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Accumulation of Chlorophyll *a*/*b*-Binding Polypeptides in *Chlamydomonas reinhardtii y-1* in the Light or Dark at 38°C[1]

Evidence for Proteolytic Control

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ABSTRACT

The kinetics of accumulation of light harvesting chlorophyll (Chl) *a*/*b*-binding polypeptides (LHCPs) in thylakoid membranes were analyzed during greening of *Chlamydomonas reinhardtii y-1* at 38°C. Initial accumulation of LHCPs in thylakoid membranes was linear; LHCP precursors or polypeptides in transit within the chloroplast stroma were not detected. The rate of accumulation in the light was at least five-fold greater than that in the dark. The relatively small amount of LHCPs that accumulated in the dark was integrated properly in the membrane, as judged by the pattern of cleavage *in vitro* by exogenous proteases, and did not turn over at a significant rate *in vivo*. The kinetic data suggested that in *y-1* cells either translation of LHCP mRNA was inhibited in the dark or newly synthesized polypeptides were degraded concurrently with transport into the chloroplast unless rescued by Chl. LHCPs accumulated in cells of the Chl *b*-deficient strain *pg-113* at the same rate in the dark or the light at 38°C, an indication that light did not affect translation of LHCP mRNA. Membrane-associated LHCPs in *pg-113* cells were completely degraded, in contrast to those in *y-1* cells, by exogenous proteases, which suggested that *pg-113* cells are deficient in a proteolytic activity. A peptidase was recovered from *y-1* cells in a membrane fraction with a buoyant density slightly less than that of thylakoid membranes. Although a role for this activity in degradation of LHCPs has not been established, the specific activity of this peptidase in *pg-113* cells was only 10 to 15% of the level in *y-1* cells.

Under well-defined conditions, degreened cells of *Chlamydomonas reinhardtii y-1* undergo light-dependent chloroplast development at a rapid and linear rate (2, 15, 22). During this process, Chl *a* and *b* initially accumulate mainly as esters of geranylgeraniol. Yet these incompletely reduced Chls are integrated into light-harvesting complexes as assayed by energy transfer from Chl *b* to Chl *a* (22). Whereas Chl is synthesized entirely within the chloroplast, LHCPs[3] are encoded in nuclear DNA, synthesized on cytoplasmic ribosomes as precursor forms, transported across the chloroplast envelope, cleaved to remove the transit sequence, and integrated into thylakoid membranes (12). At 25°C, light induces these *cab* genes in degreened cells of *C. reinhardtii y-1* (13, 21). Elevated temperature (38°C) elicits maximal levels of LHCP mRNA in these cells in the dark, but LHCPs do not accumulate until cells are exposed to light (13, 15). Thus, greening at 38°C provides a system with which the role of light in control of accumulation of LHCPs at posttranscriptional steps can be analyzed. A correlation between accumulation of LHCPs and Chl synthesis has been amply demonstrated (3, 4, 16). In higher plants, a specific role for Chl *b* in rescue of LHCPs from degradation seems apparent in leaves exposed to light (31) and in isolated chloroplasts (3). It is less clear whether the lack of accumulation in *y-1* cells in the dark results from degradation in the absence of Chl synthesis or specific inhibition of translation of LHCP mRNA.

In experiments reported here, we measured *in vivo* short-term kinetics of accumulation of LHCPs in *y-1* cells. The results suggest that in *y-1* cells LHCPs are degraded at the level of transport into the chloroplast unless rescued by Chl. This conclusion was supported by studies with mutant strain *pg-113* of *C. reinhardtii*, which lacks Chl *b* (26). These cells accumulate LHCPs in the light or dark, evidence that translation of mRNA is not affected by light, and are deficient in a membrane-bound peptidase.

MATERIALS AND METHODS

Greening of Cells

The *y-1* strain of *Chlamydomonas reinhardtii* used in this work was from a stock maintained in this laboratory. *C. reinhardtii pg-113* [*arg-2*, *mt*$^+$, *cbn*] cells (26) were kindly provided by Dr. A. Boschetti. The locus for Chl *b* deficiency is probably allelic with *cbn-1* (8). Cells were grown at 25°C in the light for 2 d and then for 3 to 4 d in the dark (2, 13, 22). Cells were harvested by centrifugation and suspended in fresh growth medium to a density of 1×10^7 cells/mL. For labeling experiments, 1 to 4 mL of the suspension were incubated in 50-mL beakers in a water-bath with shaking at 38°C. Cells were labeled with 37 KBq/mL L-[U-^{14}C]arginine (12.8 GBq/mmol, Amersham Corp., Arlington Heights, IL) or 160 KBq/mL L-[2,3,4,5-^{3}H]arginine (1.85 TBq/mmol, Amersham

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[3] Abbreviations: LHCP, light-harvesting Chl *a*/*b*-binding polypeptide; RbcL and RbcS, large and small subunits of ribulose bisphosphate carboxylase/oxygenase, respectively; NA, *p*-nitroanilide.

Corp.) at the end of a preincubation period (1.5–2 h) in the dark or immediately after such preincubated cells were exposed to incandescent light (15, 22).

Cell Fractionation

At the end of a labeling period, 1-mL samples of cell suspension were injected into 9 mL ice-cold 50 mM Hepes-NaOH (pH 7.5), containing 50 μg/mL cycloheximide. The cells were collected by centrifugation, washed with 5 mL of Hepes buffer, suspended in 0.5 mL of the buffer, and broken by sonication for 20 s with an ultrasonic cleaner (model B-12, Branson, Shelton, CT) filled with ice-cooled water. Homogenates were transferred with an additional 0.5 mL of 50 mM Hepes buffer to 1.5-mL microcentrifuge tubes, which were suspended in water-filled, 10-mL Oak Ridge-type tubes, and centrifuged 40 to 60 min at 80,000*g* in a Spinco Type 50 rotor (Beckman Instruments, Inc., Palo Alto, CA) at 2°C. Soluble protein was precipitated by mixing the resulting supernatant fluids with 0.25 mL 50% (w/v) trichloroacetic acid and was collected by centrifugation.

Protease Treatment

Total cellular membrane pellets were suspended in 50 mM Hepes-NaOH (pH 7.5), (200 μL per pellet from 10^7 cells) and digested 30 min on ice with 100 to 150 μg/mL thermolysin (protease type X), 50 μg/mL trypsin (TPCK-treated), or 15 μg/mL proteinase K (protease type XI) obtained from Sigma Chemical Co., St. Louis, MO. Digestion by thermolysin was stopped by addition of sodium EGTA (pH 7.2) to 20 mM; control samples received EGTA before protease was added. Digestion by trypsin or proteinase K was stopped by addition of PMSF to 10 mM. Protein was then collected by centrifugation after addition of TCA to 10% (w/v).

Immunoprecipitation

Intact and protease-generated fragments of LHCPs and RbcS were immunoprecipitated after solubilization of proteins in SDS as described previously (23).

Sucrose Gradient Analysis

Pellets containing 1 to 2 × 10^9 cells were suspended in 10 mL cold 20 mM Tricene-KOH (pH 8.0), containing 2 mM $MgCl_2$, broken by passage through a French pressure cell at 5,000 p.s.i., centrifuged at 5,000*g* for 2 min to remove unbroken cells and then at 120,000*g* for 60 min at 2°C. The membrane pellet was suspended in 20 mM Tricene-KOH (pH 8.0), containing 1 mM $MgCl_2$, applied to a 0.5 to 1.4 M continuous sucrose gradient (10 mL) containing the same buffer, and centrifuged 18 h at 38,000 rpm in a Spinco SW41 rotor (Beckman Instruments, Inc.) at 2°C. Gradients were fractionated into 1.0 mL fractions.

Assays

Reaction mixtures for assay of peptidase activity included 20 mM Tricene-KOH (pH 8.0), and 0.7 mM amino acyl-*p*-nitroanilide. Activity was monitored by a change in absorbance at 410 nm (ϵ = 8400 L·mol^{-1} cm^{-1}) (32). Protein was measured with the bicinchoninic acid reagent as described by the supplier (Pierce Chemical Co., Rockford, IL). Chl was measured in 80% acetone extracts (22).

Electrophoresis and Radioautography

Electrophoresis in the presence of 0.15% SDS on 10 to 20% polyacrylamide gels and radioautography were performed as described previously (14). For fluorography, gels were soaked 15 min in Amplify (Amersham Corp.) before they were dried. Although the gel system used here did not separate polypeptides 16 and 17, it provided good resolution of LHCPs from other polypeptides of similar size. These characteristics permitted better quantitation by densitometry of stained gels and radioautograms (23). Gels and films were scanned with a GS 300 scanning densitometer (Hoefer Scientific Instruments, San Francisco, CA) and peak areas were integrated with the Hoefer GS 350 computer software.

RESULTS

Synthesis of LHCPs in *y-1* Cells in Light or Dark

Figure 1 compares the patterns of radioactivity in membrane polypeptides from *y-1* cells pulse-labeled with [^{14}C] arginine for 15 min in the dark or light at 38°C. Major differences in the patterns are the amounts of labeled LHCPs, designated as 11, 16, and 17 (6), which resulted from different initial kinetics of accumulation in the light or dark (Fig. 2a). In the light, ^{14}C in immunoprecipitated polypeptide 11 increased at a linear rate, with a lag of about 1 min. Because

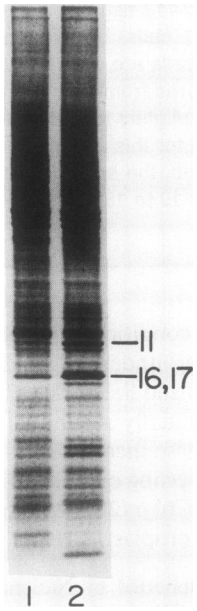

Figure 1. Effect of light on accumulation of membrane polypeptides in *y-1* cells. Degreened cells were preincubated in the dark at 38°C for 1.5 h and then labeled with [^{14}C]arginine for 15 min in the dark (lane 1) or the light (lane 2). Membrane fractions were prepared and subjected to electrophoresis and radioautography as described in "Materials and Methods." The positions of polypeptides 11, 16, and 17 are indicated.

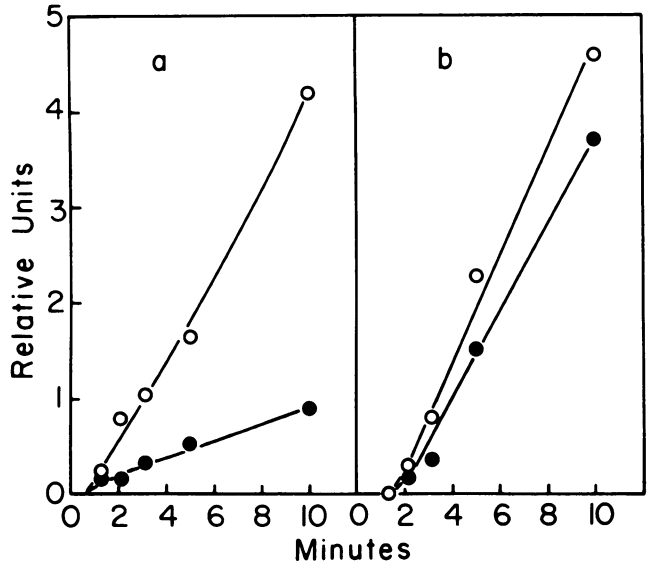

Figure 2. Accumulation of labeled (a) polypeptide 11 and (b) RbcS in the light or dark in *y-1* cells. Degreened cells were preincubated 1 h in the dark at 38°C and then labeled with [^{14}C]arginine in the light (○) or the dark (●). At the times indicated, 1-mL aliquots of the cell suspension were mixed with 5 mL of cold 10% (w/v) trichloroacetic acid. Cells were collected by centrifugation, dissolved in 0.1 M Tris base containing 2% SDS, and treated with antibodies against polypeptide 11 (13). Supernatants from the resulting immunoprecipitates were treated with antibodies against RbcS (23). Relative radioactivity in each polypeptide was determined by densitometry of radioautograms after electrophoresis of immunoprecipitates (23).

this lag was observed in the light or dark and in labeling of RbcS shown in Figure 2b, it probably was due only to the time required for cellular uptake of [^{14}C]arginine and incorporation into protein. In this experiment, polypeptide 11 increased in the dark at a rate about 20% of that in the light (Fig. 2a). Similar differences in rates of accumulation of labeled polypeptides 16 and 17 in the light or dark were found by scanning radioautograms after electrophoresis of membrane protein (*e.g.* Fig. 1). Unprocessed, precursor forms of LHCPs were not detected in immunoprecipitates of cell extracts.

The high rate of accumulation in the light produced a differential increase in bulk amount of LHCPs relative to other membrane polypeptides, observed by protein stain after electrophoresis. No change was observed in the pattern of membrane polypeptides in *y-1* cells in the dark; LHCPs accumulated no more rapidly than other polypeptides.

Labeled polypeptides 11, 16, and 17, as indicated in Figure 1, were recovered with membranes after centrifugation of broken-cell preparations. The membranes were resolved on sucrose gradients and newly synthesized polypeptides 11, 16, and 17 were detected by radioautography after electrophoresis of protein in each fraction of the gradients. Even with a pulse of labeled arginine as short as 3 min, distribution of labeled 11, 16, and 17 paralleled that of Chl and of the thylakoid polypeptides detected by protein stain. The same distribution in gradients was found regardless of whether cells were labeled in the light or dark. No detectable amount of the polypeptides was found in the soluble fraction of the cell or in a chloroplast envelope fraction prepared as described by Mendiola-Morgenthaler *et al.* (25).

By comparison, accumulation of [^{14}C]arginine in RbcS, another chloroplast polypeptide synthesized on cytoplasmic ribosomes (12, 29), was nearly the same in the dark or the light in *y-1* cells (Fig. 2b). After cell fractionation, RbcS was recovered predominantly, as expected, in the soluble fraction. RbcL, which is synthesized in the chloroplast, also accumulated in the soluble fraction in the dark or the light at similar rates (data not shown). There was essentially no effect of light on total protein synthesis.

Integration of LHCPs into Membrane

Since polypeptides 11, 16, and 17 were recovered with thylakoid membranes, even in the dark, we determined whether they were integrated into, and not simply associated with, membranes by assaying their sensitivity to proteases. LHCPs that are integrated into the membrane are not degraded by proteases except for cleavage of an N-terminal fragment, about 20 amino acids in length, from polypeptides 11 and 17 (7; also ref. 12 for review). If simply associated with membrane surfaces, the polypeptides should be extensively digested (17). To verify this assumption, precursors of polypeptides 11, 16, and 17, produced by *in vitro* translation (13, 23), were mixed with the membrane fraction from degreened cells. As assayed by electrophoretic analysis of the incubation mixture and immunoprecipitated products, the precursors of 11, 16, and 17 were extensively digested by proteolytic activity associated with membranes. Addition of thermolysin, trypsin, or proteinase K to the mixture resulted in complete digestion of polypeptides immunoreactive to antibodies against polypeptide 11.

Attempts to achieve complete digestion of mature, integrated polypeptides 11, 16, and 17 after treatment of membranes with detergents were less successful. Addition of SDS to mixtures of membranes with proteinase K did not lead to complete digestion, as found also by Herrin *et al.* (11). Addition of Triton X-100 to 1% (w/v) in the reaction mixture allowed nearly complete digestion of polypeptides 16 and 17 but did not promote further digestion of polypeptide 11. This resistance to digestion apparently results from changes that occur due to integration of the polypeptides into the membrane and their association with Chl and other lipids.

Evidence that polypeptides 11, 16, and 17 are not inherently resistant to proteolysis was obtained with membranes from cells of strain *pg-113* of *C. reinhardtii* (26). We confirmed by HPLC that Chl *b* is undetectable in these cells (22). However, in contrast to other Chl *b*-deficient strains of *C. reinhardtii* (8, 28) and higher plants (3, 24, 31), *pg-113* cells grown in the light at 25°C accumulate nearly normal amounts of the LHCPs (26). When we treated membranes from light-grown *pg-113* cells with thermolysin or trypsin, essentially complete digestion of the LHCPs occurred (Fig. 3). In the absence of Chl *b* the LHCPs apparently were not properly integrated into the membrane and thus were not protected by the membrane from proteolysis.

We then tested the resistance to thermolysin of newly synthesized polypeptides in membranes from *y-1* cells labeled

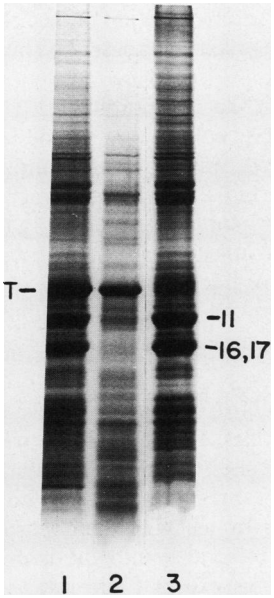

Figure 3. Sensitivity to proteolytic digestion of LHCPs in membranes from *pg-113* cells. Membranes were prepared from cells grown in the light at 25°C for 2 d and divided into two portions; each portion (20 μg of Chl) was treated with thermolysin on ice for 30 min as described in "Methods and Materials." EGTA was added to one portion before addition of protease (lane 1) and to the other at the end of the treatment (lane 2). An untreated control is shown in lane 3. T, band of thermolysin.

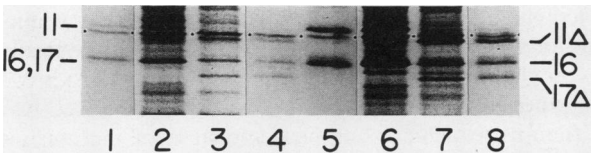

Figure 4. Immunoprecipitation of LHCPs from *y-1* cells and protease-generated fragments. Degreened cells were preincubated 1.5 h at 38°C in the dark, then labeled in the dark or light for 15 min. Membranes were prepared, divided into two portions, and treated with thermolysin as described under Figure 3. The figure shows the midportion of the radioautogram of the gel after electrophoresis of the samples. For lanes 1 to 4, cells were labeled in the dark. Lane 1, immunoprecipitate of the undigested membrane sample in lane 2; lane 4, immunoprecipitate of the digested sample in lane 3. For lanes 5 to 8, cells were labeled in the light. Lane 5, immunoprecipitate of the undigested sample in lane 6; lane 8, immunoprecipitate of the digested sample in lane 7. The positions of polypeptides 11, 16, and 17, and the products of digestion 11Δ and 17Δ are indicated. The contaminating polypeptide (see text), which was not digested by thermolysin, was used as a marker in the immunoprecipitated samples and is marked with dots.

for 15 min in the dark or light (Fig. 4). At the end of the digestion period the band corresponding to labeled polypeptide 11 had disappeared and was quantitatively replaced by a new band designated 11Δ. Likewise, the intensity of a new band, designated 17Δ, corresponded to the decrease in the polypeptide 16 + 17 pair. Since these new species reacted with antibodies to polypeptide 11, which also reacts with 16 and 17 (13), these products were identified as fragments of these polypeptides. The pattern shown in Figure 4 was also obtained after digestion with trypsin and proteinase K. The time course of digestion was the same with membranes labeled in the dark or in the light. These results indicated that 11, 16, and 17 entered membranes and achieved their correct orientation in the dark (Fig. 4, lanes 1–4) as well as in the light (Fig. 4, lanes 5–8).

A minor membrane polypeptide, which migrated just ahead of polypeptide 11, was also immunoprecipitated in these experiments, as observed previously (13). The original antigen preparation, from which the antibodies were prepared, apparently also contained polypeptide 12 as a contaminant. This polypeptide was resistant to thermolysin and was used as a reference (marked with dots in Fig. 4).

Analysis of Turnover of LHCPs

Degreened *y-1* cells contain the same level of translatable LHCP mRNA in the light or dark at 38°C (13). Thus, the disparate kinetics of accumulation of LHCPs shown in Figure 2a could result from selective inhibition of translation of LHCP mRNA or subsequent rapid degradation of most of the newly synthesized polypeptides. Because LHCPs within *pg-113* cells are not degraded in the absence of Chl *b* (26) yet are susceptible to exogenous proteases incubated with isolated membranes (Fig. 3), *pg-113* cells possibly are deficient in a proteolytic mechanism. Consequently, this strain should be a suitable organism to examine whether light affects accumulation of LHCPs at the translational level. LHCPs were barely detectable in the membrane fraction from *pg-113* cells grown at 25°C in the dark for 4 d, which suggested that light was required to induce synthesis of LHCPs at 25°C in these cells just as in *y-1* cells (13). At 38°C, *pg-113* cells also behaved similarly to *y-1* cells in that LHCP mRNA became abundant in the dark, as assayed by *in vitro* translation. Figure 5 shows that, in the dark, recovery of labeled polypeptide 11, 16, and 17 with membranes was about 95% of that in the light. Thus, in *pg-113* cells translation of LHCP mRNA was not affected by light. The amount of Chl did not increase during incubation of *pg-113* cells in the dark at 38°C.

If the rate of translation of LHCP mRNA in *y-1* cells in the dark is similar to that in *pg-113* cells, then the different rates of accumulation of these polypeptides in *y-1* cells in the light or dark (*e.g.* Fig. 2a) probably reflected different rates of degradation. To test whether degradation occurs after LHCPs associate with thylakoid membranes, pulse-chase experiments were carried out. Dark-grown cells were labeled with [^{14}C] arginine for 10 min and then a 160-fold excess of [^{12}C]arginine was added along with cycloheximide to inhibit further incorporation of [^{14}C]arginine. These additions decreased incorporation of ^{14}C into cytoplasmic proteins below levels detectable by radioautography, as shown in lane 1 of Figure 6. When labeling was done in the light, no decrease in the amount of ^{14}C in polypeptides 11, 16, and 17 was detected during 45 min of chase in the light (not shown). Thus, the polypeptides that became integrated into the membranes in the light, when Chl was actively synthesized, were stable.

Pulse-chase experiments were also done with degreened cells in the dark. Because of the low rate of accumulation in the dark, polypeptides 11, 16, and 17 were partially obscured

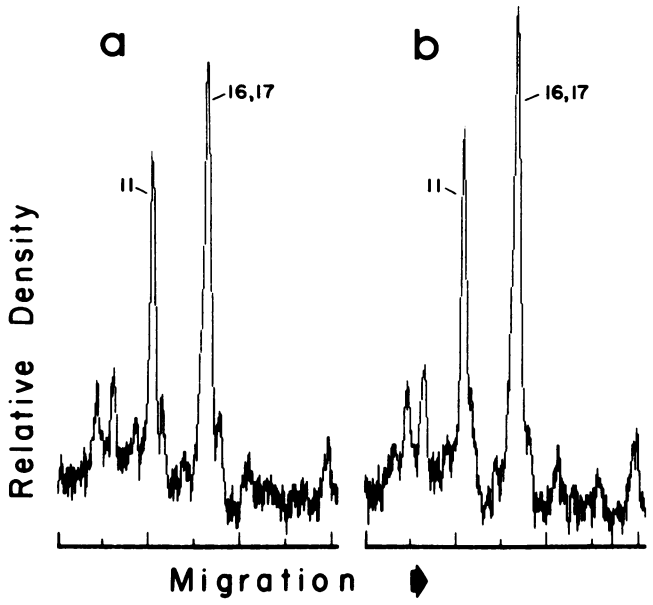

Figure 5. Effect of light on accumulation of LHCPs in *pg-113* cells. Cells were grown in the dark for 3 d, preincubated in the dark at 38°C for 1.5 h and then labeled with [^{14}C]arginine for 15 min (a) in the dark or (b) in the light. Membrane fractions were prepared and subjected to electrophoresis. Radioautograms were scanned, and areas under the peaks were integrated to compare quantitatively the amount of ^{14}C in polypeptides 11, 16, and 17 (labeled in figure). The figure shows densitometric scans of the mid-portion of the patterns.

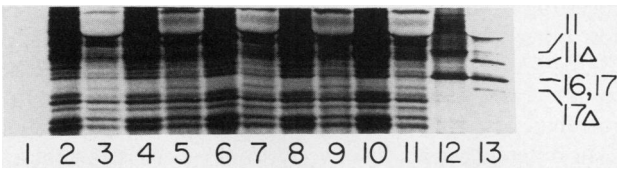

Figure 6. Pulse-chase labeling of LHCPs in *y-1* cells in the dark. Cells, grown for 3 d in the dark, were preincubated 1.5 h in the dark at 38°C and then labeled 10 min with [^{14}C]arginine. Cycloheximide (50 μg/mL) and [^{12}C]arginine (1 mM) were added to stop incorporation of ^{14}C. Samples were removed 0, 10, 20, 30, and 45 min after the pulse. Another sample was labeled 10 min in the light. The membrane fraction was prepared from each sample, one-half of each was treated with thermolysin as described under Figure 3, and then all samples were subjected to electrophoresis. Lane 1, cycloheximide and [^{12}C] arginine were added at the beginning of the pulse; lanes 2 and 3, sample removed at the end of the 10 min pulse; lanes 4 and 5, 10 min of chase; lanes 6 and 7, 20 min of chase; lanes 8 and 9, 30 min of chase; lanes 10 and 11, 45 min of chase; lanes 12 and 13, sample labeled in the light. Odd-numbered lanes contained membranes treated with thermolysin.

by other polypeptides that migrated during electrophoresis at similar rates. To enhance detection, particularly of polypeptide 11, the membranes were treated with thermolysin to shift their mobility as illustrated in Figure 4. As shown in Figure 6, no detectable decay in the amount of labeled polypeptides occurred during 45 min of chase in the dark. Shortening the pulse-labeling period from 10 to 5 min produced the same results. Thus, those polypeptides that were integrated into the membrane in the dark, albeit at a diminished level, also were stable.

Peptidase Activity in Cell Fractions

Accumulation of LHCPs in *pg-113* cells in a protease-sensitive form suggested that these cells are deficient in the activity that normally restricts accumulation of these polypeptides. We confirmed the report by Lang *et al.* (19) that the soluble fraction of *Chlamydomonas* cells contains several peptidase activities. However, when LHCP precursors were incubated with the soluble fraction from cells, only processing to the mature LHCPs was observed. Further degradation of these polypeptides did not occur (23). In contrast, as stated above, incubation of the LHCP precursors with membranes resulted in extensive degradation. Therefore, we examined in particular membrane-bound proteases.

The total membrane and soluble fractions of broken *y-1* cells were applied to sucrose gradients to examine the distribution of peptidase activities. Peptidase activities in the soluble fraction were recovered near the top of a 0.5 to 1.4 M continuous sucrose gradient (Fig. 7a). With the soluble fraction, activity with alanyl-*p*-nitroanilide (Ala-NA) as substrate was greater than that with leucyl-*p*-nitroanilide (Leu-NA). In contrast, membrane-bound activity with Ala-NA was low compared to that with Leu-NA (Fig. 7b). An additional wash of the membrane preparation before loading onto the gradients did not change the recovery of peptidase activities, which indicated that activity with Ala-NA from the region of the gradient containing membranes (fractions 2–6 in Fig. 7b) probably reflected that of a membrane-bound enzyme rather than soluble enzymes that were trapped in the membrane pellet. No activity was detected in membranes with *N*-benzoyl-L-Arg-NA, a substrate for trypsin-like enzymes, or with peptidyl-*p*-nitroanilides such as *N*-succinyl-Ala-Ala-Pro-Leu-NA.

A relatively high amount of peptidase activity, assayed by hydrolysis of Leu-NA, was found associated with membranes in these gradients (Fig. 7b). The bulk of this activity had a buoyant density somewhat lower than that of thylakoid membranes, which in Figure 7b is indicated by the distribution of Chl. In other experiments, in which membranes from degreened cells were applied to a discontinuous gradient containing layers of 0.5, 1.0, and 1.5 M sucrose, the specific activity (nmol Leu-NA hydrolyzed min^{-1} mg protein^{-1}) of the fraction recovered at the 0.5/1.0 M sucrose interface was two- to three-fold greater than that of thylakoid membranes collected at the 1.0/1.5 M sucrose interface. Because the lower density fraction contained carotenoid pigments but very little Chl, it possibly included membranes of the chloroplast envelope (25).

To further investigate whether the membrane-associated peptidase may be involved in degradation of LHCPs, the level of this activity was examined in *pg-113* cells. As shown in Figure 7c, the maximal peptidase specific activity with Leu-NA as substrate in membranes from dark-grown cells was about 15 nmol min^{-1} mg protein^{-1}, which was 10 to 15% of that in the *y-1* strain. The specific activity in the membrane preparation from light-grown *pg-113* cells was the same as that found with membranes from dark-grown cells. With the

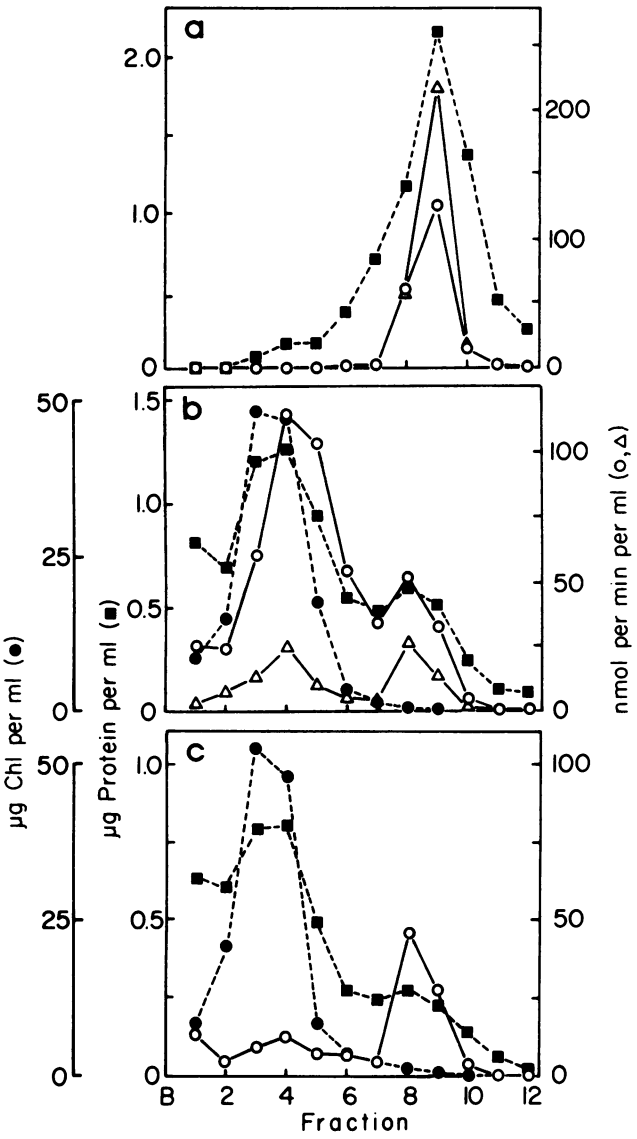

Figure 7. Analysis of peptidase activities in sucrose gradients. Cells grown in the dark for 3 d at 25°C were preincubated 1 h in the dark at 38°C and then exposed to light for 30 min. The (a) soluble and (b) membrane fractions from *y-1* cells and the (c) membrane fraction from *pg-113* cells were applied to 0.5 to 1.4 M continuous sucrose gradients and centrifuged as described in "Materials and Methods." Fractions were assayed for: (O), peptidase activity with Leu-NA as substrate; (Δ), peptidase activity with Ala-NA as substrate; (●), Chl; and (■), protein. B, bottom of gradient. No Chl was present in soluble fractions. Peptidase activity with Ala-NA as substrate was not detected in membranes from *pg-113* cells with the amount of sample used for panel (c).

amounts of sample applied to gradients in these experiments, activity with Ala-NA as substrate was not detected in the membrane fractions. It was not clear from these experiments whether the *pg-113* cells are deficient in the peptidase or simply contain less of the membrane-associated form.

DISCUSSION

Although degreened *y-1* cells at 38°C contain a full complement of LHCP mRNA (13), the polypeptides accumulate at a low rate in the dark. Our experiments were designed to test kinetically at which stage this control is exercised. LHCPs were recovered essentially quantitatively with membranes in the light or dark, which suggested that a significant amount did not exist as a soluble pool within the stroma. We also found no evidence that the polypeptides resided within envelope membranes for a detectable length of time. Processing of LHCP precursors was rapid, even in extensively degreened *Chlamydomonas* cells, since no precursors were detected in immunoprecipitates of pulse-labeled cells. LHCPs that entered membranes either in the light or the dark were integrated properly into thylakoid membranes in a stable form. In these respects, our *in vivo* kinetic data correspond with results obtained from uptake *in vitro* of precursors of the polypeptides by isolated chloroplasts from higher plants (3, 5, 16, 17). The two most likely possibilities for the minimal accumulation in the absence of Chl synthesis, therefore, were inhibition of mRNA translation or posttranslational degradation of the LHCPs.

Wild-type *Chlamydomonas reinhardtii* cells accumulated LHCPs in the dark at 38°C but at a rate considerably less than that in the light (15). Stronger evidence for synthesis of LHCPs in the dark was obtained with cells of the *pg-113* strain. As in dark-grown *y-1* cells, translatable LHCP mRNAs were abundant in *pg-113* cells at 38°C (data not shown). However, in contrast to *y-1* cells, the polypeptide accumulated in the dark at a rate similar to that in the light (Fig. 5), even though no increase in Chl was detected in the dark. In *pg-113* cells, only mature LHCPs accumulated which indicated that processing of LHCP precursors occurred normally. Whereas most other Chl *b*-less strains accumulate very little LHCPs (28), *pg-113* cells accumulate LHCPs (26) but in a protease-sensitive form, which suggests that this strain is deficient in a proteolytic activity.

The difference in ability of *pg-113* and *y-1* cells to synthesize Chl *b* should be considered as a factor that affects accumulation of LHCPs. It could be argued that in *pg-113* cells, Chl(ide) *b* is not available to stimulate LHCP degradation in the dark. This seems unlikely because Chl *b* seems to be required for stabilization of Chl *a*/*b*-protein complexes (31). Residual thylakoid membranes in degreened *y-1* cells are enriched in Chl *b* (22), which may explain the stability of the LHCPs that reach thylakoid membranes in the dark (Fig. 6). Alternatively, Chl(ide) *b* could selectively suppress translation of LHCP mRNA in *y-1* cells in the dark. This possibility is less easily resolved, but such a process would be expected to attenuate LHCP synthesis in the light, when synthesis of Chl is rapid. From our results, we cannot unequivocally exclude translational control in *y-1* cells. However, the accumulation of these proteins in *pg-113* cells, coupled with the inverse correlation of LHCP accumulation and peptidase activity in the two strains, suggest that rather than light affecting translation of mRNA, accumulation is determined at the level of proteolysis. Thus, the low availability of Chl in the dark may allow LHCPs to be degraded in *y-1* cells.

Degradation of LHCPs also occurs when interaction of LHCPs and Chl is diminished by alterations in the proteins. Kohorn *et al.* (16, 17) found that deleting a segment of the C-terminus of a *Lemna* LHCP prevented stable interaction with thylakoid membranes. Such truncated polypeptides,

which apparently lack the domains required for interaction with Chl and/or membranes (10, 17), were not integrated into membranes when incubated with stromal proteins and ATP, as occurs with full-length LHCPs (5, 9). Cline *et al.* (10) extended these observations to show that both truncated and full-length LHCPs of pea were transported across the chloroplast envelope into the stroma but then were degraded when integration into thylakoids was blocked.

In higher plants, degradation of LHCPs or their precursors seems to occur on thylakoid membranes. It is unlikely that proteolytic enzymes in the stroma (19, 20) are involved in degradation, because incubation of LHCP precursors with these enzymes did not result in significant proteolysis beyond cleavage of the transit sequence (23). In contrast, degradation of LHCPs and Chl *b*, already incorporated into functional thylakoid Chl-protein complexes during a period of greening of bean seedlings, occurred when partially greened seedlings were transferred to the dark (1). In the *chlorina-f2* mutant of barley the polypeptides are synthesized, imported into the chloroplast, integrated into thylakoids, and processed (3). The polypeptides in such Chl *b*-deficient mutants are subsequently degraded (31). Pea seedlings, in which synthesis of LHCP mRNA was induced, synthesized but also degraded the polypeptides in the dark (4). In these higher plant systems a pool of labeled LHCPs, which decayed during a chase period of several hours, was associated with membrane and easily detectable. Nevertheless, extensive degradation of LHCP precursors did not occur when they were incubated with purified thylakoid membranes plus stroma, a system that promotes integration of the polypeptides into membranes in association with Chl (5, 9).

Turnover in *C. reinhardtii* cells of proteins such as the thylakoid polypeptide D1 in *nac* mutant strains (18), D1 and D2 in photoinhibited cells (30), and RbcS in chloramphenicol-treated cells (29) has been detected. In these cells, transient pools of the proteins, with half-lives of several minutes to an hour, were measured after pulse-labeling. However, in our experiments, we could not detect an unstable pool of LHCPs in these algal cells, even with pulse-labeling periods shorter than used with other systems. Assuming equal rates of synthesis in the light or dark, we estimated that a $t_{1/2}$ for LHCPs of less than 1 min would be necessary to cause the lower rate of accumulation in the dark (*e.g.* Fig. 2a). It is possible that a mechanism operates at the level of entry into the chloroplast in *Chlamydomonas* to achieve such rapid degradation of any LHCPs not rescued through interaction with Chl. LHCP precursors may be particularly susceptible to proteolysis if transport across the envelope requires an unfolded conformation, as occurs with import into mitochondria (27). Presence of peptidase activity in a membrane fraction with a buoyant density expected for envelope membranes (Fig. 7) supports this suggestion.

Preliminary studies (JK Hoober, unpublished data) have indicated that properties of the peptidase activity associated with membranes do not correspond with those of other chloroplast-associated peptidases previously described (19, 20). Although involvement of the membrane-associated peptidase in degradation of LHCPs has not been established, support for this possibility is the low level at which this activity occurs in *pg-113* cells, which may be the reason for accumulation of LHCPs in these cells (26). Furthermore, a peptidase with remarkably similar substrate specificity occurs on the surface of *Arthrobacter* cells that is capable of rapidly degrading a polypeptide during transport to the cell surface (DG Phinney, JK Hoober, unpublished results). A full characterization of the *Chlamydomonas* membrane-bound peptidase and definition of its intracellular location will be necessary to determine whether this enzyme plays an important role in controlling accumulation of LHCPs.

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