Small Cab-like Proteins Retard Degradation of Photosystem II-associated Chlorophyll in Synechocystis sp. PCC 6803

KINETIC ANALYSIS OF PIGMENT LABELING WITH $^{15}$N AND $^{13}$C

Received for publication, August 24, 2007, and in revised form, October 30, 2007

Published, JBC Papers in Press, October 30, 2007

DOI 10.1074/jbc.M707133200

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Isotope (Na$^{15}$NO$_3$, (15NH$_4$)SO$_4$ or [13C]glucose) labeling was used to analyze chlorophyll synthesis and degradation rates in a set of Synechocystis mutants that lacked single or multiple small Cab-like proteins (SCPs), as well as photosystem I or II. When all five small Cab-like proteins were inactivated in the wild-type background, chlorophyll stability was not affected unless the scpABCDE$^{-}$ strain was grown at a moderately high light intensity of 100–300 μmol photons m$^{-2}$ s$^{-1}$. However, the half-life time of chlorophyll was 5-fold shorter in the photosystem I-less/ scpABCDE$^{-}$ strain than in the photosystem I-less strain even when grown at low light intensity (~3 μmol photons m$^{-2}$ s$^{-1}$) (32 ± 5 and 161 ± 25 h, respectively). In other photosystem I-less mutants that lacked one to four of the scp genes the chlorophyll lifetime was in between these two values, with the chlorophyll lifetime generally decreasing with an increasing number of inactivated scps. In contrast, the chlorophyll biosynthesis rate was only marginally affected by inactivation of scps except when all five scp genes were deleted. Small Cab-like protein deficiency did not significantly affect photoinhibition or turnover of photosystem II-associated β-carotene. It is concluded that SCPs do not alter the stability of functional photosystem II complexes but retard the degradation of photosystem II-associated chlorophyll, consistent with the proposed involvement of SCPs in photosystem II re-assembly or/and repair processes by temporarily binding chlorophyll while photosystem II protein components are being replaced.

In oxygenic photosynthetic organisms two pigment-protein complexes, photosystem I (PS I)$^2$ and photosystem II (PS II), work in concert to extract electrons from water and produce NADPH, using energy from light. The light-capturing capacity of the two photosystems is enhanced by peripheral antenna pigment-protein complexes, which are different in various groups of photosynthetic organisms (1). In plants and green algae these antenna complexes are constituted by polypeptides encoded by a multigene family of cab genes (2) and contain chlorophyll a, chlorophyll b, and carotenoids as light-harvesting cofactors. Most of the cab gene family members code for proteins with three transmembrane helices, of which the sequences of the first and third membrane-spanning regions including their chlorophyll-binding sites are similar (Cab-family proteins). Other Cab-family members in plants include the four-transmembrane helix PsbS protein of PS II that is involved in non-photochemical energy dissipation (see Ref. 3 for review) and early light-inducible proteins (ELIPs) that have one, two, or three predicted transmembrane helices and that accumulate in thylakoids under stress conditions (reviewed in Ref. 4).

In contrast to plants, cyanobacteria do not have Cab proteins with multiple transmembrane helices; instead, members of the cyanobacterial phylum have either phycobilisomes in the cytoplasm or, in prochlorophytes, chlorophyll a/b-binding transmembrane Pcb proteins (5) as major peripheral antenna complexes. However, cyanobacteria contain single-helix proteins of the Cab family, named SCPs (small Cab-like proteins) or HlI (high light-induced proteins) (6). We will use the SCP designation here, as the corresponding genes are induced not only at high light intensity (7–10), but also in other stress conditions, such as at low temperature (11), low pH (12), nitrogen and sulfur starvation (8, 13), salt and hyperosmotic stresses (11, 14–16), presence of hydrogen peroxide (17), or inhibitors of photosynthetic electron transport (18).

Similar to other Cab proteins, the sequence of SCPs includes conserved motifs of residues involved in chlorophyll binding, although association of SCPs with chlorophyll has never been demonstrated experimentally despite considerable effort in various laboratories. Multiple scp genes are present in all sequenced cyanobacteria, including prochlorophytes. The genome of the cyanobacterium Synechocystis sp. PCC 6803 (the organism will be referred to in this article as Synechocystis) contains four scp genes that have been named scpB-scpE (7). In addition, ferrochelatase in many cyanobacteria and the chloroplast-targeted isozyme in plants have a ~60-residue C-terminal extension that is similar to the SCPs. For this reason, the C-terminal extension has been named ScpA and this SCP extension does not seem to be necessary for activity of ferrochelatase in Synechocystis (7).

The current working hypothesis is that members of the SCP family are involved in processes of PS II assembly/repair and may serve as a temporary pigment reservoir with relatively low affinity for pigments while PS II components are being replaced (19–21). Inactivation of scp genes in Synechocystis alters cell pigmentation (22), reducing the amount of chlorophyll, carote-
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Optical density at 730 nm in a 1-cm cuvette using a Shimadzu UV-160 spectrophotometer.

Isotope Labeling—Stable isotope labeling experiments were performed essentially as described in Ref. 27, 28. In brief, to label cells with $^{15}$N, Synechocystis cultures were grown to OD$_{730}$ $\sim$ 0.7 (late exponential phase) in BG-11 medium containing regular NaNO$_3$ as fixed-nitrogen source and were then diluted with fresh BG-11 medium without NaNO$_3$, to a final OD$_{730}$ of 0.1 for the wild-type strain and PS II-less mutants and 0.2 for the slower growing PS I-less mutants. At the time of dilution ($t = 0$), the cultures were supplemented with a mixture of ($^{15}$NH$_4$)$_2$SO$_4$ and Na$^{15}$NO$_3$ to a final concentration of 2 mM and 9 mM, respectively, and grown for the time specified. Glucose (10 mM final concentration) was added to all cultures except for the wild type, HT-3, and scpABCDE$^{-}$ strains incubated at 45–300 $\mu$mol photons m$^{-2}$ s$^{-1}$ to support cell growth.

For $^{13}$C labeling of chlorophyll and carotenoids, Synechocystis cells were grown from OD$_{730}$ $\sim$ 0.2 to an OD$_{730}$ of about 0.6 in 10 mM TES/NaOH (pH 8.2)-buffered BG-11 medium supplemented with 1.5 mM glucose and then resuspended to an OD$_{730}$ of 0.15 in freshly autoclaved 10 mM TES/NaOH (pH 8.2)-buffered BG-11 medium from which NaHCO$_3$ had been omitted. The medium was supplemented with a mixture of 2 mM [$^{13}$C]glucose and 2.4 mM Na$_2^{13}$CO$_3$. Every 24 h, the cultures were supplemented with an additional 1 mM of [$^{13}$C]glucose and 2.4 mM of Na$_2^{13}$CO$_3$ to maintain a high concentration of labeled bicarbonate and exponential growth of the cultures.

All chemicals enriched in $^{12}$C and $^{15}$N isotopes were purchased from Cambridge Isotope Laboratories Inc. (Andover, MA). $^{13}$C enrichment in [$^{13}$C]glucose and Na$_2^{13}$CO$_3$ was at least 99% and $^{15}$N enrichment in ($^{15}$NH$_4$)$_2$SO$_4$ and Na$^{15}$NO$_3$ was more than 98%.

Pigment Extraction and Purification—The extraction of pigments from Synechocystis cells and their purification by HPLC (using a Waters Spherisorb S10ODS2 (250 x 10 mm) Semi-Prep column eluted with a water/methanol-acetone gradient) was performed essentially as described earlier (27, 28). Pigment detection upon elution was performed by continuously recording absorption at 440 and 665 nm; the integrated peak areas at these two wavelengths were used to quantify carotenoids and chlorophyll (chlorophyllide), respectively. The fractions containing pigments were collected and dried under vacuum. Chlorophyll and chlorophyllide labeled with $^{14}$N were used for MS measurements without further purification. To perform mass spectrometry analysis of $^{13}$C-labeled chlorophyll, the pigment was first converted to phaeophytin by dissolving it into 0.4 ml of an acetone/water mixture (9:1 v/v) containing 20 $\mu$l of 0.1% (v/v) HCl and then chlorophyll-derived phaeophytin was subjected to a second round of HPLC using the elution conditions described above. Considering the significantly increased retention time of phaeophytin relative to that of chlorophyll, this procedure allowed to obtain pigment that is free of contaminants that co-elute with chlorophyll. Before mass spectrometric analysis of $^{13}$C-labeled carotenoids, the HPLC-separated pigments were re-dissolved in a small volume of methanol and subjected to a second round of HPLC purification using a YMC Carotenoid S-5 column (Waters) eluted with a linear methanol/acetone
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gradients for 15 min. After drying the pigments were stored at −80 °C until further mass spectrometric analysis.

**Mass Spectroscopy**—Positive ion mass spectra of 15N- or 13C-labeled pigments were obtained by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry on a Voyager Biospectrometry Work Station (Foster City, CA) using a tertithiophene matrix. Pigment samples were excited by nitrogen laser excitation pulses at a frequency of 3 Hz. The laser power was optimized to obtain a good signal-to-noise ratio after averaging 200–800 single-shot spectra.

**Analysis of Pigment Mass Spectra**—Mass spectra of 15N-labeled chlorophyll and chlorophyllide were analyzed as described in Ref. 27. In brief, experimentally measured mass spectra of the porphyrins composed of a mixture of 14N and 15N isotopes were simulated by a linear combination of five theoretical spectra of chlorophyll or chlorophyllide assumed to contain zero, one, two, three, or four 15N atoms (the balance being 14N atoms), whereas isotopes of all other elements were present according to their natural relative abundance. Using this analysis, relative amounts of chlorophyll with different numbers of 15N atoms per molecule were calculated for every pigment sample, and the obtained ratios were related to the total concentration of the corresponding pigment present in the growing *Synechocystis* culture.

Chlorophyll stability in different strains was determined from changes in the concentration of the pool of chlorophyll molecules in which all four nitrogen atoms were 14N (unlabeled chlorophyll). Assuming that chlorophyll degradation is monoeponential with a rate constant of k_d, changes in the total chlorophyll concentration C_{chl} (labeled plus unlabeled) in exponentially growing *Synechocystis* culture can be expressed as Equation 1,

$$\frac{dC_{chl}}{dt} = f_{chl}m_0e^{\mu t} - k_dC_{chl} \quad \text{(Eq. 1)}$$

where \( \mu \) is the specific growth rate of the culture, and \( m_0 \) is the concentration of cell biomass \( m \) at \( t = 0 \). The parameter \( f_{chl} \) reflects the fraction of biomass converted to chlorophyll per unit time and formally corresponds to the rate constant of chlorophyll synthesis (27). In the exponential growth phase, when the chlorophyll-to-biomass ratio \( (C_{chl}/m) \) remains constant in Equation 2,

$$f_{chl} = (k_d + \mu) \times C_{chl}/m \quad \text{(Eq. 2)}$$

The parameter \( f_{chl} \) calculated on the basis of Equation 2 was used to compare rates of chlorophyll synthesis in different cyanobacterial mutants.

To determine the stability of \( \beta \)-carotene, mass spectra of this carotenoid isolated from 13C-labeled cells were viewed as an overlay of mass spectra produced by (i) the pool of unlabeled \( \beta \)-carotene containing 13C at natural abundance (1.1%) and represented by four mass peaks with \( m/z \) ratios ranging from 536.4 to 539.4 (\( m/z = 536.4 \) corresponds to the M\(^+\) mass of monoisotopic \( \beta \)-carotene) and (ii) the pool of labeled \( \beta \)-carotene with \( m/z \) ratios ranging from 560.5 to 576.6 due to significant enrichment in 13C in molecules synthesized after administering the label. Relative amounts of the two pools of \( \beta \)-carotene were determined by measuring the total intensities of the mass peaks corresponding to the two groups of pigments; the absolute concentrations of labeled and unlabeled carotenoids were calculated from the total content of this pigment in the sample.

**Isolation of PS I and PS II Complexes**—PS II and PS I were isolated from the HT-3 strain of *Synechocystis* (30), which contains a His\( \text{a} \) tag at the C-terminal end of the CP47 protein, using procedures described in detail in (31, 32) with modifications as described below. To reduce possible photophytinization of chlorophyll, the pH of all buffers used to isolate PS II was maintained at 7.0 using 40 mM HEPES-NaOH. Considering that thylakoids were isolated from a small volume of 15N-labeled cells, thylakoid membranes were resuspended to a final chlorophyll concentration of 0.25 mg/ml and solubilized with dodecyl maltoside that was added to a final concentration of 0.25%, as compared with 1.0 mg chlorophyll ml\(^{-1}\) and 0.8% dodecyl maltoside used in (31). After passing through a column containing 1 ml of Ni-NTA resin (Qiagen) that retained PS II complexes, the eluant was applied to a second 1.0 × 10-cm column filled with DEAE-cellulose to purify PS I by ion-exchange chromatography. PS I was eluted from the second column by applying a 0–200 mM NaCl gradient in 40 mM HEPES-NaOH (pH 7.0) buffer.

**Oxygen Evolution**—Steady-state rates of oxygen evolution were measured at 25 °C using an oxygraph (Hansatech, Cambridge, UK) equipped with a Clark-type electrode. The suspension of *Synechocystis* cells at 1.2 \( \mu \)g chlorophyll ml\(^{-1}\) was supplemented with artificial electron acceptors 2,5-dimethyl-p-benzoquinone (DMBQ) and potassium ferricyanide to a final concentration of 0.4 mM and 2.0 mM, respectively. Oxygen production was measured upon exposure of cells to a saturating light intensity (Xe lamp) of about 2500 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\) passed through an orange cutoff filter (Corning) transmitting light at \( \lambda \geq 570 \text{ nm} \).

**RESULTS**

**15N Labeling of Chlorophyll in SCP-less Mutants**—Growth of *Synechocystis* cells in the presence of 15NO\(_3\) and 15NH\(_4\) caused gradual accumulation of chlorophyll molecules enriched in 15N, whereas the amount of chlorophyll containing only 14N remained stable or decreased gradually due to degradation of the pigment (Fig. 1). Fixed nitrogen is taken up very readily in cyanobacteria (33) and incorporation of nitrogen into chlorophyll is rapid (27), thus minimizing lag times in labeled N incorporation. Table 1 compares half-life times of chlorophyll molecules in different *Synechocystis* strains calculated from the kinetics of disappearance of unlabeled (14N) chlorophyll in cultures growing in the presence of 15N-labeled nitrate and ammonium.

In the wild type, the effect of SCP deletion on chlorophyll stability was observed only at moderately high light intensity (100 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\) and above), whereas in PS I-less strains acceleration of chlorophyll degradation after deletion of SCPs could be detected even in cells growing at low light intensity. In PS I-less cells, chlorophyll degradation generally was faster as the number of inactivated SCP genes increased. Of the single *scp* deletions in the PS I-less strain, inactivation of the *scp\( \beta \)* gene had the most profound effect on chlorophyll stability,
reducing the lifetime of chlorophyll by a factor of 2.5 compared with the PS I-less control strain (Table 1). In contrast, deletion of either scpA, scpC, scpD, or scpE had no significant effect on the chlorophyll life-time (not shown) and inactivation of the scpA, scpC, and scpD genes together reduced the chlorophyll half-life time by only about 1.5-fold (Table 1). In the absence of all five SCPs (i.e., in the PS I-less/scpABCDE− strain) chlorophyll degradation occurred about five times faster than in the PS I-less strain. In contrast, the effects of SCP deletions on chlorophyll stability in the wild type and the PS II-less strain were rather unremarkable. At a light intensity of 300 μmol photons m−2 s−1 deletion of all five SCPs decreased the chlorophyll lifetime by only a factor of two (Table 1). Therefore, the results are suggestive of SCPs affecting primarily the lifetime of chlorophyll associated with PS II, which is most chlorophyll in the PS I-less strain but only a small fraction of the chlorophyll in the wild type.

Chlorophyll biosynthesis can also be monitored by the 15N-labeling method (Table 1). In general, the rate of chlorophyll synthesis in PS I-less strains is decreased due to the reduced chlorophyll content of these strains. However, of the three strains studied (wild type, PS II-less, and PS I-less), upon deletion of the five SCP genes the PS I-less strain showed the largest decrease in the chlorophyll biosynthesis rate (almost 4-fold versus about 15% in the wild type and the PS II-less strain).

Labeling of Chlorophyll Associated with PS I and PS II—The data presented in Table 1 show that chlorophyll degradation in Synechocystis was faster when cells were grown at high light intensity and in strains containing only PS II. This observation suggests that particularly chlorophyll bound to PS II degrades more rapidly than PS I-associated chlorophyll. To verify this interpretation, PS I and PS II complexes were isolated from the HT-3 Synechocystis mutant, which contained a His6 tag at the C terminus of the CP47 protein of PS II (30). SCP genes were not inactivated in this strain. Before isolation of the photosystems, HT-3 cells were grown in the presence of 15N for 15 h at a light intensity of about 150 μmol photons m−2 s−1. Pigments were extracted from both photosystems and the 15N labeling pattern of purified chlorophyll from the two types of preparations was analyzed by mass spectrometry. The replacement of unlabeled chlorophyll molecules with 15N-labeled ones occurred faster in pigments extracted from PS II complexes (Fig. 2), and at the end of the labeling period unlabeled (14N) chlorophyll constituted about 29% of the total chlorophyll amount extracted from PS II, whereas in chlorophyll extracted from PS I nearly 43% of chlo-

![FIGURE 1. Kinetics of 15N labeling of chlorophyll in Synechocystis strains lacking SCPs.](image)

**TABLE 1**

Chlorophyll synthesis and degradation rates in Synechocystis strains with various deletions in SCPs and/or photosystems

10 mM glucose was added to all cultures except for the wild type and scpABCDE− strains incubated at 45–300 μmol photons m−2 s−1. The cell doubling time in the cultures was determined by measuring changes in OD_730. The chlorophyll half-life time _t_1/2 was determined from changes in the concentration of unlabeled chlorophyll. The rate constant of chlorophyll synthesis, _f_Chl<sub>syn</sub>_ was calculated according to Equation 2 using the parameters shown in this table. Listed are the average results of two to seven independent experiments ± S.D.

| Strain | Growth light intensity | Chlorophyll half-life time | Cell doubling time | Chlorophyll content | Rate of chlorophyll synthesis, _f_Chl<sub>syn</sub> | % WT level |
|--------|------------------------|---------------------------|-------------------|---------------------|---------------------------------|------------|
| Wild type | 2–4 | >200 | ND<sup>a</sup> | ND | ND |
| 45 | >200 | 15.2 ± 2.1 | 2.40 ± 0.22 | 100 |
| 100 | >200 | ND | ND | ND | ND |
| 300 | 49 ± 5 | ND | ND | ND | ND |
| scpABCDE− | 2–4 | >200 | ND | ND | ND |
| 45 | >200 | 15.8 ± 2.0 | 2.10 ± 0.15 | 84 |
| 100 | 152 ± 21 | ND | ND | ND | ND |
| 300 | 24 ± 2 | ND | ND | ND | ND |
| PS II-less | 45 | >200 | 20.4 ± 1.8 | 2.15 ± 0.12 | 68 |
| 45 | >200 | 18.3 ± 3.0 | 1.68 ± 0.08 | 59 |
| PS I-less | 2–4 | 161 ± 25 | 21.3 ± 3.3 | 0.48 ± 0.03 | 15 |
| 2–4 | 64 ± 3 | 26.8 ± 0.33 | 0.43 ± 0.03 | 13 |
| 2–4 | 149 ± 14 | 22.8 ± 2.2 | 0.50 ± 0.04 | 16 |
| 2–4 | 65 ± 4 | 25.5 ± 2.6 | 0.44 ± 0.04 | 14 |
| 2–4 | 71 ± 10 | 27.1 ± 3.1 | 0.41 ± 0.02 | 12 |
| 2–4 | 113 ± 19 | 20.6 ± 1.1 | 0.44 ± 0.03 | 15 |
| 2–4 | 46 ± 7 | 22.9 ± 3.5 | 0.33 ± 0.03 | 13 |
| 2–4 | 48 ± 4 | 21.4 ± 1.0 | 0.39 ± 0.05 | 16 |
| 2–4 | 32 ± 5 | 53.3 ± 5.4 | 0.13 ± 0.02 | 4 |

<sup>a</sup> ND, not determined.
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![Graph showing the relative amount of chlorophyll in PS II and PS I](image)

The relative amount of unlabeled chlorophyll in pigments extracted from intact cells used to isolate PS I and PS II was rather similar to that of chlorophyll extracted from PS I due to the fact that chlorophyll bound to PS I constitutes as much as 90% of the total amount of this pigment in *Synechocystis* (34).

By the end of the 15-h growth period the total chlorophyll concentration in the culture increased by a factor of 2.2 due to growth of the culture (the doubling time is 12–14 h). Assuming that the PS I/PS II ratio remained constant during the experiment, which is reasonable as the growth conditions did not change, from the data presented in Fig. 2 one can estimate that nearly 35% of the PS II-associated chlorophyll that was present at $t = 0$ has degraded during the labeling period, whereas there is a less than 5% loss in the unlabeled chlorophyll associated with PS I during the same labeling period. Therefore, in agreement with the data shown in Table 1, these results demonstrate that PS II-associated chlorophyll turns over much faster than that associated with PS I, even in the presence of SCPs. The half-life time of the D1 protein of PS II in *Synechocystis* cells growing at light intensity of 100 μmol photons m$^{-2}$ s$^{-1}$ was reported to be about 2 h (35), and the half-life time of CP43 and D2 proteins in the HT-3 strain grown at 150 μmol photons m$^{-2}$ s$^{-1}$ was in the range of 5–7 h (data not shown). As in more than twice this time only a third of the chlorophyll is replaced, this indicates that a significant part of PS II-associated chlorophyll is recycled upon complete degradation of PS II.

There is no reason to expect that the accelerated turnover of PS II-bound chlorophyll shown in Fig. 2 was due to the presence of histidine residues fused to the CP47 protein. In fact, earlier studies have demonstrated that the introduction of the His$_n$ tag affects neither the physiology of HT-3 cells nor the properties of the isolated PS II complex (30, 36).

Chlorophyllide Recycling—The experimental data presented so far demonstrate that chlorophyll turnover in *Synechocystis* cells is mostly associated with PS II rather than PS I and that SCPs slow down the degradation of PS II-associated chlorophyll. The next question to be addressed is whether SCPs also are involved in the newly discovered chlorophyll/chlorophyllide cycle (28), in which chlorophyll is “degraded” to chlorophyllide but then is often reutilized to be converted to chlorophyll again.

To be able to focus on PS II-associated chlorophyll, which is affected by SCPs and which turns over relatively rapidly, subsequent experiments were performed with PS I-less cells. In the PS I-less strain, which was used as a control, the molar ratio of chlorophyll to pheophytin was $39 \pm 1$ to 2. Considering that two pheophytin molecules are present in every assembled PS II center and that preparations of isolated PS II centers generally contain 35–42 chlorophylls (31, 34 and references therein), nearly all chlorophyll in the PS I-less *Synechocystis* strain is expected to be associated with PS II.

PS I-less and PS I-less/SCP-less strains of *Synechocystis* accumulate easily detectable amounts of chlorophyllide (24). Previous measurements have shown that chlorophyllide labeling with $^{15}$N in the PS I-less strain occurs only slightly faster than the labeling of chlorophyll suggesting that chlorophyllide in the cell consists of a mixture of pools of molecules in the process of chlorophyll biosynthesis and those formed upon chlorophyll dephytylation (27, 28). To determine whether SCPs affect labeling kinetics of the chlorophyllide pool, $^{15}$N labeling of chlorophyll and chlorophyllide was monitored in the PS I-less/SCPABCDE$^{-}$ and PS I-less/SCPABCDE$^{-}$/SCP$^{-}$ strains (Fig. 3). Particularly in the PS I-less/SCPABCDE$^{-}$ strain the differences between chlorophyll and chlorophyllide labeling were very similar to those in the control. Whereas in the PS I-less/SCPABCDE$^{-}$ strain unlabeled chlorophyllide disappeared more rapidly, possibly signifying some inhibition of chlorophyllide conversion to chlorophyll in the absence of ScpE, deletion of SCPs did not appear to have a major effect on chlorophyllide lifetimes.

A substantial part of chlorophyllide and phytol released upon the de-esterification of PS II-associated chlorophyll in *Synechocystis* cells is recycled for the biosynthesis of new chlorophyll molecules (28). As presented in the previous paragraph, SCP proteins do not appear to greatly affect chlorophyllide conversion. To test the validity of this indication, we directly monitored the levels of labeled and unlabeled chlorophyll as well as chlorophyll with a labeled ring and unlabeled tail, and chlorophyll with a labeled tail and unlabeled ring (Fig. 4A) in PS I-less control cells and in the PS I-less/SCPABCDE$^{-}$ strain. Fig. 4A shows a typical mass spectrum of pheophytinized chlorophyll isolated from PS I-less/SCPABCDE$^{-}$ *Synechocystis* cells grown in the presence of $^{13}$C-labeled glucose for 48 h. The four groups of peaks with $m/z$ ranging from 870 to 875, 887 to 895, 895 to 907, and 908 to 927 represent unlabeled chlorophyll ($^{12}$Por$^13$Phy), chlorophyll in which the porphyrin constituent of the pigment is unlabeled and the phytol constituent is $^{13}$C-labeled ($^{12}$Por$^{13}$Phy), chlorophyll in which the phytol constituent is labeled while the phytol constituent is unlabeled ($^{13}$Por$^{12}$Phy), and fully $^{13}$C-labeled chlorophyll ($^{12}$Por$^{12}$Phy), respectively. The pools of $^{12}$Por$^{13}$Phy and $^{13}$Por$^{13}$Phy pools represent chlorophyll molecules that have been formed from a “new” ring and an “old” tail or *vice versa*, after a dephytylation/phytylation cycle. Fig. 4B compares concentrations of differentially labeled chlorophyll pools in PS I-less and PS I-less/SCPABCDE$^{-}$...
cells at different time points. These data were obtained by integrating the intensities of the four groups of pheophytin mass peaks to calculate the relative amounts of 12Por13Phy, 13Por12Phy, and 12Por13Phy; the absolute concentrations of the four pools of pigments were determined from the known values of the total chlorophyll content in the corresponding Synechocystis cultures. As chlorophyll isolated from the PS I-less/scpABCDE− strain contained significant amounts of 12Por13Phy and 13Por12Phy, the results of this experiment demonstrated that rather effective recycling of chlorophyllide can occur in the absence of the SCPs, thus essentially excluding the possibility that SCPs perform their chlorophyll-stabilizing function primarily by enhancing chlorophyllide reutilization.

13C Labeling of Carotenoids—Besides the reduction in chlorophyll concentration, deletion of SCP proteins caused an overall decrease in carotenoid level in Synechocystis strains, which was especially evident in mutants lacking PS I (24). The decrease in β-carotene was roughly proportional to the decrease in the amount of chlorophyll, and the chlorophyll-to-β-carotene ratio therefore changed little upon the deletion of SCPs. Thus, according to HPLC analysis the molar ratio of chlorophyll to β-carotene was 4.7 ± 0.4, 5.4 ± 0.2, and 4.4 ± 0.3 (mean ± S.D. of three independent measurements) in the PS I-less, PS I-less/scpABCDE−, and PS I-less/scpABCDE− mutants, respectively.

The stability of β-carotene was measured in several SCP-less strains by analyzing the kinetics of 13C incorporation into this pigment in cells growing in the presence of [13C]glucose (Fig. 5). When normalized to the initial β-carotene concentration, accumulation of 13C-enriched β-carotene was substantially slower in the PS I-less/scpABCDE− strain than in the PS I-less and PS I-less/scpABCDE− strains due to the much lower growth rate of cells missing all five SCPs (see Table 1). However, the pool of unlabeled β-carotene disappeared at approximately the same rate in the three strains (Fig. 5). Interestingly, the half-life time of unlabeled β-carotene in the three strains was within the range of 12–15 h, which is much shorter than the half-life time of unlabeled chlorophyll, even in the PS I-less/scpABCDE− cells (see Table 1). These data corroborate the results of our earlier kinetic modeling of 13C incorporation into major cyanobacterial carotenoids isolated from cells grown in the presence of a...
mixture of unlabeled and uniformly labeled $^{13}$C glucose; these results showed that without SCPs the rates of β-carotene and myxoxanthophyll synthesis normalized to the cell concentration were slowed down markedly but degradation rates of myxoxanthophyll, β-carotene, zeaxanthin, and echinenone were not significantly affected (37). Apparently, upon “degradation” β-carotene was nearly quantitatively converted to zeaxanthin and echinenone in PS I-less cells (37).

Considering that the molar ratio of chlorophyll to β-carotene measured in PS I-less Synechocystis cells (~5:1) was similar or even slightly higher than the ratio reported for isolated PS II centers (31, 34), it is likely that most or all β-carotene in PS I-less cells is associated with PS II. The β-carotene turnover rate measured in whole-cell pigment extracts therefore reflects the turnover rate of β-carotene associated with PS II in PS I-less cells (Fig. 5). If this rate also correlates with the PS II lifetime (β-carotene may need to be released from PS II in order to be converted or degraded), the inactivation of scp genes would not alter PS II stability, at least at the low light intensity used to grow PS I-less cultures.

**Oxygen Evolution**—Fig. 6 compares the loss of oxygen-evolving activity in PS I-less and PS I-less/SCP-less cells exposed to a relatively high light intensity (500 μmol photons m $^{-2}$ s $^{-1}$) in the presence of the protein synthesis inhibitor gentamicin (50 μg/ml final concentration; an order of magnitude more than needed for full inhibition of cell growth). Gentamicin was added to the cells 30 min prior to actinic illumination to prevent the recovery of PS II from the light-induced damage. Gentamicin was added to the cells 30 min prior to actinic illumination to prevent the recovery of PS II from the light-induced damage. The kinetics of light-induced PS II inactivation was similar in the PS I-less, PS I-less/scpABCD $^{-}$, and PS I-less/scpABCD $^{-}$ strains indicating that inactivation of the scp genes has little effect on the sensitivity of PS II to photodamage induced by high light intensity.

**DISCUSSION**

All cyanobacterial genomes sequenced to date contain genes for single helix SCP polypeptides. The SCPs carry domains that are also conserved in Cab proteins and that are known to bind chlorophyll in these Cab proteins constituting the light-harvesting complexes (LHCs) in higher plants (38). The emerging concept is that in cyanobacteria the single helix SCP proteins are important for stabilizing damaged PS II centers and presumably serve as a temporary pigment reservoir while PS II components are being replaced (20, 21, 24). We have now performed a detailed analysis of chlorophyll and β-carotene turnover in a set of SCP-less Synechocystis mutants using the $^{15}$N and $^{13}$C pigment labeling technique we developed to measure synthesis and degradation rates of photosynthetic pigments in cyanobacteria (27, 28).

**Pigment Turnover in SCP-containing Cells**—PS II is considered to be the main target of light-induced oxidative damage leading to the loss of oxygen-evolving activity of this complex (39, 40). In intact cells, damaged PS II centers can be repaired via a complex process that includes proteolytic degradation of the D1 protein and subsequent integration of a newly synthesized D1 into the damaged PS II. As a consequence, the D1 protein has a high turnover rate in the light. Other chlorophyll-binding proteins comprising the PS II core -D2, CP43, and CP47- are turning over more slowly (41). Our results show that chlorophyll associated with PS II apparently turns over much slower than major PS II proteins (Fig. 2, Table 1) indicating that most of the chlorophyll molecules are recycled/reused during the process of PS II reassembly/repair.

As shown in $^{15}$N-labeling experiments, degradation of the porphyrin macrocycle of chlorophyll was slow but measurable (half-life time of about 160 h) in the PS I-less Synechocystis strain grown at a very low light intensity (2–4 μmol photons m $^{-2}$ s $^{-1}$; Table 1). When PS I-less cells were incubated in the presence of $^{13}$C glucose, we observed relatively fast (half-life
time of about 48 h) disappearance of $^{12}$Por$^{13}$Phy chlorophyll molecules containing unlabeled porphyrin and phytol moieties (Fig. 4) due to a continuous chlorophyll de-esterification/cho-
rophyllide re-esterification cycle in cyanobacterial cells (28). This cycle likely includes de-esterification of some chlorophyll molecules taking place upon dissociation and repair of dam-
aged PS II. In the absence of SCPs, cells were able to recycle (phytylate) chlorophyllide molecules formed upon chlorophyll de-esterification, although the efficiency of this recycling appeared to be slightly lower without SCPs (Figs. 3 and 4).

$^{13}$C-labeling experiments demonstrated that depletion of the pool of unlabeled $\beta$-carotene occurred with kinetics that were much faster than chlorophyll degradation/dephytylation under the same conditions (half-life time of about 15 h). Judging from the pigment stoichiometry in PS I-less Synechocystis cells (the chlorophyll/$\beta$-carotene ratio is about 5:1), nearly all $\beta$-carotene in these cells is expected to be associated with PS II. Rapid conversion of PS II-associated $\beta$-carotene into zeaxanthin due to D1 protein turnover has been observed in Chlamydomonas reinhardtii cells exposed to high light intensity (42). In that study synthesis of new $\beta$-carotene was found to be required for reassembly of photodamaged PS II. As $\beta$-carotene most likely is unable to be converted into other carotenoids if embedded in the PS II complex (no access for $\beta$-carotene conversion enzymes), our measurements of the $\beta$-carotene lifetime may suggest that PS II protein turnover takes place in Synechocystis thylakoids even at low light intensity and at about the same rate in SCP-containing and SCP-less cells. Alternatively, $\beta$-carotene molecules may readily diffuse in and out of the PS II complex (thereby becoming temporarily available to $\beta$-carotene process-
ing enzymes) similar to the diffusion of PS II-bound plasto-
quinone $Q_{b}$. In support of this possibility, a rapid exchange of D1/D2-associated chlorophylls with the pool of the exog-
enously added pigments has been demonstrated in isolated preparations of PS II reaction centers (43), although we are currently unaware of experimental data indicating that a simi-
lar exchange can occur between carotenoids. Regardless of the exact mechanism responsible for the rather short $\beta$-carotene lifetime in PS I-less Synechocystis cells, our results indicate that the dynamic metabolism of this PS II-associated carotenoid is not affected by SCPs.

**SCPs Retard Degradation of PS II-associated Chlorophyll**—
According to the $^{13}$N-labeling data, breakdown of chlorophyll (or at least of the N-containing porphyrin macrocycle that was tracked) accelerated in mutants with inactivated $scp$ genes (Table 1). The chlorophyll lifetime was shorter in the $scp$ABCDEF$^{-}$ strain than in the wild type when cells of both strains were grown at high light intensity that stimulates PS II photodamage and expression of the $scp$ genes (8). The lifetime of chlorophyll was also shorter in many PS I-less/SCP-less strains than in the corresponding PS I-less control with intact $scps$. Note that even at low light intensity $scp$ genes are expressed at relatively high levels in PS I-less cells (7). More-
over, we have shown that inactivation of $scp$ genes in a PS I-less background causes faster chlorophyll degradation in cells grown under light-activated heterotrophic growth (LAHG) conditions, when cells are exposed to light for only 15 min every 24 h (22, 24). In contrast, PS I-associated chlorophyll appears to be stable (PS II-less strain; Table 1) and to be little affected by the presence or absence of SCPs as the chlorophyll lifetime in the PS II-less and PS II-less/SCPABCD$^{-}$ strains grown at mod-
erate light intensity was indistinguishable from each other and from that in wild type. Together, the data provide strong evi-
dence that SCP proteins can impede degradation of PS II-associated chlorophyll molecules.

$^{15}$N-labeling experiments performed with PS I-less strains showed a general trend of decreasing chlorophyll stability with a decreasing number of intact $scp$ genes, although the effect of subsequent $scp$ deletions was not strictly additive. Chlorophyll degradation was fastest in the PS I-less mutant lacking all five SCPs, and was somewhat slower in mutants with inactivated $scp$ABCD or $scp$ACDE gene sets. Inactivation of single $scp$ genes had only a minor effect on chlorophyll stability, except for the $scp$B deletion mutant (Table 1). Deletion of $scp$E is expected to have an equally significant effect as in the PS I-less/ $scp$ACDE$^{-}$ strain chlorophyll degraded $\sim 2.5$ faster than in the PS I-less/SCPACD$^{-}$ strain although in the PS I-less/SCP$E^{-}$ strain the chlorophyll degradation rate was about the same as in the PS I-less control. Selected SCP proteins may functionally com-
penstate for each other (24) and/or inactivation of specific $scp$ genes or combinations thereof may alter the expression of the remaining ones. In any case, the rate of chlorophyll degradation dropped to the same extent when $scp$B or $scp$E deletions were introduced into the PS I-less/SCPACD$^{-}$ background strain (Table 1).

**Stability of Active PS II Is Not Influenced by SCPs**—
The observed effect of SCPs on chlorophyll stability in principle may be explained in two different ways: (i) SCP proteins stabilize active PS II complexes making them more resistant to (photo) damage and thereby reducing the frequency of PS II reassembly or degradation events that can lead to chlorophyll breakdown; or (ii) SCPs prevent chlorophyll degradation upon repair of damaged PS II centers. We strongly favor the second explana-
tion, for the following reasons. First, the loss of $O_{2}$ evolution was similar in PS I-less and PS I-less/SCP-less cells upon exposure to high light intensity (Fig. 6). Also, in the quadruple $scp$BCD$^{-}$ (hiAABC$^{-}$) mutant containing normal PS I, PS II was nearly as sensitive to photodamage as PS II in the wild-type strain (23). Moreover, the loss of unlabeled $\beta$-carotene during the $^{13}$C-labeling experiment occurred at similar rates in the PS I-less, PS I-less/SCPABCD$^{-}$, and PS I-less/SCPABCD$^{-}$ strains (Fig. 5), suggesting that at low light intensity the PS II dynamics are independent of SCPs. In further support of the argument that SCPs protect chlorophyll upon degradation and repair of PS II complexes, ScpD/PS II complexes can be co-isolated with several FtsH proteases (21) that are involved in the repair of photodamaged PS II. In addition, functional $scps$ were neces-
sary to ensure a high rate of chlorophyll and PS II accumulation in PS I-less/chl$^{-}$ cells exposed to light after incubation under LAHG conditions (22, 24), supporting a role of SCPs in PS II assembly and repair processes.

In conclusion, in this article we have demonstrated that SCP proteins inhibit degradation of PS II-associated chlorophyll. Chlorophyll-binding motifs in the transmembrane region of the SCPs may be instrumental in temporarily accommodating chlorophyll, thereby making chlorophyll molecules less acces-
Anomalously high levels of the structural proteins DidB, DidC, and DidD are associated with the HT-3 Synechocystis sp. PCC 6803 strain. We also thank Shaw Shahriari for help with the isolation and analysis of His-tagged PS II.

Acknowledgments—We thank Prof. Terry M. Bricker for the gift of the HT-3 Synechocystis sp. PCC 6803 strain. We also thank Shaw Shahriari for help with the isolation and analysis of His-tagged PS II.

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