 20] including wheat germ translation products of AB30 RNA), and *th* (a thylakoid preparation from chloroplasts incubated with pLHCP). * indicates the position of LHCP.

native LHCP was present only in the green complex, some of the introduced labeled LHCP in the thylakoid migrated to the position of polypeptide not in complex (Fig. 4B).

LHCP Mutants

Having established that exogenous pLHCP can be processed and incorporated into the correct LHC, we determined whether the removal of specific portions of the mature LHCP coding sequence would disrupt the normal assembly process. We chose to delete various parts of the polypeptide chain which corresponded to regions defined by our model for the folding of LHCP in the thylakoid membrane. For example, we removed segments that purportedly span the lipid bilayer (mutants E, F, or J), or ones that protrude into either the lumen or stroma (mutants D, I, or K) (Fig. 5).

We created deletions in the mature LHCP by removing selected DNA sequences from the coding region of the clone pAB30 (see Materials and Methods). All deletions are in the mature portion of the protein; the transit peptide is kept intact to enable import into the chloroplast (34, 43, 50). These mutant coding sequences were then cloned into a plasmid so that the transcription unit would be downstream of the SP6 RNA polymerase promoter. The reconstructed LHCP coding regions served as templates for in vitro RNA synthesis using

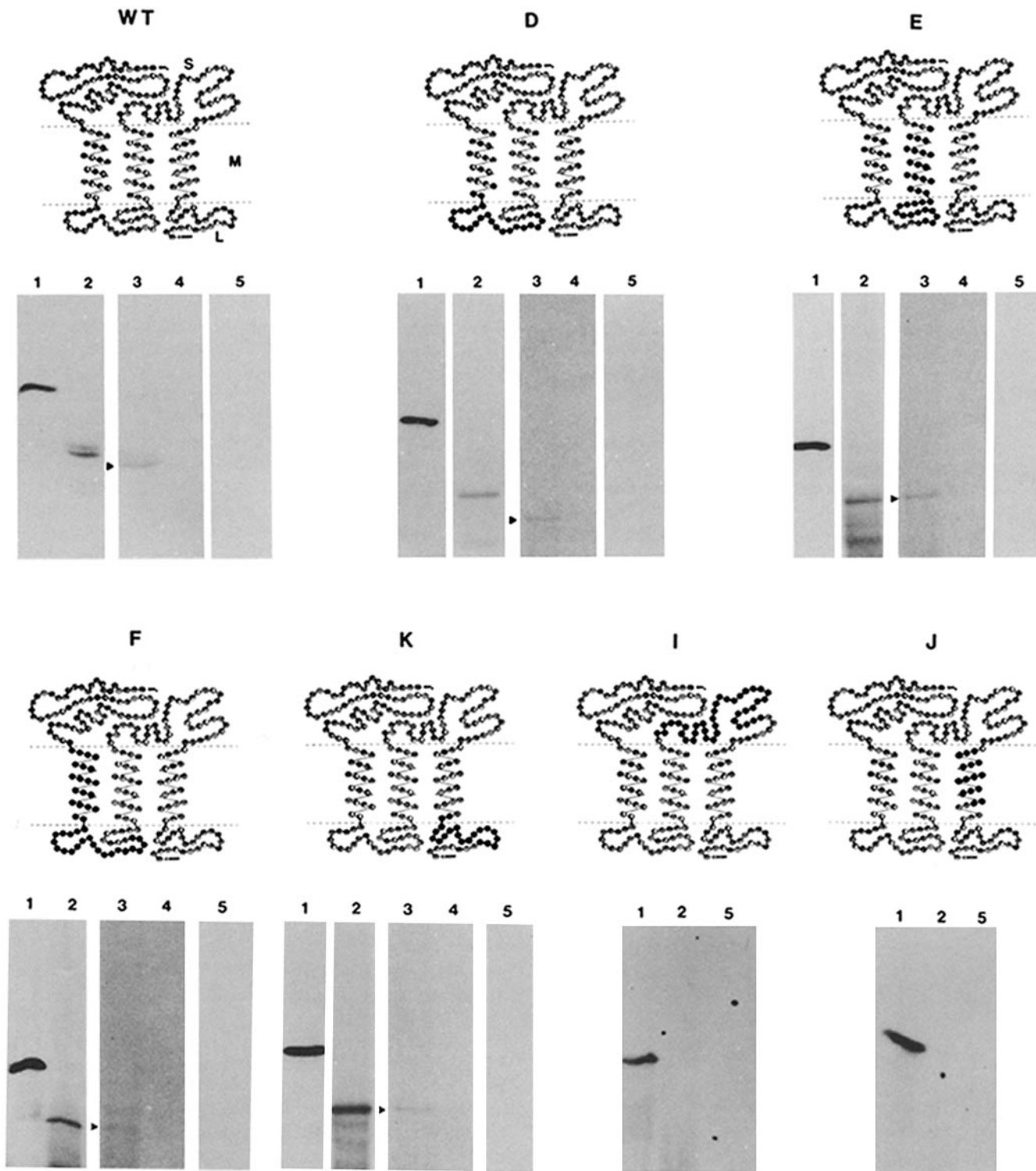


Figure 5. Fractionation and tryptic digestion of compartments isolated from chloroplasts incubated with wild-type and six mutant LHCPs. Above each gel is a model for the folding of LHCP with respect to the thylakoid membrane (25). Open circles represent amino acids, and filled circles indicate the region deleted from each mutant construction (*D*, *E*, *F*, *K*, *I*, and *J*). The extent of the deletions, with nucleotide numbers and corresponding amino acids (Fig. 1) for each are as follows: *D*, 741 (E)–821 (G); *E*, 803 (I)–932 (M); *F*, 687 (E)–821 (V); *K*, 1109 (P)–1168 (F); *I*, 895 (G)–1008 (K); and *J*, 1053 (A)–1098 (T). (See Materials and Methods for a complete description of the constructions.) *WT* refers to the wild-type AB30 polypeptide; *S* denotes stroma; *M*, membrane; and *L*, lumen. The fluorograms show mutant or wild-type pLHCP synthesized in vitro (lanes 1), and thylakoid (lanes 2) and stroma (lanes 5) fractions isolated after the incubation of the precursors with isolated chloroplasts. Thylakoid fractions (lanes 3, arrow) and pLHCP (lanes 4) after incubation with trypsin are also shown. Lanes 3 and 4 were exposed two times as long as the other lanes.

SP6 RNA polymerase, and proteins of the expected size were synthesized from this mutant AB30 RNA in a wheat germ extract in the presence of [³⁵S]methionine (Fig. 5, lanes 1).

Analysis of Mutants

Each labeled mutant protein was incubated with *Lemna* chloroplasts. Fig. 5 displays thylakoid (lanes 2) and stroma (lanes 5) fractions isolated from plastids incubated with the pLHCP of each of the six mutants. The same quantities (cpm) of translation products were incubated with the chloroplasts, and equal amounts of the uptake reactions were loaded on the gels for each of the deletions. Thus, the relative intensities reflect the relative abundance of the mutant polypeptides in the thylakoid membranes. We did not quantitate the differences, so these comparisons are only qualitative. We find that with several mutants, F, D, E, and K, the thylakoid but not the stroma fraction contains a labeled protein having the expected size of a processed mature species. Several other mutants, including I and J, cannot be detected in either the stroma or thylakoid fractions (Fig. 5).

To ensure that the processed mutants are indeed associated with thylakoids, and not with envelope membranes, we isolated envelopes from chloroplasts which had been incubated with mutant pLHCPs by flotation on sucrose gradients (11). Approximately 95% of either the newly introduced mutant or wild-type LHCP pelleted with the thylakoid membranes, while no more than 5% could be found in the envelope fraction (data not shown; see also reference 8). The small amount found in the envelope fraction presumably represents a contamination with thylakoids also noted in other reports (11). A similar distribution is seen when we obtain sufficient *Lemna* chloroplast material to allow for Coomassie Blue and antibody staining. These large preparations show that envelope and thylakoid proteins fractionate to the expected location on the gradients.

Accessibility of Wild-Type and Mutant Peptides to Trypsin

Trypsin has been shown to cleave a 2-kD segment from the amino terminus of the mature LHCP in pea thylakoids; this portion is exposed on the stromal side (1, 35, 42, 45). A similar result has been obtained for *L. gibba*: trypsin treatment of thylakoid membranes that have incorporated wild-type LHCP in vitro reduces the major labeled protein band by ~2 kD (Fig. 5, WT, lane 3), indicating that the AB30 polypeptide does indeed have a terminus that is accessible to trypsin cleavage in a similar location.

Trypsin treatment of the mutant proteins after incorporation should indicate whether they are similarly accessible to trypsin and, thus, whether the location of the amino terminus with respect to the thylakoid membrane is similar to that of the wild-type protein. The results of such experiments are shown in lanes 3 of Fig. 5 (arrows). Trypsin treatment of membranes containing the processed mutant D shows that this polypeptide also has a ~2 kD segment cleaved. However, mutants E, F, and K, which are found in the thylakoids, do not have an amino terminus that is accessible to trypsin.

To ensure that any proteolytic digestion observed was due to exogenous trypsin and was not, in fact, due to an endogenous protease, we also incubated thylakoids in the absence of trypsin for a similar time (Fig. 5, lanes 2). No 2-kD shift was observed for the bands that correspond to mature mutant

LHCPs. Moreover, pLHCPs are not susceptible to a similar cleavage or protection from cleavage when not associated with the lipid bilayer; no radioactivity was detected on the gel after a comparable amount of precursor translation product was incubated with trypsin (Fig. 5, lanes 4). Thus the trypsin susceptibility patterns of F, K, D, E, and wild-type thylakoid-associated proteins are due to their association with the membrane. We note that the additional lower molecular mass bands, seen in the thylakoid preparations containing mutant polypeptides, are digested by trypsin. These bands could represent peptides that are not integral membrane proteins and that are sensitive to trypsin.

The fluorographs of the tryptic digests (lanes 3 and 4) have been exposed twice as long as the other lanes to clearly visualize these less radioactive products. Although added protease reduces the total amount of mutant LHCP recovered, we still see totally protected (i.e., undigested) labeled peptide. The reduction in the amount of labeled protein after trypsin treatment is also observed for the wild-type LHCP and mutant D, yet there is also a concurrent 2-kD shift. We conclude, therefore, that at least a fraction of the mutant proteins E, F, and K are resistant to protease treatment when they are associated with the thylakoids. We have yet to determine the nature of the loss of radioactivity, but we consider it probable that the protease treatment has a general disruptive effect on our membrane preparations rendering much of the protein accessible to digestion. Indeed, the temperature of incubation and the concentration of trypsin are critical factors in such analyses (45), and a further probing of these parameters should provide more detail of the protein's orientation in the membrane.

Mutants Are Not Detected In LHC II

We next sought to determine whether the mutant polypeptides that were found in thylakoid membranes were also contained in LHC II. Fig. 6 shows the resolution of thylakoid complexes from chloroplasts incubated with the mutant LHCPs. All of the processed mutant protein migrates as a monomeric protein near the electrophoretic front in the first dimension of the gel (region F, Fig. 6) and not as a member of LHC II (region C, Fig. 6). In a separate experiment (data not shown), each uptake reaction also included wild-type precursor to ensure that mature LHCP would be detected in the complex. When the wild type was present, radioactivity arising from this peptide migrated with the front (Fig. 6, region F) as well as in the LHC II position, and therefore obscured any other protein which might run as monomeric LHCP. We show, therefore, in Fig. 6 an experiment that included only the mutant polypeptide. We have never been able to see any of our mutant peptides associated with complexes. Thus although precursor mutants F, D, E, and K are processed and thylakoid bound, they are not associated with LHC II.

Discussion

We have characterized a second chlorophyll *a/b*-binding protein gene, AB30, from *Lemna gibba* which does not contain an intron (cf. AB19, reference 25) and which is highly homologous in its amino acid sequence to Cab genes of other species. We have used this cloned gene to synthesize pure pLHCP and analyze its uptake and incorporation into LHCS

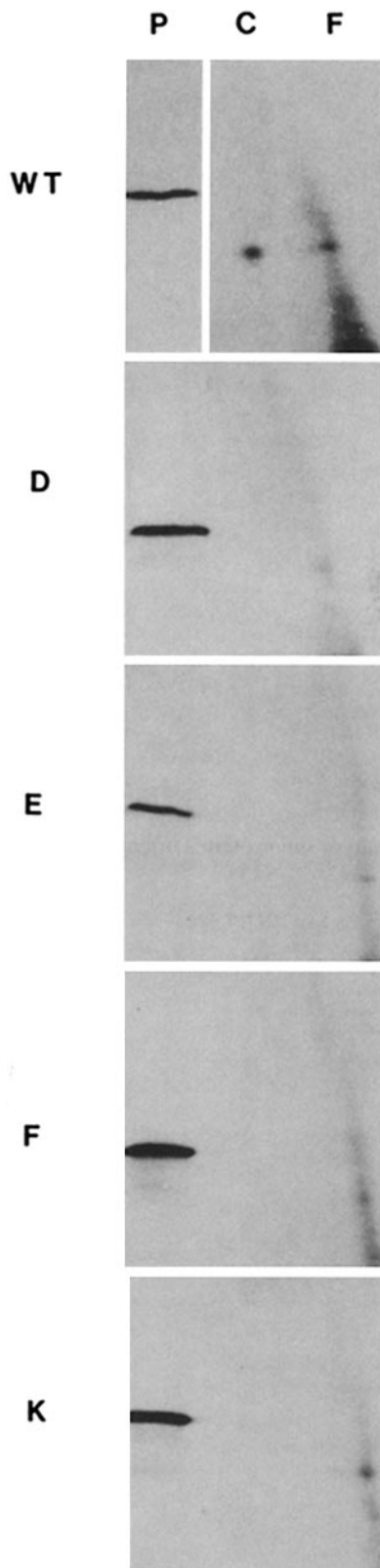


Figure 6. Mutant LHCPs are not assembled into LHC II. Fluorograms are shown of thylakoid membrane, from chloroplasts incubated with mutant LHCPs, subjected to electrophoresis in a nondenaturing gel (first dimension), followed by fractionation in a denaturing gel system (see Fig. 4). Mutant and wild-type pLHCP translation products (*P*) were also run in the denaturing dimension. Only the the fluorographs of the denaturing gels are shown. *WT*, wild-type AB30 pLHCP; *D*, *E*, *F*, and *K*, mutant pLHCP. The letters *C* and *F* above the fluoro-

in the thylakoid membranes of isolated *L. gibba* chloroplasts. The amount of mature LHCP found in the thylakoids is greatly increased by the addition to the reaction of a co-factor, SAM, and a precursor, ALA, of chlorophyll synthesis (Fig. 2). Our results suggest, but do not prove, a requirement for concurrent synthesis of chlorophyll for integration of LHCP into the thylakoid membranes of the isolated *Lemna* chloroplasts. A similar observation has been made for maize, but not for barley (8), and we believe that the differing results reflect the available chlorophyll and chlorophyll precursor pool sizes within the isolated chloroplasts of the different species. We are currently attempting to determine the nature of this requirement.

Multiple Forms of LHCP

Our experiments demonstrate that a single pLHCP can be converted into several distinct processed forms, each of which corresponds in electrophoretic mobility to a form seen in native thylakoid membranes. It has been previously suggested that several different LHCP forms may arise from the different members of the LHCP gene family (7, 14, 15, 25, 42, 47). We do not exclude this likely possibility as the source of at least some of the variations seen. However, we do show here that for *Lemna* the observed LHCP pattern can be mimicked by the processing of a single gene product *in vitro*. We think that the three forms do not arise through a nonspecific proteolytic event because (a) analysis of proteins before chloroplast lysis and fractionation shows that the three radioactive bands are already present at this time and thus do not occur as breakdown products during these procedures; (b) the addition of pLHCP to the thylakoid preparations during membrane isolation does not result in any degradation of the added protein; (c) the fastest migrating forms do not accumulate with increasing times of chloroplast incubation in a manner that would suggest a precursor-product relationship between the fast and slow forms; and (d) mutant *D*, which can be cleaved by trypsin after insertion into the thylakoid, is processed to only one form. We do not know how the three forms arise, but we and others (4, 32) think it a reasonable possibility that an LHCP-specific protease or modification enzyme is acting during the uptake reaction.

We have noted that while incorporated wild-type LHCP is found in multiple bands, only one band is observed in the region where a correctly processed mutant polypeptide would be expected to migrate. Thus, as only the wild-type LHCP is found in the LHC, there is a relationship between association with LHC II and the occurrence of the multiple forms.

Cleavage by trypsin of the multiple forms of LHCP in membranes of *L. gibba* produces a single electrophoretic band (Fig. 5). Similar experiments with pea, which has two readily resolved LHCP bands, also results in a single band (45). Since the tryptic digestion is known to cleave an amino-terminal segment (35, 45), it is possible that the three LHCP forms differ at their amino termini. Alternatively, although all three bands are incorporated into LHC II, only one may be resistant to trypsin, while the others may be digested to fragments too small to detect. Perhaps knowledge of the conditions that influence the appearance and relative amounts of the different forms will be of help in distinguishing the various possibilities. The variation in the number of radioactive bands that we

gram denote the positions in the gels of the LHC II complex and monomeric polypeptides (electrophoretic front), respectively.

sometimes observe between different experiments may originate with the tissue itself; we do note a variation in the degree of greening of individual fronds with a single culture of the plants. Further characterization of the multigene families from several species, and the use of antibodies against oligopeptides specific for individual gene products may also help to determine the nature of the observed LHCP heterogeneity.

Analysis of the Mutant Proteins

Through our initial analysis of the mutant proteins, we have learned that some altered LHCPs are found in a processed form in thylakoid membranes, but are not incorporated into LHC. Thus, stable complex formation is not required for the integration of altered LHCPs into thylakoid membranes. Any transient association of mutant LHCPs with LHC II would not have been detected in our experiments. Although the mutant peptides cannot be detected in LHC II, we have yet to determine whether they are capable of binding chlorophyll or carotenoid.

Mutants D, E, F, and K all appear in the thylakoid fraction at a size expected of a normally processed form. We have not determined whether the processing activity occurs before or after the integration of LHCP into thylakoid membranes. Here we have presented a qualitative analysis, and our results suggest that different mutant polypeptides are accumulated to different degrees (Fig. 5). These differing levels of accumulation might result either from differing uptake or from differential stabilities within the chloroplast. The latter, if true, might reflect a differential pigment binding.

We have attempted to probe at least one aspect of the conformation of the mutant LHCPs in the thylakoid membrane by mild trypsin digestion. We find that trypsin can not cleave the amino terminus from several of the mutants (E, F, and K) when they are incorporated into the thylakoids (Fig. 5). This finding suggests that these altered polypeptides could have a different orientation with respect to the membrane than the wild-type protein, perhaps with the amino terminus on the luminal rather than the stromal side. We have considered the possibility that the insensitivity to trypsin is a result of their being encapsulated by vesicles formed from disrupted membranes which fractionate with the thylakoids. However, we view this possibility as unlikely because any newly introduced LHCP or endogenous stromal proteins would also be expected to fractionate with the thylakoid membranes, and this is not observed; we find that I and J do not fractionate in this way, and our thylakoid fractions have no detectable stromal peptides (e.g., the large subunit of ribulose biphosphate carboxylase/oxygenase).

We must consider with reference to the model how the deletions created in the mutant polypeptides could lead to such an altered orientation as we propose above. Mutations F and E both remove a putative membrane-spanning sequence, and this could limit the LHCP chain to cross the membrane only twice rather than three times, resulting in the amino and carboxy termini being on the same side of the membrane. Indeed, trypsin does not cleave the amino terminus from F or E, and this is compatible with the amino terminus now being within the lumen and inaccessible to the protease. The absence of the region deleted in mutation D may still allow the membrane-spanning regions near the deletion to cross the membrane and thus leave the amino terminus in the stroma. This interpretation is consistent with the tryptic cleavage of a 2-kD fragment from the mutant D

polypeptide in thylakoids. If it proves possible to obtain enough material, these ideas will be further tested by a tryptic digestion of "inside-out" thylakoids (i.e., with the luminal rather than the stromal surface exposed) isolated from the chloroplasts after the uptake reaction. Another possible approach would be to attempt such an analysis on plants transformed with the mutant genes.

We have previously proposed that the carboxy-proximal regions deleted in mutants I, J, and K are involved in the insertion of LHCP into the thylakoid; the positively and negatively charged residues within these sequences could interact with the stromal surface and help to "lock" the protein in the proper orientation (25). Similar mechanisms have been proposed for prokaryotic membrane polypeptides (51, 52). Neither mutant J nor I is detected in the chloroplast, yet since their precursor forms carry the complete transit peptide, they should be imported; other LHCP mutants (see above) and a bacterial protein coupled to the pea SSU transit sequence (43, 50) can be imported into chloroplasts. Upon longer exposures of the thylakoid fractions from chloroplasts incubated with mutant polypeptides J and I, we see faint bands that migrate faster than LHCP which may be degraded mutant protein. Therefore, we think that both the J and I mutant polypeptides are imported but rapidly degraded once within the chloroplast. Indeed, there is no report of detectable wild-type LHCP (or its precursor) *in vivo* outside of thylakoid membranes. Thus, we suggest that regions deleted in mutants J and I may be involved in the initial association with or integration of LHCP into the thylakoid membrane.

We would predict from the model that mutation K, which removes the carboxy-terminal portion of LHCP, would also influence the initial insertion event, but not necessarily inhibit it, as part of the carboxy-, negatively charged tail is still intact. The mutant K polypeptide is found in a processed form, indicating that the membrane insertion event is not inhibited. However, the protein is in an orientation that renders the amino terminus inaccessible to trypsin. We did not predict this lack of trypsin sensitivity from consideration of the model, but it may reflect the consequence of a disrupted initial insertion event.

Our results are consistent with the previously proposed model, but certainly do not prove it to be correct. It is still possible, for example, that mutants F and E do not place the amino terminus in the lumen, but simply move this region closer to the membrane surface or change the secondary structure of the region rendering it inaccessible to trypsin. Regions covered by deletion I and J may contain domains essential for correct protein compartmentalization within the chloroplast (41).

Further understanding will require additional mutations and a more refined analysis of their conformations. The qualitative observations of LHCP mutants that we present here have shown that the formation of stable LHC II is not required for (altered) LHCP insertion into the membrane, and suggest that this process is affected by the synthesis of chlorophyll. The results represent a step toward an understanding of the processes that lead to the formation of mature LHCs in higher plants.

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