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Photoregulation of the Light-Harvesting Chlorophyll Protein Complex Associated with Photosystem II in *Dunaliella tertiolecta*[1]

Evidence that Apoprotein Abundance but Not Stability Requires Chlorophyll Synthesis

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ABSTRACT

The marine chlorophyte *Dunaliella tertiolecta* Butcher responds to a one-step transition from a high growth irradiance level (700 micromoles quanta per square meter per second) to a low growth irradiance level (70 micromoles quanta per square meter per second) by increasing the total amount of light-harvesting chlorophyll (Chl) *a*/*b* binding protein associated with photosystem II (LHC II), and by modifying the relative abundance of individual LHC II apoproteins. When high light-adapted cells were incubated with gabaculine, which inhibits Chl synthesis, and transferred to low light, the LHC II apoproteins were still synthesized and the ^{35}S-labeled LHC II apoproteins remained stable after a 24 hour chase. These results suggest that Chl synthesis is not required for stability of the LHC II apoproteins in this alga. However, when the control cells are transferred from high light to low light, the amount of the four LHC II apoproteins per cell increases, whereas it does not in the presence of gabaculine. These results suggest that Chl synthesis is required for a photoadaptive increase in the cellular level of LHC II.

The unicellular green alga, *Dunaliella tertiolecta* Butcher, responds to a decrease in growth irradiance by increasing the number and density of thylakoid membranes and membrane proteins (9, 27, 28). The photoadaptive increase in thylakoid protein synthesis is rapid, with half-times on the order of a few hours (8, 29), and does not appear to be mediated by phytochrome. Among the first proteins which increase following a HL[4] to LL shift are the LHC II apoproteins (29). The increase in LHC II is accompanied by an increase in Chl *a* and *b* (27, 28).

In *D. tertiolecta*, like higher plants, LHC II apoproteins are encoded in a nuclear gene family. The transcripts are translated in the cytoplasm as precursors that are larger than the mature proteins, and transported to the chloroplast where specific proteases cleave a transit peptide from the N terminal (1, 5, 19, 20). The mature apoproteins bind Chl *a* and *b* as well as some xanthophylls, at specific, but as yet unidentified binding sites, and the complexes are inserted into the thylakoid membrane (5, 26–28). The synthesis of pigments is coordinated with that of the apoproteins such that normally no excess pigment is synthesized which is not bound to protein and no significant excess protein is synthesized without the simultaneous synthesis of pigments. The mechanism of coordination of these two distinctly different biosynthetic pathways is unclear (12, 15).

One possible mechanism for coordinating the two components of the pigment-protein complex is based on the post-translational stabilization of nascent LHC II molecules by Chl (2, 10, 12, 21). In higher plant seedlings and *Chlamydomonas*, Chl *a* synthesis is essential for the stable accumulation of LHC II (3, 16, 21, 22, 24). We hypothesized, therefore, that if Chl synthesis were blocked in *D. tertiolecta*, newly synthesized LHC II apoproteins would rapidly degrade, and cells would be unable to increase the cellular pool of the apoproteins following a shift from HL to LL. Using gabaculine to inhibit Chl biosynthesis and ^{35}[S]SO_4 incorporation to follow protein synthesis, we examined how Chl synthesis affects the accumulation and stability of LHC II apoproteins following a shift from HL to LL. Our results suggest that Chl synthesis is not required to stabilize LHC II apoproteins but is required to elicit the photoadaptive accumulation of the pigment protein complex.

MATERIALS AND METHODS

Culture Conditions

Dunaliella tertiolecta (Woods Hole Clone DUN), was grown at 18 ± 1°C in turbidostat culture with ASW (8), containing a final SO_4^{-2} concentration of 200 μM (compared

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[4] Abbreviations: HL, high light (700 μmol m^{-2} s^{-1}); ASW, artificial seawater; LL, low light (70 μmol m^{-2} s^{-1}); LHC II, light-harvesting Chl protein complex associated with PSII.

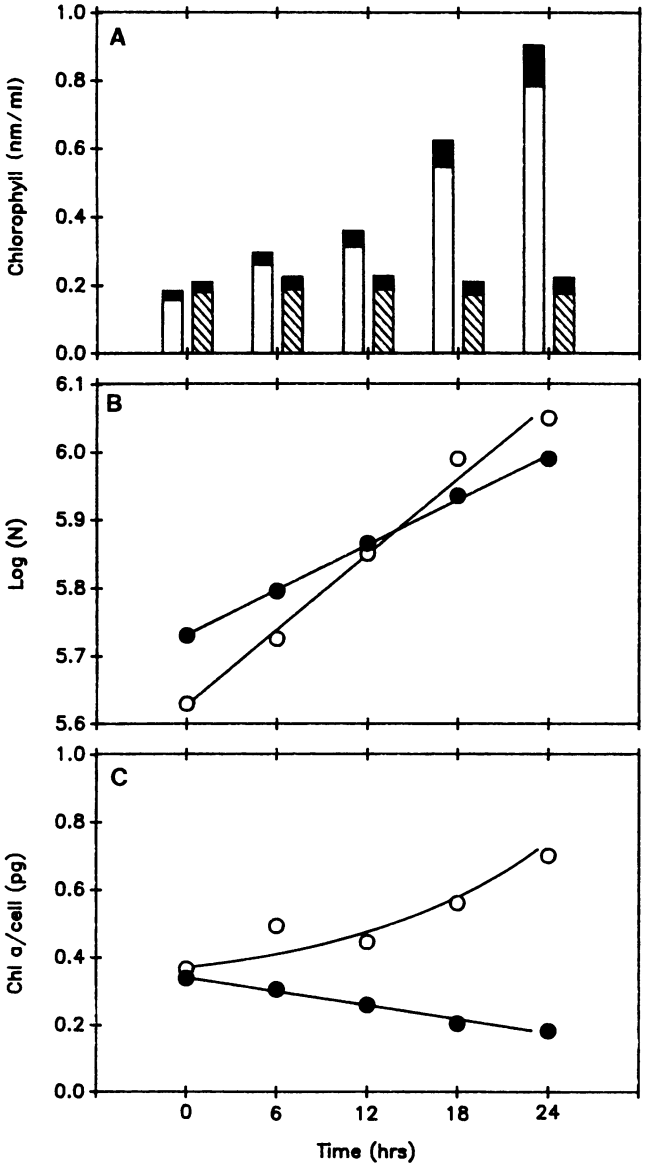

Figure 1. A, Effect of 500 μM gabaculine on Chl *a* and *b* accumulation in *D. tertiolecta*, following a shift from HL to LL. Chl *a* in control cells (open bar), Chl *a* in gabaculine-treated cells (stippled bar), Chl *b* (filled bar). B, The effect of 500 μM gabaculine on cell division ([○], control; [●], gabaculine-treated) N is cell number/mL. C, The cellular pool sizes of Chl *a* in control (○) and gabaculine-treated (●) cells. Cells were grown in continuous light at 700 μmol quanta m^{-2} s^{-1}. At time 0 light was reduced to 70 μmol quanta m^{-2} s^{-1}, with or without 500 μM gabaculine. Results are the means of three replicate experiments.

with 27 mM in natural seawater), and supplemented with F/2 nutrients (11). These conditions allowed relatively high rates of incorporation of $^{35}[S]SO_4$ without limiting growth by SO_4 (see 23). The culture vessel consisted of a 3.2 L jacketed glass cylinder which was continuously bubbled with sterile air. Continuous light was provided by banks of VHO fluorescent tubes and growth irradiance was shifted in a single step from 700 (HL) to 70 (LL) μmol quanta m^{-2} s^{-1} by changing the number of tubes. Cells were counted with a haemocytometer and a mean count of eight fields was used for calculating cellular pool sizes of constituents.

Pigment Determination

Photosynthetic pigments were extracted by homogenizing 10 mL samples filtered on Whatman GF/C glass fiber filters in 90% acetone. Corrected spectra were recorded on an Aminco DW-2c spectrophotometer and Chl *a* and *b* were calculated using the equations of Jeffrey and Humphrey (13).

Thylakoid Protein Synthesis

The time course of thylakoid protein synthesis following a transition from HL to LL was determined by following the incorporation of $^{35}[S]SO_4$ into proteins in the presence and absence of gabaculine. Gabaculine (3-amino-2,3-dihydrobenzoic acid), purchased from Fluka Chemical Corp., blocks the formation of δ-amino levulinic acid (6). Twenty-four hours prior to the light transition, 500 μM gabaculine dissolved in 500 μM DMSO (final concentration of each) was added to a turbidostat. The DMSO increases the efficiency of the inhibitor, presumably by increasing its permeability, and yet by itself (in a control culture) has no noticeable effects on growth, Chl or protein synthesis over the course of the experiment. Several hours prior to, and at selected times thereafter, cells from 500 mL subsamples were harvested by centrifugation at 5,000*g* for 5 min. The pellet was resuspended in 100 mL of SO_4-free ASW, with or without gabaculine, and incubated at LL with 1 mCi $^{35}[S]SO_4$ for 1 h. After the incubation, the sample was split into two equal volumes. Thylakoids membranes were immediately isolated from one (pulse). To the other, $MgSO_4$ was added to a final concentration of 20 mM (chase) and subsamples were taken at selected times for thylakoid membrane isolation.

Thylakoids were prepared as previously described (28). All buffers contained 200 μM PMSF. Cells were harvested by centrifugation and resuspended in 0.4 M sucrose, 50 mM Hepes (pH 7.4), and 5 mM EDTA. The suspension was sonicated in an ice bath for 90 s with a Kontes microprobe sonicator, and centrifuged at 45,000*g* for 20 min. The pellet was washed three times in 6.2 mM Tris, 48 mM glycine (pH 7.8), and 20 mM EDTA, and resuspended in 50 μL of 100 mM DTT, and 100 mM Na_2CO_3. Twenty μL of 4% SDS, 15% glycerol, and 0.05% bromothymol blue were then added and the sample was heated for 2 min at 95°C. Radioactivity was measured on triplicate 10 μL aliquots of the membrane preparation. Aliquots containing either equivalent amounts of radioactivity or corresponding to equivalent cell numbers were loaded on a 15% polyacrylamide gel containing SDS

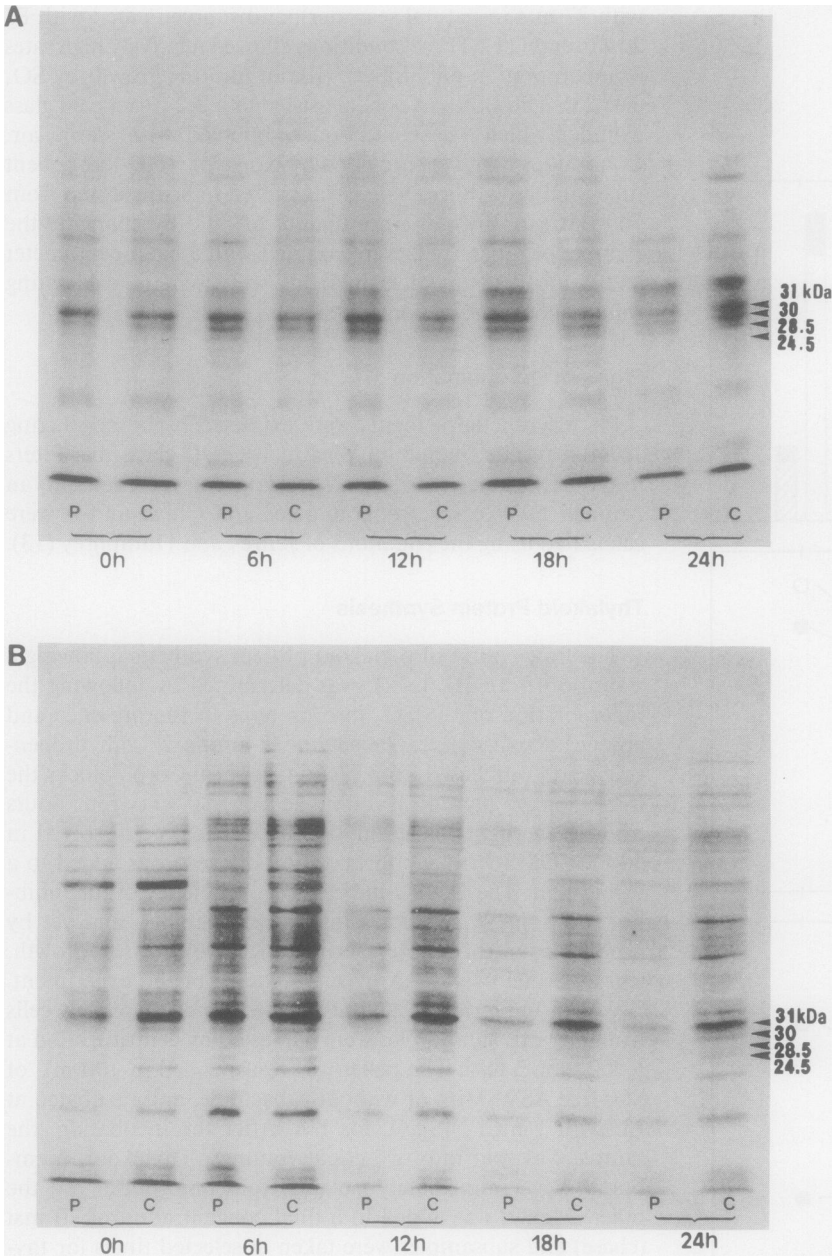

Figure 2. Effects of gabaculine on synthesis of LHC II apoproteins in *D. tertiolecta.* Cells were grown in continuous light at 700 μmol quanta m^{-2} s^{-1}. At time 0 light was reduced to 70 μmol quanta m^{-2} s^{-1}, without (A) and in the presence of (B) 500 μM gabaculine. At 6 h intervals, 100 mL of culture were incubated for 1 h with a pulse (P) 1 m Ci of $^{35}[S]SO_4$, followed by a 2 h chase (C) with 20 mM cold SO_4. Each lane was loaded with 60,000 cpm. Proteins were separated on 15% SDS-PAGE and electrophoretically transferred to nitrocellulose. In control cells, synthesis of the four proteins which correspond in mol wt to LHC II apoproteins (indicated with arrows) increased threefold relative to other thylakoid proteins over the first 18 h. In gabaculine-treated cells, bands corresponding to LHC II apoproteins were labeled and remained labeled after the chase; however, there was no significant change in the relative synthesis of individual apoproteins following the light transition.

and the proteins were separated electrophoretically as described (17). Proteins were electrophoretically transferred to nitrocellulose and $^{35}[S]SO_4$ incorporation was visualized by autoradiography (26, 30). The autoradiograms were scanned with a densitometer to provide quantitation. LHC II apoproteins were detected on the nitrocellulose by immunoassay with polyclonal antibodies raised against pea LHC II (4, 27) and visualized with a peroxidase reaction.

To confirm that radiolabeling of LHC II apoproteins was not an artifact of comigration of other labeled proteins, two-dimensional gel electrophoresis was performed. The pellet containing thylakoids was washed three times and resuspended in 6.2 mM Tris, 48 mM glycine, 20 mM EDTA to give 0.5 mg Chl/mL. Membranes were partially solubilized in 60 mM octyl-β-D-glucopyranoside for 45 min at 4°C and in the dark. Undissolved membranes were removed by centrifugation for 15 min at 48,000*g*. Solubilized thylakoid membranes were loaded on a 10% LiDS polyacrylamide gel (28). The gel was run in the dark at 4°C for 6 h at constant current of 10 mA. The bands corresponding to each well were then cut and either stained with Coomassie blue or prepared for electrophoresis in the second dimension. In the latter case, the green band corresponding to LHCP3, the monomeric form of LHC II, was excised, incubated in 50 mM Na_2CO_3, 2% SDS, 5 mM mercaptoethanol, heated 4 min at 70°C, and set on the top of a 15% SDS polyacrylamide gel. The gel was run at room

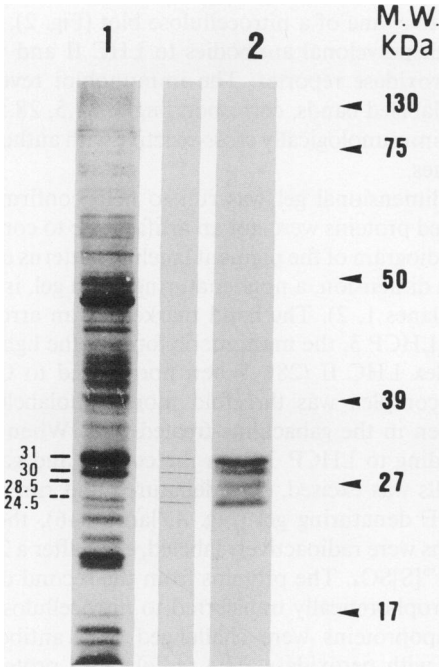

Figure 3. Characterization of LHC II apoproteins from *D. tertiolecta*. Lane 1, autoradiogram of ^{35}S-labeled thylakoid proteins after transfer to nitrocellulose. The four LHC II apoproteins (indicated with arrows) were detected by incubation of the nitrocellulose with polyclonal antibodies raised against pea LHC II and visualized with a peroxidase stain (lane 2).

temperature for 16 h at 20 mA. Proteins were transferred to nitrocellulose and visualized by autoradiography and antibody reaction with horseradish peroxidase (26).

RESULTS

The effect of gabaculine on Chl synthesis is shown in Figure 1. In the control culture, pigment synthesis increased rapidly following the transfer from HL to LL. Within 24 h after the light shift, the total amount of Chl *a* and Chl *b* per volume of culture increased 5- and 7.6-fold, respectively (Fig. 1A); consequently, the Chl *a*/*b* ratio decreased from about 11 to 7. In contrast, in the gabaculine-treated culture, transferred from HL to LL, Chl *a* synthesis was completely inhibited, while the amount of the Chl *b* per volume culture doubled.

Cells continued to divide, even after a 24 h exposure to gabaculine (Fig. 1B). The division rate of control cells was 1.4 d^{-1} while gabaculine-treated cells divided at 0.9 d^{-1}. Because cells continued to divide in the presence of gabaculine, while Chl *a* synthesis ceased, Chl *a* per cell decreased from 3.4 to 1.8 ($\times 10^{-16}$) mol within 24 h following the shift, whereas it increased from 3.7 to 7 ($\times 10^{-16}$) mol in the control (Fig 1C).

We examined the synthesis and turnover of LHC II apoproteins in the presence and absence of gabaculine in pulse-chase studies over a 24 h period following a shift in light from HL to LL. After a 1 h exposure to ^{35}[S]SO_4, the four bands which corresponded in mol wt to LHC II apoproteins (26) were radiolabeled in both gabaculine and control cells (Fig. 2). In both control and gabaculine-treated cells, these bands were labeled following a 2 h chase with cold SO_4^{2-}. Inspection of the autoradiograms reveals that the relative rate of synthesis of the putative LHC II apoproteins increased approximately twofold relative to other thylakoid proteins in the control cells but remained remarkably constant in gabaculine-treated cells.

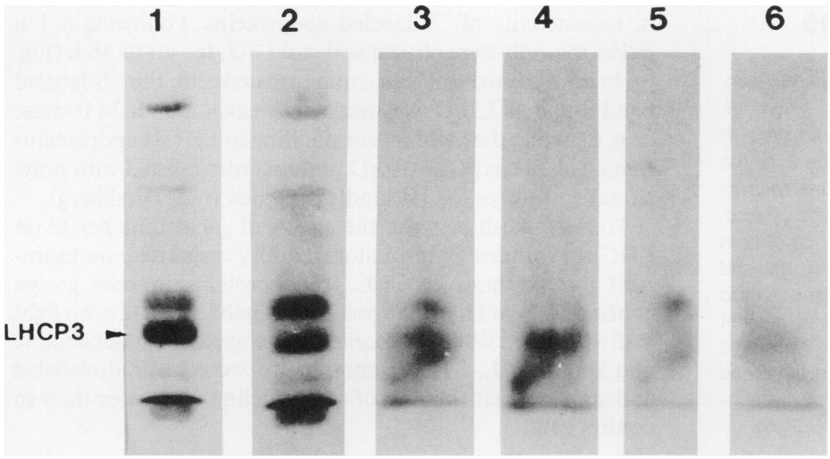

Figure 4. Radiolabeled LHC II apoproteins from *D. tertiolecta* in the absence and presence of 500 μM gabaculine on a two-dimensional gel. Lanes 1–2, Coomassie blue stained gel pigment proteins on a nondenaturing gel. Thylakoid membranes were solubilized in octylglucoside and equal amounts of Chl *a* were loaded and separated in the first dimension on an LiDS PAGE at 4°C. After separation in the first dimension, the bands corresponding to LHCP 3 were cut and the proteins were heat denatured and separated in the second dimension by a SDS 15% polyacrylamide gel. The ^{35}S-labeled apoproteins were transferred to nitrocellulose and visualized by autoradiography. The blot was probed with polyclonal antibodies raised against pea LHC II and visualized with peroxidase. Lane 3, the control LHCP following a 1 h pulse with 1 m Ci ^{35}[S]SO_4. Lane 4, following a 24 h incubation with 20 mM $MgSO_4$. Lane 5, a gabaculine-treated sample following a 1 h pulse with 1 m Ci ^{35}[S]-SO. Lane 6, following a 24 h chase with 20 mM $MgSO_4$.

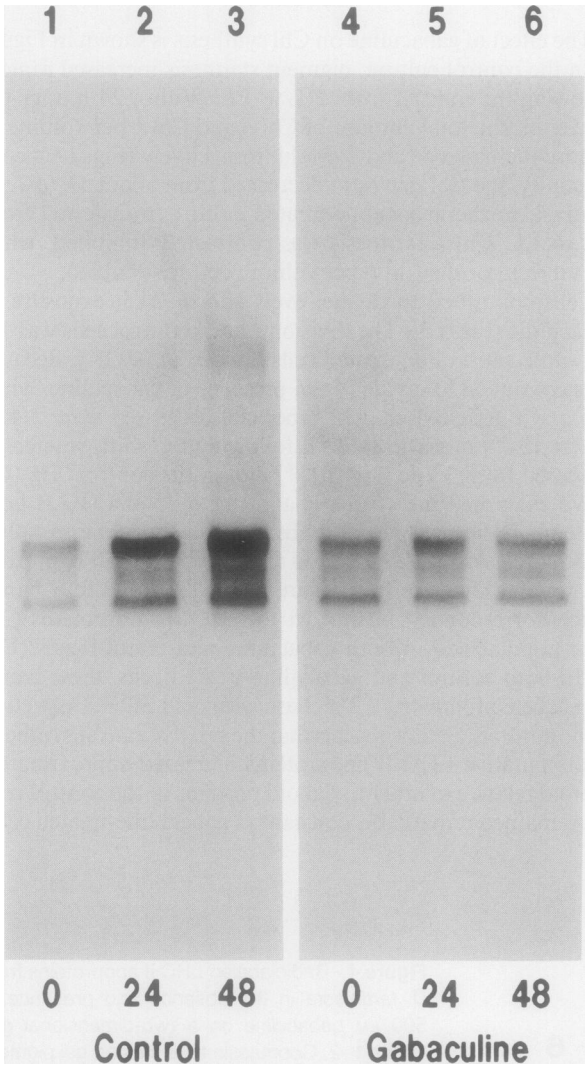

Figure 5. Effect of gabaculine on LHC II apoprotein accumulation. Cells were grown in continuous light at 700 μmol m^{-2} s^{-1}. At time 0, light was reduced to 70 μmol m^{-2} s^{-1} without (lane 1) or with (lane 4) 500 μM gabaculine. Twenty-four and 48 h later (lanes 2 and 3 for the control and lanes 5 and 6 for the gabaculine-treated culture, respectively), cells were harvested and thylakoid membranes prepared. Proteins were loaded on the equivalent of equal number of cells in each lane and separated on a 15% SDS polyacrylamide gel and transferred to nitrocellulose. The LHC II apoproteins were detected after incubation of the nitrocellulose with antibodies raised against pea LHC II. In the control culture, the LHC II apoproteins increased threefold within 48 h following the decrease in light intensity, whereas in the gabaculine-treated culture the relative abundance of the apoproteins remained constant, while cell number increased 2.5-fold.

The identity of the radiolabeled proteins was partially established by immunoassay (Fig. 3). An autoradiogram of a representative lane of a nitrocellulose blot (Fig. 2), was challenged with polyclonal antibodies to LHC II and visualized with a peroxidase reporter. The immunoblot revealed that four radiolabeled bands, corresponding to 24.5, 28.5, 30, and 31 kD are immunologically cross-reactive with authentic LHC II antibodies.

A two-dimensional gel was run to help confirm that the radiolabeled proteins were not an artifact due to comigration. An autoradiogram of the pigment labeling patterns of proteins in the first dimension, a nondenaturing green gel, is shown in Figure 4 (lanes 1, 2). The band marked by an arrow, corresponds to LHCP 3, the monomeric form of the light harvesting complex LHC II (28). When normalized to Chl *a*, the LHCP 3 complex was threefold more radiolabeled in the control then in the gabaculine-treated cells. When the band corresponding to LHCP 3 from the control and gabaculine-treated cells was excised, heat denatured and run on a 15% SDS-PAGE denaturing gel (Fig. 4, lanes 3–6), the LHC II apoproteins were radioactively labeled, even after a 24 h chase with cold $^{35}[S]SO_4$. The proteins from the second dimension were electrophoretically transferred to nitrocellulose and the LHC II apoproteins were challenged with antibodies and visualized with peroxidase. The radiolabeled proteins corresponding to the 24 to 31 kD on the autoradiogram cross reacted with LHC II antibodies. In both the control and gabaculine-treated cells, a 32 kD protein, which comigrated with LHCP3 on the green gel, was labeled initially and lost its radioactivity following a 24 h chase. The 32 kD protein did not react when challenged antibodies to LHC II.

The affect of gabaculine on the accumulation of LHC II apoproteins was assessed by measuring apoprotein levels on Western blots (Fig. 5). In control cells the total amount of LHC II apoproteins per cell increased 24 h and 48 h after a shift from HL to LL, whereas in the presence of gabaculine the LHC II apoproteins remained relatively constant, despite an almost 50% decrease in Chl per cell (Fig. 1c).

The stability of LHC II apoproteins, synthesized in gabaculine-treated cells, was examined by following the reduction in radioactivity of ^{35}S-labeled apoproteins. Following a 1 h pulse, the cells were chased with cold SO_4 for up to 48 h (Fig. 6). In both control and gabaculine-treated cells, the ^{35}S-labeled band found at 32 kD was markedly weaker after a 24 h chase (Fig. 6), while the bands corresponding to LHC II apoproteins remained labeled. The 34 kD protein cross-reacted with polyclonal antibodies to D1 (kindly provided by J. Hirshberg).

To distinguish between the effects of gabaculine *per se* on LHC II synthesis from photoregulatory responses, we examined the synthesis of LHC II apoproteins in cells grown continuously in HL in the presence of gabaculine (*i.e.* no light shift) (Fig. 7). When HL cells were treated with gabaculine and kept in HL, LHC II apoproteins were still radiolabeled and stable, albeit the rate of radiolabeling was lower than in control cells.

DISCUSSION

Gabaculine prevented *D. tertiolecta* from shade adapting by inhibiting the accumulation of LHC II apoproteins. Fol-

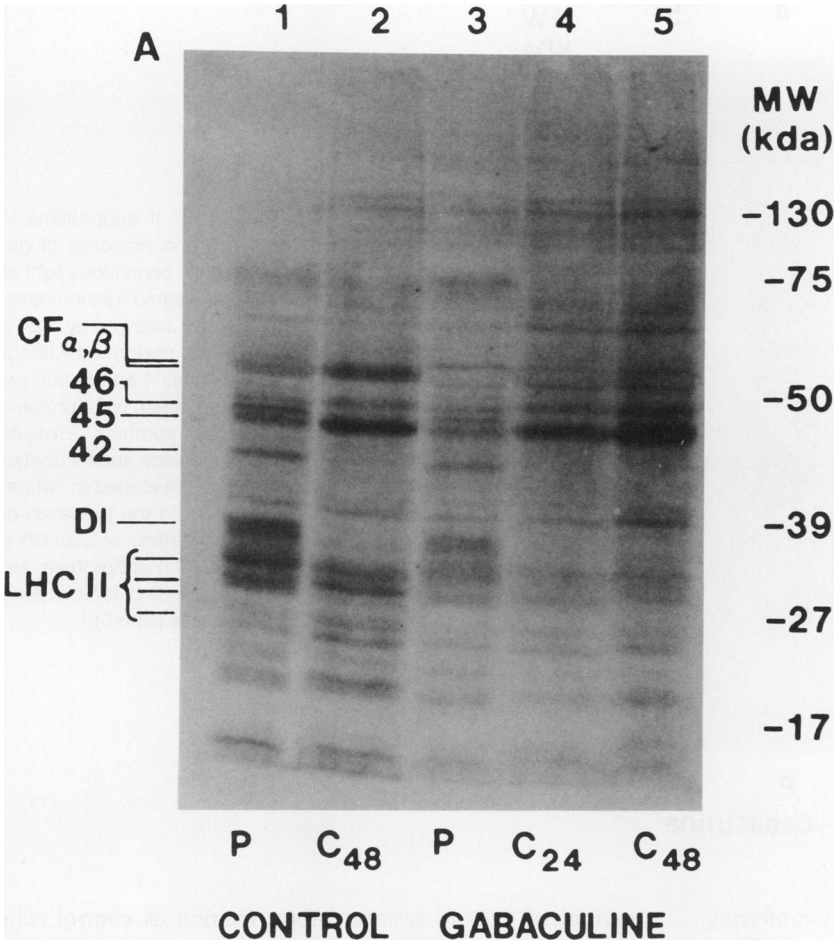

Figure 6. Effects of gabaculine on the stability of LHC II apoproteins. A, Cells were incubated for 1 h with 1 mCi of $^{35}[S]SO_4$, followed by a chase with 20 mM cold $MgSO_4$. Lanes were loaded with proteins corresponding to equal cell numbers. In control cells bands corresponding to the LHC II apoproteins are radiolabeled after a 48 h chase (C_{48}). Twenty-four hours after the light transition and in the presence of gabaculine, $^{35}[S]SO_4$ was still incorporated into LHC II apoproteins and remained stable even after 24 h (C_{24}) and 48 h chases (C_{48}) with cold SO_4.

lowing a shift from HL to LL, Chl per cell decreased in gabaculine-treated cells, while it increased in the control (Fig. 1c). Moreover, the relative abundance of LHC II apoproteins per cell remained constant (Fig. 5). These results suggest that Chl biosynthesis is essential for the light-intensity dependent accumulation of LHC II apoproteins in *D. tertiolecta*. The mechanism of signal transduction for this response remains elusive. One possible hypothesis is based on the posttranslational stabilization of LHC II apoproteins by Chl *a* or *b*. In this hypothesis Chl synthesis limits the stability of LHC II. Irradiance level influences the relative rate of Chl synthesis but not that of LHC II apoproteins. When irradiance is decreased (but the cells are not put in the dark), the rate of Chl synthesis is increased relative to growth. The increased relative rate of Chl synthesis stabilizes newly synthesized LHC II apoproteins, and the pool of the pigment-protein complex increases until a new steady-state is reached.

When Chl *a* synthesis was blocked by gabaculine in *D. tertiolecta*, all four LHC II apoproteins were synthesized (Fig. 2) and remained labeled after a 48 h chase (Fig. 6). If Chl synthesis were required to stabilize the LHC II apoproteins we would expect to observe a turnover of proteins in the absence of Chl synthesis (7). If LHC II polypeptides turned over, radioactively labeled apoproteins should be diluted by a cold chase. It follows that isotope dilution should increase with increasing length of the chase. Data obtained at different chase times, following a shift from HL to LL (Fig. 6), or on HL-adapted cells (Fig. 7) are inconsistent with this hypothesis since after a 4, 10, 18, or 24 h chase the total level of radiolabeled LHC II apoproteins remained constant while D1 turned over. Moreover, the relative abundance of individual LHC II apoproteins remained constant 48 h after the light shift (Fig. 5), suggesting that none of the four apoproteins is preferentially degraded.

Why do the LHC II apoproteins appear to be stable in the absence of Chl *a* biosynthesis in *D. tertiolecta*? In higher plant LHCP complexes Chl *a* could protect apoproteins from proteolytic attack by blocking specific recognition sites; one possibility is that the amino acid sequence of the LHC II apoporteins in *D. tertiolecta* may differ. The amino acid sequence of the 28.5 kD LHC II apoprotein from *D. tertiolecta* is 80% homologous with that of pea and tomato (19), suggesting that the amino acid sequence *per se* probably does not account for the difference in the stability between the green alga and higher plants. A second possibility is that LHC II proteases are much more active in greening higher plant plastids than in *D. tertiolecta* which have fully developed chloroplasts. Our data do not address that hypothesis. A third possibility is that a fraction of previously synthesized Chl molecules, bound to other Chl protein complexes, could

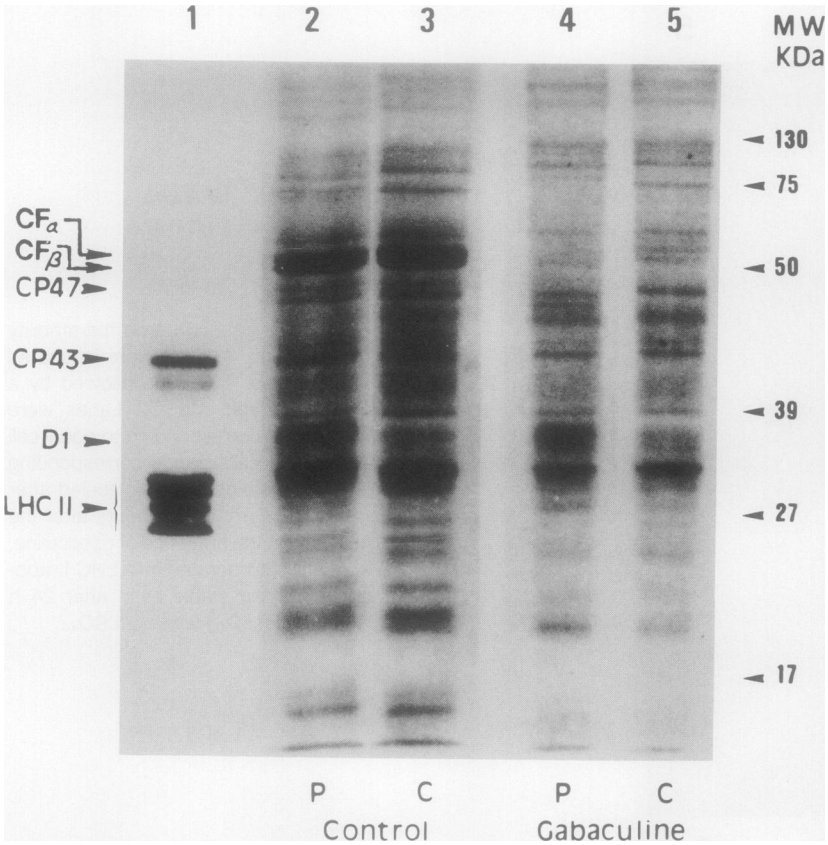

Figure 7. Synthesis of LHC II apoproteins in high light, in the absence and presence of gabaculine. Cells were grown in continuous light at 700 μmol m^{-2} s^{-1}. Under the same light intensity, a 1 h pulse (P) with $^{35}[S]SO_4$, followed by a 24 h chase (C) with $MgSO_4$, were performed without (lanes 2 and 3) and with (lanes 4 and 5) 500 μM gabaculine. Lanes were loaded with proteins corresponding to equal cell numbers. Proteins were transferred to nitrocellulose and incubated with different antibodies as described in "Materials and Methods" (lane 1). In the presence of gabaculine, although the synthesis of both Chl *a* and *b* is blocked, the 4 LHC II apoproteins are synthesized (lane 4), and no loss of radiolabeling is detected after a 24 h chase (lane 5).

exchange with nascent LHC II apoproteins, thereby conferring stability. If this were a large-scale phenomenon, LHC II apoproteins would still be synthesized but no significant amounts of LHC II apoproteins could accumulate. If apoproteins, labeled during a pulse, obtained pigments from previously synthesized pigment-protein complexes and thus were stabilized, then during a chase, unlabeled apoproteins should acquire pigments from labeled complexes resulting in a turnover of the labeled complex and a decrease in the amount of ^{35}S-labeled apoprotein. Thus, if pigment exchange accounted for the apparent stability of LHC II apoproteins in gabaculine-treated cells, we would still expect to observe a decrease in labeled LHC II apoproteins after a prolonged chase. We did not. We could also consider the possibility of an exchange with other pigments-proteins complexes, such as CPa or LHC I. CPa does not seem to turn over (Fig. 7). Nitrocellulose filters challenged with polyclonal antibodies to LHC I revealed no change in the pool size of that apoprotein in gabaculine treated cells (data not shown). These results strongly suggest, but do not unequivocally prove, that the exchange of pigments between previously synthesized pigment protein complexes and nascent LHC II apoproteins is minor.

The amount of total LHC II apoprotein synthesized after a 1 h pulse in gabaculine treated cells was lower than in the control; however, the total amount of $^{35}[S]SO_4$ incorporated by the cells in the presence of gabaculine was also about 40% lower than that observed in the control (data not shown). In the absence of Chl synthesis, cells cannot harvest the same amount of light to sustain carbon fixation as control cells which photoadapt. Thus, a decrease in growth irradiance is reflected in a reduction in overall protein synthesis and growth rate. A similar result has been reported for *Euglena* (6).

When HL cells treated with gabaculine were kept in HL, the cellular level of both Chl *a* and *b* decreased, while LHC II apoproteins remained constant suggesting that Chl *b* synthesis is not a prerequisite for LHC II synthesis or stability. Additionally, when Chl *a* synthesis was blocked and cells transferred from HL to LL, a significant portion of Chl *a* was converted to Chl *b*. A similar conversion has been reported in *Dunaliella salina* submitted to a dark exposure (23). These results suggest that in *D. tertiolecta* Chl *b* synthesis is light-intensity dependent and not an artifact of gabaculine, nor dependent on the rate of supply of Chl *a*. The change in the Chl *a*/*b* ratio in *D. tertiolecta* is independent of either a change in the cellular level of total LHC II or in population of the apoproteins (26).

The results of this study confirm that Chl synthesis is required for a shade-adaptative increase in LHC II. A similar conclusion has been reached by others with regard to the light induction of LHCP mRNA accumulation in *Chlamydomonas reinhardtii* and higher plants (2, 14, 15, 22). Slovin and Tobin (25) have provided evidence consistent with translational control of LHCP mRNA in *Lemna gibba*, and Sheperd *et al.* (24) found that LHCP synthesis during the cell cycle of *C. reinhardii* is governed largely by the availability of LHCP mRNA. In *Dunaliella tertiolecta* LHC II transcripts increase

three-fold within 9 h following a shift from HL to LL (18), suggesting that, in part, the photoadaptive response is transcriptionally or posttranscriptionally regulated.

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