Multiple Deletions of Small Cab-like Proteins in the Cyanobacterium *Synechocystis* sp. PCC 6803

**CONSEQUENCES FOR PIGMENT BIOSYNTHESIS AND ACCUMULATION**

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Deletion of the genes for four or five small Cab-like proteins (SCPs) in photosystem (PS) I-less and PS I-less/PS II-less strains of *Synechocystis* sp. PCC 6803 caused a large decrease in the chlorophyll and carotenoid content of the cells without accumulation of early intermediates in the chlorophyll biosynthesis pathway, suggesting limited chlorophyll availability. The PS II/PS I ratio increased upon deletion of multiple SCPs in a wild type background, similar to what is observed in the presence of subsaturating concentrations of gabcuelin, an inhibitor of an early step in the tetrapyrrole biosynthesis pathway. Upon deletion of multiple SCPs, neither 77 K fluorescence emission properties of phycobilisome-less thylakoids from the PS I-less/PS II-less strain nor the energy trapping efficiency of PS II were affected, indicating that under steady-state conditions SCPs do not bind much chlorophyll and do not serve as PS II antenna. Under conditions where protochlorophyllide reduction and thus chlorophyll synthesis were inhibited, chlorophyll disappeared quickly in a mutant lacking all five SCPs. This implies a role of SCPs in stabilization of chlorophyll-binding proteins and/or in release of chlorophylls. Under these conditions of inhibited reduction of protochlorophyllide, the accumulation kinetics of this intermediate were greatly altered in the absence of the five SCPs. This indicates an alteration of tetrapyrrole biosynthesis kinetics by SCPs. Based on this and other evidence, we propose that SCPs bind carotenoids and transiently bind chlorophyll, aiding in the supply of chlorophyll to nascent or reassembling photosynthetic complexes, and regulate the tetrapyrrole biosynthesis pathway as a function of the demand for chlorophyll.

In oxygenic photosynthesis, chlorophyll plays a crucial role in light harvesting as well as in transformation of absorbed light energy to chemical energy. However, the combination of chlorophyll and light is both a blessing and a curse for photosynthetic organisms. Whereas both are required for photosynthesis, chlorophyll excited by light can be converted to the triplet state, which in the presence of oxygen may lead to the formation of potentially damaging singlet oxygen (1O2) (1). The photodestructive potential of chlorophyll is greatly reduced if the chlorophyll is adjacent to carotenoids, which are efficient quenchers of both triplet chlorophyll and 1O2 (2). *In vivo* this is accomplished through pigment-binding proteins, which provide adjacency between chlorophylls and carotenoids and which allow efficient transfer of excitations eventually to the reaction center chlorophyll. In higher plants, the major chlorophyll-binding proteins are those that are part of the core complex of photosystem (PS)I and PS II as well as the light-harvesting antenna complexes, which are encoded by a multigene family of *cab* genes (3). Most of the *cab* gene family members code for chlorophyll *a/b*-binding proteins with three transmembrane helices, of which the sequences of the first and third membrane-spanning regions are similar and where chlorophyll-binding sites are highly conserved. Other members of the *cab* gene family in plants are *psbS*, the product of which is predicted to have four membrane-spanning regions (4) and affects nonphotochemical quenching (5), as well as genes for single-helix (6) and two-helix (7) members.

In contrast to plants, cyanobacteria use phycobilisomes as the major light-harvesting apparatus, and they do not contain multihelix Cab proteins (light-harvesting antenna complexes or related chlorophyll *a/b*-binding peripheral antenna proteins). However, numerous genes potentially coding for small proteins with a single membrane-spanning region similar to the first and third transmembrane regions of Cab proteins and with conserved residues involved in chlorophyll binding were found in genomes of many cyanobacteria including prochlorophytes (see Refs. 8 and 9). For example, the *Prochlorococcus marinus* MED4 genome contains as many as 23 such genes (9). The genome of the cyanobacterium *Synechocystis* sp. PCC 6803 contains genes for four small Cab-like proteins (SCPs) that have been named StpB–StpE (10). In addition, ferrochelatase in cyanobacteria and the chloroplast-targeted isozyme in plants has a ~60-residue C-terminal extension (absent in other ferrochelatases) that is similar to the SCPs. For this reason, the C-terminal extension has been named StpA and this SCP extension does not seem to be necessary for the activity of ferrochelatase in *Synechocystis* (10). Since one of the conditions under which the small *scp* genes are expressed is high light intensity, these genes also have been named *hli* (high light-inducible) by other authors (8, 11).

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| || The abbreviations used are: PS, photosystem; PChlide, protochlorophyllide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; LAHG, light-activated heterotrophic growth; SCP, small Cab-like protein; TES, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethylamino]ethanesulfonic acid; HPLC, high pressure liquid chromatography. |

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The function of SCPs is still largely unclear, although the number of existing scp genes in different species of cyanobacteria seems to be related to their ecological niches (9). In *Synechocystis* sp. PCC 6803, levels of ScpB, which are regulated under different stress conditions, and the mutant lacking four SCPs (ScpB-ScpC) was unable to survive at high light intensity (11). Therefore, it was suggested that expression of scp genes is critical in the adaptation of cyanobacteria to various light intensities (11, 12). Upon deletion of ScpC, dark-grown PS I-less/chlL cells showed a decrease in protophorphyrin (pPchlide), phycoerythrin, and hem levels (ChlL is a subunit of the light-independent Pchlide reductase, and therefore chlL mutants are unable to synthesize chlorophyll in the dark). Moreover, the chlorophyll synthesis rate in the PS I-less/chlL/scpC-strains decreased when these two mutants were transferred from darkness to light (13), implying that at least some of the SCPs may be involved in the regulation of chlorophyll biosynthesis. Considering that single-deletion SCP mutants in a wild-type background do not exhibit a strong phenotype, in the present paper, we focus on studies of mutants lacking multiple SCPs in different backgrounds (PS I-less, PS II-less, and/or chlL-less). The results of these studies support the idea of SCP involvement in regulation of various steps of tetrapyrrole biosynthesis coupled to transient chlorophyll binding by SCPs and provide evidence that SCPs may aid in the supply of chlorophyll to nascent or reassembling photosynthetic units.

**EXPERIMENTAL PROCEDURES**

**Growth Conditions**—*Synechocystis* sp. PCC 6803 wild-type and mutant strains were grown at 30 °C in BG-11 medium (14), buffered with N-tris(hydroxymethyl)-2-aminoethanesulfonic acid (TES)-NaOH (pH 8.0). For photomixotrophic and photoheterotrophic growth, the medium was supplemented with 5 mM glucose. Unless indicated otherwise, strains containing PS I were grown with continuous illumination at an intensity of 50 µmol of photons m⁻² s⁻¹, whereas those in a PS I-less background were grown at a lower light intensity (2–3 µmol of photons m⁻² s⁻¹). When strains were grown in liquid culture under LAHG conditions (15), cells were kept in complete darkness except for one 15-min light period (white light at 20 µmol of 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. No antibiotics were added to the liquid growth media to avoid any influence posed by these compounds. Growth was monitored by measuring the optical density of the cells at 770 nm using a Shimadzu UV-160 spectrophotometer.

**DNA Manipulation**—To introduce a scpB deletion, a 261-bp NcoI to KpnI fragment (covering the entire *Synechocystis* sp. PCC 6803 scpB coding region and 46 bp downstream of the stop codon) was deleted from the plasmid containing the scpB gene with 440 and 820 bp upstream and downstream, respectively, of this gene (13) and was replaced by a zeocin resistance cassette (16). Plasmids containing *Synechocystis* sp. PCC 6803 scpC, scpD, and scpE genes interrupted by erythromycin resistance/ sacB, spectinomycin, and erythromycin resistance cassettes, respectively, have been described in Ref. 13. The sacB gene coding for levulinic acid and leading to sucrose sensitivity (17) was introduced together with the erythromycin resistance cassette to replace part of scpC, so that the cassette could be removed and reused for scpE deletion. To be able to recycle and reuse the erythromycin resistance cassette, a markerless scpC deletion plasmid was also made. A 153-bp PhaAI (34 bp downstream of the start codon) to BsaHI (26 bp upstream of the stop codon) fragment was deleted from the plasmid containing scpC (13). The construction of the scpA interruption plasmid (with a kanamycin resistance cartridge inserted at the border of the ferrochelatase domain and the scp extension of ScpA to obtain a truncated ferrochelatase without the Cab domain), the psbB deletion plasmid (with streptomycin resistance) and the psaAB deletion plasmid (with chloramphenicol resistance) have been described by Funk and Vermaas (10), Vermaas et al., and Boussiba and Vermaas (19), respectively. To delete chlL, an 810-bp Accl-Nhel fragment (from 85 bp downstream of the start codon of the chlL gene to 33 bp upstream of its stop codon) was deleted from the pFQ2 plasmid (20) and replaced by a gentamycin resistance cassette.

The *Synechocystis* sp. PCC 6803 pshB gene along with the 3’ end of *psbD* was amplified by PCR using primers with the sequence 5’-CCCAACGGAGAAGGACCTATCAGCAGTGG-3’ (referred to as 5’ pshB) and 5’-AGCAGAAATCTTGACAGAAAGG-3’ (named 3’ *pshB*). The sequence of these primers corresponds to nucleotides 1349186–1349160 and 1347440–1347466 in CyanoBase (available on the World Wide Web at www.kazusa.or.jp/cyano/cyano.html), respectively. Nucleotides in lowercase represent changes made to the genome sequence to introduce a synthetic unit.

Transformation of *Synechocystis* sp. PCC 6803—Transformation of *Synechocystis* sp. PCC 6803 with the plasmids described above was carried out according to Vermaas et al. (23). Transforms were selected by screening for resistance to appropriate antibiotics and then tested at increasing concentrations of antibiotics to create a deletion-carrying strain. The plasmid pUC19 was then cloned in pUC19 using the BamHI and PstI restriction sites in the primers. A 582-bp Accl-BsaIII fragment was deleted and replaced with a 1.9-kb gentamycin resistance marker (21). This fragment starts in *psbD* (67 bp upstream of the *psbD* coding region, which starts at a GGT (22) and 82 bp 5’ of the *psbD* stop codon) and includes the 5’ 1515 bp of the 1380-bp *psbD* coding region. Upon introduction of this deletion into the *Synechocystis* sp. PCC 6803 genome, both the 3’ end of *psbD* and the 5’ end of *psbC* are deleted. However, since a second *psbD* gene, *psbDII*, remains present in the cyanobacterium, the phenotype associated with a lack of this gene region is caused by the lack of *psbC*.

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**Thylakoid Membrane Preparation**—Thylakoid membrane preparations were carried out as described before (26). For 77 K fluorescence measurements, isolated thylakoids were suspended in pyrophosphate buffer (50 mM Mes-NaOH (pH 6.5) and 20 mM sodium pyrophosphate), followed by centrifugation, and finally resuspended in thylakoid buffer (20 mM Mes-NaOH (pH 6.5), 5 mM MgCl₂, 5 mM CaCl₂, and 20% glycerol (v/v)). This additional wash step helped to remove most phycobiliprotein contamination in the thylakoid suspensions and subtracting concentration of chlorophyll for about 1 day to OD₅₇₀ ~0.4 and were then used for measurements.

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**Fluorescence Spectroscopy**—Fluorescence emission spectra were determined using a Fluorolog 2 instrument (Spex Industries, Metuchen, NJ). Measurements were carried out at 77 K with excitation and emission slit widths of 1 and 0.25 mm, respectively, which correspond to bandwidths of 4 and 1 nm. Fluorescence induction using intact cells was measured in the presence of 5 µM DCMU using the same instrument equipped with a manually triggered UNIBLITZ-28L AOTF electronic shutter (3-ms opening time). The emission wavelength was set at 680 nm (the bandwidth at half-maximum was 8 nm), and the excitation wavelength was 435 nm. Cell densities from different strains were adjusted to a concentration of 0.2 µg of chlorophyll/ml.

**Gaculin Treatment**—Wild-type cells were grown in continuous light to early exponential growth phase (OD₅₇₀ ~0.2), and then 5 µM gaculin, an inhibitor of the tetrapyrrole biosynthesis pathway at the level of glutamate-1-semialdehyde aminotransferase, was added to the cultures. Cells were allowed to grow in the presence of this substratating concentration of gaculin for about 1 day to OD₅₇₀ ~0.4 and were then used for measurements.
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RESULTS

Construction of Multiple scp Deletion Mutants—The scpC interruption plasmid carrying an erythromycin resistance cassette and the sacB gene was introduced into wild type Synechocystis sp. PCC 6803, and homozygous transformants lacking intact scpC were obtained. This strain was then transformed with the markerless scpC deletion plasmid to remove the erythromycin resistance-sacB cassette, and sucrose-resistant colonies lacking SacB activity were selected. Transformants were confirmed to lack the erythromycin resistance marker and sacB and to contain the scpC deletion. The scpC– strain thus created was then transformed sequentially with scpA, scpD, and scpB and/or scpE interruption/deletion plasmids, leading to the scpABCDE–, scpACDE–, and scpABCD– strains. Complete segregation at the scp loci was verified by PCR. Fig. 1 illustrates the result for the scpABCDE– strain in comparison with the wild type.

To generate the PS II-less strains lacking four or five scp genes, the scpAC– mutant was used for transformation with deletion plasmids for psbB and psbC, which encode the two major chlorophyll-binding proteins in PS II, CP47 and CP43. This transformation order was chosen because the spectinomycin resistance marker conveys streptomycin resistance as well (27). Therefore, the streptomycin resistance marker was introduced, and homozygous segregants (psbB–) were obtained before constructs with the spectinomycin resistance marker were introduced into this strain. The resulting PS II-less/psbA–mutant was then transformed sequentially with scpD and scpB and/or scpE interruption/deletion plasmids, leading to the PS II-less/psbABC–, PS II-less/psbACDE–, and PS II-less/psbABCD– strains. The psaAB cluster coding for the PSI reaction center proteins was deleted in SCP mutants or in PS II-less/SCP mutant strains to add the PS I-less phenotype. The scp deletion mutants generated in this study and their markers are listed in Table I.

All multiple SCP deletion mutants in various backgrounds except for the PS I-less/psbABCDE– strain (which will be described later) grew equally well as their corresponding control strains in liquid medium without supplementation with antibiotics under continuous light conditions (data not shown).

TABLE I

Synechocystis sp. PCC 6803 mutants lacking multiple scp genes and the markers used for interruption or deletion

| Strains | Background | SCP inactivations | Markers |
|---------|------------|-------------------|---------|
| Wild type | scpABCD | Km, Zeo, Sp |
| PS II-less | scpABC | Str, Km, Zeo, Sp |
| PS I-less | scpAD | Cm, Km, Zeo, Sp |
| PS I-less/psbA | scpB | Km, Zeo, Sp |
| PS I-less/psbA | scpC | Sp, Zeo, Sp |
| PS I-less/psbA | scpD | Em, Erm |

Pigment Composition—To determine the effect of deletion of SCPs in the wild type and the PS I-less, PS II-less, and PS I-less/PS II-less strains in terms of their pigment content, cells were extracted with basic methanol, and the extracts were subjected to HPLC analysis. As shown in Table II, the chlorophyll content of the wild type and PS II-less strains showed a small decrease (5–20%) upon deletion of four SCPs. Upon deletion of five SCPs in these two background strains, the chlorophyll decrease was larger (25–30%). Interestingly, the total carotenoid level in the multiple scp deletion mutants showed a parallel 30–40% decrease as compared with wild type (Table II). Among the four major carotenoids present in wild type Synechocystis, zeaxanthin and echinenone levels remained more constant upon deletion of scp genes than the levels of myxoxanthophyll and β-carotene, which in the scpABCDE–
Cells were grown in continuous light to OD$_{730}$ ~0.4–0.6. Values given are the averages from three different determinations. ND, not determined.

| Strains         | Chlorophyll (µg/ml)/OD$_{730}$ | Carotenoids (µg/ml)/OD$_{730}$ | Chlorophyllide (ng/ml)/OD$_{730}$ |
|-----------------|---------------------------------|---------------------------------|-----------------------------------|
| Wild type       | 4.05 ± 0.04                     | 2.17 ± 0.03                     | ND                                |
| scpABCD$^-$     | 3.87 ± 0.03                     | 1.62 ± 0.04                     | ND                                |
| scpACDE$^-$     | 3.75 ± 0.04                     | 1.59 ± 0.05                     | ND                                |
| scpABCDE$^-$    | 3.06 ± 0.05                     | 1.41 ± 0.03                     | ND                                |
| PS II-less      | 3.56 ± 0.07                     | 1.85 ± 0.05                     | ND                                |
| scpABCD$^-$     | 2.94 ± 0.04                     | 1.72 ± 0.04                     | ND                                |
| scpACDE$^-$     | 3.13 ± 0.02                     | 1.68 ± 0.04                     | ND                                |
| scpABCDE$^-$    | 2.58 ± 0.05                     | 1.48 ± 0.03                     | 0.1 ± 0.1                         |
| PS I-less       | 0.81 ± 0.02                     | 0.75 ± 0.06                     | 1.2 ± 0.1                         |
| scpABCD$^-$     | 0.62 ± 0.02                     | 0.67 ± 0.03                     | 2.6 ± 0.2                         |
| scpACDE$^-$     | 0.58 ± 0.02                     | 0.58 ± 0.02                     | 3.2 ± 0.3                         |
| scpABCDE$^-$    | 0.28 ± 0.02                     | 0.43 ± 0.02                     | 3.5 ± 0.3                         |
| PS I-less/PS II-less | 0.18 ± 0.02                  | 0.75 ± 0.01                     | 1.8 ± 0.2                         |

Table II

Carotenoid levels in Synechocystis sp. PCC 6803 strains

Cells were grown in continuous light to an OD$_{730}$ of ~0.4. Pigments were extracted from cells with methanol, and methanol extracts were analyzed by HPLC. The myxoxanthophyll, zeaxanthin, echinenone, and β-carotene levels in wild type were 0.60 ± 0.04, 0.52 ± 0.02, 0.33 ± 0.02, and 0.71 ± 0.03 (µg/ml)/OD$_{730}$, respectively. Values are averages from three determinations.

| Strains          | Chlorophyllide (ng/ml)/OD$_{730}$ |
|------------------|-----------------------------------|
| Wild type        | 0.001 ± 2.0                       |
| scpABCD$^-$      | 0.02 ± 1.0                        |
| scpACDE$^-$      | 0.1 ± 0.1                         |
| scpABCDE$^-$     | ND                                |
| PS I-less/PS II-less | 0.0 ± 0.1                       |

Table III

Carotenoid content (percentage of wild type)

To determine whether the changes in chlorophyll content led to accumulation of chlorophyll biosynthesis precursors, methanol extracts from PS I-less and PS I-less/PS II-less strains lacking four or five SCPs and grown in continuous light were subjected to HPLC analysis using an elution gradient that can resolve hydrophilic compounds, including chlorophyllide, PChlide, and Mg-protoporphyrin IX. As shown in Table II, in strains carrying multiple scp deletions, chlorophyllide accumulated to 2–3-fold higher levels than in the corresponding parental strains. However, no accumulation of PChlide or Mg-protoporphyrin IX was detected within our detection limits (1 nM/OD$_{730}$ for PChlide and 0.02 nM/OD$_{730}$ for Mg-protoporphyrin IX). Interestingly, the growth rate of the PS I-less/scpABCD$^-$ strain in continuous light was about 2 times slower than that of the PS I-less strain (doubling times of 52 ± 3 and 25 ± 2 h, respectively), suggesting that the rate of synthesis of tetrapyrroles (such as heme) is limiting or that as yet unidentified compounds early in the tetrapyrrole pathway may accumulate.

Fluorescence Induction—The observation that chlorophyll levels dropped upon deletion of scp genes in all background strains in a way similar to what was observed upon the deletion of PS I and/or PS II that bind the majority of chlorophyll in the wild type Synechocystis suggests that SCPs may bind chlorophyll as well. To investigate whether the decrease in chlorophyll content upon deletion of multiple scp genes affects the antenna size of PS II, fluorescence induction curves of the PS I-less strain and derivatives lacking four or all SCPs were
measured upon 435-nm excitation, a wavelength of light that is absorbed mostly by chlorophyll. Although the kinetics of variable fluorescence appeared to be 2–9% slower in strains with SCP deletions as compared with the PS I-less control (Fig. 2), these changes were small, and therefore antenna properties of PS II have not been altered significantly in the SCP-less mutants. However, the absolute values of initial ($F_{0}$) and/or variable ($F_{v}$) fluorescence measured on a per chlorophyll basis changed in several of the strains (see legend to Fig. 2), which may reflect varying levels of phycobilisomes enzymatically disconnected from PS II and contributing to the room temperature fluorescence around 680 nm, the presence of inactive PS II centers, and accumulation of chlorophyllide in the absence of SCPs.

Low Temperature Fluorescence Emission—In order to determine the effects of deletion of most or all SCPs on low temperature fluorescence characteristics of chlorophyll, 77 K fluorescence emission spectra of whole cells from wild type and scpABCDe−, scpACDe−, and scpABCDe− strains were measured upon excitation at 435 nm (Fig. 3A). The wild type showed a major peak at 725 nm, which is characteristic for PS I-associated chlorophyll, and two smaller peaks at 685 and 695 nm, which correspond to PS II-associated chlorophyll. Part of the emission around 685 nm comes from phycobilisome components such as allophycocyanin B. Particularly upon deletion of all five SCPs but also upon deletion of ScpACDe or of ScaACDe, the relative fluorescence emission at 685 and 695 nm increased, indicating an increase in the PS II/PS I ratio, in line with previous observations (12). Similar changes in the PS II/PS I ratio were observed if wild type was grown in the presence of a subsaturating concentration of gabaculin (Fig. 3B), which inhibits chlorophyll biosynthesis at the step before δ-aminolevulinic acid formation. Under these conditions, chlorophyll on a per cell basis decreased 30% compared with the control (data not shown), similar to what is seen upon deletion of the scp genes (Table II).

77 K fluorescence emission spectra upon excitation of chlorophyll at 435 nm were also measured using whole cells of the PS I-less and of the PS I-less/PS II-less strains with or without scp deletions. Since particularly in the SCP-less mutants the chlorophyll content was reduced significantly without a concomitant decrease in the amount of phycobilins, the 77 K fluorescence emission from these cells displayed a strong phycobilisome component emitting around 685 nm (Fig. 4A). In fact, the 685-nm peak was the only fluorescence peak observed in PS I/PS II-less cells (data not shown). To determine which fluorescence components may be potentially associated with chlorophyll, emission spectra were determined using isolated thylakoids that were substantially depleted of phycobilisomes during isolation. Fig. 4B compares 77 K fluorescence emission spectra of thylakoid membranes from PS I-less and PS I-less/scpABCDe− cells. The traces are fairly similar, and differences are due to a somewhat higher contamination of the PS I-less/scpABCDe− thylakoids by phycobilisomes, as determined by experiments with excitation at 625 nm (data not shown). In contrast, fluorescence emission spectra from thylakoids isolated from the SCP-containing and SCP-less strains in a PS I-less/PS II-less background were qualitatively different (Fig. 4C). Upon 435-nm excitation, the emission maximum in the PS I-less/PS II-less/scpABCDe− mutant peaked at about 678 nm, whereas in the corresponding PS I-less/PS II-less control strain, the maximum was detected at 690 nm with a shoulder around 683 nm. Excitation of phycobilisomes, a small quantity of which remained in both preparations, caused a shift in the emission maximum from 678 to 680 nm and from 690 to 683 nm in the PS I-less/PS II-less/scpABCDe− and PS I-less/PS II-less mutants, respectively, indicating that the fluorescence around 678 and 690 nm is indeed associated with chlorophylls contained in the thylakoid membranes of the two strains.

PChlide Accumulation and Chlorophyll Degradation—In cyanobacteria, the ChlBLN complex catalyzes light-independent conversion of PChlide to chlorophyllide, an immediate precursor of chlorophyll. When chlL− cells are grown in darkness or under LAHG conditions, PChlide accumulates as PChl reduction is inhibited (20), and existing chlorophyll is diluted out by growth of the culture that is unable to synthesize chlorophyll. In order to investigate whether the deletion of five SCPs affected the accumulation of PChlide and the presence and persistence of chlorophyll during LAHG growth, cultures of PS I-less/chlL− and PS I-less/chlL−/scpABCDe− strains were transferred to LAHG conditions, and samples from these cul-
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Fig. 4. 77 K fluorescence emission spectra of Synechocystis sp. PCC 6803 cells and isolated thylakoids. A, spectra were recorded for intact cells of the PS I-less (solid line) and PS I-less/scpABCDE− (dashed line) strains upon excitation at 435 nm. B, spectra were recorded for thylakoids isolated from the PS I-less (solid line) and PS I-less/scpABCDE− (dashed line) strains. Excitation was at 435 nm. C, spectra were recorded for thylakoids isolated from the PS I-less/PS II-less (solid line and filled circles) and PS I/PS II-less/scpABCDE− (dashed line and open squares) strains. Excitation was at 435 (solid line and dashed line) or at 625 nm (filled circles and open squares). All spectra were normalized to the maximum fluorescence emitted in the 670–700-nm wavelength range; in C, the fluorescence intensity emitted upon excitation at 625 nm was reduced 1.5 times for better visualization of the data. a.u., arbitrary units.

Fig. 5. PChlide accumulation and chlorophyll levels in cells after transfer to LAHG conditions. A, chlorophyll levels monitored in the PS I-less/chIL− (○) and the PS I-less/chIL−/scpABCDE− (○) strains upon transfer to LAHG conditions at time 0. B, PChlide amounts measured in the PS I-less/chIL− (○) and the PS I-less/chIL−/scpABCDE− (○) strains upon transfer to LAHG conditions at time 0. Cells were diluted to OD730~0.3 with fresh medium every day to maintain logarithmic growth. Data shown are the average of three experiments.

conditions for at least 2 weeks. However, the accumulation of PChlide in the PS I-less/chIL−/scpABCDE− strain followed a much different pattern; the PChlide level increased rapidly within the first 3 days after transfer to LAHG conditions and decreased gradually after day 4. Interestingly, the maximum PChlide level in the PS I-less/chIL−/scpABCDE− strain grown under LAHG conditions for 3 days was about the same as that in the control strain after LAHG growth for 2 weeks (Fig. 5B). At the end of the 2-week period of growth under LAHG conditions, the PChlide level in the PS I-less/chIL−/scpABCDE− strain was about one-eighth of that in the PS I-less/chIL− strain. Together, the results demonstrate significant changes in regulation of the tetrapyrrole biosynthesis pathway in the absence of SCPs and possibly a destabilization of chlorophyll-binding proteins and/or impairment in reuse of chlorophyll.

Kinetics of Pigment Synthesis—Both phycobilins (data not shown, but see Ref. 19 for data on scpB− and scpE− mutants) and chlorophyll (Fig. 5) disappeared in the PS I-less/chIL−/scpABCDE− mutant upon growth under LAHG conditions. To examine whether the biosynthesis rate of these pigments was affected by deletion of scp genes, the chlorophyll and phycobilin content was determined in PS I-less/chIL− and PS I-less/chIL−/scpABCDE− strains that had been grown under LAHG conditions for 2 weeks and then were continuously illuminated at 2–3 μmol of photons m−2 s−1. As shown in Fig. 6, deletion of the five SCPs caused a large decrease in both the chlorophyll and phycobilin biosynthesis rate during the early stages of greening; the time for this mutant strain to reach half of the eventual chlorophyll content is ~50 h versus less than 10 h in the control strain. In Xu et al. (13), a somewhat slower chlorophyll biosynthesis rate upon deletion of either scpB or scpE already had been shown, whereas deletion of either scpC or scpD, coding for very similar peptides, had little effect. However, upon deletion of both scpC and scpD, the time to reach half of the eventual chlorophyll content is about 25 h (Fig. 6A). Combined with the results obtained with the PS I-less/chIL−/scpABCDE− mutant, these data indicate that a decrease in the number of active SCPs causes a progressive decrease in the overall rate of tetrapyrrole biosynthesis.
progressive decrease in chlorophyll content with the increase in some degree. Therefore, in the current study, multiple (four or natively, SCPs may be involved in regulation of chlorophyll deletion of SCPs in various backgrounds may suggest that II (29) (see also Table II), the large decrease in chlorophyll upon deletion of chlorophyll-binding proteins in PS I and/or PS II (31), as expected for chlorophyll-binding proteins. Wild type Synechocystis cells grown under control conditions have very low levels of SCPs (11). However, in a PS I-less mutant, a significant increase in scpB, scpC, scpD, and scpE transcripts has been detected, and the transcript data has been correlated with the results of immunodetection of ScpC and ScpD (10). Quantification of the PS II amount in PS I-less Synechocystis cells based on 14C-DMCu binding studies suggests that PS I-less cells contain 75–110 chlorophyll molecules per PS II (28, 32), whereas determination of the chlorophyll/phoeythin ratio suggests that the number of chlorophylls per PS II (per 2 pheophytins) can be as low as 37 in this mutant (33). Considering that purified PS II core particles from cyanobacteria contain 37–41 chlorophylls (33–35), anywhere between 0 and 60% of the total chlorophyll in PS I-less cells might be bound to SCPs, depending on which determination is more reliable.

We favor the interpretation of little chlorophyll to be associated with SCPs for the following reasons. (i) Normalized 77 K fluorescence emission spectra of thylakoids isolated from the PS I-less and PS I-less/scpABCDE− strains were very similar (Fig. 4B), despite a significant difference in the chlorophyll content of the two mutants. (ii) Upon SCP removal, fluorescence induction of DCMU-treated PS II centers remains essentially unchanged in PS I-less strains (Fig. 3), indicating that antenna properties have not been altered. These considerations argue strongly against binding of a significant amount of chlorophyll to SCP proteins in the PS I-less Synechocystis strain. Consequently, the decrease in chlorophyll amount on a per cell basis upon SCP deletion in the PS I-less strain is interpreted as a decrease in the number of PS II centers per cell.

Chlorophyll Synthesis Is Misregulated in the SCP-less Mutants—The presented results clearly show that SCPs have a major positive influence on chlorophyll accumulation, particularly in the PS I-less and PS I-less/PS II-less strains that have a very low chlorophyll level (Table II). However, even in the wild type, the chlorophyll level decreased upon SCP deletion, leading to a decrease in the PS I/PS II ratio in the scpABCDE− strain as determined from the steady-state fluorescence emission spectrum at 77 K (Fig. 3A). A similar change in the PS I/PS II ratio was observed when chlorophyll synthesis was inhibited by gabaculin (Fig. 3B). Taking into account that SCP proteins are unlikely to bind a considerable amount of chlorophyll under steady-state growth conditions, the observed decrease in the chlorophyll content in Synechocystis cells lacking SCPs most likely is a consequence of changes in chlorophyll synthesis and/or degradation kinetics.

The recovery of the chlorophyll content in Synechocystis cells upon transfer of an LAHG-grown PS I-less/chlL− culture to continuous light was much slower in the absence of the SCPs (Fig. 6), indicating a lower rate of chlorophyll synthesis in the SCP-less strain, at least after the dark-to-light transition. The initial lack of PChlide characteristic of the PS I-less/chlL−/scpABCDE− mutant grown under the LAHG conditions for several days (Fig. 5B) is not the sole reason for slow chlorophyll biosynthesis in this strain; phycobilin synthesis is equally slow.

Fig. 6. Light-dependent chlorophyll and phycobilin biosynthesis. A, chlorophyll a biosynthesis upon illumination (light intensity, 2 μmol of photons m−2 s−1) was monitored in the PS I-less/chlL− (●), PS I-less/chlL−/scpCD− (△), and PS I-less/chlL−/scpABCDE− (○) strains that had been propagated under LAHG conditions for 2 weeks. B, phycobilin biosynthesis upon illumination (light intensity, 2 μmol photons m−2 s−1) was monitored in the PS I-less/chlL− (●) and PS I-less/chlL−/scpABCDE− (○) strains that had been propagated under LAHG conditions for 2 weeks. Cultures were transferred to continuous light at time 0. The inset in B shows how ΔODscp taken as a measure of the relative phycobilin amount, was determined. Data shown are the average of three experiments. The eventual levels of chlorophyll and phycobilin in the PS I-less/chlL−/scpABCDE− strain were 0.24 ± 0.03 and 0.08 ± 0.02 (μg/ml)/OD730 respectively.

DISCUSSION

In a previous paper devoted to the function of SCPs in Synechocystis, we hypothesized that SCPs may be involved in regulation of the tetrapyrrole biosynthesis pathway depending on the occupancy of their pigment binding sites (13). This interpretation was based on the observations that PChlide and phycobilin levels decreased in the single ScpB or ScpE deletion mutants grown under LAHG conditions, and recovery of chlorophyll and phycobilin levels was slowed down in the deletion mutants compared with the control strain after the cultures were transferred to continuous light. Similarly slow pigment synthesis upon transfer of LAHG-grown PS I-less/chlL− cells to continuous light was found in the PS I-less/chlL− mutant that lacked both scpC and scpD (Fig. 6A) but not when either scpC or scpD was missing, suggesting that these two proteins, which share about 87% identity in amino acid sequence, have a complementary regulatory function in chlorophyll synthesis. Considering the high similarity between the primary sequences of all five SCPs in Synechocystis, it is probable that these proteins may functionally compensate for each other to some degree. Therefore, in the current study, multiple (four or all five) SCPs were deleted in different background strains.

One of the most striking observations of this study is a progressive decrease in chlorophyll content with the increase in the number of deleted SCPs in Synechocystis (Table II). As a similar reduction in chlorophyll content in cells was observed upon deletion of chlorophyll-binding proteins in PS I and/or PS II (29) (see also Table II), the large decrease in chlorophyll upon deletion of SCPs in various backgrounds may suggest that these proteins bind a substantial amount of chlorophyll. Alternatively, SCPs may be involved in regulation of chlorophyll synthesis and/or degradation, and the virtual disappearance of chlorophyll in the PS I-less/PS II-less strain could be a consequence of an inhibition of chlorophyll biosynthesis and/or acceleration of chlorophyll degradation rather than the disappearance of chlorophyll-binding sites. These possibilities will be discussed below.

SCP Have Limited Capacity to Bind Chlorophyll in PS I-less Cells—Considering the sequence similarity of SCPs to the first and third transmembrane helices of Cab proteins with pigment-binding residues strictly conserved (10), SCP proteins are good candidates to bind chlorophyll. In fact, ScpC and ScpD were found to be localized in the thylakoid membrane of Synechocystis cells (31), as expected for chlorophyll-binding proteins. Wild type Synechocystis cells grown under control conditions have very low levels of SCPs (11). However, in a PS I-less mutant, a significant increase in scpB, scpC, scpD, and scpE transcripts has been detected, and the transcript data has been correlated with the results of immunodetection of ScpC and ScpD (10). Quantification of the PS II amount in PS I-less Synechocystis cells based on 14C-DMCu binding studies suggests that PS I-less cells contain 75–110 chlorophyll molecules per PS II (28, 32), whereas determination of the chlorophyll/phoeythin ratio suggests that the number of chlorophylls per PS II (per 2 pheophytins) can be as low as 37 in this mutant (33). Considering that purified PS II core particles from cyanobacteria contain 37–41 chlorophylls (33–35), anywhere between 0 and 60% of the total chlorophyll in PS I-less cells might be bound to SCPs, depending on which determination is more reliable.

We favor the interpretation of little chlorophyll to be associated with SCPs for the following reasons. (i) Normalized 77 K fluorescence emission spectra of thylakoids isolated from the PS I-less and PS I-less/scpABCDE− strains were very similar (Fig. 4B), despite a significant difference in the chlorophyll content of the two mutants. (ii) Upon SCP removal, fluorescence induction of DCMU-treated PS II centers remains essentially unchanged in PS I-less strains (Fig. 3), indicating that antenna properties have not been altered. These considerations argue strongly against binding of a significant amount of chlorophyll to SCP proteins in the PS I-less Synechocystis strain. Consequently, the decrease in chlorophyll amount on a per cell basis upon SCP deletion in the PS I-less strain is interpreted as a decrease in the number of PS II centers per cell.

Chlorophyll Synthesis Is Misregulated in the SCP-less Mutants—The presented results clearly show that SCPs have a major positive influence on chlorophyll accumulation, particularly in the PS I-less and PS I-less/PS II-less strains that have a very low chlorophyll level (Table II). However, even in the wild type, the chlorophyll level decreased upon SCP deletion, leading to a decrease in the PS I/PS II ratio in the scpABCDE− strain as determined from the steady-state fluorescence emission spectrum at 77 K (Fig. 3A). A similar change in the PS I/PS II ratio was observed when chlorophyll synthesis was inhibited by gabaculin (Fig. 3B). Taking into account that SCP proteins are unlikely to bind a considerable amount of chlorophyll under steady-state growth conditions, the observed decrease in the chlorophyll content in Synechocystis cells lacking SCPs most likely is a consequence of changes in chlorophyll synthesis and/or degradation kinetics.

The recovery of the chlorophyll content in Synechocystis cells upon transfer of an LAHG-grown PS I-less/chlL− culture to continuous light was much slower in the absence of the SCPs (Fig. 6), indicating a lower rate of chlorophyll synthesis in the SCP-less strain, at least after the dark-to-light transition. The initial lack of PChlide characteristic of the PS I-less/chlL−/scpABCDE− mutant grown under the LAHG conditions for several days (Fig. 5B) is not the sole reason for slow chlorophyll biosynthesis in this strain; phycobilin synthesis is equally slow.

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suggesting a decreased flux through a large portion of the tetrapyrole biosynthesis pathway. However, the results regarding PChlide accumulation upon transfer of the PS I-less/chlL/scpABCDE" strain to LAHG conditions indicate that an inhibition of tetrapyrole biosynthesis upon SCP deletion is a major oversimplification; the flux through the chlorophyll biosynthesis pathway in the PS I-less/chlL/scpABCDE" strain appears to be very rapid (almost 10-fold faster than in the control) during the first several days of growth under LAHG conditions and then slows down very much after longer LAHG incubation (Fig. 5B). In contrast, in the PS I-less/chlL" strain, the PChlide level continues to increase upon growth under LAHG conditions, indicating a constant flux through the chlorophyll biosynthesis pathway. Interestingly, the pattern of chlorophyll degradation and PChlide accumulation seen in the PS I-less/chlL"/scpABCDE" strain is similar to that observed in a Synechocystis mutant that synthesized significant amounts of chlorophyll b instead of chlorophyll a due to introduction of the chlorophyll a oxygenase (cao) gene from a higher plant (36). It was hypothesized that in this cao-containing mutant, regulation of the tetrapyrole biosynthesis pathway was altered by the presence of a variety of b-type pigments.

In a previous study using single-deletion SCP mutants, we have shown that the addition of δ-aminolevulinic acid in darkness drastically increased the level of Mg-protoporphyrin IX and Mg-protoporphyrin IX monomethyl ester in the PS I-less/chlL"/scpE" strain, whereas PChlide accumulated in the PS I-less/chlL/"scpB" strain (13). In the control PS I-less/chlL" strain, δ-aminolevulinic acid supplementation did not lead to large changes in the levels of tetrapyrole intermediates. Together with the observed accumulation of chlorophyllide in different SCP-less mutants (Table II), these data seem to be indicative of a major, rather complex misregulation of the tetrapyrole biosynthesis pathway when SCPs are absent. Since many intermediates in the chlorophyll biosynthesis pathway are phototoxic, inadequate regulation of chlorophyll synthesis and degradation represents a risk to photosynthetic cells, especially at high light intensity. Indeed, strains lacking scpC or scpB/ scpE were unable to compete with wild-type cells during co-cultivation at high light intensity, whereas the mutant lacking four scp genes (scpBCDE") died at such an intensity (11).

Involvement of SCPs in Chlorophyll Stabilization—A large difference in chlorophyll stability was found upon deletion of five scp genes: in the PS I-less/chlL" strain grown in darkness, the chlorophyll content on a per-cell basis decreased with a half-time of about 2 days (primarily because of dilution due to cell division, indicating that chlorophyll is stable for many days), whereas in the PS I-less/chlL"/scpABCDE" strain, the chlorophyll content disappeared much faster. Moreover, the amount of chlorophyll remaining in the culture was significant (5–10% of the amount present in continuous light) for the dark-grown PS I-less/chlL"/scpABCDE" strain but was negligible after deletion of the scp genes. The reason for the decreased chlorophyll stability and chlorophyll level upon growth under LAHG conditions may be a destabilization of chlorophyll-binding protein complexes upon deletion of SCPs and/or a decrease in the efficiency of recycling chlorophyll that is freed up upon degradation of protein complexes and is moved to newly synthesized ones. Considering that the most significant effects of SCP deletion on chlorophyll content were observed in strains lacking PS I, SCP proteins may be particularly important for stabilization or reassembly of PS II pigment-protein complexes.

Upon deletion of all five SCPs in the PS I/PS II-less mutant, a significant shift in the 77 K chlorophyll fluorescence maximum from 690 to ~678 nm was observed. In the present study, inactivation of PS II was accomplished by deletion of psbB and psbC encoding CP47 and CP43, respectively. Although the stability of other PS II subunits may be reduced greatly by these deletions (29), trace amounts of D1 and D2 proteins may still remain in thylakoids, potentially contributing to the fluorescence emission around 680 nm. If so, SCP proteins may have a role in temporary stabilization of the D1/D2 aggregate during assembly and/or disassembly of PS II.

Inhibition of Carotenoid Biosynthesis in SCP-less Mutants—Upon deletion of all five SCPs, the carotenoid level decreased by 0.3–0.7 (μg/ml)/OD600 in all backgrounds (Table II). The content of all four major carotenoids had been affected (Table III), with myoxanthophyll and β-carotene showing the largest decrease. The simplest explanation of these data is that SCPs bind carotenoids and that upon SCP deletion less of these carotenoids are bound in cells. The molecular stoichiometry between carotenoids and chlorophyll in the amount lost upon deletion of SCPs is 0.6–1.2 in the wild type, PS II-less, and PS I-less strains, in line with the hypothesis that SCPs may be able to bind both chlorophyll and carotenoids. A similar observation of a concomitant loss of both types of pigments was reported when chlorophyll biosynthesis in plants was reduced by means of antisense RNA (37). In any case, the apparent ability of SCPs to bind carotenoids is consistent with the well-established ability of C4b-light-harvesting antenna complex proteins to bind a variety of carotenoids (38).

In conclusion, we propose that SCPs are involved in the regulation of chlorophyll biosynthesis at different stages and also increase the lifetime of chlorophyll under in vivo conditions by either stabilizing chlorophyll-protein complexes or stabilizing chlorophyll until it can be reused. These functions probably are related to each other, and the chlorophyll occupancy state of SCPs may be a signal in regulation of tetrapyrole biosynthesis.

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