The Presence of Chlorophyll b in Synechocystis sp. PCC 6803 Disturbs Tetrapyrrole Biosynthesis and Enhances Chlorophyll Degradation*

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Both chlorophyll (Chl) a and b accumulate in the light in a Synechocystis sp. PCC 6803 strain that expresses higher plant genes coding for a light-harvesting complex II protein and Chl a oxygenase. This cyanobacterial strain also lacks photosystem (PS) I and cannot synthesize Chl in darkness because of the lack of chlL. When this PS I-less/chlL/lhcb/cao⁺ strain was grown in darkness, Chl degradation was much slower than in the PS I-less/chlL/lhcb/cao⁺ strain, suggesting that the presence of Chl b leads to more rapid turnover of Chl-binding proteins and/or a more active Chl degradation pathway. Levels and biosynthesis kinetics of Chl and of its biosynthetic intermediates are very different in the PS I-less/chlL/lhcb/cao⁺ strain versus the control. Moreover, when grown in darkness for 14 days, upon the addition of 3-aminolevulinic acid, the level of magnesium-protoporphyrin IX increased 60-fold in the PS I-less/chlL/lhcb/cao⁺ strain (only ~2-fold in the PS I-less/chlL control strain), whereas the PChlide and protoheme levels remained fairly constant. We propose that a b-type PChlide, Chl, or pheide in the PS I-less/chlL/lhcb/cao⁺ strain may bind to tetrapyrrole biosynthesis regulatory protein(s) (for example, the small Cab-like proteins) and thus affect the regulation of this pathway.

Chlorophyll (Chl)¹ is vital for photosynthesis. Chl a is the primary electron donor in the reaction center of the two photosystems and also serves as a light-harvesting pigment. In higher plants, both Chl a and b are bound to the light-harvesting complex (LHC), which is encoded by a multi-gene family of cab genes (1, 2). However, cyanobacteria synthesize Chl a but not Chl b, and they do not contain Cab proteins (LHC or related Chl a/b-binding peripheral antenna proteins), with the exception of small Cab-like proteins (SCPs) that have a single membrane-spanning region similar to the first and third transmembrane regions of Cab proteins (3).

Chl b is synthesized from Chl a or its precursors through the activity of Chl a oxygenase (CAO), and the gene encoding CAO has been identified (4, 5). The in vivo substrate of CAO remains unknown. Chl a, Chl b, and PChlide a and Chl a are all possible candidates (6). Some enzymes in the chlorophyll biosynthesis pathway have a rather low substrate specificity. For example, NADPH:protochlorophyllide oxidoreductase can tolerate several modifications of the substrate at rings A and B (7–9).

Therefore, oxygenation of any of the possible substrates may lead to Chl b as the final product.

Hemes, phycobilins, and Chls are derived from the tetrapyrrole protoporphyrin IX that is produced from 3-aminolevulinic acid (ALA). In plants and some bacteria (including cyanobacteria), ALA is synthesized via a pathway starting from glutamate (10). Ferrochelatase catalyzes the introduction of iron into protoporphyrin IX, leading to the production of heme and phycobilins, whereas the introduction of magnesium by magnesium chelatase is the first committed step on the Chl biosynthesis pathway. The enzymes catalyzing porphyrin synthesis reactions generally have been identified, but the mechanisms regulating the flow of metabolites through these pathways in response to changing environmental conditions remain unclear. Recently, several SCPs in Synechocystis sp. PCC 6803 were shown to regulate the early steps of tetrapyrrole biosynthesis as a function of pigment availability (11).

The Chl biosynthesis pathway leads to the synthesis of protochlorophyllide (PChlide) via magnesium-protoporphyrin IX (Mg-proto IX) and magnesium-protoporphyrin IX methyl ester (Mg-proto IX ME). PChlide is reduced to chlorophyllide, which is phytlated to form Chl. In cyanobacteria, PChlide reduction proceeds via two pathways: NADPH:protochlorophyllide oxidoreductase catalyzes the light-dependent reduction, and the ChlB/L complex catalyzes this reduction independent of the presence of light (12, 13, 14).

Chl degradation takes place during the turnover of Chl or upon cell death (15). Rapid degradation of free Chl or its colored derivatives is necessary to avoid cell damage by their photodynamic action. The mechanism and regulation of this degradation...
tion is largely unknown, although much progress regarding the identification of degradation intermediates has been made in the past several years (16, 17).

A Synechocystis sp. PCC 6803 mutant has been generated that lacks the light-independent pathway of PChlide reduction, and therefore, this strain synthesizes Chl in a light-dependent manner (18). This mutant has also been generated in a photosystem (PS) I-less background, which contains only 15–20% of the amount of Chl present in wild type (19). Upon introduction and overexpression of pea lhcb and Arabidopsis cao genes into this Synechocystis sp. PCC 6803 strain, a PS I-less/lhcb/L hcb/icao strain resulted. This strain contained more Chl b than a and had a large amount of Chl b in the PS II core complex (20). Judging from the growth rate, the oxygen evolving ability, and the Chl content on a per-cell basis, the presence of Chl b poses little difficulty under normal growth conditions. Some if not most of the Chl binding sites in the PS II core complex appear to have little substrate specificity and are able to functionally accommodate Chl b. We use this system to study the regulation of Chl biosynthesis and Chl degradation in the presence of Chl b. In this study, we demonstrate that the presence of Chl b disturbs tetrpyrrole biosynthesis and enhances Chl degradation.

EXPERIMENTAL PROCEDURES

Growth Conditions—The Synechocystis sp. PCC 6803 PS I-less/lhcb/L hcb/icao control strain and the Chl b-containing PS I-less/lhcb/L hcb/icao strain (20) were grown at 30 °C at a light intensity of 5-μmol photons m⁻² s⁻¹ (unless indicated otherwise) in BG-11 medium (21) buffered with 5 mM TES-NaOH (pH 8.0) supplemented with 5 mM glucose. When strains were grown in liquid culture under light-activated heterotrophic growth (LAHG) conditions (22), cells were kept in complete darkness with the exception of one 15-min light period (white light at 20-μmol photons m⁻² s⁻¹) every 24 h. For growth on plates, 1.5% (w/v) Difco agar and 0.3% (w/v) sodium thiosulfate were added, and BG-11 was supplemented with antibiotics appropriate for the particular strain. Growth was monitored by measuring the optical density of the cells at 730 nm using a Shimadzu UV-160 spectrophotometer.

ALA Supplementation—Cells were propagated for 2 weeks under LAHG conditions and were taken in the middle of the 23.75-h dark period. Wearing their exponential growth phase (Aₜ₀₀ 0.5-0.6), 4 ml ALA and 40 mM TES-NaOH (pH 8.2) buffer were added to the cultures. A given period of dark incubation in the presence of ALA, cells were harvested by centrifugation, frozen in liquid nitrogen, and stored at -80 °C until further analysis.

Pigment Analysis—Pigments were extracted from cell pellets concentrated from 90-ml cultures by three successive extractions with methanol containing 0.1% (w/v) ammonium hydroxide (25). Supernatants were combined, and the solvent was evaporated under a stream of nitrogen until the samples were dry. Dried samples were stored at -80 °C for up to 24 h. The extracts were redissolved in a small volume of NH₄OH containing methanol and immediately subjected to high-performance liquid chromatography (HPLC) analysis on an HP-1100 Chemstation using a Waters Spherisorb SSODS2 (250 × 4.0 mm) column filled with C-18 reverse phase silica gel. A 15-min gradient of ethyl acetate (0-100%) in acetonitrile:water:triethyamine (9:1:0.01, v/v/v) at a flow rate of 1.5 ml/min was used to elute the HPLC column for Chl detection. To detect less hydroporphic tetrpyrrole compounds, a 28-min linear gradient of 30-100% acetonitrile:water:triethyamine (9:1:0.01, v/v/v) in water at a flow rate of 1.0 ml/min was added before a 6-min gradient of ethyl acetate (0-100%) in acetonitrile:water:triethyamine (9:1:0.01, v/v/v) at a flow rate of 1.0 ml/min. The spectra of the eluted pigments were recorded continuously in the range of 350-710 nm. An additional 1-min wash with 100% acetonitrile:water:triethyamine (9:1:0.01, v/v/v) was carried out between the two gradients.

Mass Spectroscopy—PChlide b was collected after HPLC analysis. Solvents were evaporated under nitrogen, and dry PChlide b was stored at -20 °C in the dark. Mass spectra were obtained by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Voyager DE STR Biospectrometry Work Station, Foster City, CA). Before analysis, 10 μg of PChlide b was mixed with terthiophene (used as a matrix) dissolved in acetone.

Protolheme Determination—Protolheme determinations were carried out essentially as described (23). Cells harvested from a 400-ml culture were broken in 3-ml basic acetone (acetone:water:0.1 N-ammonium hydroxide (9:2.1 v/v/v)) with a BeadBeater using six breaking cycles (30-s shaking followed by 3-5-min cooling on ice). Subsequently, the sample was centrifuged, and the supernatant containing extracted Chl, carotenoids, and other non-covalently bound pigments was discarded. The pelleting containing protolheme, cell debris, and glass beads was extracted with 3-ml acetone containing 2% HCI three times; each extraction included 10-s shaking with a BeadBeater and centrifugation at 4 °C for 2 min. The supernatants were combined to which 4.5 ml of peroxide-free diethyl ether was added followed by 23 ml of deionized water. The ether phase was carefully taken and dried under a stream of nitrogen. Subsequently, 0.4 ml of 1 N NaOH and deionized water were added to a final volume of 4 ml. The protolheme concentration was determined by recording the reduced-minus-oxidized difference spectrum on a Shimadzu UV-160 spectrophotometer with an alkaline-pyridine solution as described previously (23).

Preparation of the Phosphorhod (Pheide b) Standard—10 mM phe- nanthraline was supplemented to Chlamydomonas cells, which had been cultured in darkness at 25 °C for 3 days. Cells were then allowed to grow for 1 h in darkness at 38 °C (24). Pheide b was extracted from the culture medium with ethyl acetate:acetic acid 3:1 (v/v) as described by Sager and Granick (25) with the exception that the concentration of phosphate (K₂HPO₄, 3.0 mM, KH₂PO₄, 7.0 mM) was higher. Because of the low pH upon extraction, pheide b was protonated, which altered the HPLC retention time. Therefore, pheide b was dried, and the dry standard was resuspended in BG-11 before it was subjected to HPLC analysis.

RESULTS

Presence of PChlide b and Phosphorhod b—The substrate specificity of CAO under in vivo conditions is still an open question. One experiment that may contribute toward its answer is to determine whether an unusual intermediate in the tetrpyrrole biosynthesis pathway appears in the PS I-less/chl L hcb/icao Synechocystis strain in which Chl b is the major Chl (20). When the PS I-less/chl L hcb/icao strain and the PS I-less/chl L hcb/icao control strain were propagated under LAHG conditions for a week, Chl was depleted and PChlide accumulated in accordance to what was observed in chl L strains (18). Cells were then extracted with basic methanol and subjected to HPLC analysis. As shown in Fig. 1A, a small peak at 6.8 min was found in the PS I-less/chl L hcb/icao strain but not in the control. Its absorption spectrum (Fig. 1B) was identical to that of PChlide b. Its abundance was ~10% of that of PChlide a in the strain. To verify the identity of PChlide b, matrix-assisted laser desorption/ionization mass spectrometry was carried out on HPLC-purified PChlide b from the PS I-less/chl L hcb/icao Synechocystis strain. As shown in Fig. 2, the major peak was at m/z 626.14, corresponding to PChlide b, and had the same isotopic distribution as expected from the chemical formula of PChlide b.

Another compound eluting at 9.1 min (Fig. 1, trace 2), the time at which the pheide b standard (24) is eluted (Fig. 1A, trace 3), and with an absorption spectrum identical to that of pheide b (Fig. 1C) was observed in the PS I-less/chl L hcb/icao strain but not in the control. On the basis of these characteristics, this new peak is assigned to pheide b.

Kineti cs of Chl Disappearance—To investigate whether the presence of Chl b affects Chl degradation kinetics, cultures of control (PS I-less/chl L) and PS I-less/chl L hcb/icao strains were transferred to LAHG conditions and samples from these cultures were harvested daily for 2 weeks and subjected to pigment analysis. The results are shown in Fig. 3A. In the control strain on a per-cell basis, Chl a was depleted with a halftime of approximately 26 h while approximately the doubling time of this strain under LAHG conditions. This suggests that Chl is diluted by the growth of the cells and not much is degraded. Alternatively, the synthesis and degradation rates of Chl a are equal at least for the first few days, assuming that some Chl synthesis takes place in chl L cells grown under LAHG conditions. In contrast, in the PS I-less/chl L hcb/icao
HPLC retention time. Peaks at 7.4, 8.0, and 8.1 min have typical durations. Pigment assignments are based on absorption spectra and was at 440 nm. Absorption spectra of the PChlide –10% of the original Chl level remained.

b strain, the total Chl content decreased with a half-time of ~15 h, even though the growth rate of this strain remained the same as the control during the first week of growth under LAHG conditions. Chl b disappeared at essentially the same rate as Chl a. Interestingly, no Chl was detectable in the PS I-less/chlL/’lhcb‘/’cao‘ strain after 5 days of growth under LAHG conditions, whereas in the control strain, approximately 5–10% of the original Chl level remained.

The reason for the rapid decrease in the Chl amount and for the lack of remaining Chl in the PS I-less/chlL/’lhcb‘/’cao‘ strain after several days of growth under LAHG conditions may be either faster Chl degradation or slower Chl synthesis in this strain. To distinguish between these two possibilities, cells were grown in total darkness for up to 4 days to eliminate the Chl biosynthesis that may occur during the daily 15-min illumination under LAHG conditions. During the incubation in total darkness, no fresh medium was added and the same volume of culture was subjected to pigment analysis every day. Under these conditions, the Chl synthesis rate in chlL strain is probably very low (26), and primarily the Chl degradation rate is measured. As shown in Fig. 3\(A\), in the control strain, Chl a was quite stable and the degradation half-time was ~4 days. In contrast, in the PS I-less/chlL/’lhcb‘/’cao‘ strain, the total Chl level decreased much faster (the half-time was <1 day); there was no significant difference in the degradation rates of Chl a and b.

Kinetics of PChlide Synthesis—PChlide is the major tetrapyrrrole intermediate that accumulates in the LAHG-grown PS I-less/chlL cells. The accumulation kinetics of total PChlide (monovinyl + divinyl PChlide a) in the PS I-less/chlL and PS I-less/chlL/’lhcb‘/’cao‘ strains during growth under LAHG conditions are shown in Fig. 4A. In the PS I-less/chlL control strain, the PChlide level exhibited a linear increase during the 13-day period of growth under LAHG conditions. Surprisingly, the accumulation of PChlide a in the PS I-less/chlL/’lhcb‘/’cao‘ strain followed a much different pattern where the PChlide a level increased rapidly within the first 3 days after transfer to LAHG conditions and decreased gradually after day...
PChlide b time 0. Cells were diluted with fresh medium every day to LAHG conditions. Cultures were transferred to LAHG conditions at I-less/chl including Mg-proto IX ME and uroporphyrin between the PS tetrapyrrole biosynthesis pathway. To determine whether the LAHG conditions may originate from altered regulation of the tetrapyrrole biosynthesis pathway, the regulation of this pathway was altered at one of the early steps, tetrapyrrole biosynthesis. Accumulation of PChlide during propagation of PS I-less/chl strains under LAHG conditions. PChlide a (A) and PChlide b (B) amounts were measured every day in the PS I-less/chl/L. control (●) and the PS I-less/chl/L/llchb'/llcao' (▲) strains grown under LAHG conditions. Cultures were transferred to LAHG conditions at time 0. Cells were diluted with fresh medium every day to $A_{730} \sim 0.3$ to maintain logarithmic growth. Data shown are the average of three experiments.

3. Interestingly, the maximum PChlide a level in the PS I-less/chl/L/llchb'/llcao' strain grown under LAHG conditions for 3 days was approximately the same as that in the control strain after LAHG growth for 2 weeks. The accumulation patterns of monovinyl- and divinyl-PChlide a were similar to that of total PChlide (data not shown).

The accumulation profile of PChlide b in the PS I-less/chl/L/llchb'/llcao' strain was very similar to that of PChlide a with the exception that it showed a 2–4-day delay and the level of accumulation was 10-fold lower than that of PChlide a (Fig. 4B). PChlide b started to accumulate 3 days after cells had been transferred to LAHG conditions. The PChlide b level reached its maximum around day 7 and then decreased gradually.

**ALA Supplementation**—The altered PChlide accumulation kinetics in the PS I-less/chl/L/llchb'/llcao' strain grown under LAHG conditions may originate from altered regulation of the tetrapyrrole biosynthesis pathway. To determine whether the regulation of this pathway was altered at one of the early steps, ALA was added to PS I-less/chl/L- and PS I-less/chl/L/llchb'/llcao' cells that had been grown under LAHG conditions for 2 weeks and changes in the levels of tetrapyrrole synthesis intermediates were monitored. As shown in Fig. 5, ALA addition to the PS I-less/chl/L- control strain caused a slow increase in the PChlide concentration, and after 2 days, the PChlide level had increased 3-fold compared with before ALA feeding, whereas the Mg-proto IX level showed an increase of 2-fold. However, the Mg-proto IX level in the PS I-less/chl/L- strain increased 60-fold in response to ALA supplementation, whereas the PChlide level did not increase more than a factor of 2. Little difference was detected in the levels of other major tetrapyrrole intermediates including Mg-proto IX ME and uroporphyrin between the PS I-less/chl/L- and PS I-less/chl/L/llchb'/llcao' strains in response to ALA supplementation (data not shown). Together, these results suggest that regulation of tetrapyrrole biosynthesis has been altered significantly as a result of the introduction of llcao and llchb genes.

As protoheme is known to be a powerful feedback inhibitor of the early steps of the tetrapyrrole biosynthesis pathway (27), protoheme levels were also determined in the PS I-less/chl/L- and PS I-less/chl/L/llchb'/llcao' strains upon ALA supplementation. As shown in Fig. 5C, the protoheme level in the PS I-less/chl/L- control strain almost doubled within 2 days of ALA addition, whereas this increase was much less pronounced in the PS I-less/chl/L/llchb'/llcao' strain. These results suggest that the relative strength of the heme biosynthesis pathway in comparison to that of the chlorophyll biosynthesis pathway is not a major factor to explain the difference in tetrapyrrole biosynthesis intermediate levels in the two strains.

**Kinetics of Chl Biosynthesis**—The data presented above indicate that the presence of Chl b, PChlide b, or other b-type pigments affects the accumulation of Chl intermediates in darkness in chl/L- strains. The next question is whether Chl synthesis upon transfer to light is affected as well. As indicated in Fig. 6, when the PS I-less/chl/L- and PS I-less/chl/L/llchb'/llcao' strains were subjected to low light, the accumulation of PChlide b in the PS I-less/chl/L- control strain doubled within 14 days, whereas this increase was much less pronounced in the PS I-less/chl/L/llchb'/llcao' strain. These results suggest that the relative strength of the heme biosynthesis pathway in comparison to that of the chlorophyll biosynthesis pathway is not a major factor to explain the difference in tetrapyrrole biosynthesis intermediate levels in the two strains.

**Fig. 4. Accumulation of PChlide during propagation of PS I-less/chl/L- strains under LAHG conditions.** PChlide a (A) and PChlide b (B) amounts were measured every day in the PS I-less/chl/L- control (●) and the PS I-less/chl/L/llchb'/llcao' (▲) strains grown under LAHG conditions. Cultures were transferred to LAHG conditions at time 0. Cells were diluted with fresh medium every day to $A_{730} \sim 0.3$ to maintain logarithmic growth. Data shown are the average of three experiments.

**Fig. 5. The effect of ALA supplementation on accumulation of tetrapyrrole biosynthesis intermediates.** A–C, accumulation of Mg-proto IX (A), PChlide (B), and protoheme (C) upon ALA supplementation to the PS I-less/chl/L- (●) and PS I-less/chl/L/llchb'/llcao' (▲) strains that had been propagated under LAHG conditions for 2 weeks and that remained in complete darkness for the duration of the experiment. Pigments were extracted and determined at specified times after the addition of 4 mM ALA. Data shown are the average of three experiments.
had been grown under LAHG conditions for 2 weeks and were then illuminated with continuous light at 0.5-μmol photons m⁻² s⁻¹ (inducing light-dependent PChlide reduction by NADPH:protochlorophyllide oxidoreductase), the rate of total Chl biosynthesis in the PS-I-less/chl-L /lhcb⁺/cao⁺ strain was ~20% of that in the control. It took the PS-I-less/chl-L /lhcb⁺/cao⁺ strain approximately 200 h of illumination to reach the steady-state Chl level observed in continuous light, whereas this process took only ~40 h in the control. Interestingly, Chl a and b were synthesized at approximately the same rate.

**DISCUSSION**

**Substrate Specificity of CAO**—Chl a and b differ only at position 3 (ring B) where Chl a carries a methyl group and Chl b carries an aldehyde group. The aldehyde group in Chl b arises from the methyl group present in Chl a and its precursors by the introduction of oxygen (28, 29). *In vitro*, CAO can catalyze the conversion of Chlide a to Chlide b although at a low rate (30). The suitability of PChlide as a substrate for CAO has been debated. Traces of PChlide b, the oxygenation product of PChlide a, were reported to occur in several green plants (31). However, no PChlide b was found in etioplasts from barley, wheat, oat, and tobacco (32), although the data of *in vitro* experiments suggested that a 5:1 ratio of PChlide b/a could be accommodated in a PChlide a/b-binding protein complex isolated from barley etioplasts (33). Therefore, there is little or no precedence for the occurrence of PChlide b under *in vivo* conditions.

No PChlide b was detected in the PS-I-less/chl-L /lhcb⁺/cao⁺ strain grown in the light. However, when the PS-I-less/chl-L /lhcb⁺/cao⁺ strain was grown under LAHG conditions, PChlide b started to accumulate around day 4 and reached a peak value at around day 7. At that time, the amount of PChlide b was ~15% of that of PChlide a (Fig. 4). In the PS-I-less/chl-L /lhcb⁻/cao⁻ strain, the accumulation kinetics of PChlide b were similar to those of PChlide a but with a delay of several days. This finding suggests that PChlide a can be a substrate for CAO, but the long delay in the conversion of PChlide a to PChlide b indicates that the conversion is very slow. Therefore, PChlide a does not appear to be an efficient substrate for CAO.

**Degradation of PChlide and Chl**—The delay of PChlide b accumulation in comparison with that of PChlide a in the PS-I-less/chl-L /lhcb⁺/cao⁺ strain transferred to LAHG conditions suggests that PChlide b is formed very slowly, and as it accumulates to some degree, it does not turn over rapidly. Both PChlides a and b disappear with a half-time of approximately 3 days in the PS-I-less/chl-L /lhcb⁻/cao⁻ strain, suggesting that this disappearance reflects the stability of PChlide in this strain if further PChlide synthesis is blocked. Indeed, the inhibition of PChlide synthesis after 4 days of LAHG growth is very probable in this mutant. PChlide levels remain very low, and Mg-proto IX levels increase after the addition of ALA (Fig. 5), indicative of a blockage between Mg-proto IX and PChlide in this strain after a few weeks of growth under LAHG conditions.

Another striking difference between the PS-I-less/chl-L⁻ and PS-I-less/chl-L⁺/lhcb⁻/cao⁻ strain is the large difference in chlorophyll stability in darkness. In the control strain, chlorophyll a is stable and has a half-time of 3–4 days (Fig. 3A), whereas in the PS-I-less/chl-L⁻/lhcb⁻/cao⁻ strain, both Chl a and Chl b disappear with a half-time of less than a day. The generally accepted pathway of Chl degradation includes dephytylation followed by dechelation and cleavage of the tetrapyrrole macrocycle by an oxygenase (referred to as pheide a oxygenase) (15). In plants, Chl b appears to enter the degradation pathway after conversion to Chlide a via chlorophyllase (leading to Chlide b) and Chlide b reductase because no final degradation products of Chl b are found (34, 35, 36). Moreover, pheide a is thought to be the sole substrate for pheide a oxygenase (37). However, in the PS-I-less/chl-L⁺/lhcb⁻/cao⁻ strain of *Synechocystis* under LAHG conditions, pheide b was found (Fig. 1), indicating that at least part of Chl b is not degraded via Chlide a, possibly because of a lack of Chl b reductase in this system. Interestingly, the Chl degradation in the PS-I-less/chl-L⁻/lhcb⁻/cao⁻ strain was faster than in the control, suggesting that the substrate specificity of pheide a oxygenase is not very high, but the accumulation of a measurable amount of pheide b and not pheide a in the PS-I-less/chl-L⁻/lhcb⁻/cao⁻ strain suggests that pheide a oxygenase does not recognize pheide b as efficiently as pheide a.

A remaining open question is the reason for the decreased chlorophyll stability in the PS-I-less/chl-L⁻/lhcb⁻/cao⁻ strain, even in darkness. One possible explanation is that the presence of Chl b in PS II complexes destabilizes these complexes to some degree and that the degrading enzymes have better access to chlorophylls (both Chl a and Chl b) than in the control. Another possibility would be a more active Chl degradation system in the PS-I-less/chl-L⁻/lhcb⁻/cao⁻ strain because of an increased abundance of the corresponding enzymes. At this moment, we cannot distinguish between these possibilities, but the former explanation seems mechanistically more attractive.

**Control of Tetrapyrrole Biosynthesis**—Tetrapyrrole derivatives including Mg²⁺-porphyrins (Chls), Fe²⁺-porphyrins (hemes), and bilins are critical components in essentially all organisms. The tetrapyrrole biosynthesis pathway is under the control of a complex regulation network, reflecting the varying needs for tetrapyrroles in response to different environmental conditions. The control can occur at several regulatory sites at different levels (38). Examples exist for regulation via end products or intermediates of the pathway or by proteins associated with these intermediates (11, 39, 40). Glutamyl-tRNA reductase (catalyzing the first committed step in the tetrapyrrole pathway) is of particular regulatory importance in photosynthetic systems. The enzyme is subject to strong feedback regulation (41) and appears to turn over rapidly (42).

PChlide is the major tetrapyrrole intermediate that accumulates in the PS-I-less/chl-L⁻ control strain, the PChlide level...
steadily increases upon growth under LAHG conditions, indicating that the biosynthesis rate exceeds the rate of degradation. However, the accumulation of PChlide in the PS I-less/chlL/hchL/lhcb+/lca0/ strain can be divided into two stages: 1) an early stage (the first several days of growth under LAHG conditions) when a much larger flux goes through the chlorophyll biosynthesis pathway in the PS I-less/chlL/hchL/lhcb+/lca0/ strain versus the control and the rate of PChlide accumulation is 2.5-fold higher than in the control and 2) a late stage (in the second week of LAHG growth) when the flux through the chlorophyll biosynthesis pathway is much slower. This finding suggests a major change in the regulation of the tetrapyrrole biosynthesis pathway, presumably mediated by the pigment-binding regulator(s) as a function of the presence of b-type pigments. During the early stage of growth under LAHG conditions, the faster Chl degradation in the PS I-less/ strain may provide a regulation mechanism for the relative activity of ferrochelatase versus magnesium-chelatase.

The protoheme level increased 2-fold in the PS I-less/chlL/hchL/lhcb+/lca0/ strain upon ALA feeding, similar to the increase of other intermediates in the magnesium branch. However, when ALA was added to the PS I-less/chlL/hchL/lhcb+/lca0/ strain grown under LAHG conditions, the protoheme level remained essentially the same, whereas the Mg-proto IX level increased 60-fold. This indicates that the regulation of the distribution of protoporphyrin IX between the two branches is altered by the presence of b-type pigments.

At least two of the SCPs have been proposed to stimulate the Chl biosynthesis pathway when they do not have pigments bound to them (e.g., after growth of chlL− strains under LAHG conditions), whereas upon Chl binding to SCPs, this stimulation may disappear (11). The PS I-less/chlL/hchL/lhcb+/lca0/ strain grown under LAHG conditions shows a phenotype consistent with the absence of stimulation by ScpB and SepE. This may suggest that in the PS I-less/chlL/hchL/lhcb+/lca0/ strain grown under LAHG conditions, an oxygenated (b-type) PChlide, Chlide, Chl, or pheide may have a higher affinity for one of the SCPs than the non-oxygenated (a-type) pigments present in the control. This may cause an altered regulation of the tetrapyrrole biosynthesis pathway in the presence of lhcb and cao. At this moment, it is unclear what may be the nature of the pigment that may be tightly bound to the SCPs. However, it is unlikely that it is Chl b itself as it has virtually disappeared after several days of growth under LAHG conditions. Instead, the pigment may be one of the degradation products (such as pheide b) or PChlide b. The exact nature of this compound and of the interaction with SCPs or other proteins will be difficult to determine because of the low levels of these compounds. In any case, the results presented here provide support for tetrapyrrole biosynthesis regulation as a function of the pigment binding site occupancy state of SCPs or other proteins of similar function.

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