Light-harvesting chlorophyll $a/b$-binding protein, LHCP, or its precursor, pLHCP, cannot be stably inserted into barley etioplast membranes in vitro. However, when these etioplast membranes are supplemented with the chlorophyll analogs Zn-pheophytin $a/b$, synthesized in situ from Zn-pheophorbide $a/b$ and di-geranyl pyrophosphate, pLHCP is inserted into a protease-resistant state. This proves that chlorophyll is the only component lacking in etioplast membranes that is necessary for stable LHCP insertion. Synthesis of Zn-pheophytin $b$ alone promotes insertion of LHCP in vitro into a protease-resistant state, whereas synthesis of Zn-pheophytin $a$ alone does not. Insertion of pLHCP into etioplast membranes can also be stimulated by adding chlorophyll $a$ and chlorophyll $b$ to the membranes, albeit at a significantly lower efficiency as compared with Zn-pheophytin $a/b$ synthesized in situ. When pLHCP is inserted into chlorophyll- or Zn-pheophytin-supplemented etioplast membranes and then assayed with protease, only the protease digestion product indicative of the monomeric major light-harvesting chlorophyll $a/b$ complex (LHCII) is found but not the one indicating trimeric complexes. In this respect, chlorophyll- or Zn-pheophytin-supplemented etioplast membranes resemble thylakoid membranes at an early greening stage: pLHCP inserted into plastid membranes from greening barley is assembled into trimeric LHCII only after more than 1 h of greening.

The major light-harvesting chlorophyll $a/b$ complex (LHCII) of photosystem II is the predominant pigment–protein complex in higher plants, comprising roughly half of the total chlorophyll (Chl) in plant cells. The LHCII apoprotein (LHCP), coded for in the nucleus, is post-translationally imported into plastids in its precursor form, pLHCP, and kept soluble in the stroma by a proteinaceous complex, possibly involving 54CP, the plastid analog of the 54-kDa subunit of the signal recognition particle (1). Finally, LHCP is inserted into thylakoids and assembled with Chls $a/b$ and the carotenoids lutein, neoxanthin, and violaxanthin (2, 3). Our knowledge about these last two steps is rather fragmentary.

At least four distinguishable pathways of protein translocation through (or insertion into) the thylakoid membrane have been identified (4, 5). The translocation of LHCP is characterized by its requirement for GTP and a pH gradient. However, the accumulation of LHCP in the membrane also requires the presence of both Chl $a$ and Chl $b$. In intermittent light (IML)-grown plants containing little or no Chl $b$, no LHCP is accumulated in the membrane, although presumably translatable mRNA is present in normal amounts. This observation led to the concept that stabilization by pigments of LHCP in the thylakoid is part of the post-translational regulation ensuring the coordinate accumulation of light-harvesting protein and light-harvesting pigments; excess protein that is not complexed with pigments is rapidly degraded by plastid proteases (6, 7).

Isolated thylakoid membranes from green or greening chloroplasts stably insert LHCP in vitro when provided with GTP (8) and stroma (9, 10). Consistently with the pigment requirement observed in vivo, etioplast membranes do not accumulate inserted LHCP even when they are supplemented with a stromal fraction from greening chloroplasts (11). One possible explanation is that the protein does insert into etioplast membranes but then fails to be stabilized by pigments, as Chl $a/b$ are absent, and thus is degraded by proteases in the etioplast membranes or in the stroma. Pigments may also play a more active role during the insertion process. Pigment binding initiates the refolding of detergent-denatured LHCP in vitro (12). This observation suggests that pigment-triggered renaturation of LHCP may play a similar role when the protein is inserted into the thylakoid, the binding of pigments and the concomitant refolding of LHCP being the driving force for translocating parts of the protein across the membrane (13). Finally, as a third possible explanation, the failure of etioplast membranes to accumulate stable membrane-inserted LHCP may have nothing to do with the lack of pigments and rather be due to the developmental state of the organelles. If there is a molecular apparatus aiding the insertion of LHCP into the thylakoid, such as a protein receptor in the membrane, components of this apparatus may be developmentally regulated and appear only during greening.

To further understand the role of Chls during the biogenesis of LHCII, we studied the insertion of LHCP into etioplast membranes complemented with Chl or its Zn analog. We did so by taking advantage of the fact that etioplast membranes can synthesize Chl or the Chl analog Zn-pheophytin (Zn-phe) from its precursor chlorophyllide (Chlide) or its analog Zn-pheophorbide (Zn-pheide) and digeranyl pyrophosphate (GGPP) (14, 15). Instead of Chlides $a/b$, we used Zn-pheides $a/b$ for practical reasons, as Zn-pheides are more stable than the Mg-containing Chlides (16). Also for experimental reasons, insertions in this study were carried out using pLHCP rather than the mature protein; the precursor is more easily distinguished from the digestion products in the protease assay for membrane inser-
tion, and we have shown previously that pLHCP becomes inserted into isolated thylakoids and assembled into LHCI trimers with comparable efficiency as the mature protein (17).

MATERIALS AND METHODS

Construction of Radiolabeled Precursor Protein—The precursor plHCP was expressed from the plasmid XLHCP-2 (18) containing the coding region of the cab gene AB80 (Lhcb1*2 according to Ref. 19) from pea (Pisum sativum) (20). Isolation of pLHCP from Escherichia coli carrying the expression plasmid pXLHCP-2 has been described elsewhere (17). Proteins were radioactively labeled in vivo by adding [35S]Met to the bacterial growth medium (17).

Preparation of Etioplasts, Etiochloroplasts, and Chloroplasts—Barley seedlings (Hordeum vulgare var. Steffi) were grown on vermiculite for 4 days at 25 °C in darkness or light (50 microeinsteins/m² s) for time periods mentioned in the text. Isolation of etioplasts, etiochloroplasts, and chloroplasts was carried out in the dark room as described (21).

Pea plants (P. sativum var. Golf) were grown on vermiculite for 9 days at 25 °C under an IML regime: 3 min of light (50 microeinsteins/m² s) and 177 min of darkness. Chloroplasts were isolated in the dark room as described (17).

Separation of Membranes and Stromal Extract—Etioplasts, etiochloroplasts, or chloroplasts were lysed, and the etiolated membranes or thylakoid membranes were separated from stromal proteins as described (17). Only membrane fractions were used in the insertion assay. Stomatal extracts were isolated from 7–9-day-old pea seedlings grown on vermiculite with 16-h light and 8-h dark periods, as described (17).

Thylakoid Protein Insertion Assay and Protease Assay—Insertion assays were carried out following the procedure described in Ref. 22, with some modifications described in Ref. 17. Stable membrane integration of the protein was verified by protease (thermolysin) treatment of membranes as described (17). Proteins of protease-treated membranes were precipitated and separated by fully denaturing SDS-polyacrylamide gel electrophoresis (15% polyacrylamide), followed by fluorography as described elsewhere (17).

Preparation of Pigments, GGPP, and PPP—Chl a/b was extracted with acetone from spinach. Zn-pheides a/b were synthesized as described by (16). Phytyl pyrophosphate (PPP) and GGPP were obtained directly by phosphorylation of the alcohols with di(triethylammonium)phosphate in the presence of trichloroacetonitrile in acetonitrile as a solvent. Phytyl and digeranyl pyrophosphate were isolated as triammonium salts as described (23, 24). GGPP or PPP were added to the in vitro insertion reactions at the 50-fold concentration of Zn-pheide a/b.

Determination of Pigments—Chl was measured according to Ref. 25. Zn-pheide a/b was separated from Zn-pheide a/b according to Ref. 26 and determined spectrophotometrically as described in Ref. 27, using the molar extinction coefficients of Ref. 28.

RESULTS

Stable Insertion of pLHCP in Etioplast Membranes Is Stimulated by Synthesis of Chl a and Chl b in Situ—Isolated thylakoid membranes are capable of stably inserting LHCP (or pLHCP) in vitro, rendering the protein largely protease-resistant. On the other hand, Chl-less membranes isolated from etioplasts do not insert LHCP in a protein-resistant form in vitro (Ref. 11; Fig. 1A, lane 1).

Fig. 1A shows the result of an insertion experiment with radioactively labeled pLHCP in which the Chl analog Zn-phe is synthesized in situ from Zn-pheide a/b and GGPP added. Upon assaying membrane-inserted protein by treating the thylakoid membrane with thermolysin, an LHCP fragment of about 20 kDa (LHCP-DP*) appeared (Fig. 1A, lane 4), which is indicative of monomeric LHClI assembled in the membrane (17). Addition of either Zn-pheide a/b or GGPP alone did not result in stably integrated LHCP (Fig. 1A, lanes 2 and 3). Hence, Chl rather than one of its precursors is required for the appearance of LHCP-DP*.

Synthesis of Zn-pheide b Alone but Not Zn-pheide a Alone Is Sufficient to Stimulate Stable Insertion of pLHCP into Etioplast Membranes—The synthesis of Zn-pheide in situ from its precursors allowed us to study the differential effect of either Chl a or Chl b on the insertion of LHCP into etioplast membranes. The insertion assay containing pLHCP and etioplast membranes was supplemented with GGPP and either Zn-pheide a or Zn-pheide b. Fig. 1B clearly shows that the synthesis of Zn-pheide b (third lane) but not Zn-pheide a (second lane) stimulates stable insertion of LHCP, as indicated by the appearance of the protease-resistant peptide LHCP-DP*.

The lanes in Fig. 1B show bands with the migration behavior of pLHCP and LHCP, which are seen, to various extents, in most of the experiments presented here. These are not due to insufficient protease digestion after the insertion reaction, as shown by control experiments in which the protease digestion has been extended (data not shown). It is unknown how the precursor and mature forms of LHCP are rendered protease-resistant in these experiments. The mature form of LHCP is sometimes but not always seen in our insertion experiments, and is probably due to some remnant activity of LHCP-processing enzyme in the stromal extract. LHCP does not seem to be an intermediate in the insertion of pLHCP into thylakoids, when membranes exhibiting mature LHCP after insertion and protease assay were subjected to a second insertion reaction with new Chl precursors but no pLHCP added, the amount of mature LHCP in the membrane did not increase (data not shown).

Stable Insertion of pLHCP into Thylakoids from IML-grown Pea Plants Is Stimulated by Synthesis of Zn-pheide b—To substantiate the observation of pigment-stimulated membrane insertion of LHCP, we repeated the experiment with thylakoid membranes from pea plants grown under an IML regime. IML-grown plants accumulate significantly reduced amounts of Chl b, depending on the light regime. In pea plants grown for 9 days under a 177-min dark/3-min light regime, we observed a Chl a/b ratio of 15. As seen in Fig. 2, even this low content in Chl b is sufficient to stimulate some stable insertion of pLHCP, as indicated by the detection of the protease-resistant LHCP fragment LHCP-DP* (Fig. 2, lane 1). Addition of Zn-pheide b to the membranes, resulting in Zn-pheide b synthesis, greatly stimulated the accumulation of protease-resistant LHCP in the membrane (Fig. 2, lane 2). Surprisingly, the synthesis of the Zn analog of Chl a by adding Zn-pheide a also exhibited a stimulating effect, although significantly lower than the one caused by the Chl b analog (Fig. 2, lane 3).

In addition to the protease fragment LHCP-DP*, another protease fragment LHCP-DP is seen in all three lanes of Fig. 2; this fragment is shorter by about 1 kDa than mature LHCP and indicates the presence of trimeric LHCP (17). This indicates that the presence even of the reduced amounts of Chl b

![Fig. 1. Zn-phe a/b synthesis stimulates the insertion of LHCP into etioplast membranes.](http://www.jbc.org/)
pLHCP Insertion into Zn-phe-supplemented Etioplast Membranes

**FIG. 2.** Synthesis of Zn-phe b stimulates insertion of pLHCP into thylakoids from IML-grown pea plants. Radiolabeled pLHC was inserted into thylakoids isolated from IML-grown pea etioplasts. For *in vitro* synthesis of the Chl analogs Zn-phe a or b, membranes were either not supplemented with Chlide analogs (first lane) or supplemented with Zn-pheides a and b (second lane) or b (third lane) and PPP. The insertion reaction was followed by protease treatment, gel electrophoresis, and fluorography as in Fig. 1. DP, protease digestion product LHCP-DP; other abbreviations are as in Fig. 1.

**FIG. 3.** Insertion of pLHCP into isolated membranes of etioplasts, etioplasts, and chloroplasts. Membranes were isolated from etiolated barley seedlings or after illumination for 1, 4, and 12 h. Insertion of radiolabeled pLHCP into these membranes was followed by protease treatment. The protease-resistant protein fragments LHCP-DP (DP) and LHCP-DP* (DP*) were separated by fully denaturing polyacrylamide gel electrophoresis and fluorographed.

LHCP Is Assembled into Monomeric and Trimeric LHCII upon Insertion into Greening Thylakoid Membranes. Depending on the Stage of Greening—Chl synthesis in etioplast membranes during the insertion of LHCP gives rise to the protease-resistant fragment LHCP-DP*, indicating the assembly of monomeric LHCII in the membrane. It does not stimulate the assembly of LHCII trimers, which would be indicated by the larger protease-resistant fragment LHCP-DP. The assembly of monomeric but not trimeric LHCII is also seen upon insertion of pLHCP into green-thylakoids at an early greening stage. We isolated membranes from barley plastids in the etiolated state and after 1, 4, or 12 h of illumination, and used these membranes for insertion of pLHCP without addition of Chl precursors (Fig. 3). As expected, after insertion into etioplast membranes, no protease-resistant LHCP fragment is detected (apart from trace amounts of pLHCP and LHCP; see above). After 1 h of greening, LHCP-DP* becomes visible, indicating the assembly of monomeric LHCII. Trimeric LHCII, as indicated by LHCP-DP, appears only after 4 h and, even more significantly, after 12 h of greening.

The Stable Insertion of LHCP into Etioplast Membranes Is Stimulated by Increasing Amounts of Chl Precursors until It Reaches Saturation. In the thylakoid insertion reaction, LHCP (or pLHCP) fails to assemble into trimeric LHCII, both in thylakoid membranes at an early greening stage and in etioplast membranes substituted with newly synthesized Chls. One possible explanation is the relatively low amount of monomeric LHCII present under these conditions, due to limiting amounts of Chls. These low levels of monomeric LHCII may be insufficient for oligomerization. The level of LHCII in the thylakoid is much higher after longer greening periods as endogenous LHCII accumulates. Therefore, we attempted to maximize the amount of pigment-stimulated LHCII accumulation in the pLHCP insertion experiments by providing increasing amounts of the Chl precursors.

Fig. 4 shows that the intensity of the LHCP-DP* band increased with increasing amounts of Zn-pheide a plus b up to 0.4 nmol, added per the equivalent of 8.5 × 10^5 plastids, and then leveled off. The addition of even higher amounts of Zn-pheide a/b led to a reduction in LHCP-DP* detected (data not shown). As shown in Table I, only about 30% of Zn-pheide added is converted into Zn-pheide. It is possible that the Zn-pheide, which cannot be assembled into LHCII, inhibits the formation of pLHCP-Zn-phe complexes at higher concentrations and therefore, limits the amount of pigment-inducible LHCII accumulation.

In Table I, the amounts of Zn-pheide a/b synthesized *in vivo* are compared with the amount of Chl a/b synthesized after various times of greening *in vivo*. The comparison is made on the basis of membranes from the same number of plastids. Clearly, the situation *in vitro* is different from that *in vivo* in that comparably low but roughly equal levels of both Chl a/b analogs are present, whereas *in vivo* there is very little Chl b compared with Chl a, at least in early stages of greening. However, assuming that Chl b is limiting *in vivo* for the assembly of LHCII, the amounts of Zn-pheide a or Zn-pheide b synthesized *in vitro* may be compared with the Chl b level in *vivo*. According to the data shown in Fig. 4, the maximum level of LHCII monomer assembly is reached when 0.2–0.4 nmol each of Zn-pheide a/b (0.4–0.8 nmol of Zn-pheide a+b) are added to the membrane equivalent of 8.5 × 10^5 plastids. The amount of Zn-pheide a and Zn-pheide b synthesized under these conditions is between 0.05 and 0.3 nmol (Table I, part B), which is equivalent to the amount of Chl b accumulated after a greening period of between 1 and 4 h.

Chl Is Required at Higher Concentrations than Zn-pheide a/b

**TABLE I**

Comparison of Chl a/b amounts accumulated during greening in vivo and amounts of Chl analogs synthesized in isolated etioplast membranes in situ

| A. Time (h) | Chl a (nmol) | Chl b (nmol) | Chl a/b |
|------------|--------------|--------------|---------|
| 1          | 2.13         | 0.043        | 50      |
| 4          | 6.12         | 1.1          | 5.5     |
| 12         | 13.6         | 3.83         | 3.5     |
| 17         | 19.04        | 6.0          | 3.2     |

| B. Zn-pheide a+b (nmol) | Zn-pheide a (nmol) | Zn-pheide b (nmol) | Zn-pheide a+b (nmol) | Esterification (%) |
|-------------------------|--------------------|--------------------|----------------------|--------------------|
| 0.15                    | 0.023              | 0.016              | 0.039                | 27.7               |
| 0.4                     | 0.059              | 0.089              | 0.128                | 32                 |
| 1.7                     | 0.174              | 0.26               | 0.434                | 27.1               |
for Stimulating Stable Insertion of pLHCP into Etioplast Membranes—In all the pigment-stimulated LHCP insertion experiments described so far, Chls were provided by their synthesis in situ in the etioplast membrane, mimicking the situation in the greening thylakoid in vivo where Chls are synthesized as LHCIIs is accumulated. However, we were interested to know whether de novo synthesis of Zn-phe a/b is necessary to stimulate assembly of LHCIIs. Therefore, we added increasing amounts of Chl a/b to etioplast membranes in the LHCP insertion assay. Fig. 5 shows that the amount of protease-resistant peptide LHCP-DP*b rises with the amount of Chl added until saturation is reached between 4 and 11 nmol of pigment. However, comparison of Figs. 4 and 5 shows that at least 10 times more Chl a/b must be added to the insertion reaction than Zn-pheide a/b to obtain about the same signal in the LHCP-DP*b band. Thus, Zn-phe synthesized in situ is much more efficient in stimulating the stable insertion of LHCP into the membrane than Chl added directly to the membrane.

**DISCUSSION**

*Isolated Plastids form Zn-phe from Zn-pheide and GGPP—*To study the role of Chls in the insertion of pLHCP into isolated thylakoids, we took advantage of the ability of lysed plastids to synthesize Chl a/b when its precursors, Chlide and GGPP, are added externally (14). As mentioned in the introduction, we used Zn-pheide a and Zn-pheide b instead of the Chlides solely for the reason that the Zn-analogs are more stable under the conditions used. It had been shown previously that (i) Chl synthetase accepts Zn-pheide a as a substrate (16), (ii) the newly synthesized Zn-pheide a can be effectively used to stabilize Chl a-binding proteins against proteolytic digestion (15), and (iii) stable LHCIIs can be reconstituted when Chl a in the reconstitution mixture is replaced with Zn-pheide a.2

*Etioplasts Contain All Components except Chls Necessary for Stably Inserting pLHCP—*Etioplast membranes do not insert pLHCP in a protease-resistant form; however, when Chls are added to the membranes or when Chls are formed in situ from its precursors, stable pLHCP is accumulated. Therefore, Chls are the only component missing in etioplast membranes to promote stable LHCP insertion. If a molecular apparatus aiding pLHCP insertion, such as a protein receptor in the thylakoid, exists, it is either already present in etioplasts or it is not absolutely required for pLHCP insertion in situ.

Neither Zn-pheides nor GGPP are sufficient by themselves to stimulate stable insertion of pLHCP, showing that Chl or its Zn-analog is required, rather than one of the precursors. However, there is a difference between de novo-synthesized Zn-pheide a and Zn-pheide b; Zn-pheide a alone does not give rise to protease-protected membrane-inserted LHCP, whereas Zn-pheide b does, albeit at lower efficiency than the combination of Zn-pheide a and Zn-pheide b. This parallels the pigment requirement for reconstituting LHCIIs in situ; whereas both Chl a and Chl b (along with xanthophylls) are required to form LHCIIs with native stability, less stable complexes can be reconstituted with Chl b as the only Chl component, but not with Chl a.3 This suggests that the addition of Chls or the formation of Zn-pheides stimulates stable LHCP insertion into etioplast membranes, because it enables these membranes to form more or less stable LHCP-pigment complexes, which are then protected against degradation in the protease assay. Alternatively, the failure of Zn-pheide a to stimulate stable LHCIIs accumulation may be explained by the presence of Chl a-binding proteins in etioplast membranes, which are thought to have a higher affinity for Chl a than LHCP (15, 29, 30) and thus would scavenge the newly formed Zn-pheide a (see below).

The notion of pigment-dependent membrane insertion of pLHCP was confirmed by experiments using thylakoid membranes of pea plants grown under an IML regime. These membranes contain limiting amounts of Chl b (Chl a/b ratio of 15) and, consistently, little or no LHCIIs. As expected, the addition of Zn-pheide b and PPP to these membranes stimulated the formation of protease-resistant LHCP. Surprisingly, Zn-pheide a also stimulated stable thylakoid insertion of pLHCP to a small but significant degree, indicating that Chl a, too, is limiting in these membranes for the formation of LHCIIs. This is in accordance with the notion that the lack of Chl b, both in IML-grown plants and in numerous Chl b-less mutants, actually is due to decreased levels of Chl a synthesized so that, upon pigmentation of Chl a-binding proteins, no surplus Chl a is available for transformation into Chl b (31). It is possible that the additional Chl a synthesized in situ eases the limitation of Chl a and thus, allows the newly inserted pLHCP to bind the small amount of Chl b available more efficiently and therefore, to accumulate more stable LHCIIs. Alternatively, the Zn-pheide a may partially stabilize some newly inserted pLHCP even if only some of the Chl b binding sites are filled with Chl b. Accumulation of LHCP has been observed in import experiments with chloroplasts from the Chl b-less barley mutant chlorina-f2 (32, 33), although stable assembly of the complex has not been shown in these experiments, and leaves from this mutant do not accumulate LHCIIs (34, 35). A third possibility is that some of the Zn-pheide a synthesized in situ is converted into Zn-pheide b. It has been shown recently that Chl b is formed from Chl a by oxidation with molecular oxygen, a reaction that requires a highly hydrophobic environment and suggests that Chl a is converted as it is already bound in a pigment-protein complex (36). We have no information as to whether part of the Zn-pheide a in our experiments is oxidized to Zn-pheide b.

Where Does the Chl Assembled into LHCIIs Come from?—As opposed to etioplast membranes, greening thylakoids assemble inserted LHCP into fully pigmented LHCIIs without externally added pigments (17). It is unclear whether substantial de novo Chl synthesis takes place in lysed greening chloroplasts unless substrates of Chl synthetase are externally added. Alternatively, Chls may be re-distributed between already existing pigment-protein complexes in the thylakoid and the newly inserted LHCP (37), or provided by putative Chl carrier proteins shuttling Chls to the site where they are assembled in newly formed pigment-protein complexes. Proteins forming rather instable complexes with Chls such as early light-induced proteins (38) and the subunit S protein of photosystem II (PsbS) (39) have been proposed to function as Chl carriers.

It is highly unlikely that the Chl to be assembled into LHCIIs resides unbound in the lipid bilayer; a pool of unbound Chl has not been observed and, more importantly, would pose a serious

**Fig. 5. Addition of Chl a/b to etioplast membranes stimulates stable integration of LHCP.** Etiolated membranes were supplemented with increasing amounts (3.2–33 nmol/8.5 × 107 plastids) of Chl a/b during an insertion reaction. Control reactions received no Chl a/b. Fluorography of protease-resistant protein fragments in a fully denaturing SDS-polyacrylamide gel electrophoresis is shown. Abbreviations are as in Fig. 1.

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2 C. Eisen and H. Paulsen, unpublished results.

3 S. Hobe and H. Paulsen, unpublished results.
risk by being excited into an unquenched triplet state, which in turn gives rise to the formation of potentially dangerous singlet oxygen. This may be an explanation for the lower efficiency of Chl a/b in stimulating the stable insertion of pLHCP into etioplast membranes as compared with Zn-phe a/b synthesized in vitro; the Chl precursor analogs Zn-pheide a/b are efficiently bound to Chl synthetase and then proceed on the biosynthetic path leading from Chl synthesis to Chl assembly. On the other hand, Chl a/b added to the thylakoids seems to enter the pathway toward LHClII assembly with much lower efficiency. However, we cannot exclude that the observed difference in stimulating stable pLHCP insertion is not a reflection of the difference between Chl and a Chl precursor but rather between Chl and its Zn analog; possibly Zn-phe is more efficiently bound to pLHCP than Chl. Chl a-binding proteins have been observed to be more efficiently stabilized by Zn-pheide a than by Chl a, both synthesized in situ in intact etioplasts (15); on the other hand, in the reconstitution of LHClII in vitro, Zn-phe a/b-containing complexes did not show an increase in stability compared with Chl a-containing complexes.2

**LHCP Inserted into Chl-supplemented Etioplast Membranes**

Is Not Assembled into Trimeric LHClII—When pLHCP is inserted into green pea thylakoids, at least part of the protein is assembled into trimeric LHClII, giving rise to a 24-kDa digestion product (LHCP-DP) in the protease assay. In monomeric LHClII, a somehow smaller section of the protein is resistant to protease, resulting in a digestion product of about 20 kDa (LHCP-DP*) (17). In all the pLHCP insertion experiments with pigment-supplemented etioplast membranes shown in this paper, the protease assays for testing the insertion reaction resulted in LHCP-DP* exclusively, demonstrating that inserted pLHCP was presumably assembled into monomeric but not trimeric LHClII. The Chl- or Zn-phe-complemented etioplast membranes in our experiments behave like thylakoid membranes at an early greening stage. In greening pea leaves, monomeric LHClII is accumulated 12–24 h prior to the appearance of trimeric complexes and then gradually disappears as the level of LHClII trimers increases (40). Consistently, when we inserted pLHCP into greening barley thylakoids at an early greening stage, without Chl or Zn-pheide added, the protease assay yielded LHCP-DP* exclusively, whereas insertions into thylakoids at later greening stages gave rise to gradually increasing amounts of trimeric LHClII as indicated by LHCP-DP.

The simplest explanation for the findings described above is that LHClII trimer formation only occurs when the monomeric complexes already assembled exceed some threshold concentration. In the barley thylakoids we studied, this threshold was apparently reached between more than 1 h and less than 4 h of greening. We assume that the amount of Zn-phe a/b synthesized in situ in etioplast membranes was not sufficient for the assembled LHClII to reach the threshold for trimerization. Clearly, the amount of esterified pigment obtained per given number of plastids in this experiments was lower than the amount of Chl a/b present in the same number of plastids after 4 h of greening, when trimer formation was observed. However, it is difficult to compare these two pigment levels, as it is impossible to compare these two pigment levels, as it is impossible to compare these two pigment levels, as it is impossible to compare these two pigment levels, as it is impossible to compare these two pigment levels, as it is impossible to compare these two pigment levels, as it is.
Light-harvesting Chlorophyll $a/b$-binding Protein Stably Inserts into Etioplast Membranes Supplemented with Zn-pheophytin $a/b$

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