Accumulation of a Light-harvesting Chlorophyll \(a/b\) Protein in the Chloroplast Grana Lamellae

**THE LATERAL MIGRATION OF THE MEMBRANE PROTEIN PRECURSOR IS INDEPENDENT OF ITS PROCESSING**

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The events that follow the import of pLHCP IIb, the apoprotein precursor of the major light-harvesting complex of photosystem II, were studied in intact pea chloroplasts. The distribution of the events of insertion into the membrane, and processing, to yield the mature form (LHCP) between stromal and granal lamellae regions of the thylakoids were followed. pLHCP was preferentially inserted into stromal lamellae (SL) from which it migrated to granal lamellae (GL). Migration occurred before or after processing, suggesting that migration and processing are independent of each other. When migration was slowed down, LHCP accumulated in SL. Prolonged inhibition of migration induced degradation of LHCP that had accumulated in SL, whereas inhibition of processing did not affect the migration of pLHCP into GL. A small difference in electrophoretic mobility was noted between LHCP in SL and in GL. The predominant mature form in SL migrated more slowly than LHCP from GL. When thylakoids were subjected to trypsin, all of the LHCP embedded in SL underwent cleavage, whereas up to 60% of the radioactive LHCP in GL was resistant to the enzyme. The possible implications of the differences in size and in the sensitivity to trypsin of LHCP are discussed.

LHClIib functions as the major light-harvesting complex of photosystem II. It constitutes about 40% of both protein and pigments of the photosynthetic membrane of higher plants and contains most of its chlorophyll \(b\) (1, 2). This complex is assumed to be responsible for the stacking of thylakoid membranes, which gives rise to grana (3, 4). Under steady-state conditions almost all of the LHClIib is located in granal lamellae (5). Lateral movement of the complex between granal and stromal lamellae, which regulates the distribution of absorbed light energy between the two photosystems, has been described. This movement is mediated by phosphorylation/dephosphorylation of specific residues in LHCPs, the apoproteins which constitute the complex (6, 7).

The apoproteins of LHClIib are encoded in the nucleus and synthesized in the cytoplasm as precursors (pLHCP) with an amino-terminal transit peptide. The latter, which is responsible for routing the protein to chloroplasts, is removed following import (2). The import processing and assembly of pLHCP have been studied extensively in recent years (8). However, several aspects of the process remain unresolved, e.g., the processing of pLHCP to LHCP. Some reports suggest that processing takes place in the stroma, before insertion into the thylakoid membrane (9–11). On the other hand, the precursor has been observed in LHClI complexes (12), and the thylakoid-bound precursor appeared to undergo processing to its mature form (13). In addition, it has been demonstrated recently that LHCP processing activity can be obtained from purified washed thylakoids (14).

Another unresolved question is the relationship between the insertion into the membrane and the processing event. In a preliminary study we have observed both the precursor and mature forms of LHCP in granal as well as stromal lamellae after the import of pLHCP into isolated pea, maize, or barley chloroplasts. The newly processed LHCP II appeared to migrate from stromal to the grana lamellae (2). Similar observations were reported for isolated pea chloroplasts (15). These authors reported that newly imported LHCP migrated from stromal to granal lamellae and that migration was not affected by light or site-directed mutation which removed the site of LHCP phosphorylation.

pLHCP has been shown to integrate into isolated thylakoids, in a process which requires MgATP and stromal protein(s) (16, 17). Using this in vitro assay, it was demonstrated that the precursor is inserted primarily into stromal lamellae before it migrates to the grana in a temperature-dependent process (18). The processing of pLHCP was reported in an essentially similar in vitro system using chloroplast lysates (19, 20). Cline (19) observed that only lysates of plastids in which thylakoid stacking has been preserved were able to process pLHCP in vitro. On the other hand in intact plastids pLHCP is processed efficiently under conditions in which few or no grana are present. Processing was observed in plastids from early stages of greening (12), in bundle sheath chloro-
plasts (21), and at low concentrations of Mg2+ at which grana become unstacked.

In the present work we have attempted to obtain a comprehensive picture of the events which follow the import of pLHCP into intact plastids and its insertion into thylakoids. We have followed the course of pLHCP import and processing in isolated plastids with emphasis on the distribution of these events between stromal and granal lamellae. We asked whether the observations on isolated thylakoids reflect the course of events in intact organelles, i.e. does insertion into stromal lamellae precede migration to the grana? When and where does processing take place? Are integration and processing independent of migration? Are the mature, processed forms observed in granal and stromal lamellae identical? Do they have the same orientation in the two types of lamellae?

MATERIALS AND METHODS

Plant Material

Pea (Pisum sativum L. cv. Alaska) was grown in vermiculite for 5 days in 13 h of light (100 microeinsteins/m2/s) at 24°C/11 h of darkness at 18°C. The plants were placed in darkness for 2 days for destarching and were illuminated again for 30 min before harvesting.

Production of pLHCP

In Vitro Transcription and Translation—The Lemma AB30 (22) gene was cloned in pSF65. It was linearized with HindIII, and in vitro transcription was carried out as described previously (22). The following changes were made: 100 µg of DNA, 80 units of RNase inhibitor (Amersham Corp.), 80 units of SP6 RNA polymerase (Amersham), 10 µg of RNase-free bovine serum albumin (Sigma, molecular biology reagent) and 10 µg of digoxigenin triphosphate (Pharmacia LKB Biotechnology Inc.) were used in a 200-µl transcription mixture. The reaction mixture was incubated at 37°C for 90 min and extracted once with phenol. An equal volume of 7.5 M ammonium acetate was added to the aqueous phase and the RNA precipitated overnight at −20°C after adding 2 volumes of ethanol. The precipitate was dissolved in 80 mM Hepes/KOH, pH 7.5, and again precipitated with ethanol. It yielded 1–3 µg of RNA/µg of DNA template. The RNA was translated in a wheat germ system as described (10, 23), with the difference that 30 mM potassium acetate was added to the reaction mixture. In most cases, 100,000–200,000 cpm of 32P-labeled pLHCP was obtained per µl of translation mixture, containing 2–4 µg of RNA.

Overexpression of pLHCP in Escherichia coli—These experiments were made with the AB80 gene of LHCIIb from pea, cloned in pDS12. Overexpression of the gene to obtain unlabeled pLHCP was performed by induction of bacteria with isopropyl-1-thio-β-D-galactopyranoside (23). Inclusion bodies which contained the protein were isolated from the bacteria (23). They were then solubilized with 8 M urea and dialyzed together with soluble pea leaf extract as described (24).

Overexpression of the gene to obtain labeled pLHCP was achieved as described above, by growing the culture in a minimal medium, in the presence of carrier-free 35S SO4 (24).

Isolation of Plastids and Subgranellar Fractions

Leaves were harvested and ground in a razor blade blender (12) in grinding buffer (50 mM Hepes/KOH, pH 7.9, 0.6 M sorbitol, 1 mM MgCl2, and 1 mM dithiothreitol). The plastids were precipitated by centrifugation for 20 s at 3,000 × g and suspended in resuspension buffer (0.1 M Hepes/KOH, pH 7.8, 0.33 M sorbitol, and 5 mM MgCl2). After 5 min on ice to allow the precipitation of aggregates of ruptured plastids, the plastids which did not aggregate were again precipitated and suspended in a small volume of resuspension buffer. Where needed, plastids were purified further by centrifugation on Percoll gradients. The band of intact organelles was washed with resuspension buffer and used for in vitro uptake of pLHCP.

Envelopes were isolated after bursting intact plastids in 10 mM Hepes/KOH, pH 8.0, by briefly shaking with a vortex, followed by immediate loading on a stepwise sucrose gradient, as described (26). The band from the 0.4–M/1.1 M sucrose interface was collected, diluted with 10 mM Hepes/KOH, pH 8.0, and precipitated at 250,000 × g for 15 min (Beckman TL 100 microcentrifuge). Thylakoids were isolated as described (12).

RESULTS

The insertion and processing of pLHCP and the distribution of these events between granal and stromal lamellae were studied in isolated, intact plastids. To minimize lateral movement of newly imported protein, import of pLHCP was carried out at 8°C for 30 min (18). Subsequently, an excess of unlabeled pLHCP was added, and the reaction was allowed to proceed at 25°C for various periods of time. The reaction was stopped by lysing the plastids at 0°C, and their thylakoids were promptly isolated and fractionated into granal and stromal lamellae (Fig. 1). The purity of the two lamellae types is evident in that the stromal lamellae fraction was enriched in 50–60-kDa proteins, corresponding to subunits Ia and Ib of photosystem I and the α and β subunits of the proton-ATP synthetase. On the other hand, in the granal lamellae fraction the 24–29-kDa polypeptides of the LHClb complex predominated (Fig. 1, left halves of both panels). The fluorograms of
Insertion and processing of pLHCP occurred in both stromal and granal lamellae. SDS-PAGE of granal and stromal lamellae proteins, isolated following the import of radioactively labeled pLHCP into intact plastids. Import reactions lasted 30 min at 8 °C. Subsequently, excess unlabeled pLHCP was added and the reaction was stopped (0 min) or continued as shown (10, 20, and 60 min) at 25 °C. Plastids were promptly reisolated, burst, and their thylakoids purified and fractionated into granal and stromal lamellae. The purified membranal fractions were dissociated and analyzed on gels which were stained (left panel) and then fluorographed (right panel). Precursor, pLHCP, and mature (LHCP) forms and molecular mass of protein markers in kDa are given. TP, in vitro translated pLHCP.

These gels suggest that the imported precursor was inserted into both stromal and granal lamellae (Fig. 1, right halves of both panels). In stromal lamellae precursor molecules were relatively more abundant. At the end of preincubation at 8 °C, both the precursor and mature forms were observed in the two membrane types, indicating that considerable processing took place during this period. During the subsequent chase at 25 °C the precursor gradually disappeared from both membrane types, with a corresponding increase in the amount of mature LHCP in the grana (Figs. 1 and 2). Thus, when lateral migration of (p)LHCP was allowed to proceed simultaneously with processing at 25 °C, a relatively rapid disappearance of the precursor from the stromal lamellae, with a concomitant accumulation of mature LHCP in the grana, became evident (Figs. 1 and 2). The overall migration from stromal to granal lamellae is even more pronounced when the amounts of precursor and mature LHCP II are expressed as fractions of the total amounts of labeled protein in the thylakoids (Fig. 2).

Figs. 1 and 2 suggest that migration from stromal to granal lamellae and processing of the precursor to the mature form are independent of each other. This conclusion is supported by further experiments. When import and chase were performed at 17 °C, a temperature at which lateral migration is slowed down, processed LHCP continued to accumulate in the stromal lamellae, whereas little change was observed in the grana (Fig. 3A). Some degradation of the mature LHCP, which had accumulated in the stromal lamellae, took place after prolonged incubation at 17 °C (Fig. 3, A, two right lanes). Furthermore, when the processing of pLHCP was inhibited by the addition of 3 mM HgCl₂ (9) after 10 min of import at 8 °C, lateral migration continued unimpaired after the plastids were transferred to 25 °C (Fig. 3, B and C). This corroborates our conclusion that migration may occur with either the precursor or mature form.

A careful comparison of the precursor and mature forms in stromal and granal lamellae revealed a small but consistent difference between the mature forms observed in the two membrane fractions. The precursor form, pLHCP, had the same apparent molecular weight in both membrane fractions (Fig. 4), whereas the mature form of LHCP in the stromal lamellae migrated in the gels somewhat more slowly (LHCP') in Figs. 3 and 4). This could be a result of either a small difference in size between the stromal and granal forms or a
Processing and Lateral Migration of (p)LHCP in Thylakoids

**Fig. 4.** Different apparent sizes of newly imported and processed LHCP in stromal and granal lamellae. pLHCP was incubated with intact pea plastids. Following the import reaction plastids were reisolated and burst. Thylakoids were isolated and a precursor coded by the AB30 gene product from the AB80 pLHCP from pea. Plastids were reisolated and burst. Thylakoids were isolated and a precursor coded by the AB30 gene product from L. gibba. A, import with labeled AB80 pLHCP from pea. B, granal lamellae and stromal lamellae were carried out as described above (Fig. 4). Membranal fractions were subjected to digestion by trypsin (+) or left untreated (−). Fractions were analyzed by SDS-PAGE. The gel was stained and fluorographed.

**Fig. 5.** Resistance to trypsin digestion of the newly imported LHCP in thylakoids. pLHCP was incubated by pea plastids which were then recovered and burst. Their thylakoids were purified and subjected to digestion by trypsin for different periods of time (5, 10, 20, or 30 min; right panel) or to different concentrations of the enzyme (left panel). The proteins were analyzed by SDS-PAGE, staining, and fluorography. The mature form (LHCP') and the trypsin cleaved polypeptide (LHCP DP) are indicated. x, trypsin concentration in standard thylakoid digestion assays (40 µg/ml).

Modification of one of the forms. Import experiments with the AB30 pLHCPPII from *Lemna gibba* demonstrate a minor difference in the mature forms present in the stromal and granal lamellae (Fig. 4A, LHCP versus LHCP'). When the precursor encoded by the AB80 from pea was used, the two processed forms were more clearly observed (Fig. 4B, LHCP' and LHCP). These resembled the 26- and 25-kDa polypeptides observed in studies which employed pLHCPs from wheat or pea (10, 15, 20). Although both forms (LHCP' and LHCP) were observed in the stromal lamellae, the former predominated. Granal lamellae contained only the smaller size processed form.

To assess whether the mature forms observed in the stromal and granal lamellae have identical orientation in the membrane, we tested their accessibility to digestion by trypsin. It is known that trypsin treatment of isolated thylakoids removes a 1.5–2-kDa fragment from the amino terminus of LHCPPII (29), a feature which has been interpreted as indicating the correct integration/orientation of the protein in the thylakoid membrane after import and processing (9).

**Fig. 6.** Higher sensitivity to digestion by trypsin of LHCP in stromal lamellae than in granal lamellae. Import of pLHCP into pea plastids, separation of plastids, and isolation of granal and stromal lamellae were carried out as described above (Fig. 4). Membranal fractions were subjected to digestion by trypsin (+) or left untreated (−). Fractions were analyzed by SDS-PAGE. The gel was stained and fluorographed.

When pea thylakoids were digested with trypsin following a short period of import and processing with AB30 pLHCP, only about one- to two-thirds of the radioactive LHCP was cleaved by the enzyme (Fig. 5). The proportion of LHCP which was resistant to trypsin varied between 30 and 60% depending on the conditions of the experiment. Similar results were observed in maize and barley using pLHCPs from either *Lemna* or barley. To eliminate the possibility that this was a result of incomplete digestion, incubation up to 30 min or increased concentrations of trypsin were employed. Both had little effect on the relative amount of the protease-resistant form (Fig. 5). We examined the possibility that resistance of LHCP to trypsin differs between stromal and granal lamellae. Following import of labeled pLHCP thylakoids were fractionated into stromal and granal lamellae, and the two lamellae types were subjected to trypsin treatment. Fig. 6 shows the results for thylakoid proteins from the two membrane types, before and after trypsin treatment. Coomassie Blue staining indicated that in both membrane types all of the native LHCP were cleaved by the protease, (Fig. 6, stained gel). However, although all of the newly imported, radioactive LHCP in stromal lamellae was cleaved (LHCP DP in Fig. 6), in the granal lamellae only about one-third of the labeled LHCP was affected. Thus, in its accessibility to trypsin digestion newly imported LHCP in the granal lamellae is heterogeneous.

**DISCUSSION**

Insertion of pLHCP into the thylakoid membrane and its processing to form LHCP were studied with intact plastids. Special attention was given to the distribution of the two processes between stromal and granal lamellae. Our results suggest that different forms of the mature protein may be involved in the assembly of LHCPPII in the two membrane types. Also, following its arrival in the grana or during assembly into the light-harvesting complex LHCP might be transiently modified or differentially folded.

In a previous report, pLHCP presented to isolated thylakoids was primarily inserted into stromal lamellae and subsequently migrated to the grana (18). The present study indicates that the observations made with isolated thylakoids indeed reflected events during import into intact organelles.
Imported pLHCP was preferentially inserted into stromal membranes and migrated to granal ones either before or after processing (Figs. 1–3). This preference in insertion was somewhat blurred by contaminant migration of the polypeptides to the grana during import or during fractionation of the membranes. Yet, the predominance of insertion into stromal lamellae became more evident when lateral migration was inhibited (Fig. 3A). Since insertion also took place into granal lamellae, preference of stromal lamellae does not result from membrane specificity but from the relative extent of exposed area (i.e. unstacked lamellae) available in the two membrane types. The available techniques did not permit distinction of lateral migration from other processes (Fig. 3). The controversy over whether pLHCP processing (or modification) may enable migration from the stacked areas. Processing can occur on either stromal or granal lamellae, although in this case too it might be restricted to unstacked regions. The two processes (migration and processing) resulted in accumulation of the mature, processed form in the grana with a corresponding disappearance of both precursor and mature forms from the stromal lamellae. Our results also show that processing and migration are independent processes (Fig. 3). The controversy over whether pLHCP is processed in the stroma or on thylakoids has not been resolved (9, 12–14, 30, 31). Our results suggest that processing takes place after insertion (membrane integration), although the possibility that some processing occurs in the stroma cannot be excluded. Furthermore, a stromal protease might remove the transit peptide while the precursor is partially embedded in the thylakoid membrane.

Observations presented in Figs. 3–5 suggest that the product of processing, LHCP, exists in different forms in the two lamellae types. The apparent difference in size may reflect intermediate processing or a modification of the LHCP in the stromal lamellae. The two forms may be related to those described by Lampma and co-workers (10, 20). Their differential distribution between stromal and granal lamellae suggests a functional aspect to the phenomenon. The stepwise processing (or modification) may enable migration from stromal lamellae to the grana or ensure trapping in the latter. Another difference between the mature LHCP in stromal and granal lamellae is the susceptibility to trypsin digestion. Although all LHCP in the stromal lamellae underwent cleavage, a significant part of the newly imported mature protein in the grana was resistant to the enzyme. A possible cause for this heterogeneity may be a difference in the manner the protein is folded in the membrane. Alternatively, it might be a result of modification of LHCP. The D1 protein of the LHCP system II exhibited transient palmitoylation which was confined to granal lamellae. Palmitoylation of LHCP was also observed in the same study (32). We suggest that the assembly of LHCP might involve such transient modification or a change in the folding state. It would mean that under the conditions of our experiments about 30–60% of the protein assembled in the grana is still in this state. The heterogeneity of the granal LHCP might thus reflect a step in the biogenesis of the complex. Its nature and possible relation to the assembly of LHCII are currently being investigated.

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REFERENCES