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Light-Harvesting Chlorophyll *a/b* Complexes: Interdependent Pigment Synthesis and Protein Assembly

F. Gerald Plumley [a,1] and Gregory W. Schmidt [b]

[a] Institute of Marine Science, University of Alaska, Fairbanks, Alaska 99775
[b] Botany Department, University of Georgia, Athens, Georgia 30602

The biogenetic interdependence of light-harvesting chlorophyll (Chl) *a/b* proteins (LHCPs) and antenna pigments has been analyzed for two nuclear mutants of Chlamydomonas that have low levels of Chl *b*, neoxanthin, and loroxanthin. In mutant PA2.1, the apoprotein precursors (pLHCP II) of the major light-harvesting complex LHC II were synthesized at approximately wild-type rates, processed to their mature size, and rapidly degraded. Because the bulk of labile LHCP II in PA2.1 was soluble, a thylakoid integration factor apparently is defective in this strain. Chl *a*, Chl *b*, neoxanthin, and loroxanthin synthesis and accumulation were coordinately reduced in PA2.1, indicating that LHCP II play important regulatory or substrate roles in de novo synthesis of these pigments. Mutant GE2.27 is impaired principally in Chl *b* synthesis but nonetheless accumulated wild-type levels of all LHCPs. Topology studies of the GE2.27 LHCP II demonstrated that their insertion into thylakoids was incomplete even though they were not structurally altered. Thus, Chl *b* formation mediates conformational changes of LHCP II after thylakoid integration is initiated. GE2.27 also exhibited very low rates of neoxanthin synthesis and was unable to accumulate loroxanthin. Revertant GE2.27 strains with varying capacities for Chl *b* formation provided additional evidence that neoxanthin synthesis and accumulation are coupled with the final steps of LHCP II integration into thylakoids. We propose that biogenesis of LHC includes interdependent pigment synthesis/assembly events that occur during LHCP integration into the thylakoid membrane and that defects in these events account for the pleiotropic characteristics of many Chl *b*–deficient mutants.

INTRODUCTION

Biogenesis of the light-harvesting chlorophyll (Chl) *a/b* complexes (LHCs) of green plants and algae requires coordinated synthesis and assembly of components from the nuclear, cytoplasmic, and plastid compartments (Taylor, 1989; Hoober et al., 1994). Assembly of LHC II, the abundant LHC associated primarily with photosystem (PS) II, involves three apoproteins (LHCP II; Peter and Thornber, 1991) that are post-translationally imported into chloroplasts, processed to their mature size, and aided in thylakoid integration by soluble factors (Cline, 1986), which may (Yalovsky et al., 1992) or may not (Yuan et al., 1993) include chloroplast heat shock protein 70 (ct-HSP70). The exact roles of integration factors in chloroplast biogenesis remain incompletely understood, in part because the phenotype of integration factor–deficient mutants has yet to be described. It is uncertain whether, in the absence of these factors, the LHCP II would still be synthesized at wild-type rates (see later discussion), processed to their mature sizes, partially integrated into thylakoids, and/or subjected to degradation in the stroma during their transit from the envelope. As part of this report, we describe the phenotype of a mutant unable to complete import and/or integration of LHCP II into thylakoids.

Assembly of LHC II also requires approximately seven Chl *a* molecules, approximately six Chl *b* molecules, two luteins, and violaxanthin and neoxanthin (Kühlbrandt et al., 1994). The molecular mechanisms that coordinate the synthesis and/or accumulation of LHCP II and the pigment moieties are highly complex (Taylor, 1989; Hoober et al., 1994). A conclusion of many studies is that neither chlorophylls nor xanthophylls are required for synthesis of the LHCP II but that pigment ligands, particularly Chl *b*, are required for stabilization of the apoproteins and assembly of the complex. Turnover of LHCP II in chlorophyll- or xanthophyll-deficient mutants occurs when they have undergone maturation and are partially integrated into thylakoids (Herrin et al., 1992), indicating that apoprotein processing and translocation through the stroma occur, although perhaps at reduced rates, in the absence of pigment ligands. Analyses of mutants unable to synthesize either chlorophylls or xanthophylls showed that pigments also affect regulation of apoprotein accumulation at the transcriptional, post-transcriptional, and translational levels (Johanningmeier, 1988; Herrin et al., 1992).

There is general consensus that Chl *b* is a stabilizing ligand for LHCP. However, only two studies (Bellemare et al., 1982; Terao and Katoh, 1989) have directly demonstrated that synthesis, thylakoid integration, and degradation of LHCP occur

[1] To whom correspondence should be addressed.

in Chl *b*–deficient mutants. Moreover, the role of Chl *b* as a stabilizer of LHCs has not been formally proven because the primary defects in the majority of Chl *b* mutants have not been rigorously defined. As reviewed by Rüdiger (1986), there has been an erroneous tendency to directly equate impaired accumulation of a pigment to its impaired synthesis. Against this assumption, Falbel and Staehelin (1994) found that wheat and barley mutants with low Chl *b* have lesions in Mg^{2+}-chelatase, slowing the overall rate of synthesis of chlorophyll precursors. Thus, preferential reduction of Chl *b* accumulation may be a secondary consequence of the higher affinity of reaction center apoproteins for Chl *a* (Tzinas and Argyroudi-Akoyunoglou, 1988), possibly depleting chloroplasts of substrate for Chl *b* synthesis. Whether other previously analyzed Chl *b* mutants are specifically impaired in Chl *b* synthesis remains to be determined. The concept that Chl *b* is specifically required for the accumulation of LHC also requires further scrutiny because many Chl *b* mutants accumulate one or more LHCPs (Michel et al., 1983; Ryrie, 1983; Chunaev et al., 1987, 1991; White and Green, 1987; Høyer-Hansen et al., 1988; Terao et al., 1988; Harrison and Melis, 1992). One Chlamydomonas mutant, *cbn*-1-113 (formerly *pg*-113; Michel et al., 1983), has a defective thylakoid protease (Hoober et al., 1990; Hoober and Hughes, 1992), and it is thought that this defect accounts for the strain's ability to accumulate LHCP in the absence of Chl *b*. It is unclear how protease deficiency could impair Chl *b* accumulation and how many other mutations might secondarily result in Chl *b*–deficient phenotypes.

Chlorophyll accumulation is also inhibited by treatments that block LHCP synthesis. For instance, cycloheximide inhibits LHCP synthesis and causes a rapid reduction in the accumulation of both Chl *a* and Chl *b* (Maloney et al., 1989). This could be due to the intrinsic lability of an enzyme or cofactor involved in chlorophyll formation (Huang and Hoffman, 1990). Alternatively, the LHCP could be directly involved in the synthesis of Chl *a* and Chl *b*, as first suggested by Fradkin et al. (1981). Maloney et al. (1989) found that the final step of Chl *a* and Chl *b* synthesis, reduction of the alcohol sidechains, occurs after the pigments have associated with the LHCP scaffolding. Huang and Hoffman (1990) analyzed chlorophyll synthesis in isolated chloroplasts and suggested that synthesis of Chl *b* takes place on LHCP. Another correlation pertains to the Chl *a*– and *b*–deficient phenotype of nitrogen-deficient cells, which coincides with the transcriptionally regulated loss of LHCP (Plumley and Schmidt, 1989). We provide additional evidence in this report that both Chl *a* and Chl *b* are synthesized in association with LHCP.

The precise roles of pigments in LHCP stabilization and the mechanisms used for calibrating the synthesis and accumulation of apoproteins and pigment ligands have been enigmatic. We assessed post-translational controls of LHC II accumulation by analyzing two Chl *b*–deficient strains of Chlamydomonas with different nuclear mutations, each of which affects the biogenetic pathways for LHCP and, subsequently, the synthesis and/or accumulation of light-harvesting pigments. From our results, we concluded that (1) the assembly of LHC II pigments and thylakoid integration of LHCP II is a multistep process, and we identify a crucial intermediate in this process; (2) Chl *b* and Chl *a* deficiencies can result from impaired integration of LHCP into thylakoids; (3) a mutant that is specifically defective in Chl *b* synthesis can still accumulate all LHCP; and (4) a control point for differential synthesis of xanthophylls is intimately associated with the capacity for both Chl *b* synthesis and LHCP II accumulation. We summarize these observations in the form of a mechanistic model that explains how the coordinated synthesis of Chl *a*, Chl *b*, and xanthophylls is dependent upon the presence of LHCP.

RESULTS

Two genetically distinct Chl *b*–deficient nuclear mutant strains of Chlamydomonas were recovered after mutagenesis and metronidazole selection. Mutants PA2.1 and GE2.27 escaped the lethal effects of metronidazole due to their low rates of photosynthetic electron transport under the low light conditions used during selection against wild-type cells. Both mutants were capable of sustained autotrophic growth under high light intensity, demonstrating that the lesions do not affect other components of the photosynthetic apparatus. Moreover, the rates of oxygen evolution, expressed on a per cell basis, were very similar for the two mutants and the wild type when assays were conducted under saturating light conditions (data not shown). Also, the long-term growth rates of the two mutant strains were similar to those of the wild-type strain (within ~15%) when grown photoheterotrophically, indicating that the lesions do not substantially affect overall metabolism. The Chl *a*/*b* ratios of PA2.1 and GE2.27 were 5.5 and 17, respectively, as compared with 2.4 for wild-type cells. The pigment composition of neither strain changed appreciably when grown photoheterotrophically, heterotrophically, or photoautotrophically (data not shown). However, the lesion in GE2.27 was temperature sensitive, with the Chl *a*/*b* ratio decreasing slowly (2 to 3 weeks) during growth at 15 to 20°C but increasing more rapidly (3 to 5 days) toward infinity as the temperature was increased to 37°C. Most of the LHCP II were also lost, presumably due to proteolysis, from GE2.27 but not the wild type at the higher temperature. Temperature dependence of Chl *b* accumulation would be expected from a missense mutation in an enzyme involved in synthesis of the pigment. Similar to the Chl *b*–deficient CD3 mutant of wheat (Allen et al., 1988), high light intensities (>400 μmol m^{-2} sec^{-1}) also increased the Chl *a*/*b* ratio and resulted in a loss of LHCP II of mutant GE2.27, indicating that Chl *b*–deficient complexes are prone to photodestruction. Only cells grown photoheterotrophically at 22 to 24°C and light of ~50 μmol m^{-2} sec^{-1} were used in this study.

On a per cell basis, the Chl *a* levels of PA2.1 were ~50% that of the wild type. GE2.27 is an unusual mutant in that it has wild-type Chl *a* levels. Although not immediately obvious from their Chl *a*/*b* ratios, mutant PA2.1 (with its lower Chl *a*/*b* ratio) accumulated slightly more Chl *b* per cell than mutant

GE2.27 (with a higher Chl *a*/*b* ratio); the significance of the accumulation of Chl *b* per cell is discussed later in regard to Chl *b* synthetic rates for each mutant. The HPLC analyses shown in Figure 1 illustrate the reduced levels of Chl *b* and other pigments in both mutant strains. PA2.1 accumulated high levels of lutein, but less than half of this xanthophyll was recovered in thylakoid membrane fractions (Figure 1). As is typical for Chl *b*–deficient mutants, neoxanthin was reduced to approximately the same extent as Chl *b* in PA2.1, whereas loroxanthin was almost absent. The coordinated accumulation of Chl *b* and neoxanthin was also evident in GE2.27. Loroxanthin levels were diminished in GE2.27 but to a lesser extent than in PA2.1. GE2.27 accumulated slightly elevated levels of violaxanthin and lutein. Another feature of the HPLC traces of GE2.27 is the occurrence of several small peaks eluting near Chl *a* and Chl *b* that have been tentatively identified as Chl *a* with incompletely reduced side chains. Preliminary data indicate that, similar to the situation in greening *y*-1 cells (Maloney et al., 1989), these pigment precursors are associated with LHCP II.

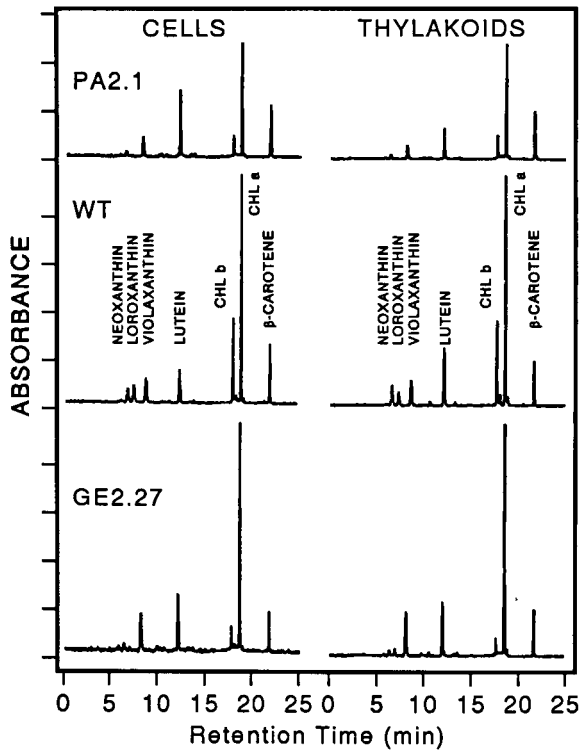

Figure 1. Pigment Composition of Mutants PA2.1, GE2.27, and the Wild Type.

HPLC traces of pigments from PA2.1, wild-type (WT), and GE2.27 strains of Chlamydomonas are shown. Extracts from an equal number of whole cells (left) and thylakoids (right) are shown. Pigments are labeled.

Chl *b* per Cell Does Not Increase when Reaction Center Apoprotein Synthesis Is Inhibited

Chloramphenicol-dependent accumulation of Chl *b* was demonstrated in a wheat mutant (Duysen et al., 1985). Falbel and Staehelin (1994) subsequently identified impaired formation of chlorophyll precursors as the basis for Chl *b* deficiency. To determine whether competition for chlorophyll by (photosystem I) PSI and PSII complexes could also account for reduced levels of Chl *b* accumulation in mutants PA2.1 and GE2.27, we grew each strain for 8 to 12 weeks (n = 3) in chloramphenicol to block synthesis of reaction center apoproteins. With high chloramphenicol concentrations (200 μg/mL), cells had doubling times of several days as compared with ~12 hr without inhibitor. Chloramphenicol reduced the total amount of chlorophyll in GE2.27 and the wild type by ~50%; this is consistent with association of ~50% of chlorophyll with reaction center apoproteins in Chlamydomonas. In PA2.1, total chlorophyll was reduced to 20% of levels in nontreated cells; this reflects the virtual absence of LHCP in this strain (as described later) and a correspondingly greater effect of chloramphenicol on depletion of the chlorophyll binding proteins in this mutant. Residual amounts of PSI apoproteins (and the PSI complex, CP I) were <5% of the uninhibited level, whereas PSII apoproteins (and the PSII complexes, CP 43/CP 47) were undetectable in both mutants and the wild-type strain (data not shown). The final Chl *a*/*b* ratios averaged 3.0 (PA2.1), 1.8 (wild type), and 5.9 (GE2.27), with only small (<10%; n = 3) variation between experiments. Chloramphenicol treatment of GE2.27 resulted in a slightly lower Chl *a*/*b* ratio than expected, but as documented later, this mutant reverts with moderate frequency. During prolonged experiments (8 to 12 weeks), revertant cells with increased Chl *b* would distort the Chl *a*/*b* ratios in the samples. Nonetheless, based on the composite of changes in the (1) Chl *a*/*b* ratios, (2) total chlorophyll per cell, and (3) fraction of chlorophyll associated with PSI and PSII, we concluded that Chl *b* accumulation per cell was not enhanced in these mutants when competition for chlorophyll was reduced by blocking synthesis of reaction center apoproteins.

Accumulation of LHC II and LHCP II in the Chl *b*-Deficient Mutants

LHC often cannot be detected by nondenaturing SDS-PAGE from Chl *b*–deficient mutants of plants and algae, and these mutants lack or are highly deficient in the major LHCP. Other work, however, has documented the accumulation of some LHCPs in Chl *b* mutants (Michel et al., 1983; Ryrie, 1983; Chunaev et al., 1987, 1991; White and Green, 1987; Hφyer-Hansen et al., 1988; Terao et al., 1988; Harrison and Melis, 1992). To determine whether PA2.1 and GE2.27 accumulate antenna complexes, thylakoids were subjected to low temperature, nondenaturing lithium dodecyl sulfate (LDS)-PAGE. As shown in Figure 2A, both PA2.1 and GE2.27 lack LHC II, but three low abundance LHCs from each strain are visible where

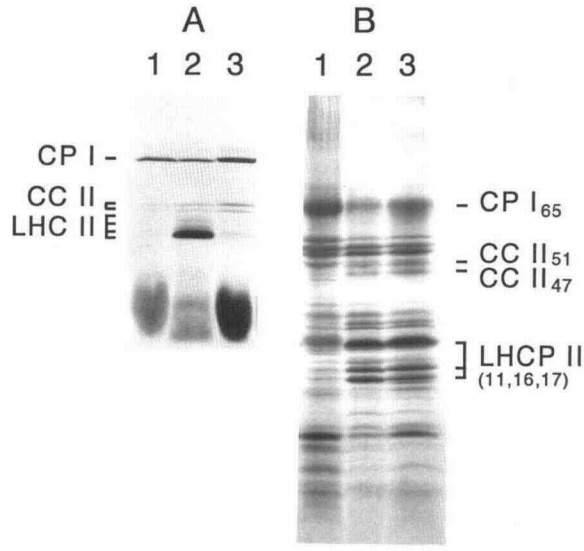

Figure 2. Pigment–Protein Complexes and Thylakoid Proteins of the Mutants and the Wild Type.

(A) and **(B)** Chlorophyll–protein complexes **(A)** or thylakoid polypeptides **(B)** of PA2.1 (lane 1), wild-type (lane 2), and GE2.27 (lane 3) strains of Chlamydomonas were resolved by low temperature LDS-PAGE. Samples were electrophoresed either without **(A)** or with **(B)** prior heat denaturation. The gel was unstained **(A)** or stained with Coomassie Brilliant Blue **(B)**.

CP I and CC II, the photosystems I and II chlorophyll–protein complexes; LHC II, the major LHC II complex plus CP 29, CP 26, and CP 24; CP I_{65}, CC II_{51}, CC II_{47}, and LHCP II (11, 16, and 17), the apoproteins of their respective chlorophyll–protein complexes.

gel lanes were overloaded. These LHCs possess high Chl *a*/*b* ratios and other characteristics typical of LHCs such as CP 24 and CP 26 and will be characterized in a subsequent communication. The pigment–protein complexes of PSI and PSII are present in both mutants; levels of the PSI core complex (CP I) were slightly elevated in both mutants on a per cell basis (Figure 2A).

Similar to most other Chl *b*–deficient mutants that have been studied, PA2.1 has low levels of virtually all LHCPs, including polypeptides 11, 16, and 17 (Figure 2B) as well as polypeptides 9, 14, 15, 17.2, and 18 (data not shown). PA2.1 accumulated almost wild-type levels of two LHCPs, polypeptides 10 and 13, the apoproteins of the complexes shown in Figure 2A. The PA2.1 sample was intentionally overloaded in lane 1 of Figure 2B to demonstrate that the LHCPs do not accumulate to appreciable levels; the major band in the vicinity of 30-kD polypeptide 11 is a non-LHCP with a slightly retarded electrophoretic mobility. A more accurate representation of the cellular levels of PA2.1 thylakoid proteins is presented later. In contrast, GE2.27 accumulated all abundant thylakoid proteins, including those of LHC II, to levels comparable with those of the wild type. The ratios of some of the LHCPs were somewhat altered in the mutant (that is, polypeptide 15 is slightly more abundant), but such minor differences are common in mutant strains. Rocket- and crossed-immunoelectrophoresis using an antibody raised against LHCP II polypeptide 11 (Plumley and Schmidt, 1983) and either thylakoid membranes or whole-cell extracts did not resolve significant quantitative or qualitative differences in the LHCP II of GE2.27 and the wild type (data not shown).

LHCP II Are Synthesized at Wild-Type Rates but Are Degraded in the Stroma of Mutant PA2.1

LHCP II synthesis occurs in at least two Chl *b*–deficient mutants, but they are subsequently degraded (Bellemare et al., 1982; Terao and Katoh, 1989). A similar process could account for the phenotype of PA2.1. In GE2.27, LHCP could accumulate to wild-type levels in the absence of Chl *b* if this mutant were deficient in a putative LHCP protease (Hoober and Hughes, 1992) or if the proteins were synthesized at elevated rates to compensate for enhanced degradation. To assess the rates of LHCP synthesis, cells were subjected to pulse–chase labeling with ^{14}C-acetate or $^{35}SO_4$ (comparable results were obtained with either isotope; see the legend for Figure 3 for details). The cells were separated into soluble/extrinsic and integral membrane fractions using NaOH as extractant. Autoradiographs of SDS-PAGE gels show that there was no difference in the rate of synthesis and stability of LHCP II in the integral membrane fractions of GE2.27 and wild-type cells, as shown in Figure 3A.

In contrast, LHCP II synthesis was nearly undetectable in PA2.1 when pulse durations of 15 min were employed (Figure 3A). Unexpectedly, when the NaOH-soluble fraction of PA2.1 was analyzed after shorter pulse labelings (for example, 5 min), substantial amounts of LHCP II were detected, especially when immunoprecipitation was employed, as shown in Figure 3B. Small amounts of polypeptides 11, 16, and 17 were immunoprecipitated from PA2.1 membranes, which also yielded all of the newly synthesized polypeptide 10. The rate of LHCP II synthesis in PA2.1 probably approaches that of wild-type cells if one compares lanes 6 and 9 (Figure 3B) and, as described later, allowance is made for rapid LHCP II degradation in the mutant. Detection of radiolabeled LHCP II proteins by immunoprecipitation of solubilized membranes was much more effective with preparations from PA2.1 (Figure 3B, lanes 5 and 6) than from wild-type cells (lanes 11 and 12), because the latter contain a large endogenous pool of unlabeled LHCP II. Although there are constraints to loading gel lanes with enough labeled protein from wild-type membranes to readily enable autoradiographic detection, a longer exposure of the gel in Figure 3B showed faint LHCP II bands in the wild-type sample (data not shown). Whereas there were essentially no detectable pools of the soluble forms of LHCP II in either the wild type (Figure 3B, lane 10) or GE2.27 (data not shown), these proteins were recovered most effectively from the soluble fraction of PA2.1 (lanes 4 and 6). The soluble LHCP II of PA2.1 were processed to a size indistinguishable from the mature form, indicating that they reside in the chloroplast stroma. The

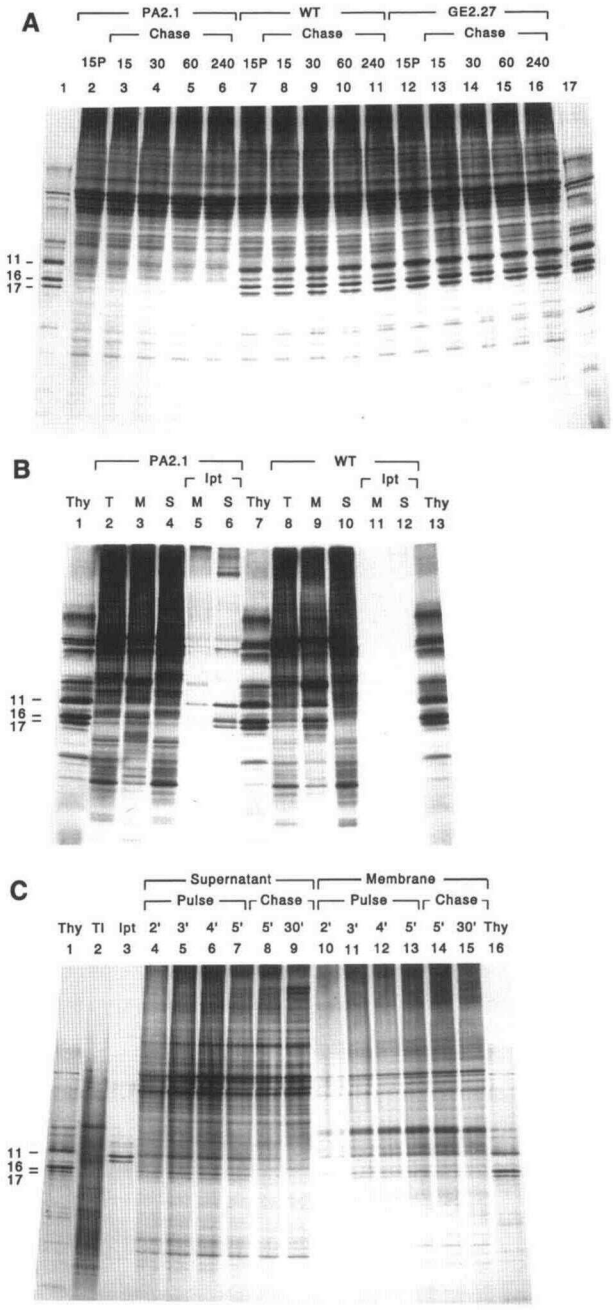

Figure 3. LHCP II Synthesis in Mutants PA2.1, GE2.27, and the Wild Type.

(A) Cells were pulse labeled with ^{14}C-acetate for 15 min (15P) (lanes 2, 7, and 12) and chased with unlabeled acetate for 15 min (lanes 3, 8, and 13), 30 min (lanes 4, 9, and 14), 60 min (lanes 5, 10, and 15), or 240 min (lanes 6, 11, and 16). At each time point, cells were lysed and fractionated into soluble/extrinsic and integral membrane proteins with 0.2 N NaOH. Equal amounts of radioactive protein from the NaOH-insoluble fractions were analyzed by SDS-PAGE. The autoradiograph shows thylakoids from PA2.1 (lanes 2 to 6), the wild type (WT; lanes 7 to 11), and GE2.27 (lanes 12 to 16). The LHCP II (11, 16, and 17) are indicated at left in purified membrane standards from wild-type cells labeled with $^{35}SO_4$ (lanes 1 and 17).

(B) Cells of PA2.1 (lanes 2 to 6) and the wild type (WT; lanes 8 to 12) were pulse labeled with $^{35}SO_4$ for 5 min. Cells were either solubilized directly in gel sample buffer as total protein (T) samples or fractionated with NaOH into soluble (S) and integral membrane (M) fractions before being analyzed as given in **(A)**. Proteins were immunoprecipitated (Ipt) from aliquots of the soluble and membrane fractions using antibodies raised against an LHCP II, polypeptide 11. $^{35}SO_4$-labeled thylakoids (Thy) from wild-type cells are in lanes 1, 7, and 13. The positions of the LHCP II (11, 16, and 17) are indicated at left.

(C) Pulse–chase labeling of PA2.1 cells with $^{35}SO_4$. Cells were labeled for 2, 3, 4, and 5 min (lanes 4 to 7 and 10 to 13) and chased for 5 and 30 min (lanes 8 and 9 and 14 and 15). Cells harvested at the indicated times were separated into NaOH-soluble (Supernatant; lanes 4 to 9) and NaOH-insoluble (Membrane, lanes 10 to 15) fractions and analyzed as given in **(A)**. Control lanes include $^{35}SO_4$-labeled thylakoids (Thy) of wild-type cells (lanes 1 and 16), in vitro translation products obtained with mRNA from wild-type cells (TI, lane 2), and precursors of the LHCP II immunoprecipitated from the translation products with antibodies raised against polypeptide 11 (Ipt, lane 3). The positions of LHCP II (11, 16, and 17) are indicated at left.

possibility that PA2.1 is blocked in LHCP II import into chloroplasts is unlikely because all endoproteases, including transit peptidase, would be inactivated immediately during the NaOH lysis procedure, and yet no precursors were detectable.

The fate of the soluble LHCP II in PA2.1 was examined by pulse–chase analysis using $^{35}SO_4$. Again, most of the LHCP II were recovered in the soluble fraction of NaOH extracts, even when pulse periods of 2 to 5 min were employed, as shown in Figure 3C. These proteins were subjected to rapid degradation with half-lives of only a few minutes. Newly synthesized LHCP II that associate with the membrane fraction of PA2.1 were more stable (Figure 3C, lanes 10 to 15), but this association was also transient because only small amounts of the LHCP II accumulated in PA2.1 thylakoids (Figure 2).

From these analyses (Figure 3), the defect in GE2.27 apparently affects LHC II assembly after insertion of LHCP II into thylakoids. The primary defect in PA2.1 appears to involve LHCP insertion into thylakoids. Although small amounts of the proteins can undergo thylakoid insertion in PA2.1, the unusual presence of high levels of soluble forms indicates a defective chloroplast component that facilitates their membrane integration.

Topological Organization of LHCP II in GE2.27 Is Altered

LHCPs normally are integrated into thylakoids with a folded conformation that is resistant to extraction by chaotropic reagents and largely refractory to protease digestion; these properties were used previously to verify the correct association of LHCP with thylakoids during in vitro import and integration studies (Schmidt et al., 1981; Cline, 1986; Huang

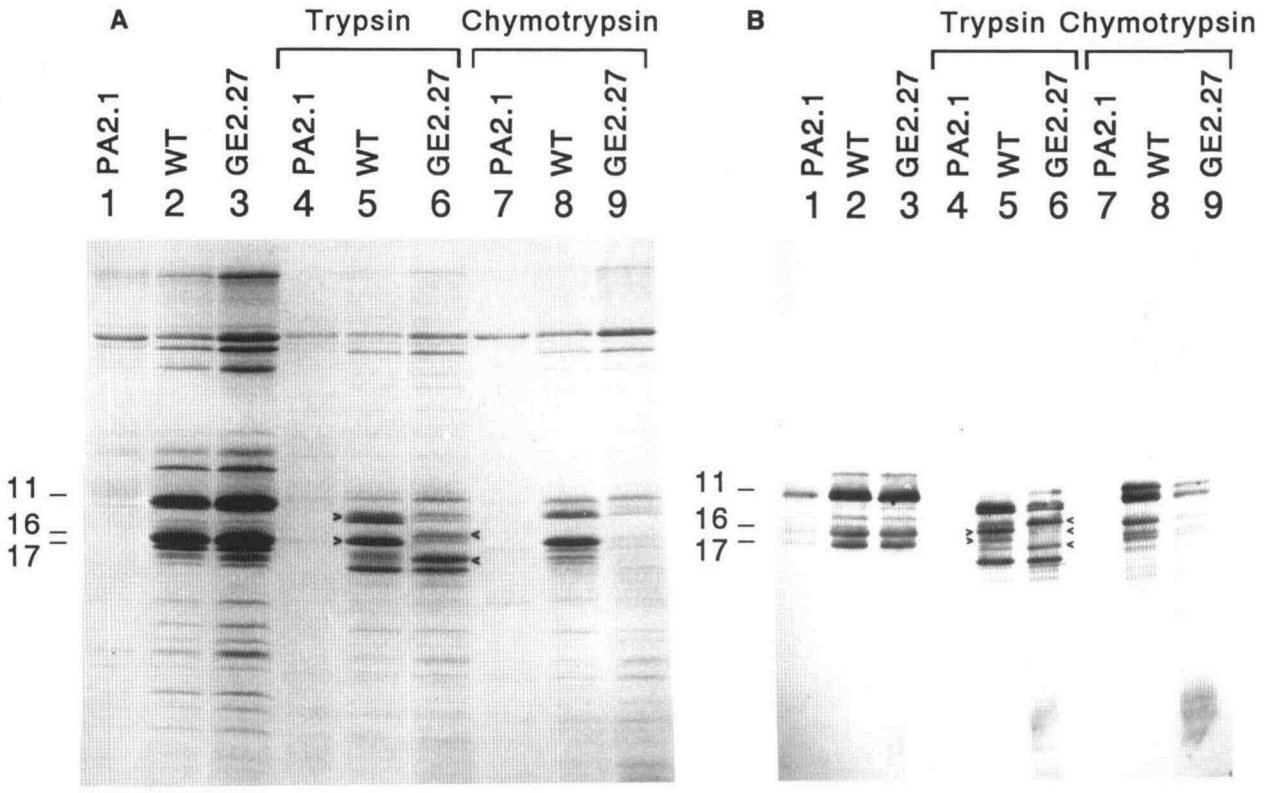

Figure 4. Topological Organization of Wild-Type and GE2.27 LHCP II Proteins.

(A) Intact thylakoids of the wild type (WT) and mutants PA2.1 and GE2.27 were treated with trypsin (lanes 4 to 6) or chymotrypsin (lanes 7 to 9) or were subjected to identical incubation conditions but in the absence of proteases (lanes 1 to 3). Membranes were recovered by centrifugation, and proteins and digestion products were resolved by SDS-PAGE and revealed by staining.

(B) Same samples as in **(A),** but proteins were detected using an antibody raised against an LHCP II, polypeptide 11. Labels are as in **(A)**. (►) and (◄), unique LHCP II fragments in wild-type and GE2.27 thylakoids, respectively.

et al., 1992; Yalovsky et al., 1992). When thylakoids from PA2.1 and GE2.27 were treated with NaOH concentrations up to five times higher than are normally used for such assays, no LHCP polypeptides were released (data not shown), demonstrating that they were firmly inserted into thylakoids.

Huang et al. (1992) found that mutated LHCP can associate with thylakoids to assume an alkali-resistant but protease-sensitive conformation. We tested the effects of several exogenously added proteases on LHCP II in thylakoids purified from PA2.1 and GE2.27. The peptide fragments protected by lipid bilayers were resolved by SDS-PAGE. The resulting peptides were visualized by staining with Coomassie blue in Figure 4A and by immunoblot analyses in Figure 4B. The LHCP II digestion products of PA2.1 were qualitatively similar to those generated from wild-type thylakoids, but because of the low abundance of LHCP II in this mutant, there could be minor differences that were not detected. Many of the LHCP II peptides of GE2.27 resulting from digestion of thylakoids with trypsin or chymotrypsin also appeared to be identical to those generated from wild-type membranes. However, several unique LHCP II digestion products were abundant in GE2.27 preparations (Figure 4). Moreover, polypeptide 16 in GE2.27, but not in the wild type, was sensitive to trypsin. Digestion of GE2.27 thylakoids with chymotrypsin (Figure 4) and thermolysin or V8 protease (data not shown) produced very few qualitatively different peptide fragments but, in all cases, led to more extensive digestion in GE2.27 than in wild-type membranes. Even though we used protease concentrations much higher than those normally used for these assays and all experiments were conducted at room temperature, complete digestion of the LHCP II of GE2.27 was never observed, as indicated by the large size of the peptide products (for example, all abundant trypsin-generated fragments of GE2.27 were larger than ~22 kD). The incomplete digestion of LHCP II indicates that at least some domains of the GE2.27 LHCP II were inaccessible to proteases and are likely to represent folded conformations, which the proteins normally achieve during their integration into thylakoids.

The immunoblots shown in Figure 4B were also reacted with antibodies raised against each of two polypeptides of the core PSII antenna (polypeptides 5 and 6) and a lumenal protein of

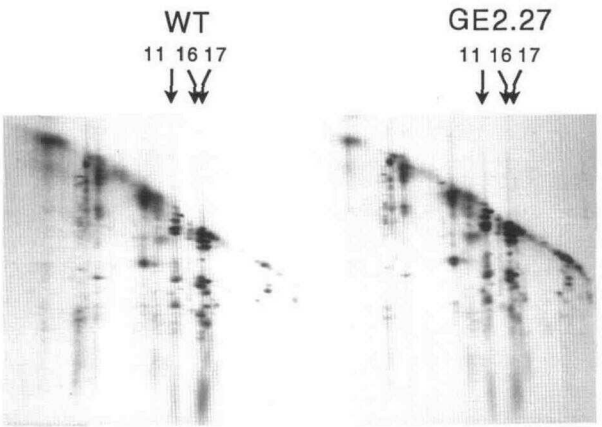

Figure 5. Peptide Mapping of LHCP II of GE2.27 and the Wild Type.

Thylakoid proteins isolated from wild-type (WT) and mutant (GE2.27) cells were subjected to SDS-PAGE. Excised gel lanes were then electrophoresed in the second dimension in the presence of trypsin. The positions of LHCP II (11, 16, and 17) in the first dimension gel are indicated above each gel. Digestion products were visualized by silver staining.

the PSII water oxidation complex (polypeptide 12). No differences in the peptide fragments of polypeptides 5 and 6 were observed in either mutant relative to the wild type, and polypeptide 12 was completely resistant to proteolysis (data not shown). Also, thylakoids from two PSI mutants (M18 and M27) and one PSII mutant (8-36c) were subjected to proteolysis. The LHCP II fragments generated were identical to those of the wild type. Finally, we detected no differences in the lipid compositions of GE2.27 and wild-type thylakoids (data not shown). Four conclusions were drawn from these control experiments. First, the orientations of thylakoid vesicles from GE2.27 and the wild type are identical. Second, the defect in GE2.27 does not result in severe alterations of thylakoid organization, as is frequently observed in Chl *b*–deficient mutants. This point has been verified by electron microscopy (data not shown). Third, the unique LHCP II fragments generated in GE2.27 by protease treatment do not result from their inability to associate with either PSI or PSII. Finally, gross alterations in lipid composition are not responsible for the changes in LHCP II topological organization observed in mutant GE2.27.

LHCP II in GE2.27 clearly are integrated into thylakoids abnormally but to a greater extent (Figure 4) than occurs upon spontaneous association of proteins with membranes (Huang et al., 1992) or than occurs in the Chl *b*–less Chlamydomonas mutant *cbn*-1-113 (Hoober et al., 1990). The failure of LHCP II to attain their normal topological organization could result from (1) defects in the primary structure of the LHCP II proteins, (2) a deficiency of pigment(s) required for proper folding of LHCP II, or (3) the absence of pigments that might shield LHCP II proteins from proteolytic attack. These issues are addressed below.

LHCP II of Mutant GE2.27 and the Wild Type Are Structurally Identical

Light-harvesting proteins accumulate in pigment-deficient mutants of photosynthetic bacteria only when secondary mutations of the apoprotein genes confer resistance to endogenous proteases (Zurdo et al., 1993). To determine whether the LHCP II of GE2.27 are altered as a result of mutations or posttranslational modifications, thylakoid polypeptides were subjected to peptide mapping, as illustrated in Figure 5. We detected no qualitative differences between the LHCP II of GE2.27 and the wild-type strain, using four different proteases and visualizing the resulting peptides by either silver staining (Figure 5) or, in the case of proteins labeled in vivo with $^{35}SO_4$, by autoradiography (data not shown). Therefore, the mutation in GE2.27, although impairing assembly of LHC II, did not result from major structural defects of the apoproteins. These results were not completely unexpected because it is unlikely that each of the multiple copies of the LHCP II genes would be mutated. Still, a defect in one LHCP II could adversely affect the assembly of other proteins of the complexes. Therefore, other methods to assess the structural integrity of the LHCP II of mutant GE2.27 were employed.

GE2.27 LHCP II Can Bind Chl *b* and Xanthophylls upon in Vitro Reconstitution

To determine whether the LHCP II of GE2.27 can bind pigments, LHC II was reconstituted in vitro with apoproteins from the mutant. Reconstitution of LHC II from wild-type thylakoids requires only the addition of Chl *b* (Plumley and Schmidt, 1987), but apoproteins from GE2.27 membranes did not form stable complexes under these conditions (data not shown). However, as shown in Figure 6, if both neoxanthin and Chl *b* were included in the reconstitution mixture with SDS-solubilized and heat-denatured thylakoids of GE2.27, the extent of LHC II formation was equivalent to that obtained with wild-type membranes. The requirement to add both Chl *b* and neoxanthin to GE2.27 thylakoids for reconstitution of LHC II is consistent with earlier studies that demonstrated a requirement for all LHC II–localized xanthophylls to achieve efficient assembly of LHC II in vitro (Plumley and Schmidt, 1987). Although GE2.27 is deficient in loroxanthin, the addition of this pigment was not required for LHC II reconstitution with GE2.27 proteins (data not shown); this is consistent with previous reconstitution assays employing xanthophylls from heterologous sources, which showed that lutein and loroxanthin are interchangeable (Cammarata et al., 1992). The absorption, fluorescence, and circular dichroism spectra of reconstituted LHC II from GE2.27 were identical to those from wild-type cells (data not shown). These data provide more conclusive evidence that the lesion in GE2.27 does not alter the structure of LHCP II because, in vitro, they are fully able to bind Chl *a*, Chl *b*, and xanthophylls.

The LHCP II fragments from trypsinized thylakoids of the

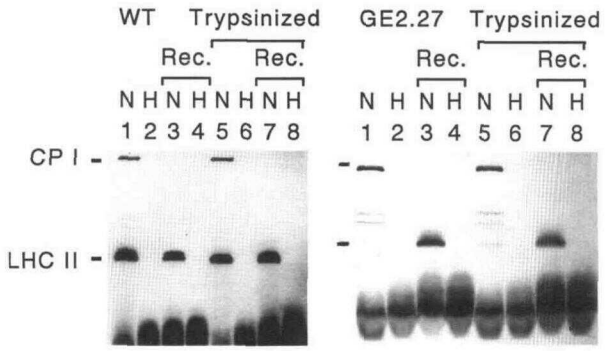

Figure 6. In Vitro LHC II Reconstitution with Apoproteins from GE2.27 and the Wild Type.

LHC II was reconstituted from SDS-solubilized and heat-denatured thylakoids from wild-type (WT) and mutant (GE2.27) cells. For lanes 5 to 8, membranes were subjected to trypsinization before solubilization and LDS-PAGE. Nonheated samples (N) containing native pigment protein complexes including LHC II and CP I are shown in lanes 1 and 5. Other samples were heated (H, lanes 2 and 6) and subjected to LHC II reconstitution (Rec., lanes 3 and 7). The reconstituted samples in lanes 4 and 8 were heated before electrophoresis. The gels were unstained. The labels are as in Figure 2A.

wild type and mutant GE2.27 alsc could be fully reconstituted (Figure 6). The ability to reconstitute LHC II in vitro has been shown to be independent of 21 N- and 57 C-terminal residues of the apoproteins (Cammarata and Schmidt, 1992), consistent with structural data indicating that all chlorophyll binding domains reside within the central, ~200–amino acid region of LHCP II (Kühlbrandt et al., 1994). Likewise, the novel trypsin-generated fragments of >22 kD of GE2.27 LHCP II appear to retain all pigment binding sites. Therefore, it is more likely that the altered proteolysis of LHCP II of GE2.27 thylakoids by trypsin is due to incomplete membrane integration of one or more domains of the proteins as opposed to the occurrence of unoccupied pigment binding sites. Forms of LHCP II with variant topology have been observed during studies of LHCP import/assembly in isolated chloroplasts (Reed et al., 1990), and it is possible that the unique trypsin products of GE2.27 LHCP II (Figure 4) are analogous to the thermolysin-derived LHCP-DP2 fragment that has been transiently detected in thylakoids (Reed et al., 1990).

Impaired Pigment Synthesis in LHC II Mutants

An additional candidate for the primary defect of GE2.27 is impaired synthesis of either Chl *b* or neoxanthin. Both of these pigments, together with Chl *a*, lutein, and violaxanthin, are required for optimal reconstitution of LHC II in vitro (Figure 6; Plumley and Schmidt, 1987) and presumably are equally important during in vivo LHC II assembly. Therefore, pigment synthesis of wild-type and mutant cells was analyzed by pulse labeling with ^{14}C-acetate, followed by thin-layer chromatography (TLC) and autoradiography. Total isotope uptake and the incorporation of label into acetone-soluble fractions were equivalent among the two mutant and wild-type strains (data not shown). Although very brief labeling periods are preferred for analyses of pigment synthesis because the half-lives of some subpopulations can be quite short (Grumbach et al., 1978; Britton, 1986), the duration of pigment pulse labeling is constrained by the degree to which sufficient amounts of carbon-14 are incorporated to enable subsequent detection. To determine whether pigment synthesis is impaired or whether pigment degradation is accelerated in GE2.27 and PA2.1, pulse-labeling periods were varied from 5 to 60 min and chases with unlabeled acetate ranged from 5 min to 70 hr.

After very short pulse labeling of wild-type cells, synthesis of Chl *a* was readily detected in autoradiographs, as indicated in Figure 7. In contrast, radioactivity did not appear in Chl *b* and neoxanthin until ~20 min and several hours, respectively,

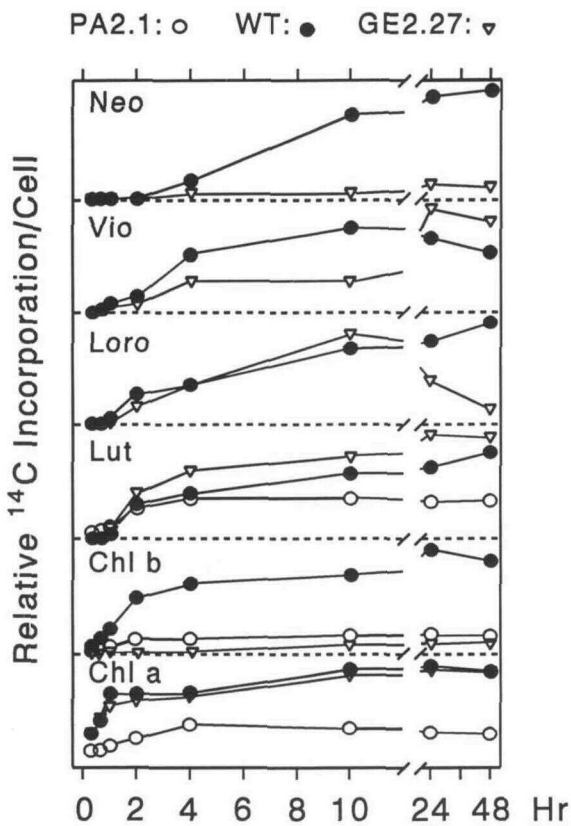

Figure 7. Synthesis of Chlorophylls and Xanthophylls in Wild-Type and Mutant Cells.

Cells were pulse labeled with ^{14}C-acetate for 20 and 40 min and diluted 10-fold in acetate-containing medium to initiate the chase. Samples were taken from the 20- and 40-min time points and at 1, 2, 4, 10, 24, and 48 hr during the chase. The relative amounts of carbon-14 per cell in neoxanthin (Neo), violaxanthin (Vio), loroxanthin (Loro), lutein (Lut), Chl *b*, and Chl *a* are plotted for PA2.1 (○), GE2.27 (▽), or wild-type (●) cells.

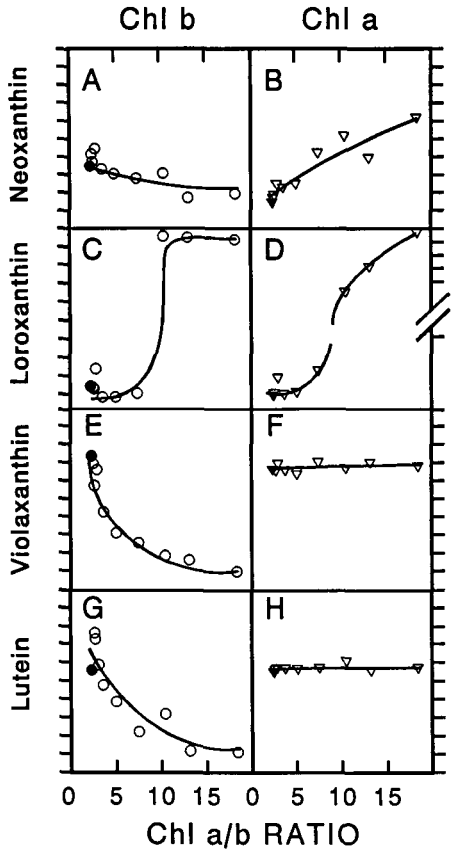

Figure 8. Correlations of Chlorophyll and Xanthophyll Formation in GE2.27 Revertants with Varying Chl *b* Levels.

Revertants of GE2.27 with Chl *a*/*b* ratios of 2.5 to 18 were obtained as described in Methods. Pigments from the wild type, the parental GE2.27 mutant, and eight revertant strains were resolved by HPLC and monitored at 460 nm. From the peak heights for each pigment, the Chl *b*/xanthophyll (left) and the Chl *a*/xanthophyll (right) ratios were plotted for the four xanthophylls as a function of the Chl *a*/*b* ratio.
(A) and **(B)** The ratio of neoxanthin to Chl *b* **(A)** or Chl *a* **(B)**.
(C) and **(D)** The ratio of loroxanthin to Chl *b* **(A)** or Chl *a* **(B)**.
(E) and **(F)** The ratio of violaxanthin to Chl *b* **(A)** or Chl *a* **(B)**.
(G) and **(H)** The ratio of lutein to Chl *b* **(A)** or Chl *a* **(B)**.
The y-axis in each plot represents a 10-fold change except in **(D)**, in which a 50-fold change in the ratio occurs. The leftmost data point in each plot (solid symbols) is from wild-type cells (Chl *a*/*b* = 2.4).

after addition of ^{14}C-acetate. Similarly, radiolabeled Chl *a* of PA2.1 and GE2.27 was visible after labelings of 5 to 15 min, but these protocols did not enable detection of either Chl *b* or neoxanthin synthesis in the mutants (data not shown). With longer labeling times (20 to 40 min; Figure 7), it was apparent that the rates of synthesis of pigments in PA2.1 are much slower (when expressed on a per cell basis) than in the wild type or GE2.27. However, because PA2.1 has ~50% as much Chl *a* per cell as the wild type (Figure 1), its rate of synthesis on a per Chl *a* basis approximates that of the other strains. In other words, the rate of Chl *a* synthesis in PA2.1 parallels the extent to which it accumulated. A low rate of Chl *a* turnover was observed in PA2.1 (Figure 7), but some turnover was occasionally observed in the other two strains and is probably not statistically significant. The rate of synthesis of Chl *b* in PA2.1 also correlated with the extent of its accumulation (that is, PA2.1 accumulated ~25% of the Chl *b* per cell as did the wild type, and incorporation of label into Chl *b* was reduced ~75% as compared with wild-type cells). We were not able to detect turnover of this pigment in PA2.1 under any condition. In accord with the very small amounts of neoxanthin and loroxanthin in PA2.1 (Figure 1), labeling of these pigments was not detectable in this strain. Overall, these results indicated that the rates of synthesis of Chl *a* and Chl *b*, and presumably of neoxanthin and loroxanthin, corresponded to the levels of pigment accumulation in PA2.1. Because turnover of pigments was insignificant in PA2.1, the synthesis of many antenna pigments appeared to be dependent upon the extent to which LHCPs were integrated into thylakoids.

The kinetics of Chl *a* synthesis in GE2.27 were indistinguishable from those of the wild type (Figure 7); this is consistent with equivalent levels of this pigment in the two strains. Likewise, labeling of lutein and loroxanthin in GE2.27 occurred with wild-type kinetics. In contrast, labeled Chl *b* was not detected in GE2.27 until 2 to 4 hr after addition of the isotope, and the extent of its labeling was reduced approximately sevenfold. In GE2.27, the rate of synthesis of neoxanthin reflected its level of accumulation, whereas, unexpectedly, it appeared that turnover of loroxanthin controls the extent of its accumulation in this mutant (Figure 1 versus Figure 7). It is unclear whether this pigment is degraded or reconverted to its lutein precursor. Although violaxanthin levels were normal in GE2.27 (Figure 1), the labeled pool of this pigment was reduced during the course of the experiment, possibly reflecting alterations of the xanthophyll epoxidation/deepoxidation cycle in this mutant. We concluded that, overall, both the rate and extent of Chl *b* formation are specifically impaired in GE2.27. Neoxanthin synthesis and loroxanthin stability seem to be diminished because of secondary effects of the primary lesion.

GE2.27 Revertants Coordinately Accumulate Chl *b* and Neoxanthin

Partial revertants of GE2.27 occur with moderate frequency and can be selected visually among colonies on agar plates. Most have a Chl *a*/*b* ratio of ~10, as opposed to 17 for nonrevertants, but by screening several hundred revertant colonies, we isolated strains with Chl *a*/*b* ratios ranging from 18 to 2.5. The revertant collection affords a novel means to correlate Chl *b* synthesis with the accumulation of xanthophylls. Pigments of the various strains were analyzed by HPLC, and their amounts are shown in Figure 8. Because the GE2.27 parent mutant has wild-type levels of Chl *a*, decreases in the Chl *a*/*b* ratio of revertants reflect predominantly an increased level of

Chl *b*, with virtually no change in Chl *a*. The Chl *b*/neoxanthin ratio (Figure 8A) changed less than twofold as cells reverted from their Chl *b*–deficient phenotype to a phenotype more like that of wild-type cells. The rather constant stoichiometry between Chl *b* and neoxanthin throughout an increase of more than sevenfold in the Chl *b* content per cell further substantiated that the accumulation of these two pigments is tightly coordinated. The Chl *b*/neoxanthin ratio is never unity, perhaps because of variations in the amounts of neoxanthin associated with different LHCs (Figure 2A; Peter and Thornber, 1991) or its occurrence in the envelope or plastoglobuli. As expected, the Chl *a*/neoxanthin ratios increased as the mutant phenotypes became more fully expressed (Figure 8B). Loroxanthin ratios behaved quite differently (Figures 8C and 8D). When Chl *a*/*b* ratios were less than ~10, the GE2.27 revertants had nearly constant Chl *b*/loroxanthin and Chl *a*/loroxanthin ratios, but these later two ratios increased ~10-fold and ~50-fold, respectively, when the Chl *a*/*b* ratios were >10. These results reflect a substantially greater loss of loroxanthin (50-fold reduction per cell) than of Chl *b* in the most severely affected mutant phenotypes. In contrast, the contents of violaxanthin and lutein per cell were relatively constant (Figures 8F and 8H), and the decreasing Chl *b*/violaxanthin and Chl *b*/lutein ratios reflected primarily a decrease in the Chl *b* per cell (Figures 8E and 8G).

The GE2.27 revertants provide strong evidence that the accumulations of Chl *b*, neoxanthin, and loroxanthin are strongly coupled. Complete or partial restoration of Chl *b* synthetic capabilities enabled neoxanthin and loroxanthin to accumulate in a nearly stoichiometric manner. Equally significant, the LHCP II of revertant strains with a Chl *a*/*b* ratio of <5 were completely integrated into thylakoids, as determined by the wild-type profiles of peptides obtained when thylakoids were treated with various proteases (data not shown). This provides additional support for the participation of Chl *b* (and perhaps xanthophylls) in the integration of LHCP II in thylakoids and provides in vivo data consistent with Cline's observations (1988) that there is a close relationship between assembly of LHC II and protease insensitivity of newly integrated LHCP.

DISCUSSION

Mutant PA2.1 Is Defective in Thylakoid Insertion of LHCP II

The pigment and protein phenotypes of PA2.1 are typical of many Chl *b*–deficient mutants. PA2.1 is almost totally deficient in polypeptides 11, 16, and 17, the apoproteins of LHC II, as well as in polypeptides 14, 15, 17.2, 18, and 22, the apoproteins of LHC I of the PSI antenna. In contrast, polypeptides 10 and 13 are accumulated to wild-type levels in PA2.1 and could be recovered as fully assembled LHC, tentatively identified as CP 24 and CP 26 (Figure 2). The preferential accumulation of minor LHC with Chl *a*/*b* ratios >2 was also observed in mutant GE2.27 (Figure 2) and is common in Chl *b* mutants (Chunaev et al., 1987, 1991; Høyer-Hansen et al., 1988; Terao et al., 1988; Harrison and Melis, 1992), possibly because they have a low Chl *b* requirement for assembly (White and Green, 1987). Our results with PA2.1 and GE2.27 support this hypothesis, whereas results from PA2.1 indicate that there are differences in the mechanisms for thylakoid insertion of the various LHCPs.

PA2.1 synthesized LHCP II at approximately wild-type rates, but these were mostly rapidly degraded as soluble but mature polypeptides (Figure 3B). This finding complements import/thylakoid integration studies with isolated chloroplasts that have detected stromal forms of the apoproteins (Reed et al., 1990). However, turnover of mature, soluble LHCP II in PA2.1 contrasts with earlier studies that showed degradation after the proteins had undergone thylakoid integration (Bennett, 1981; Bellemare et al., 1982; Terao and Katoh, 1989). Impaired integration of LHCP II in PA2.1 could be due to a defect in one of several factors. For instance, it could be due to a reduced rate of total chlorophyll synthesis, as suggested by data from Falbel and Staehelin (1994), but this is unlikely because LHCP II are much more completely integrated into thylakoids in Chlamydomonas mutants that are unable to synthesize Chl *a* and Chl *b* (Herrin et al., 1992) than occurs in PA2.1 (Figure 3). Reed et al. (1990) demonstrated the accumulation of mature LHCP in the stromal fraction when isolated chloroplasts were treated with ionophores, but it is unlikely that the lesion in PA2.1 affects a thylakoid ion channel because PA2.1 grows photoautotrophically.

It is possible that PA2.1 has a defect in either the ct-HSP70 homolog (Yalovsky et al., 1992), another factor required for membrane integration (Cline, 1986; Yuan et al., 1993), or an unidentified thylakoid-localized receptor for imported proteins (Keegstra, 1989). In any case, the defective component seems to be exquisitely specific for LHCP, whereas other proteins, such as ferredoxin-$NADP^+$ reductase (Tsugeki and Nishimura, 1993), that also interact with ct-HSP70 are faithfully imported into chloroplasts of PA2.1, as deduced from its autotrophic growth capability. Perhaps only one ct-HSP70 homolog (Marshall et al., 1990) is required for LHCP II import and/or integration, whereas other homologs are required for the minor LHCP and the other imported proteins that accumulate in PA2.1. Alternatively, a defective import/integration factor in PA2.1 may have partial activity, allowing functional interactions with some but not all LHCPs. Such factors may not be required for the import and integration of minor LHCP and/or other imported proteins; unassisted membrane integration might occur, with varying degrees of efficiency, for all imported proteins. Finally, different imported proteins rely upon different pathways for sorting within the chloroplast (Michl et al., 1994), and only one of these pathways may be defective in PA2.1. Regardless of the precise lesion, PA2.1 represents the only known Chl *b*–deficient mutant whose defect involves a specific defect in translocation through the stroma and/or integration of LHCP II into thylakoids.

Defective Chl *b* Synthesis in Mutant GE2.27

Chl *b* has been repeatedly hypothesized to play a role in the stabilization of LHCPs, especially those of LHC II, yet mutants GE2.27 (Figure 2B) and *cbn*-1-113 (Michel et al., 1983) accumulate wild-type levels of all LHCP. As discerned from studies of *cbn*-1-113 (Michel et al., 1983; Eichenberger et al., 1986) and our published (Figure 1) and unpublished data of GE2.27, these two mutants differ markedly in their carotenoid compositions, amount of Chl *b* per cell, circular dichroism spectra, rates of light-dependent oxygen evolution, and thylakoid ultrastructure. Therefore, we conclude that these mutants have different lesions. Mutant *cbn*-1-113 is also protease deficient (Hoober et al., 1990; Hoober and Hughes, 1992), and this may account for the accumulation of LHCP in the absence of Chl *b* in this mutant. It is unlikely that this protease is missing in GE2.27 because LHCP II are rapidly degraded when this strain is grown under high light or at elevated temperatures (see Results) or when thylakoids are placed in low salt buffers (data not shown). LHCP II turnover in GE2.27 under these conditions may be mediated by a different protease than the one defective in mutant *cbn*-1-113, but this seems unlikely. Although *cbn*-1-113 and GE2.27 are different mutations, both demonstrate that in Chlamydomonas the ability to accumulate wild-type levels of LHCP is independent of the ability to accumulate Chl *b*.

The data presented in Figures 5 and 6 are inconsistent with a lesion in LHCP II structural genes in GE2.27. However, proteolysis of thylakoids revealed an altered topological organization of LHCP II in this strain (Figure 4). LHCP II form three transmembrane helices, two of which have extensions into the stromal compartment (Kühlbrandt et al., 1994). Previous studies have shown that the domains in the stroma are unexpectedly resistant to digestion by exogenous proteases. Moreover, because protease resistance can be demonstrated with purified LHC II (data not shown), some sites might be occluded directly or indirectly by pigment ligation. In import studies with intact chloroplasts, Adam and Hoffman (1993) and Reed et al. (1990) identified thylakoid-localized intermediates of LHCP with increased susceptibility to trypsin cleavage. It was proposed that augmented proteolysis could be due to delayed pigment assembly. The results obtained with GE2.27 support this hypothesis, but the novel profiles of LHCP II digestion products obtained with GE2.27 thylakoids are not due to the absence of pigment shields per se. Because LHCP II from trypsinized thylakoids of mutant GE2.27 can be reconstituted with pigments in vitro as efficiently as intact apoproteins (Figure 6), it is more likely that pigment binding induces conformational changes of the proteins after membrane insertion. This interpretation would be consistent with data obtained from chloroplast import studies that demonstrated that protease insensitivity of newly integrated LHCP coincided with formation of LHC II (Cline, 1988).

The role of xanthophylls in the topological organization of the LHCP II also warrants consideration because neoxanthin and loroxanthin are deficient in GE2.27. Neoxanthin is likely to be located peripherally, whereas loroxanthin is functionally interchangeable with lutein (Cammarata et al., 1992). Also, lutein, which is proposed to be centrally organized within the membrane-spanning helices of the LHCP II (Kühlbrandt et al., 1994), is present at wild-type levels in GE2.27. Therefore, Chl *b* is favored as the entity needed for completion of the topological organization of LHCP II in mutant GE2.27. Although the structural model for LHC II by Kühlbrandt et al. (1994) suggests a nearly equal distribution of Chl *b* on both the stromal and lumenal planes of the lipid bilayer, the energy transfer kinetic measurements of Pälsson et al. (1994) indicate that the two chlorophyll species are partitioned toward opposite sides of the membrane. It follows that synthesis/assembly of Chl *b* would be topologically constrained unless antenna proteins undergo a multistep integration process. LHCP II appear to assemble low levels of Chl *b* in mutant GE2.27 (F.G. Plumley and G.W. Schmidt, manuscript in preparation) and therefore are at an intermediate step of complex formation that provides at least partial protection from exogenous proteases (Figure 4). In contrast, the LHCP II of mutant *cbn*-1-113, which is totally devoid of Chl *b*, seem to be arrested at a still earlier stage of integration, as reflected in their more complete digestion by exogenous proteases (Hoober et al., 1990). In any case, it seems certain that pigments play important roles in the folding of LHCP II in vivo and in vitro and that the LHCP II of GE2.27 are able to complete this process only partially because of inadequate levels of Chl *b*.

Pigment pulse–chase labeling data (Figure 7) indicated that GE2.27 is defective in Chl *b* and neoxanthin synthesis. Low levels of Chl *b* accumulation indicated that the mutation is leaky. It is possible that the bulk of newly synthesized Chl *b* is rapidly degraded in GE2.27, but this seems unlikely for several reasons. First, ample levels of two minor LHCPs were present and bind pigments (Figure 2A), and the LHCP II proteins of GE2.27 could bind Chl *b* in vitro (Figure 6) and partially do so in vivo (F.G. Plumley and G.W. Schmidt, manuscript in preparation). Thus, unless the defect is in an LHC II–specific factor for Chl *b* assembly with apoproteins, it appears that all Chl *b* molecules synthesized are rapidly assimilated into developing complexes. Second, with pulse labeling as short as 5 min, the small amounts of Chl *b* synthesized in GE2.27 (and PA2.1) did not turn over, regardless of the duration of the chase. In contrast, Chl *b* (and Chl *a*) turnover was easily detected in carotenoid mutants of Chlamydomonas in experiments using a 10-min pulse labeling (Herrin et al., 1992). Third, the kinetics of Chl *b* synthesis in PA2.1 and GE2.27 were different (Figure 7), and rapid turnover does not easily account for the reduced rates of labeling of Chl *b* in both mutants. Fourth, using the same protocols employed by Falbel and Staehelin (1994), we recently found that GE2.27 accumulated two chlorophyll precursors, protochlorophyllide and Chlide *a*. We tentatively interpret these results as an indication that Chl *b* synthesis is blocked at the level of Chlide *a* → Chlide *b* in GE2.27.

Currently, the defect in GE2.27 cannot be definitively ascribed to a mutated gene for a Chl *b* biosynthesis enzyme,

but the temperature sensitivity of the phenotype and moderate frequency of revertants are consistent with a missense mutation that diminishes catalytic activity of Chl *b* oxygenation (Schneegurt and Beale, 1992). We suggest that the defect involves either a monooxygenase that converts the 3-methyl group on ring B to a hydroxyl intermediate or an NADP-dependent dehydrogenase that oxidizes this intermediate to the 3-formyl group unique to Chl *b*. It is possible, however, that the defect in GE2.27 involves a defective chlorophyll synthetase (Rüdiger and Schoch, 1991). A defect in this enzyme would account for the aberrant accumulation of Chlide *a* in this mutant and would provide support, in a general fashion, for the model of Falbel and Staehelin (1994), as a defect at this point would retard the overall rate of chlorophyll synthesis. We discount this hypothesis because decreased Chl *b* accumulation due to an impaired chlorophyll synthetase should have been ameliorated when GE2.27 was grown in chloramphenicol and/or darkness to decrease the demand for chlorophyll by PSI and PSII proteins (Akoyunoglou et al., 1978; Herrin et al., 1992). Under these growth conditions, formation of esterified Chl *b* should have been stimulated but instead was still specifically inhibited in GE2.27. It is also feasible that the defect in GE2.27 involves an enzyme required for neoxanthin or loroxanthin synthesis and that the lack of Chl *b* synthesis is a secondary consequence of these lesions. These hypotheses are inconsistent with the findings of Rock and Zeevaart (1991) that neoxanthin-deficient mutants still accumulate Chl *b* and documentation that loroxanthin is not required for in vitro (Figure 6; Cammarata et al., 1992) or in vivo (Plumley et al., 1989) assembly of LHC II. Therefore, the lesion in mutant GE2.27 most likely specifically affects Chl(ide) *b* biosynthesis, and failure to synthesize neoxanthin, stabilize loroxanthin, and esterify Chlide *a* are all secondary consequences of an altered Chl *a* oxygenase or dehydrogenase in GE2.27.

Overall, our results indicated little futile pigment synthesis (with the possible exception of loroxanthin) in GE2.27 and PA2.1, because their rates of pigment synthesis mirrored rates of pigment accumulation. Moreover, the results from GE2.27 and PA2.1 may be extended to many previously described Chl *b*–deficient mutants as well as to the chlorotic phenotypes arising from nitrogen deficiency, which has been correlated with severe depletion of mRNAs for LHC apoproteins (Plumley and Schmidt, 1989). Some Chl *b*–deficient mutants may have impaired ability to synthesize Chl *b*, but importantly, the results from PA2.1 and the barley and wheat mutants (Falbel and Staehelin, 1994) clearly indicate that defects in the Chl *b* synthetic pathway per se are not necessarily the primary lesions in many of these mutants.

Interrelationships between Pigment Biosynthesis and LHCP II Accumulation

The studies of chlorophyll and xanthophyll synthesis in GE2.27 and PA2.1 apparently represent the initial attempts to differentiate between pigment synthesis and pigment turnover in Chl *b*–deficient mutants. The surprising outcome is that LHCP II are implicated directly in the synthesis of Chl *a*, Chl *b*, neoxanthin, and loroxanthin. PA2.1 was severely affected in LHCP accumulation and correspondingly synthesized each chlorophyll and xanthophyll moiety (Figure 7) only to the extent that they were recovered in PSI/PSII complexes, in minor LHC, and/or in envelopes/plastoglobuli. Also, the series of partial revertants of GE2.27 with gradations in the degree of Chl *b* deficiency illustrated a positive relationship between the levels of Chl *b*, completely integrated LHCP II, and neoxanthin (Figure 8). Moreover, incomplete integration of LHCP II in GE2.27 correlated with an apparent failure to convert violaxanthin, which accumulated to higher levels in this mutant, to neoxanthin (Parry et al., 1992), as well as with maintenance of loroxanthin after its formation from lutein (Figures 7 and 8). To account for these observations, we favor a hypothesis that extends earlier observations of Fradkin et al. (1981), Maloney et al. (1989), and Huang and Hoffman (1990). We suggest that LHCPs could be sites at which synthesis of Chl *a*, Chl *b*, and two xanthophylls occurs most efficiently, depending on the extent to which LHCPs are folded into thylakoids. Also, Chl *b* formation, which we suggest has Chlide *a* as its precursor, could occur when LHCP are in a partially integrated state such that the enzymes interact not only with the porphyrin substrates but also with domains of the binding proteins. This would place the Chl *b* precursor in the hydrophobic environment thought to be required for synthesis of a formyl group without a *gem*-diol intermediate (Porra et al., 1994). Finally, translation of chloroplast-encoded chlorophyll binding proteins also is attendant with chlorophyll synthesis (Eichacker et al., 1990, 1992), suggesting that interlinking the final steps of pigment synthesis with formation of apoprotein binding sites is a physiological necessity.

METHODS

Strains and Culture Conditions

Chlamydomonas reinhardtii 137 (GB125, mt$^+$) is available from the Chlamydomonas Genetics Center at Duke University (Durham, NC). The two mutant strains, GE2.27 and PA2.1, were obtained by ethyl methanesulfonate and azide mutagenesis, respectively, and metronidazole selection (Schmidt et al., 1977). Half of the progeny obtained with repeated backcrosses between each mutant and parental cells had the wild-type phenotype, which is indicative of a single, nuclear gene lesion. Only mutant clones obtained from such backcrosses were used in this study. Cultures were grown in Tris-acetate-phosphate (TAP) medium (Harris, 1989) at 22 to 24°C on an orbital shaker (~150 rpm) with constant illumination (50 μmol m^{-2} sec^{-1}) from cool-white fluorescent bulbs. When present, chloramphenicol was added to TAP at 200 μg/mL. Cells were grown for 8 to 12 weeks; at this concentration, cells grew at ~20% of their normal growth rate. GE2.27 frequently reverts to a phenotype with higher levels of chlorophyll (Chl) *b*; mutant colonies were picked from agar plates every 2 to 4 weeks, and all experiments were conducted with cultures having the mutant phenotype

as verified by a Chl *a*/*b* ratio of ~17. Revertant strains, visually distinguished on agar plates by their pigmentation, were also selected and characterized.

Pigment and Acyl Lipid Analyses

Whole cells or thylakoids (prepared as described later) were extracted in 80% acetone for chlorophyll determinations using the equations of MacKinney (1941). Cell concentration was determined by fixation with iodide-potassium iodide (Lugol's solution) and use of a hemacytometer. For HPLC analyses, pigments were extracted with 90% acetone (containing 0.01 N NH_4OH) overnight at −20°C and centrifuged at 14,000*g* for 10 min; the pellet was reextracted in 80% acetone. Pigments were resolved by HPLC using a 4.6 mm × 25 cm Microsorb-MV C18 column (Rainin, Woburn, MA) and an acetone/water mobile phase (Plumley et al., 1989).

For pulse–chase labeling of pigments, log phase cells were harvested at 5000*g* for 5 min, washed in MIN growth medium (TAP, but Tris-HCl replaces Tris-acetate), resuspended in MIN plus 0.6 mM $NaHCO_3$ to 10 μg/mL chlorophyll, and incubated in the light for 30 min to deplete intracellular acetate pools. ^{14}C-acetate (2 mCi/mmol; DuPont NEN, Boston, MA) was added to 1.5 μCi/mL, and cells were pulse labeled in the light for 5 to 60 min. Cultures were diluted 10-fold in TAP to initiate the chase period and were subsequently diluted as necessary to keep the cell density below ~10^5/mL. Samples were taken immediately after adding the TAP chase and at subsequent intervals ranging from 5 min to 70 hr. Labeled cells were placed in an ice-water bath for 15 min, collected by centrifugation at 5000*g* for 5 min, transferred to microcentrifuge tubes with TAP, and centrifuged at 13,000*g* for 5 min. Cell pellets were resuspended in seven volumes of ice-cold water followed by cold acetone to a concentration of 80% (v/v). Pigments were extracted overnight at −20°C, and insoluble material was removed by centrifugation at 13,000*g* for 10 min. Pigments were transferred to ether with NaCl and water (Plumley and Schmidt, 1987), dried under N_2 gas, and resuspended in a minimal volume (15 to 50 μL) of acetone.

Pigment solutions were spotted under an N_2 gas stream onto silica gel 60 thin-layer chromatography plates (TLC; Merck, Darmstadt, Germany), placed in a tank flushed with N_2 gas, and resolved with a mobile phase of benzene/isopropanol/water (100:10:0.25). TLC plates were exposed to Kodak X-Omat film at −60°C after spraying with En^{3}Hance (DuPont NEN). This TLC procedure is essentially as described by Janero and Barnett (1981), except that Chl *a* breakdown on the TLC plates was prevented by performing all steps under an N_2 atmosphere. All pigments were resolved as single bands, but the relative positions of the closely migrating loroxanthin and violaxanthin were reversed relative to the chromatographs of Janero and Barnett (1981), and a few nonpigmented bands were evident in our autoradiographs. The density of the pigment signals on the autoradiographs was digitally recorded via CCD-based camera, and quantification was performed with the MCID software package (Imaging Research, Inc., Brock University, St. Catharines, Ontario, Canada).

Analysis of Thylakoid Proteins

Thylakoids were purified by flotation through sucrose step gradients (Chua and Bennoun, 1975). To obtain sufficient quantities of thylakoids from PA2.1, membranes from the lower (1.5/1.8 M) sucrose interface were combined with those from the upper (0.5/1.5 M) fraction; SDS-PAGE revealed only slight differences in polypeptide composition between these two fractions. The recalcitrance of PA2.1 thylakoids to rise to the upper interface appears to be directly related to effects of light-harvesting complex (LHC) deficiency on the buoyant density of thylakoid fragments.

Freshly prepared thylakoids were digested at 300 μg/mL chlorophyll in 50 mM Hepes-NaOH, pH 7.5, 100 mM NaCl with 50 μg/mL of either trypsin, chymotrypsin, thermolysin (plus 10 mM $CaCl_2$), or *Staphylococcus aureus* V8 protease for 30 min at 25°C in darkness. Proteolysis was terminated with either 1 mM phenylmethylsulfonyl fluoride (trypsin, chymotrypsin, and V8 protease) or 10 mM each EGTA and EDTA (thermolysin). Thylakoids were centrifuged for 30 min at 140,000*g*, and pellets were resuspended in sample buffer for PAGE (see later discussion); boiling sample buffer was added to the V8 protease–treated thylakoids to prevent proteolysis in the lithium dodecyl sulfate (LDS)-denatured samples.

In Vivo and in Vitro Labeling of Proteins

In vivo labeling of Chlamydomonas with $^{35}SO_4$ (carrier free; ICN, Costa Mesa, CA) has been described by Schmidt and Mishkind (1983), and labeling with ^{14}C-acetate (~50 mCi/mM; DuPont NEN) was performed similarly, except that cells were resuspended and preincubated for 30 min in MIN before labeling was initiated. The chase was with 15 mM sodium acetate, pH 7.0. In some cases (see legends to Figures 3A to 3C), cells were lysed, and integral membrane proteins were separated from extrinsic and soluble proteins by adding equal volumes of 0.2 N NaOH to aliquots of the labeled cultures and centrifuging at 13,000*g* for 10 min (Jensen et al., 1986).

Proteins were synthesized in vitro using the wheat germ cell-free system and ^{35}S-methionine (DuPont NEN) (Mishkind et al., 1987). Proteins were immunoprecipitated with antibodies raised against Chlamydomonas polypeptide 11 (Plumley and Schmidt, 1983). Samples of membranes and trichloroacetic acid precipitates of soluble proteins were resuspended in 50 to 100 μL of 2% (w/v) LDS, 100 mM Tris-HCl, pH 7.6, 100 mM DTT and diluted with 1 mL of 1% Triton X-100, 0.3 M LiCl, 5 mM EDTA, 100 mM Tris-HCl, pH 7.6. Protease inhibitors (5 mM ε-amino-*n*-caproic acid, 1 mM benzamidine) were added, and insoluble material was removed by centrifugation at 140,000*g* for 15 min. Phenylmethylsulfonyl fluoride was added to 1 mM, and anti-polypeptide 11 IgG was added to 1 mg/mL of the starting extract. After 1 hr at 37°C, a twofold excess of protein A–Sepharose was added, and incubation was continued with mixing at 37°C for 1 hr. Samples were centrifuged at 13,000*g* for 1 min, and the pellets were washed three times with 1 mL of dilution buffer (see previous discussion), which was followed by two washes with 1 mL of 10 mM Tris-HCl, pH 8.6. Samples were resuspended in LDS gel sample buffer (see later discussion) and boiled for 1 min. Freshly prepared iodoacetamide was added to 25 mM, and the sample was incubated for 1 hr in the dark at 37°C before resolution by SDS-PAGE (see later discussion). All radiolabeled proteins were detected after LDS-PAGE (see next section) by fluorography, as described by Laskey and Mills (1976).

LDS-PAGE and Protein Blotting

Samples were resuspended in gel sample buffer (60 mM Tris-HCl, pH 8.6, 60 mM DTT, 5 mM ε-amino-*n*-caproic-acid, 1 mM benzamidine, 2% LDS, 12.5% sucrose) and resolved by PAGE, as described by Mishkind and Schmidt (1983). Proteins were detected with Coomassie Brilliant Blue R 250. Protein blotting employed nitrocellulose

membranes, and six different primary polyclonal IgG fractions were obtained from New Zealand white rabbits (Plumley and Schmidt, 1983). The antibodies were raised against either the 30-kD light-harvesting chlorophyll protein (LHCP) II polypeptide 11, the 29-kD water oxidation complex protein (polypeptide 12), the 48- and 42-kD core antenna proteins (polypeptides 5 and 6, respectively) of the photosystem (PS) II reaction center (all from Chlamydomonas), or the α and β subunits of thylakoid ATP synthetase from lettuce. Each antibody has been characterized previously (Plumley and Schmidt, 1983; Greer et al., 1986; Jensen et al., 1986; Plumley et al., 1989). Immunoreactive polypeptides were visualized after addition of goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma) and the substrates 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

Peptide Mapping

The method of Jensen et al. (1986), modified from Cleveland et al. (1977), was used for peptide mapping of LDS-PAGE–resolved proteins. Digestions were with trypsin (100 μg of protease per cm of gel), chymotrypsin (50 μg per cm of gel), V8 protease (5 μg per cm of gel), or papain (1 μg per cm of gel).

Reconstitution of LHC II

Reconstitution of LDS/heat-denatured LHC II was achieved as described by Plumley and Schmidt (1987). Reconstitution of LHC II from wild-type thylakoids required the addition of only Chl *b*, whereas reassembly of LHC II from mutant GE2.27 required supplements of pigments other than Chl *b* (see Results for details).

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