Kinetics of Chlorophyll Accumulation and Formation of Chlorophyll-Protein Complexes during Greening of *Chlamydomonas reinhardtii y*-1 at 38°C[1]

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ABSTRACT

The initial kinetics of accumulation of chlorophylls (Chl) were analyzed during optimal greening of *Chlamydomonas reinhardtii y*-1 at 38°C. Acetate was required for maximal synthesis of Chl, which occurred at a linear rate when degreened cells were exposed to light. During the first hour Chl *a* and *b* accumulated predominantly as geranylgeraniol esters, with lesser amounts of the species with more reduced alcohol side chains. When Chl synthesis was blocked either by treatment with gabaculine or by transfer to the dark, the distribution shifted to the more reduced forms. Similar kinetic patterns indicated that a common pool of chlorophyllides *a* and *b* provided substrate for the enzymatic system that performs esterification and reduction of the sidechain for each group of Chl. Chl *b* was essentially quantitatively integrated into light-harvesting complexes as indicated by energy transfer to Chl *a*. In the presence of cycloheximide, an inhibitor of cytoplasmic protein synthesis, Chl *b* did not accumulate and Chl *a* production was reduced about one-half. The results demonstrate that Chl *a/b*-protein complexes assemble rapidly during greening and that reduction of the alcohol side chain of the Chl is not required for assembly of these complexes.

The rapid and linear greening of yellow cells of *Chlamydomonas reinhardtii y*-1 at 38°C offers unique opportunities to examine kinetically light-dependent aspects of chloroplast development. This system, uncomplicated by the lag in greening that occurs in these cells at 25°C (15, 25) can be used to analyze, for example, synthesis of major components of thylakoid membranes such as the light-harvesting Chl *a/b*-protein complex (LHC2[3]). This complex in higher plants recently was described as an aggregate of three polypeptides (LHCPs) each associated with eight Chl *a* molecules, seven Chl *b* molecules, and several carotenoids (7). Degreened *C. reinhardtii y*-1 cells contain a barely detectable level of translatable mRNA for the LHCPs at 25°C (14, 22) but develop a full complement of these mRNAs during a 2-h incubation in the dark at 38°C (14). As shown by the response to phenanthro-

lines, these cells are fully capable of synthesizing Chlide *b* in the dark (3–5). Yet synthesis of LHCPs proceeds at a low rate and no Chl is made until cells are exposed to light (16). As a continuation of this work, we have examined the kinetics of Chl accumulation under conditions that support a maximal rate of greening when degreened cells are exposed to light. This analysis showed that the species that appeared initially were those in which the esterified alcohol is incompletely reduced. Since we found that essentially all the Chl *b* is associated with light-harvesting complexes, these results indicate that reduction of the alcohol side chain is not essential for assembly of Chl-protein complexes.

MATERIALS AND METHODS

Greening of Cells

Chlamydomonas reinhardtii y-1 used for these experiments was a strain maintained in this laboratory. Strain *pg*113 (23) was kindly provided by Dr. A. Boschetti. Green *y*-1 cells, after growth in the light for 1 d, were transferred to the dark and growth was continued for 3 to 4 d at 25°C (3, 16). The degreened cells were harvested by centrifugation and suspended in fresh growth medium (15, 16) to a density of 1 × 10^7 cells/mL. For assays of the effects of acetate, degreened cells were suspended in a medium containing 3 mM K$_2$HPO$_4$, 7 mM KH$_2$PO$_4$, 7.5 mM NH$_4$Cl, 1.0 mM MgSO$_4$, 0.1 mM CaCl$_2$, and 10 mM NaCl; sodium acetate was added as desired. Cells were incubated with shaking in a water-bath maintained at 38°C and exposed to light from incandescent lamps at a fluence, measured with a model 65A radiometer (Yellow Springs Instrument Co., Yellow Springs, OH), of 200 ± 10 W/m^2.

For analysis of Chl during greening, up to 50 mL of cell suspension were incubated in 600-mL beakers. Chl was extracted from pellets of 1 × 10^8 cells with 80% acetone and measured spectrophotometrically (2).

HPLC

Chl in 2 × 10^8 cells was extracted with 10 mL 80% acetone. The extract was mixed vigorously with 10 mL diethyl ether and 20 mL 1 M KCl in a separatory funnel (6). The ether layer was collected, cooled on dry ice to freeze out water, and then evaporated under a stream of nitrogen. The residue was dissolved in 0.20 mL acetone. Samples (10–20 µL) were analyzed by HPLC on an ODS-C$_{18}$ reverse-phase column

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[3] Abbreviations: LHC2, light-harvesting complex of PSII; LHCP, light-harvesting Chl *a/b*-binding apoprotein of LHC2; Chlide, chlorophyllide.

(0.46 × 25 cm; bead size, 5 μm; Altex Division, Beckman Instruments, Inc., San Ramon, CA) eluted at room temperature with 100% methanol at a flow rate of 1.5 mL/min (26). The effluent was monitored by absorbance at 652 nm, a wavelength at which Chls *a* and *b* have approximately the same absorption coefficient (21). The elution profile was processed by a model 427 integrator (Beckman Instruments, Inc.) to obtain retention times and the relative amount of each species.

Spectrofluorimetry of Membrane Samples

Suspensions of greening cells (1 × 10⁸ cells/sample) were mixed with an equal volume of ice-cold 20 mM Tricine-NaOH (pH 8.0) containing 100 μg/mL cycloheximide. The cells were collected by centrifugation, suspended in 500 μL of 20 mM Tricine buffer, and broken by sonication. The suspension was centrifuged at 3,000*g* for 30 s and the supernatant fluid was further centrifuged at 75,000*g* for 20 min at 2°C. The membrane pellet was suspended in 5 mL of 20 mM Tricine buffer. Excitation and emission spectra were determined at room temperature with a Perkin-Elmer model 650-105 fluorescence spectrophotometer as described previously (3) after adding Triton X-100 to final concentrations of 0.01 and 0.5% (w/v).

RESULTS

Effect of Acetate on Accumulation of Chl

To examine the kinetics of specific processes during greening, we sought conditions that would support an optimal rate of Chl synthesis. Acetate is the carbon source required for growth of *C. reinhardtii y*-1 cells in the dark (24) and, as shown in Figure 1, strongly affected the kinetics of total Chl accumulation during greening. In these experiments degreened cells were exposed to light, after a 1.5-h preincubation in the dark, at 38°C. Acetate was added at various times during preincubation or after illumination. Chl accumulated at a maximal, linear rate when sufficient acetate was present throughout the experiment (Fig. 1, curve 1). Removal of acetate 5 min before exposure to light reduced the subsequent rate of Chl accumulation in the light to less than 10% of the maximal rate (Fig. 1, curve 2, first 15 min). Chl synthesis began immediately at nearly the maximal rate when acetate was added back 15 min after the start of illumination (Fig. 1, curve 2). In contrast, when acetate was not present either during the preincubation period or after exposure to light, accumulation of Chl was barely detectable during the initial hour of illumination (Fig. 1, curve 4), and a lag of about 15 min elapsed before Chl synthesis was observed then acetate was added back after 15 min of exposure to light (Fig. 1, curve 3). Although 2 mM acetate was sufficient to produce the maximal rate of Chl synthesis, acetate was routinely included in subsequent experiments at 7.5 mM, the concentration in the growth medium (24).

Accumulation of Chl with Incompletely Reduced Alcohol Side Chains

Chl were recovered from degreened cells grown in the dark at 25°C as the phytyl species, with an *a/b* ratio of 0.6 to 0.7
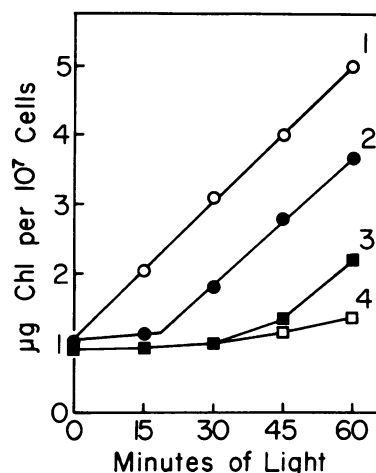

Figure 1. Effect of acetate on Chl synthesis. Degreened cells were suspended in medium lacking acetate and preincubated in the dark at 38°C for 1.5 h prior to exposure to light. At various times, 1 M sodium acetate was added to provide a final concentration of 10 mM. Curve 1, acetate was added at the beginning of preincubation. Curve 2, acetate was added as for curve 1; at the end of the preincubation, cells were removed from the medium by centrifugation, washed with acetate-free medium, suspended in acetate-free medium, and then exposed to light. Acetate was restored after 15 min of light. Curve 3, cells were preincubated without acetate; acetate was added after 15 min of light. Curve 4, acetate was absent throughout the experiment.

as determined by HPLC (Fig. 2a). Chl *b* eluted from the column at an elution volume about half that of Chl *a*. The amounts of Chl *a* or *b* did not change during 2 h of preincubation in the dark at 38°C (data not shown). Samples from greening cells, however, contained eight major species as shown in Figure 2b. The absorption spectrum of material in each of peaks 1 to 4 had maxima at 467 and 652 nm, a characteristic of Chl *b*. The absorption spectrum of material in each of peaks 5 to 8 had maxima at 430 and 666 nm, property of Chl *a*. Peaks 4 and 8 were identified as phytol-containing Chl *b* and Chl *a*, respectively, by comparison with Chls from green cells grown in the light at 25°C for 2 d (Fig. 2d). To identify the additional species present during greening, elution parameters for each component were obtained (Table I). The ratios (α_1) of the capacity factors (k') for the various Chl species (peaks 1–7) relative to that of Chl a_p (peak 8) were the same as found by Shioi *et al.* (26, 27) for the forms of Chl listed in Table I. The ratios (α_2) of the capacity factors within the Chl *b* group were coincident to those for the Chl *a* group (Table I), which indicated that both groups were generated by the same series of side chains. Furthermore, a linear relation was found between log k' *versus* the number of double bonds in the alcohol sidechain (26) for each group, with a slope for the Chl *b* series (peaks 1–4) identical to that for the Chl *a* series (peaks 5–8) (not shown). These results support identification of Chl in each peak as given in Table I.

Figure 3 shows the time course of accumulation of each form during a 2-h greening period. As shown in the insets of Figure 3, geranylgeraniol Chlides were predominant initially but decreased in relation to other forms with time in light. On the other hand, the relative amount of tetrahydrogeran-
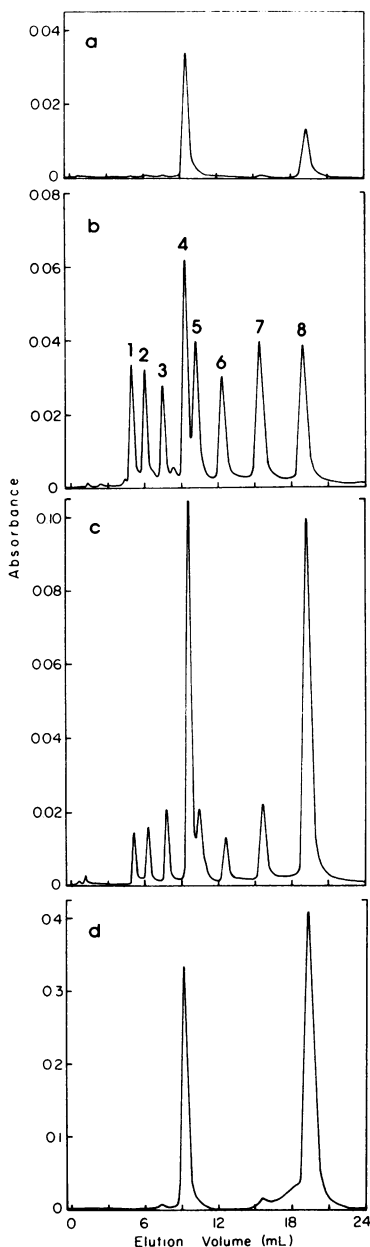
Table I. *Chromatographic Parameters for Chl from Greening Cells of C. reinhardtii y-1*

Chl were eluted with 100% methanol at a flow rate of 1.5 mL/min at room temperature. $k' = (t_R - t_0)/t_0$, where t_R and t_0 are retention times of retained and unretained solutes, respectively, $\alpha_1 = k'$ (Chl x)/k' (Chl a_p), and $\alpha_2 = k'$ (Chl)/k' (Chl$_p$) within each of the Chl a and Chl b groups.

Peak	Chl[a]	Retention Time	k'	α_1	α_2
		min			
1	Chl b_{GG}	4.53	1.66	0.26 (0.27)[b]	0.52 (0.55)
2	Chl b_{DHGG}	5.17	2.04	0.32 (0.33)	0.65 (0.67)
3	Chl b_{THGG}	6.03	2.55	0.40 (0.40)	0.81 (0.82)
4	Chl b_P	7.08	3.16	0.49 (0.49)	1.00 (1.00)
5	Chl a_{GG}	7.59	3.46	0.54 (0.56)	0.54 (0.56)
6	Chl a_{DHGG}	8.83	4.19	0.65 (0.67)	0.65 (0.67)
7	Chl a_{THGG}	10.60	5.24	0.81 (0.83)	0.81 (0.83)
8	Chl a_P	12.69	6.46	1.00 (1.00)	1.00 (1.00)

[a] Chl a_{GG}, Chl a_{DHGG}, Chl a_{THGG}, and Chl a_p (same for Chl b series) refer to Chl with, respectively, geranylgeraniol, dihydrogeranylgeraniol, tetrahydrogeranylgeraniol, and phytol as the esterified alcohol.   [b] Values in parentheses are from Shioi *et al.* (26, 27).

Figure 2. HPLC analysis of Chl during greening. Chl was extracted and analyzed as described in "Materials and Methods" from de-greened cells in growth medium (a) at the end of a 2-h preincubation in the dark at 38°C, (b) after 1 h of light, (c) after 1.5 h of light but with gabaculine added to 1 mM at 0.5 h into the light period, and (d) after 2 d of growth at 25°C.

ylgeranyl and phytyl Chlides increased with time. A plot of the actual amount of each species, and the amount as a percentage of total newly synthesized Chl, *versus* time were similar for Chl *a* and *b* (Fig. 3). Synthesis of Chl *b* occurred without an apparent lag. The samples did not contain a significant amount of free Chlides (*e.g.* Fig. 2a–d), which should appear in the first 3 mL of eluate because they are not strongly retained by the reverse phase column during HPLC. The *a/b* ratio of newly synthesized Chl during greening was

approximately 2.0 throughout the 2 h of greening (Fig. 3). The Chl ratio in fully green cells was 2.1 ± 0.1 (Fig. 2d).

The amounts of Chl with incompletely reduced sidechains correlated with the rate of greening. The patterns shown in Figure 2b were obtained with a greening rate represented by curve 1 in Figure 1. In experiments in which the cells greened more slowly, a greater portion of the newly synthesized Chl was recovered as the phytyl forms.

To test whether the Chl that appeared initially could be subsequently converted to the phytyl forms, conditions were established in which further Chl synthesis was blocked. Ga-baculine (3-amino-2,3-dihydrobenzoic acid) is an irreversible inhibitor of glutamate 1-semialdehyde aminotransferase (13) and at relatively high concentrations inhibits tetrapyrrole synthesis in higher plants (10, 13), cyanobacteria (19), and algae (18), including wild-type *C. reinhardtii* at 25°C (20). When added to suspensions of *C. reinhardtii* y-1 cells at 38°C 1 h before exposure to light, gabaculine (1 mM) completely blocked subsequent Chl accumulation but did not affect the incorporation of [^{14}C]arginine into cellular protein (data not shown). We then examined in subsequent experiments the effect of gabaculine on cells that were exposed to light for 30 min to first allow accumulation of Chl precursors before the inhibitor was added. The ensuing slow onset of inhibition of Chl synthesis (Fig. 4) resembled the kinetics of inhibition by gabaculine of the aminotransferase extracted from barley (13). When Chl synthesis eventually ceased, the level of Chl was similar to that in untreated cells in light for 60 min (Fig. 4). As shown in Figure 2c, 1 h after gabaculine was added (*i.e.*, 90 min after the start of illumination), the amounts of peaks 1 to 3 and 5 to 7 were low, relative to the pattern for untreated cells shown in Figure 2b. The decreases in these peaks were accounted for by commensurate increases in phytyl Chlide *b* (peak 4) and phytyl Chlide *a* (peak 8), respectively, in gaba-culine-treated cells.

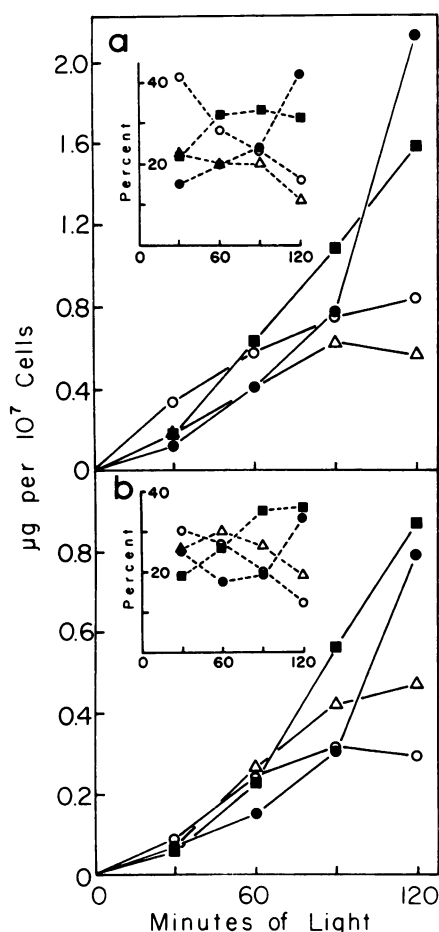Although in the presence of gabaculine the changes in

Figure 3. Amounts of Chl precursors as a function of time of greening. At the times indicated, samples of greening cells were extracted and Chl was analyzed by HPLC as described in "Materials and Methods." (a) The increase in amount of the forms of Chl *a* (peaks 5–8 in Fig. 2b) with time. *Inset*: Presentation of each form as percent of total Chl *a*. (b) The increase in amount of the forms of Chl *b* (peaks 1–4 in Fig. 2b) with time. *Inset*: Presentation of each form as percent of total Chl *b*. O, Geranylgeraniol Chlides; △, dihydrogeranylgeraniol Chlides; ■ tetrahydrogeranylgeraniol Chlides; ● phytyl Chlides.
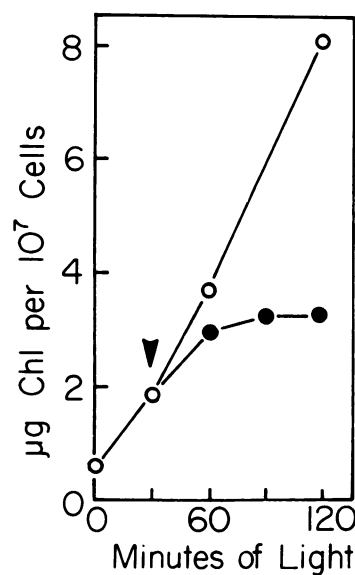

Figure 4. Effect of gabaculine on Chl synthesis in greening cells. Gabaculine was added to a final concentration of 1 mM after 30 min of exposure of cells to light. O, Control cells; ●, cells treated with gabaculine.

distribution of Chl suggested conversion to the more reduced forms, supporting evidence was sought with another experimental design. Thus, Chlide synthesis was blocked by transferring cells to the dark. Figure 5a shows the pattern obtained after cells had greened for 1 h in the light at 38°C. The cells were then placed in the dark for another hour before the sample shown in Figure 5b was taken. Interestingly, the *a*/*b* ratio for total Chl, determined from the sums of all the forms of Chl *a* and *b* decreased from 1.65 to 1.15 during the hour in the dark. However, a shift toward the forms with more reduced sidechains during the hour in the dark was still obvious. In particular, the geranylgeraniol species (peaks 1 and 5) in Figure 5b were markedly reduced in comparison to the pattern shown in Figure 5a, while the phytol-containing Chl (peaks 4 and 8) increased in the dark.

The same precursor forms, in smaller amounts, were found in cells greening at 25°C (Fig. 5c), a temperature at which the cells green more slowly (15, 16, 25). The stability of the

chromatographic patterns with storage of the samples and the lack of significant amounts of these additional forms of Chl in fully green cells grown at 25°C (Fig. 2d) indicated that they were not altered derivatives of Chl or chromatographic artifacts. The use of acetone to prepare and store the samples minimized alterations of the Chl (30). As an additional control, only a single component, phytyl Chlide *a*, was detected in extracts of *pg*113 cells, a Chl *b*-less mutant strain (23), which were grown in the light at 25°C.

Recovery of Chl in Light-Harvesting Complexes

To determine whether cytoplasmically synthesized proteins such as the LHCPs are required for synthesis of the precursors of Chl, cycloheximide was added to cells 5 min before exposure to light to inhibit cytoplasmic protein synthesis. In the presence of the inhibitor, the rate of Chl accumulation was still approximately 40% of control values. Analysis by HPLC revealed, however, that only Chl *a* accumulated under these conditions, including small amounts of the incompletely reduced precursors. Only traces of precursors of Chl *b* were detected (data not shown).

This effect of cycloheximide suggested that Chl *b* accumulated only to the extent that it could be accommodated in light-harvesting complexes. To examine this point directly, we took advantage of the fact that in LHC2, Chl *a* and *b* are in sufficiently close juxtaposition that energy absorbed by Chl *b* is efficiently transferred to Chl *a* (see ref. 29 for review). As illustrated in Figure 6a, in membranes dissociated with 0.5% Triton X-100 both Chl *a* and Chl *b* exhibited their expected fluorescence emission spectra, with a maximum for Chl *b* at 652 nm under these conditions. In contrast, at detergent concentrations too low to cause dissociation of complexes (0.01% Triton X-100), excitation of Chl *b* at its maximum of 472 nm resulted in an emission spectrum with a maximum
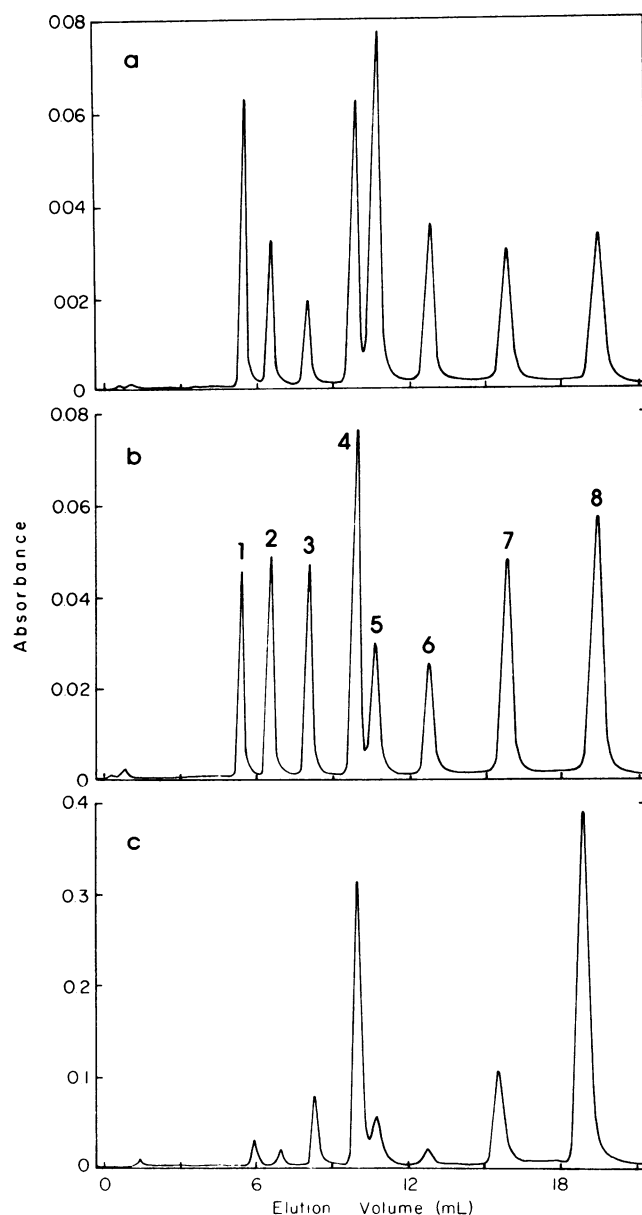
In excitation spectrum 1 in Figure 6b the maxima at 438 nm and 472 nm were approximately equal in intensity when emission was measured at 678 nm. If all Chl *b* species were contained in Chl-protein complexes, the same excitation spectrum, with reduced fluorescence intensity, should be obtained when emission was measured at 652 nm. Instead, as shown by excitation spectrum 2 in Figure 6b, a slight increase in the maximum at 472 nm relative to that at 438 nm was found when fluorescence was measured at 652 nm. The extent of this relative increase was 3 to 5% of the total fluorescence of Chl *b* at 652 nm after dissociation of the Chls with detergent. This value represents the maximal amount of free Chl *b*, assuming 100% energy transfer, in this sample. However, since the efficiency of energy transfer is usually less than 100%, as indicated by measurements on isolated LHC2 (29), the amount of free Chl *b* was probably negligible.

As the concentration of Triton X-100 was progressively increased above 0.03%, emission from Chl *b* at 652 nm gradually increased. About 10-fold higher concentrations of
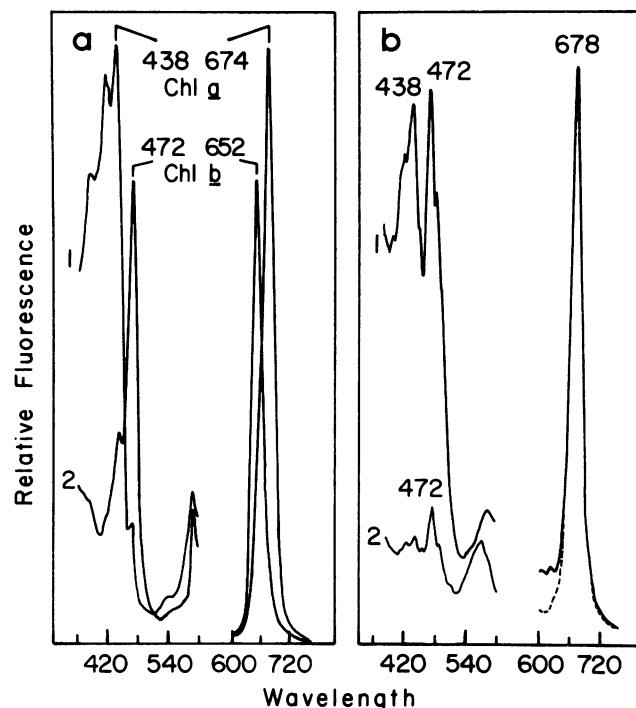

Figure 5. HPLC analysis of Chl from greening cells. (a) Chl from cells greening for 1 h in the light at 38°C was analyzed as described in "Materials and Methods." For (b) companion suspension of cells was further incubated 1 h in the dark at 38°C before Chl was extracted and analyzed. Peaks are numbered as in Figure 2. (c) Degreened cells were exposed at 25°C to white fluorescent light (15 W m⁻²) for 5 h as described previously (15) before Chl was extracted and analyzed by HPLC.

at 678 nm that was essentially superimposable on the spectrum obtained when Chl *a* was excited at 438 nm. The shift in emission of Chl *a* from 674 to 678 nm also reflects integration into complexes (11, 29). In the membrane samples used to obtain these spectra, the geranylgeraniol, dihydrogeranylgeraniol, and tetrahydrogeranylgeraniol species accounted for 60% of the total Chl *b* and 73% of the total Chl *a*, with a chromatographic pattern similar to that shown in Figure 2b.

Figure 6. Assay of Chl in LHC2 by energy transfer. Membranes were prepared from degreened cells that were incubated 2 h at 38°C in the dark and then exposed to light for 30 min. Chl increased from 1.1 to 3.3 μg per 10⁷ cells during illumination. Excitation (380-600 nm) and emission (600-740 nm) spectra were obtained after addition of Triton X-100 to (a) 0.5% (w/v), to dissociate Chl-protein complexes, or to (b) 0.01% (w/v). The fluorescence intensity from the sample in (a) was about threefold greater than from that in (b). For (a) traces 1 and 2 show excitation spectra obtained with emission wavelengths of 674 nm and 652 nm, respectively. The emission spectra were recorded with excitation wavelengths at the respective maxima. For (b), traces 1 and 2 show excitation spectra obtained with emission wavelengths of 678 nm and 652 nm, respectively. The solid tracing with maximum at 678 nm represents the emission spectrum with excitation at 472 nm, whereas the dotted curve represents the emission spectrum with excitation at 438 nm.

n-octyl-glucopyranoside were required to achieve dissociation of the complexes, with detectable free Chl *b* at 0.5 and nearly complete dissociation at 0.9% (w/v) detergent (data not shown). Therefore, although the bulk of the Chl contained incompletely reduced alcohol moieties, this assay indicated that nearly all the Chl were integrated into functional complexes.

As assayed by energy transfer, we found that in membranes from dark-incubated cells, with a Chl *a/b* ratio of 0.7, at least 90% of the Chl *b* occurred in Chl-protein complexes. In such samples, a small emission peak at 635 nm was observed, which we attribute to free protochlorophyll(ide) (data not shown).

DISCUSSION

C. reinhardtii y-1 cells at 38°C have the capability of synthesizing Chlide *b* in the dark (3). However, this ability is suppressed under normal conditions. Interestingly, degreened cells have a marked enrichment in Chl *b* relative to the amount of Chl *a* (Fig. 2a). It is not known whether this results from a slow rate of *de novo* synthesis of Chl *b* in the dark, a conversion of Chl *a* to Chl *b* (1), or a preferential degradation of Chl *a* during degreening. The possibility that Chl *a* can be converted to Chl *b* in the dark is supported by the decrease in the ratio when greening cells were transferred to the dark (Fig. 5). However, this reaction may not be significant during greening, because the *a/b* ratio did not change when Chl synthesis was inhibited in the light with gabaculine (Fig. 2c). The residual level of LHCPs and Chls that exist in degreened cells occur in Chl-protein complexes, as determined by energy transfer assays, but we have been unsuccessful in detecting the low level of these complexes in degreened cells by electrophoretic methods (8) designed for this purpose (JK Hoober, unpublished results).

Measurable Chl synthesis does not occur until these cells are exposed to light (3, 16). Although degreened cells contain a considerable quantity of starch, which is metabolized when cells are exposed to light (24, 25), initial rapid greening is dependent upon an adequate amount of acetate in the medium. These results suggest that acetate provides a better carbon and/or energy source for this process than does endogenous starch. During greening the initial rates of synthesis and esterification of Chlides with geranylgeranyl pyrophosphate apparently exceed the capacity to reduce the sidechain to the phytol moiety. Soll *et al.* (28) showed that two pathways exist in spinach for generation of the sidechain of Chl. In the first, geranylgeraniol pyrophosphate, synthesized in the chloroplast stroma, is esterified to Chlides by Chl synthetase in thylakoid membranes and then reduced stepwise to produce the phytol-containing Chl. In the second pathway, geranylgeraniol pyrophosphate is first reduced in the chloroplast envelope membranes to phytyl pyrophosphate, which possibly is transferred to thylakoid membranes, where Chl synthetase generates phytyl Chlides directly. In *C. reinhardtii*, the amounts of phytyl Chlides increased relatively slowly during the first hour of greening at 38°C. Instead, the predominant forms during this time were geranylgeraniol Chlides. Thus our results with the algal cells confirm the conclusion reached for etioplasts of higher plants (27, 28) that the first pathway

described above is used essentially exclusively during the early period of greening.

In fully green cells the precursor forms of the Chl were not detected, with the exception of small amounts of tetrahydrogeranylgeraniol Chlides (Fig. 2d) which may result from a greater capacity of green cells to reduce the alcohol sidechain. When Chl synthesis was blocked either with gabaculine or by transfer of cells to the dark, a marked shift in Chl toward the reduced forms occurred (Figs. 2c and 5b). The more pronounced shift in gabaculine-treated cells, as compared with that in cells transferred to the dark, may again reflect a greater reducing capacity in the light. Since our results indicate that the incompletely reduced Chl became integrated into Chl-protein complexes, reduction of the sidechain apparently can continue after assembly of the complexes.

From these data we cannot determine whether Chl *b* is derived from Chl *a* (1, but see also refs. 5 and 12), which could be the basis for the similarities in patterns during greening, or whether Chlide *b* is generated prior to esterification. The existence of Chlide *b* in higher plants (9) and our previously obtained evidence that Chlide *b* is derived directly from protochlorophyllide (3, 4) suggest that during greening the *a/b* ratio was established prior to esterification. Moreover, the similarities in their kinetic patterns suggest that Chlides *a* and *b* enter a common pool and are esterified and reduced by the same enzymatic system (28).

Cells of *C. reinhardtii y*-1 become depleted of thylakoid membranes during growth in the dark (24). Assuming that Chl synthetase in the alga resides in thylakoid membranes, as it does in spinach (28), these results suggest that during greening the final stages of Chl synthesis and assembly of Chl-protein complexes in the alga occur in the residual thylakoid membranes. As further support for this conclusion, most of the protochlorophyll(ide) in dark-grown algal cells is recovered in membranes with the same buoyant density as thylakoid membranes after sucrose gradient separation (5). The protochlorophyll(ide) is reduced to Chl(ide) *a* when cells are exposed to light and is converted to Chl(ide) *b* when membranes are treated in the dark with phenanthrolines (4, 5).

Accumulation of Chl *b*, and about half the Chl *a*, is dependent upon availability of cytoplasmically synthesized proteins. The immediate onset of the effects of inhibition of cytoplasmic protein synthesis suggests that cessation of Chl *b* synthesis is not caused by loss of an enzyme required for synthesis of Chl *b*. Furthermore, the absence of protein synthesis *per se* was not responsible for the near absence of Chl *b*, because *Chlamydomonas* cells synthesized Chlide *b* when treated with *o*-phenanthroline, which itself inhibited protein synthesis (3). The additional presence of cycloheximide did not interfere with this effect of phenanthrolines (DP Bednarik, JK Hoober, unpublished data). The continued synthesis of Chl *a* also indicated that cycloheximide did not directly suppress the biosynthetic pathway for Chl. A requirement of the cytoplasmically synthesized Chl *a/b*-binding proteins and formation of Chl-protein complexes for continued synthesis of Chl *b* (and an equal amount of Chl *a*) is a more likely explanation for the effect of cycloheximide. This should not be surprising, because most of the Chl *b*, and a nearly equiv-

alent amount of Chl a, is associated with LHCPs made on cytoplasmic ribosomes (7, 8, 12). The substantial amount of Chl a that accumulated in the presence of cycloheximide most likely was associated with Chl a-binding proteins synthesized within the chloroplast (12).

The immediate and rapid increase in the amount of Chl with onset of exposure of the algal cells to light is correlated with the prior accumulation of mRNA for LHCPs (14). In this regard, this situation is similar to those higher plants in which a pulse of red light induces synthesis of LHCP mRNA through activation of phytochrome, thereby eliminating the lag in Chl accumulation (17). We have verified by direct measurement (LR Asbury, DB Marks, JK Hoober, unpublished results) that in *Chlamydomonas* the LHCPs also increase in parallel with Chl upon exposure of cells to light. As illustrated in Figure 6, we have demonstrated that during greening, the consequent assembly of Chl-protein complexes occurs rapidly, with little if any Chl b remaining unassociated with complexes.

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