Light-Harvesting Chlorophyll a/b-Binding Protein Inserted into Isolated Thylakoids Binds Pigments and Is Assembled into Trimeric Light-Harvesting Complex¹

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The light-harvesting chlorophyll a/b-binding protein (LHCP) is largely protected against protease (except for about 1 kD on the N terminus) in the thylakoid membrane; this protease resistance is often used to assay successful insertion of LHCP into isolated thylakoids in vitro. In this paper we show that this protease resistance is exhibited by trimeric light-harvesting complex of photosystem II (LHCII) but not by monomeric LHCII in which about 5 kD on the N terminus of LHCP are cleaved off by protease. When a mutant version of LHCP that is unable to trimerize in an in vitro reconstitution assay is inserted into isolated thylakoids, it gives rise to only the shorter protease digestion product indicative of monomeric LHCP. We conclude that more of the N-terminal domain of LHCP is shielded in trimeric than in monomeric LHCII and that this difference in protease sensitivity can be used to distinguish between LHCP assembled in LHCII monomers or trimers. The data presented prove that upon insertion of LHCP into isolated thylakoids at least part of the protein spontaneously binds pigments to form LHCII, which then is assembled in trimers. The dependence of the protease sensitivity of thylakoid-inserted LHCP on the oligomerization state of the newly formed LHCII justifies caution when using a protease assay to verify successful insertion of LHCP into the membrane.

The biogenesis of LHCII in higher plants involves a number of different cell compartments (Hohe, et al., 1994; Jansson, 1994; Thorner, et al., 1994). The apoprotein LHCP is coded for in the nucleus and translated by cytoplasmic ribosomes in a precursor form. The precursor pLHCP is transported across the outer and inner plastid envelope membrane, traverses the stroma, probably as a soluble complex with other protein(s) (Cline, 1986; Chitnis, et al., 1987). Recently, the stromal factor has been suggested to be a chloroplast analog of the 54-kD subunit of the signal recognition particle (Hoober, et al., 1994). The trimerization of LHCII monomers can also be performed in vitro, either by using reversibly dissociated native LHCII monomers (Nussberger, et al., 1992) or LHCII monomers reconstituted in vitro from LHCP and pigments, which then trimerize in the presence of lipids (Hobe, et al., 1994).

Probably after the insertion of LHCP into the thylakoid membrane and certainly prior to the assembly of LHCII trimers, pigments are bound to the protein. The mechanism of this step has been more of an enigma until now than those of many of the other steps in LHCII biogenesis. Since Chl biosynthesis, at least the last steps involved, is thought to be located in the thylakoid (Rüdiger, 1993), it is likely that the binding of pigments to LHCP also takes place in this compartment, although other possibilities have been discussed (Hoober, et al., 1994).

LHCP reconstitutes with pigments in detergent solution to yield complexes that are structurally very similar to monomeric LHCII isolated from the thylakoid (Plumley and Schmidt, 1987; Paulsen, et al., 1990). The reconstitution of mutated versions of LHCP has been used to distinguish between protein domains that are and others that are not involved in pigment binding or stabilization of LHCP-pigment complexes (Cammarata and Schmidt, 1992; Paulsen and Hobe, 1992). Moreover, reconstitution experiments with LHCP in detergent solution showed that bound pigments stabilize the apoprotein toward the attack of proteases, which is also thought to be the case in the thylakoid (Apel and Kloppstech, 1980; Bennett, 1981) and that pigment binding triggers the folding of previously largely unfolded LHCP into its native conformation (Paulsen, et al., 1993). The latter observation suggests that in the thylakoid, too, LHCP folding and maybe even insertion is coupled with the binding of pigments. However, the formation of LHCP-pigment complexes in detergent solution may be mechanistically different from the process taking place in the lipid bilayer of the thylakoid membrane. Therefore, it is desirable to establish an in vitro system for

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Abbreviations: LHCII, light-harvesting complex of PSII; LHCP, light-harvesting Chl a/b-binding protein; LHCP-DP/DP*, deletion products of LHCP; pLHCP, precursor form of LHCP.
studying the binding of pigments to LHCP in the environment of the thylakoid membrane.

After insertion of LHCP into isolated thylakoids and subsequent analysis of the pigment-protein complexes on a partially denaturing “green” gel, the inserted LHCP has been observed to migrate with the LHCII fraction in partially denaturing gel electrophoresis (Chitnis et al., 1986; Cline, 1988). However, these observations do not prove pigment binding to inserted LHCP in these in vitro experiments, since the inserted LHCP may have interacted nonspecifically with the endogenous LHCII. The insertion of LHCP into isolated thylakoids is often assayed by treating the thylakoids with protease, which is thought to degrade protein peripherally attached to the membrane, whereas pigment binding as a step in the biogenesis of LHCII.

The results demonstrate that the in vitro insertion of LHCP into isolated thylakoids will be useful for studying the step of spontaneously organized into trimeric complexes. These data confirm earlier suggestions that the shielding of the protein in the thylakoid membrane or in detergent solution. These data confirm earlier suggestions that the shielding of the protein in LHCII is due to bound pigments rather than to the membrane environment (Paulsen et al., 1993).

Taking advantage of the ability to overexpress pea LHCP derivatives with internal mutations were: WY16,17AV, the amino acid Arg at position 21 is replaced by Gln (Hobe et al., 1995). C-terminal deletion mutants of the precursor protein (pLHCP) were: ΔC-219 (corresponds to ΔC-9 in Paulsen and Hobe, 1992), a stop codon after the amino acid Ala at position 219 in the original LHCP sequence and an additional exchange of the last three amino acids, Val, Asn, and Asn, in the sequence against Ala, Gln, and Ala, respectively; ΔC-221 (corresponds to ΔC-6 in Paulsen and Hobe, 1992), and ΔC-222, and ΔC-223 (corresponding to ΔpLHCP-259 and ΔpLHCP-260 in Paulsen and Kuttkat, 1993), which are missing the last 11, 10, and 9 amino acids, respectively. The latter three mutants were constructed as described by Paulsen and Kuttkat (1993).

Preparation of Radiolabeled Precursor Proteins

Radiolabeled pLHCP and its derivatives were prepared by overexpressing pea cab gene constructs (see above) in Escherichia coli strain JM101 as described earlier (Paulsen et al., 1990), except the growth medium contained 0.1 mCi of [35S]Met and 2.5 μg mL⁻¹ thiamine. The purification of overexpressed pLHCP was carried out as described earlier (Paulsen et al., 1990; Waegemann et al., 1990). The protein was solubilized in 8 M urea, 8 mM DTT for 10 min immediately prior to the insertion reaction. Nonsolubilized protein was removed by centrifugation at 15,000g for 3 min.

Preparation of Chloroplasts, Lysates, Thylakoids, and Stroma

Chloroplasts were isolated from 7- to 9-d-old pea seedlings (P. sativum var Golf) grown on vermiculite with 16-h light and 8-h dark periods. Chloroplasts were isolated in a medium containing 0.33 m sorbitol, 50 mM Hepes-KOH (pH 8.0), and 1.5 mM MgCl₂, and were purified by centrifugation on a Percoll gradient (Schindler et al., 1987). Stromal extract was prepared by resuspending chloroplast pellets to a Chl concentration of 3 mg mL⁻¹ in lysis buffer (10 mM Hepes-KOH [pH 8.0], 10 mM MgCl₂), incubating on ice for 10 min, and centrifuging for 10 min (4°C) at 8000g. Thylakoid pellets were washed twice with lysis buffer and resuspended to a Chl concentration of 2 mg mL⁻¹ in 0.33 m sorbitol, 50 mM Hepes-KOH (pH 8.0), and 10 mM MgCl₂. The stromal extract was stored in liquid N₂.

Thylakoid Protein Insertion Assay

Integration assays were carried out following the procedure of Yuan et al. (1993) with some modifications. Integration assays (100 μL) received thylakoids to a final Chl concentration of 0.33 mg mL⁻¹, 5 to 10 mg mL⁻¹ stromal proteins, 10 mM Mg-ATP, 10 mM MgCl₂, 55 mM sorbitol, 23 mM Hepes-KOH (pH 8.0), 1 mM DTT, and 160 mM urea. Insertion reactions were started by adding urea-denatured pLHCP or derivatives (0.2-0.5 μM in 8 μM urea) to the insertion mixture. Insertion was carried out for 30 min at 25°C under continuous illumination (60 μE m⁻² s⁻¹) and gentle shaking. The reaction was terminated by adding 0.5 mL of insertion buffer (0.33 m sorbitol, 50 mM Hepes-KOH [pH 8.0]) and centrifuging at 8000g for 10 min at 4°C.

Thermolysin Treatment of Isolated Thylakoids

Thylakoids (equivalent to 33 μg of Chl) were resuspended in 450 μL of thermolysin buffer (10 mM Hepes-
KOH [pH 8.0], 0.5 mM CaCl₂), and a freshly prepared thermolysin solution (1 mg mL⁻¹) in thermolysin buffer was then added to a final concentration of 0.1 mg mL⁻¹. After the thylakoids were incubated for 30 min at 25°C, protease treatment was terminated by adding 10 mM EDTA and 0.5 mL of cold stop buffer (10 mM Hepes-KOH [pH 8.0], 5 mM EDTA). Thylakoids were recovered by centrifugation at 8000g for 10 min at 4°C.

Heat Treatment of Thylakoids and Native LHCII Trimers

Thylakoids were dissolved in thermolysin buffer to a final Chl concentration of 70 μg mL⁻¹, and native LHCII trimers in thermolysin buffer, containing 0.6% (w/v) nonylglycoside, to a final Chl concentration of 0.5 mg mL⁻¹ and incubated for the times mentioned in the text at 60°C in a gently shaking water bath.

Isolation and Purification of Native LHC II Trimers

LHCII trimers were isolated from pea chloroplasts according to the method of Burke et al. (1978) with some modifications. The chloroplast pellets were resuspended in 100 mL of 1 mM Tris, 10 mM NaCl, 5 mM EDTA, incubated on ice for 10 min, and centrifuged (13,500g for 10 min). The pellet was resuspended in 40 mL of 0.1 M sorbitol, 5 mM EDTA, centrifuged (13,500g for 10 min), and then resuspended in 40 mL of 0.1 M sorbitol. The suspension (equivalent to 24 mg of Chl) was centrifuged for 10 min at 13,500g. The pellet was solubilized in 0.5% (v/v) Triton X-100 at a Chl concentration of 0.5 mg mL⁻¹ and stirred at room temperature for 30 min. LHCII trimers were purified by centrifugation on a linear Suc gradient (3.4-34.2% [w/v] Suc, 0.5% [v/v] Triton X-100) for 16 h at 95,000g (4°C). The resulting red fluorescing band (trimeric LHCII) was collected, adjusted to 0.3 M KCl, and kept at room temperature for 20 min. After the sample was centrifuged at 13,500g for 5 min, the pellet was washed twice with 4 mL of 0.1 M KCl and centrifuged again (13,500g for 5 min). The pellet was resuspended in H₂O and centrifuged (13,500g for 5 min). The LHCII trimer pellets were stored at -20°C.

Protein Sequencing

Edman degradation was carried out on the Hewlett-Packard G1005A protein-sequencing system using the routine 3.0 chemistry. Coomassie blue-stained protein bands were excised from the gel, mashed, and incubated with 0.1% (v/v) SDS and 50 mM Tris (pH 8.0). Protein samples were then directly loaded onto the hydrophobic part of the biphasic sequencer support.

Analysis of Samples

For analysis by fully denaturing SDS-PAGE (15% polyacrylamide) followed by fluorography, equal amounts of Chl were loaded on the gel. For analysis on partially denaturing 15% polyacrylamide gels (Paulsen et al., 1990; Paulsen and Hobe, 1992), thylakoids were solubilized to a final Chl concentration of 0.5 mg mL⁻¹ in a buffer containing 0.45% (w/v) decyl maltoside, 0.1% (w/v) lithium dodecyl sulfate, 10% (v/v) glycerol, and 2 mM Tris maleate (pH 7.0) (described by Allen and Staehelin, 1991).

Miscellaneous Methods

Chl concentrations were measured according to the method of Porra et al. (1989). Protein assays were performed using the Bio-Rad protein assay with BSA as a standard.

RESULTS

Protease Treatment of pLHCP Integrated into Isolated Thylakoids Results in Two Different Protein Fragments

We are interested in identifying the molecular events that accompany the insertion of LHCP into the thylakoid membrane and the complexation of LHCII with pigments to yield functional LHClI. LHCP insertion into the thylakoid can be studied by using an experimental system in vitro, in which the protein is exposed to isolated thylakoids in the presence of Mg-ATP or light as an energy donor and of stromal extract (Cline, 1986; Chitnis et al., 1987). We inserted LHCP (apparent molecular mass 25 kD) or pLHCP (apparent molecular mass 30 kD) from pea, overexpressed and ³⁵S-labeled in E. coli, into isolated pea thylakoids and assayed the insertion reaction by removing all noninserted material with protease. We found, as expected, a 24-kD fragment termed LHCP-DP, indicating that the major part of inserted LHCP polypeptides, except for a 1- or 6-kD fragment on the N terminus of LHCP or pLHCP, respectively, became protease resistant (Cline, 1988; Reed et al., 1990). In addition to the 24-kD fragment, we consistently observed a shorter fragment of about 20 kD (Fig. 1), which was also observed earlier (Reed et al., 1990) and termed LHCP-DP*. This shorter fragment does not represent an intermediate state during LHCP insertion, since during the first 30 min of the insertion reaction both the 20- and 24-kD fragments appeared and increased simultaneously (Fig. 1). The 24-kD fragment was not a partial digestion product during the generation of the 20-kD protease fragment ei-

![Figure 1](https://example.com/figure1.png)  
**Figure 1.** The relative amounts of the two protease digestion products of LHCP inserted into isolated thylakoids do not change during the time course of an insertion experiment. Radiolabeled pLHCP was incubated with isolated thylakoids under insertion conditions for the times indicated and then assayed by protease as described in Materials and Methods." The same amounts of thylakoid membranes (15 μg of Chl) were applied to lanes 1 to 8, electrophoresed, and fluorographed (see "Materials and Methods"). DP, LHCP-DP; DP*,...
tested in the thylakoid insertion assay in comparison with pigments in vitro. Can or cannot form LHCII trimers after their reconstitution?

To test this hypothesis, we used different LHCP mutants in the insertion reaction that constitute into monomeric but not into trimeric LHCII in solution also, at least partially, assemble into trimeric LHCCI upon insertion into isolated thylakoids and that only these trimeric complexes in the membrane give rise to the protease digestion product LHCP-DP. Other LHCP derivatives that reconstitute into monomeric but not into trimeric LHCCI in solution presumably assemble into LHCCI monomers after thylakoid insertion, which then are digested into LHCP-DP*. Further experiments performed to test this conclusion are described below.

LHCP-DP Is Formed Only by LHCP Derivatives That Are Able to Assemble into LHCII Trimers

Earlier, we showed that LHCCI reconstituted in detergent solution forms structurally authentic trimers in the presence of lipids (Hobe et al., 1994). By using this in vitro trimerization, we characterized LHCP mutants in which a trimerization motif WYxxxR in position 16 to 21 or part thereof was deleted or point-specifically mutagenized (Hobe et al., 1995). These mutants, which are no longer able to trimerize after their complexation with pigments, were tested in the thylakoid insertion assay in comparison with nonaltered pLHCP or mutants that are still capable of forming trimers after reconstitution with pigments (Figs. 3-5). The various mutants used are summarized in Table I and II.

Figure 3 shows that after integration of pLHCP and subsequent analysis of the pigment-protein complexes on a partially denaturing gel, the radioactively labeled protein co-migrates mostly with the LHCCI trimer band. On the other hand, the nontrimerizing LHCP mutant WY16,17AV co-migrates exclusively with the monomeric but not with the trimeric LHCCI band. Similarly, further LHCP mutants that do not trimerize after pigmentation in detergent solution (Table I) never appeared in the trimeric LHCCI fraction after their insertion into isolated thylakoids, whereas LHCP and its trimerizing mutants did (not shown).

When thylakoid membranes were treated with protease after the insertion experiments with various LHCP derivatives described above and the digestion products were subsequently analyzed on a fully denaturing gel, only trimerizing LHCP derivatives gave rise to both LHCP-DP and LHCP-DP* (Fig. 4, pLHCP, ΔN-15; Fig. 5, pLHCP, ΔN-11) whereas nontrimerizing LHCP derivatives were all digested to LHCP-DP* exclusively (Fig. 4, R21Q, WY16,17AV; Fig. 5, ΔN-53, ΔC-xxx). Taken together, these results suggest that LHCP and its derivatives that can be reconstituted into LHCCI trimers in detergent solution also, at least partially, assemble into trimeric LHCCI upon insertion into isolated thylakoids and that only these trimeric complexes in the membrane give rise to the protease digestion product LHCP-DP. Other LHCP derivatives that reconstitute into monomeric but not into trimeric LHCCI in solution presumably assemble into LHCCI monomers after thylakoid insertion, which then are digested into LHCP-DP*. Further experiments performed to test this conclusion are described below.

LHCP-DP and LHCP-DP* Result from Differential Proteolytic Digestion of the N Terminus

In thylakoid-bound LHCCI, only the N terminus of the protein is thought to be exposed to the solvent so that it is accessible to proteases. Therefore, we presumed that LHCP-DP and LHCP-DP* are products of differential protease digestion on the N terminus of LHCP. Theoretically, LHCP-DP* could also be due to C-terminal cleavage of a 5-kD polypeptide from LHCP-DP, caused, for instance, by a leak in some of the thylakoid membrane vesicles making the C terminus on the luminal side accessible to protease. To exclude this possibility, we inserted a number of C-terminal deletion mutants of pLHCP into thylakoids that are lacking between 9 and 13 amino acids. As can be seen in Figure 5 (ΔC-xxx), LHCP-DP* of these mutants was shorter than the digestion product LHCP-DP* of nonmodified pLHCP (lane 1) by about 1 kD, which proves that the difference in length between LHCP-DP and LHCP-DP* is not due to different C termini. Conversely, in LHCP versions lacking 10 or 52 amino acids on the N terminus (lanes ΔN-11 and ΔN-53, respectively), LHCP-DP* has the same length as LHCP-DP* from nonmodified pLHCP.

Proteolytic Digestion of the N Terminus

Table 2. The relative amounts of the two protease digestion products of thylakoid-inserted LHCP are not dependent on the duration or temperature of the protease digestion. Radiolabeled pLHCP was inserted into thylakoids as described in “Materials and Methods.”

<table>
<thead>
<tr>
<th>Protease</th>
<th>25°C (min)</th>
<th>0°C (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1  2  3  4  5</td>
<td>5  10  15  30  -  -  -  -  30</td>
</tr>
<tr>
<td>DP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DP*</td>
<td></td>
<td></td>
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</tbody>
</table>

Figure 2. The relative amounts of the two protease digestion products of thylakoid-inserted LHCP are not dependent on the duration or temperature of the protease digestion. Radiolabeled pLHCP was inserted into thylakoids as described in “Materials and Methods.” Protease treatment of the membranes was carried out either at 25°C for the times indicated (lanes 1-4) or at 0°C for 30 min (lane 5). Samples (containing 15 μg of Chl each) were loaded on a polyacrylamide gel and fluorographed. Abbreviations are as in Figure 1.
Assembly of Light-Harvesting Complex II in Isolated Thylakoids

Table I. Derivatives of LHCP

<table>
<thead>
<tr>
<th>Derivative</th>
<th>N-Terminal Region of LHCP</th>
<th>R^a</th>
<th>T^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>LHCP</td>
<td>MRKSATTKVVASSGSPWYGPRVYKLGP.</td>
<td>+^c</td>
<td>+</td>
</tr>
<tr>
<td>WY16, 17AV</td>
<td>M.........................AV. ................</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>R21Q</td>
<td>M.........................Q. ................</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>∆N-11</td>
<td>MRGSAWY. ................</td>
<td>+</td>
<td>n.d.^d</td>
</tr>
<tr>
<td>∆N-15</td>
<td>MY. .......................</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LHCP</td>
<td>PGDYGWDTAGLSADPETFSKRMRELEVIH.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>∆N-53</td>
<td>MSPE. .......................</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>∆N-66</td>
<td>MIH. .......................</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

^a R, In vitro reconstitution of LHCP monomers.  
^b T, In vitro trimerization.  
^c +/-, Yields does not yield stable complexes.  
^d n.d., Not determined.

tensive digestion of the N terminus of LHCP compared to LHCP-DP.

Final proof for the two LHCP digestion products differing at their N termini came from peptide sequencing. Nussberger et al. (1993) found that trypsin removes the first 8 amino acids of LHCP in native trimeric LHClI. This is consistent with the shortening of LHCP by about 1 kDa, which we observed when treating isolated LHClI trimers or thylakoid membranes with either trypsin or thermolysin. We identified the N-terminal amino acid sequence of LHCP-DP* as LSADPETFSDKRELEVIH5RMAL; thus, the N terminus of LHCP-DP* is Leu in position 51. Therefore, the difference in length between the N termini of LHCP-DP and LHCP-DP* is 43 amino acids, which may easily account for the observed difference in apparent molecular mass. The N terminus of LHCP-DP* appears to be close to the cleavage site of chymotrypsin in trimeric LHClI (Nussberger et al., 1993; see “Discussion”).

In a control insertion experiment (Fig. 5, ∆N-66), we used the LHCP mutant ∆N-66, whose N-terminal deletion extends into the N-proximal membrane-spanning domain and with which no stable LHClI can be reconstituted in vitro (mutant ∆N-5 in Paulsen and Hobe [1992]). This mutant gave rise to neither LHCP-DP nor LHCP-DP*, indicating either that it does not insert at all into the membrane or that it does insert but then is not pigmented and, thus, not protease resistant. The differences in the intensity of LHCP-DP and LHCP-DP* bands in Figure 5 (lanes 1–8) are probably not due to different insertion efficiencies but rather to different specific radioactivities of the proteins.

In Trimeric LHClI More of the N-Terminal Domain Is Protected against Protease than in Monomeric LHClI

If, as suggested above, LHCP-DP and LHCP-DP* are formed by protease digestion of trimeric and monomeric LHClI, respectively, then it should be possible to generate LHCP-DP* by deliberately dissociating native LHClI trimers in thylakoids into the monomeric complexes before treating the membranes with protease. Figure 6 shows that this is indeed the case. During heat treatment of native thylakoids at 60°C for up to 10 min, followed by proteolytic digestion, the LHClI trimer band on a partially denaturing gel gradually disappeared as the LHClI monomer band increased, demonstrating progressive dissociation of trimeric LHClI (Fig. 6A). A concomitant increase of LHCP-DP* at the expense of the originally predominant LHCP-DP was also observed when membranes were analyzed on a fully denaturing SDS-polyacrylamide gel (Fig. 6B). The same results were obtained when the dissociation of thylakoid-bound LHClI into monomers was triggered by the action of phospholipase A as described by Remy et al. (1982) (not shown). These results confirm the previous assignment of the longer and shorter protease fragments to the digestion of trimeric and monomeric LHClI, respectively.

Table II. Derivatives of pLHCP

<table>
<thead>
<tr>
<th>Derivative</th>
<th>C-Terminal Region of pLHCP</th>
<th>R^a</th>
<th>T^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLHCP</td>
<td>ENLADHLADPVNNNAWSYATNFPGK</td>
<td>+^c</td>
<td>+</td>
</tr>
<tr>
<td>∆C-219</td>
<td>..........................DPAQN</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>∆C-222</td>
<td>..........................DPVNNNAW</td>
<td>+</td>
<td>n.d.^d</td>
</tr>
<tr>
<td>∆C-223</td>
<td>..........................DPVNNNAWS</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

^a R, In vitro reconstitution of LHClI monomers.  
^b T, In vitro trimerization.  
^c +/-, Yields does not yield stable complexes.  
^d n.d., Not determined.
We repeated this experiment after inserting radioactively labeled pLHCP into the thylakoids. Analysis of heat-treated and protease-digested membranes in a partially denaturing gel system showed that the amount of radioactively labeled protein in the LHCII trimer band gradually decreased, whereas it increased in the LHCII monomeric band (Fig. 7A). The relative amounts of radioactive LHCP-DP and LHCP-DP*, when separated by denaturing gel electrophoresis (Fig. 7B), were very similar to those generated from the endogenous total LHCII (Fig. 6B), further confirming that LHCP (or pLHCP) inserted into isolated thylakoids is assembled, at least in part, into trimeric LHCII.

It should be noted that during heat treatment of thylakoid membranes the amount of unbound pigment increased and, concomitantly, the total amount of LHCII visible in the LHCII trimer plus monomer bands decreased, indicating partial dissociation of LHCII into LHCP and pigments (Fig. 6A). Upon prolonged heat denaturation,
LHCCI bands disappeared altogether from the partially denaturing gel; at the same time, protease treatment of these membranes no longer generated LHCP-DP* (not shown). The most likely explanation is that dissociation of LHCCI monomers renders LHCP more protease sensitive so that it will be digested into shorter fragments. This strongly suggests that LHCP-DP* is the protease digestion product of LHCCI monomers rather than the nonpigmented protein integrated in the membrane, although we cannot exclude the latter possibility.

**Different Protease Sensitivities of the N Terminus of LHCP Are also Visible in Detergent-Solubilized LHCCI Trimmers and Monomers**

The observation that more than the N-terminal domain of LHCP becomes protease sensitive upon heat dissociating LHCCI monomers suggests that it is not so much the membrane environment of the protein that is responsible for protection of most of LHCP against protease than it is the pigments bound. This notion is further confirmed by the finding that protease treatment of isolated LHCCI monomers and trimers in solution, either isolated from the thylakoid membrane (Fig. 8) or reconstituted in vitro, yields the same LHCP fragments as are obtained from thylakoid-bound LHCCI (Fig. 8). It should be noted that LHCP-DP* from thylakoid-bound LHCCI on the one hand and solubilized LHCCI on the other hand appear to migrate somewhat differently (Fig. 8, lanes 1 and 2, respectively), although for both of these bands the N-terminal protein sequences were identical. This may be the same. One possible explanation is that in solubilized LHCCI but not in thylakoid-bound LHCCI the C terminus of LHCP is exposed to the protease and thus may be shortened.

**DISCUSSION**

**Trimerization Motif**

In an attempt to understand which structural elements in LHCP are essential for the formation of trimeric LHCCI, we recently identified the trimerization motif WYxxxR in position 16 to 21 in the N-terminal domain of LHCP. If either WY or R in this motif is deleted or exchanged, the reconstituted monomeric LHCCI containing the respective LHCP derivatives fail to trimerize (Hobe et al., 1995). In this paper we confirm that the trimerization motif is significant for the trimerization, not only for in vitro reconstituted LHCCI but also for LHCCI located in the thylakoid membrane. When LHCP is inserted into isolated thylakoid membranes, it forms trimeric LHCCI, whereas LHCP mutants containing an impaired trimerization motif form monomeric but not trimeric LHCCI. The assembly of inserted LHCP into trimeric LHCCI is assessed by its co-migration with the band of LHCCI trimers in partially denaturing PAGE of the solubilized membranes and by differential protease resistance of monomeric and trimeric LHCCI (see below).

**LHCP Inserted in Isolated Thylakoids Is Pigmented and Organized into Trimeric LHCCI**

It has been observed before that LHCP inserted into isolated thylakoids would co-migrate with LHCCI after solubilizing the membranes and separating pigment-protein complexes on a partially denaturing gel (Chitnis et al., 1986; Kohorn et al., 1986; Cline, 1988). However, co-migration of the inserted LHCP with LHCCI did not prove that the newly inserted protein had indeed been complexed with pigments, since the possibility could not be ruled out that the protein was only nonspecifically associated with LHCCI trimers.

Successful insertion of LHCP into the thylakoid is commonly assayed by protease resistance of the inserted protein except for a 1- to 2-kD N-terminal fragment (Schmidt
by about 50 amino acids at its N terminus. Consistently, but not complexed with pigments may also arrive at a distance but only give rise to the shorter digestion product, (LHCP-DP*), we observe that the amounts of both LHCII and finally disappear altogether. The most likely interpretation is that upon prolonged heat treatment LHCII complexed with a bacterial signal sequence was integrated into the bacterial membrane and gave rise to a protease-resistant fragment of 26 kD (Kohorn and Auchincloss, 1991). We are presently testing whether the integration of LHCP in the thylakoid is mechanistically coupled with its complexation with pigments as was proposed earlier (Paulsen et al., 1993).

A Larger Part of the N-Terminal LHCP Domain Is Buried in Trimeric LHCII than in Monomeric LHCII

The data presented in this paper demonstrate a distinct difference between monomeric and trimeric LHCII concerning the molecular environment of the N terminus of LHCP. In monomeric LHCII, 50 N-terminal amino acids can be removed by thermolysin or trypsin, whereas in the trimeric form, only 10 amino acids are accessible to these proteases. The crystal structure of LHCII trimers suggests that the N-terminal hydrophilic domain of LHCII is involved in contacts between the monomers (Kühlbrandt and Wang, 1991; Kühlbrandt et al., 1994). Our observation that about 50 amino acids in the N-terminal LHCP domain are protected in trimeric but not in monomeric LHCII is in agreement with the notion that this protein domain is located at the interface between monomeric complexes. Consistently, when part of this protein domain is removed by chymotrypsin treatment of native LHCII, the trimeric complexes irreversibly dissociate into monomers (Nussberger et al., 1993). Why chymotrypsin cleaves trimeric LHCII in a domain where trypsin and thermolysin have no access is not understood but is probably due to structural differences among these proteases. Taken together, these observations strongly suggest that the N-terminal domain of LHCP is directly involved in monomer-monomer interactions in LHCII trimers.

Recently, LHCII in a Chl b-deficient *Chlamydomonas reinhardtii* strain was shown to yield a shorter fragment upon trypsin digestion than in the wild type (Plumley and Schmidt, 1995). This finding has been interpreted as an incomplete insertion of LHCP in the thylakoid. An alternative explanation may be that Chl b deficiency impairs LHCII oligomerization in the mutant algae, causing more extensive protease digestion of the monomers.

Protease Digestion as an Assay for Thylakoid Insertion of LHCP

In many studies on LHCP integration in organello or in thylakoids, the localization of LHCP in the membrane has been assayed by washing the membrane with NaBr (Chitnis et al., 1987) or NaOH (Schmidt et al., 1981; Chitnis et al., 1986; Cline, 1986; Yalovsky et al., 1992); this treatment supposedly removes all protein from the membrane that is not stably integrated. In other studies, treatment of the thylakoids with protease was used rather than a washing step to distinguish integral LHCP from protein that is only nonspecifically attached to the membrane (Kohorn et al., 1986; Cline, 1988; Auchincloss et al., 1992; Huang et al., 1992; Yuan et al., 1993). This assay for membrane insertion was found to be more stringent than the NaBr or NaOH wash and, therefore, thought to be superior (Auchincloss et al., 1992; Huang et al., 1992; Yuan et al., 1993).

Considering the data presented in this paper it appears that the higher stringency observed for the protease
 assay compared to the wash assay may be due to the fact that the largely protease-resistant LHCP fragment (LHCP-DP) is observed only when LHCP is not only integrated into the thylakoid but also complexed with pigments and even organized in trimeric LHCII. This is certainly a sufficient condition of successful LHCP integration in the membrane, since it is hard to imagine that LHCP would be pigmented and trimerized anywhere else. However, it might not be a necessary condition. Whether LHCP can be integrated into the thylakoid without being pigmented still needs to be established. As long as this is unclear, we suggest that care be taken when comparing studies of the insertion of LHCP into the thylakoid using different assays for the localization of the protein in the membrane and that the protease assay for LHCP integration be interpreted with caution, since it is dependent not only on the actual integration of LHCP into the membrane but also on the assembly of trimeric LHCII.

Very recently, Li et al. (1995) presented evidence that the insertion of LHCP into the thylakoid membrane is dependent on a plastid analog (54CP) of the mammalian signal recognition particle (Franklin and Hoffman, 1993) and thus might be mechanistically related to the signal recognition particle-mediated translocation process in other organisms. This novel aspect prompts a number of new experimental approaches to elucidate the events in the thylakoid leading to the productive integration of LHCP in the membrane, its assembly into LHCII, and the role played by pigments in these events.

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**LITERATURE CITED**


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