Long-Term Acclimation of the Cyanobacterium Synechocystis sp. PCC 6803 to High Light Is Accompanied by an Enhanced Production of Chlorophyll That Is Preferentially Channeled to Trimeric Photosystem I1[W]

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Cyanobacteria acclimate to high-light conditions by adjusting photosystem stoichiometry through a decrease of photosystem I (PSI) abundance in thylakoid membranes. As PSI complexes bind the majority of chlorophyll (Chl) in cyanobacterial cells, it is accepted that the mechanism controlling PSI level/synthesis is tightly associated with the Chl biosynthetic pathway. However, how Chl is distributed to photosystems under different light conditions remains unknown. Using radioactive labeling by 35S and by 14C combined with native/two-dimensional electrophoresis, we assessed the synthesis and accumulation of photosynthetic complexes in parallel with the synthesis of Chl in Synechocystis sp. PCC 6803 cells acclimated to different light intensities. Although cells acclimated to higher irradiances (150 and 300 μE m−2 s−1) exhibited markedly reduced PSI content when compared with cells grown at lower irradiances (10 and 40 μE m−2 s−1), they grew much faster and synthesized significantly more Chl, as well as both photosystems. Interestingly, even under high irradiance, almost all labeled de novo Chl was localized in the trimeric PSI, whereas only a weak Chl labeling in photosystem II (PSII) was accompanied by the intensive 35S protein labeling, which was much stronger than in PSI. These results suggest that PSII subunits are mostly synthesized using recycled Chl molecules previously released during PSII repair-driven protein degradation. In contrast, most of the fresh Chl is utilized for synthesis of PSI complexes likely to maintain a constant level of PSI during cell proliferation.

Photosynthetic autotrophs are completely dependent on light as the only source of energy for their proliferation. However, light intensity can swiftly change due to variable environmental conditions, sometimes being too low to sufficiently drive photosynthetic reactions, and other times being even higher than can be utilized, or at least safely dissipated, by a photosynthetic apparatus. In the latter case, the excessive part of the absorbed energy can be ultimately transformed into energy of reactive oxygen species, which has a destructive impact on key cellular components: nucleic acids, lipids, pigments, and proteins. To cope with light fluctuations, cyanobacteria as well as algae and plants possess complex regulatory machinery to optimize the utilization of light energy and to protect photosynthetic apparatus against the damage induced by excessive light. This machinery involves the regulation of size and number of light-harvesting antennas (Anderson et al., 1995; Walters, 2005), dissipation of light energy by non-photochemical quenching (El Bissati et al., 2000; Müller et al., 2001), redistribution of light energy between photosystems by state transition (Mullineaux and Emlyn-Jones, 2005; Fujimori et al., 2005), or adapting the capacity of carbon dioxide (CO2) fixation (Demmig-Adams and Adams, 1992).

One of the most prominent responses to light intensity, and also to its spectral quality, is a selective regulation of the abundance of PSI in the thylakoid membrane, which controls the frequency of excitation of photosystems to optimize the entire photosynthetic electron flow (Chow et al., 1990; Muramatsu and Hihara, 2012). Given that the PSII level is much more stable, this process establishes a specific stoichiometry between both photosystems (PSI/PSII ratio) depending on particular light conditions (Neale and Melis, 1986; Murakami and Fujita, 1991; Walters and Horton, 1994). A dynamic adjustment of the PSI/PSII ratio has been shown to be required for maintaining a high quantum efficiency of photosynthesis in plants (Chow et al., 1990) and algae (Melis et al., 1996). In cyanobacteria, a physiological significance of photosystem stoichiometry was demonstrated on mutants of the cyanobacterium Synechocystis sp. PCC 6803 (hereafter, Synechocystis). The pmgA mutant has lost the ability to

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selectively decrease the PSI content during acclimation to high light (HL) and its growth is severely inhibited under prolonged HL conditions (Hihara et al., 1998; Sonoike et al., 2001). A similar defect in maintaining reduced PSI content in HL has been demonstrated in a Synechocystis strain lacking the putative chlorophyll (Chl)-binding domain of ferrochelatase. The growth of this mutant has been completely abolished by HL (Sobotka et al., 2011). The mechanism responsible for the HL-induced selective decrease in PSI content is not clear; however, the process appears to be under control of the redox state of the cytochrome b_{6f} complex (Murakami and Fujita, 1993). In a proposed model, a signal from cytochrome b_{6f} downregulates Chl biosynthesis, particularly formation of an early Chl intermediate aminolevulinic acid (ALA), and it seems to be a limited Chl availability that determines the PSI content (Fujita et al., 1990; Muramatsu et al., 2009).

Consistently with this model, transcription of psaA/B genes for large Chl-binding core subunits is not a rate-limiting step in the PSI accumulation, while de novo Chl synthesis is blocked immediately after being transferred to HL conditions (Muramatsu et al., 2009). On the other hand, Chl is required during the repair/replacement of PSI complexes that are photodamaged much faster than PSI complexes (Nowaczyk et al., 2006; Nixon et al., 2010). Therefore, the synthesis of Chl-binding PSI subunits, especially the fast turning over D1 subunit, should be much faster in comparison with PSI subunits (Yao et al., 2012a, 2012b). It raises the question of how an enhanced synthesis of PSI subunits is achieved if less de novo-synthesized Chl is produced under HL conditions to keep PSI at a reduced level. Chl is produced via a quite long and branched pathway together with heme and other tetrapyrroles, and the formation of ALA is a particularly critical step controlling the accumulation of the whole spectrum of phototoxic intermediates of Chl/heme biosynthesis (for review, see Czarnecki and Grimm, 2012; see Supplemental Fig. S1 for a scheme of tetrapyrrole pathway). However, controlling PSI content by the metabolic flow through the whole tetrapyrrole pathway does not seem to be flexible enough to balance the formation of all tetrapyrrole cofactors. The de novo Chl biosynthesis is expected to be tightly synchronized with synthesis of Chl-binding proteins (Komenda et al., 2012) but a cross talk between Chl and Chl-protein biosyntheses has to also reflect the fact that the lifetime of Chl molecules is much longer than that of proteins (Vavilin et al., 2005; Vavilin and Vermaas 2007; Yao et al., 2012a, 2012b); therefore, a potential role of the recycled Chl in controlling Chl-protein biogenesis must be addressed as well. In this study, we focused on the correlation between synthesis of both photosystems and Chl biosynthesis in Synechocystis cells fully acclimated to different light intensities. Using radioactive labeling of proteins and Chl by \textsuperscript{35}S and \textsuperscript{14}C, respectively, we found that the rate of de novo Chl formation, as well as synthesis of both photosystems, is significantly enhanced in HL, despite the markedly reduced cellular level of PSI. Our data suggest that there is no simple correlation between the actual synthesis of PSI and PSII core subunits and the synthesis and distribution of de novo Chl; de novo Chl was found to be predominantly directed to the PSI trimer, whereas the Chl-binding PSII subunits seem to be mostly synthesized using the recycled Chl molecules previously released during Chl-protein degradation. It appears that the particular level of PSI needed for the optimal photosynthetic performance at a given light intensity is reached by a precise equilibrium between the rate of cell proliferation, the rate of Chl biosynthesis, and the distribution of Chl into individual Chl-proteins.

RESULTS

Acclimation of the Synechocystis Cells to Various Irradiances

To understand how Chl biosynthesis is synchronized with the varying demand for Chl-binding subunits of PSI/PSII, we analyzed the Synechocystis wild type acclimated to different light intensities. Cells were first grown for 5 d under continuous illumination at 40 \(\mu\)E m\(^{-2}\) s\(^{-1}\) (moderate light [ML]) and subsequently moved for 24 h to either lower light (LL; 10 \(\mu\)E m\(^{-2}\) s\(^{-1}\)) or higher light intensities (HL1, 150 \(\mu\)E m\(^{-2}\) s\(^{-1}\); HL2, 300 \(\mu\)E m\(^{-2}\) s\(^{-1}\)). Absorption spectra of cells normalized per optical density at 750 nm (OD\(_{750}\) are shown in Figure 1A. Whereas the shift to LL did not result in significant changes in the cell pigmentation (data not shown; see Table I for Chl level), amounts of Chl and phycobilisomes per cell were markedly lowered as a response to higher irradiance (Fig. 1A). Chl concentration at HL2 reached about 50% of that observed at ML (Table I; Supplemental Fig. S2). Levels of carotenoids also decreased, except for myxoxanthophyll, the level of which more than doubled after 24 h at HL2 (Supplemental Fig. S2). Growth rates of the cultures were accelerating with light intensity reaching the maximum at HL2 (Table I), a further increase in light intensity to 600 \(\mu\)E m\(^{-2}\) s\(^{-1}\) started to inhibit growth (data not shown), although cell pigmentation did not significantly alter when compared with HL2 (see Supplemental Fig. S3 for the whole-cell spectra). Supplementing growth media with 5 mM NaHCO\(_3\) did not affect the growth rate or Chl level at HL2 (Table I), implying that under our growth conditions with a higher concentration of CO\(_2\) in the growth chamber, the proliferation of Synechocystis cells was not significantly limited by CO\(_2\) availability. To gather information about the functional status of PSII during acclimation to HL, we also determined the ratio of variable to maximal fluorescence (\(F_v/F_m\)). When cells grown at ML were moved to HL2, the \(F_v/F_m\) ratio declined rapidly (Supplemental Fig. S4); nevertheless, in the following 6 h it recovered to the initial value. This suggests that even under HL2, cells coped relatively quickly with the initial damage of PSII, and fully acclimated cells did not contain a significant portion of nonfunctional PSII complexes and grew much faster than at ML.
Accumulation and Synthesis of PSI and PSII in HL-Acclimated Cells

In the Synechocystis cells, practically all Chl is associated with PSI under ML (Shen et al., 1993). In order to determine how total levels of PSI and PSII are correlated with the drop in the cellular Chl level under HL, we first separated membranes from cultures acclimated to LL, ML, HL1, and HL2 by SDS-PAGE and then probed with antibodies against PSII and PSI subunits (Fig. 3A). As expected, an increase in light intensities reduced the amount of both PSI and PSII per cell (Fig. 3A). However, a decrease in the PSI level was much more pronounced. The following separation of photosynthetic complexes using clear-native electrophoresis (CN-PAGE) combined with detection of Chl fluorescence in gel allowed us to assess the content of particular oligomeric forms of PSI and PSII (Fig. 3B). Interestingly, the amount of both PSI and PSII monomers per cell was not significantly altered by HL, which contrasts with the dramatically downregulated amount of trimeric PSI and the clearly lowered level of PSII dimer (Fig. 3B). To assess how de novo synthesis of PSI/PSII complexes corresponds to changes in their accumulation at a cellular level, cells acclimated to ML and HL2 were radioactively labeled using [35S]Met/Cys mixture for 30 min and separated by CN-PAGE. After exposing the CN gel to a phosphor imager plate, we found rather similar labeling of the PSI trimer in both cultures while the labeling of the PSII dimer and both PSI and PSII monomers was at least doubled (Fig. 4A). Taking into account a much lower level of PSI trimer per cell in the HL2 cells, the results indicate significantly faster synthesis of all four complexes at this light intensity. To see how the individual Chl-binding PSI core subunits D1, D2, CP43, and CP47 contributed to the PSII monomer

### Table 1. Growth rate and Chl content of Synechocystis cells under different growth regimes

| Light Intensity | Doubling Time (hours ± so) | Chl (μg ml⁻¹ OD₅₇₀⁻¹) |
|-----------------|---------------------------|----------------|---|
| LL (10 μE m⁻² s⁻¹) | 32.8 ± 1.3 | 5.9 ± 0.08 |
| ML (40 μE m⁻² s⁻¹) | 15.8 ± 0.8 | 5.5 ± 0.12 |
| HL1 (150 μE m⁻² s⁻¹) | 10.4 ± 1.3 | 3.6 ± 0.12 |
| HL2 (300 μE m⁻² s⁻¹) | 7.2 ± 0.3 | 2.5 ± 0.09 |
| HL2 + 5 mM NaHCO₃ | 7.1 ± 0.3 | 2.4 ± 0.10 |
and dimer labeling, we separated them in a second dimension using SDS-PAGE (Fig. 4B). Although their total level was lower at HL2, particularly in the dimeric PSII, the synthesis of all four proteins was clearly enhanced in both PSII complexes. We also observed a higher accumulation and synthesis of components of ATPase and FtsH2 and FtsH3 proteases at HL2 (Fig. 4B).

Light-Driven Up-Regulation of the Tetrapyrrole Biosynthetic Pathway

Acclimation of the cells to HL2 led to a fast down-regulation of PSI and phycobilisome levels (Fig. 1) and resulted in a reduction of the thylakoid membrane system (Fig. 2). On the other hand, synthesis of Chl-proteins was accelerated (Fig. 4). To evaluate Chl biosynthesis in the acclimated cells, we analyzed this metabolic pathway in detail. First, using a set of specific antibodies, we estimated levels of enzymes involved in the synthesis of heme and Chl in the cells acclimated to the particular light intensities by western blot (Fig. 5; see Supplemental Fig. S1 for a scheme of the tetrapyrrole pathway). Total levels of almost all enzymes, including the heme-producing ferrochelatase, were upregulated by light and light also induced association of enzymes with membranes. The enzymes reached the highest level at HL1 but further increase in irradiance to HL2 resulted in a drop of certain enzymes/enzyme subunits (Mg-protoporphyrin monomethyl ester cyclase, light-dependent protochlorophyllide oxidoreductase, Gun4, and geranylgeranyl reductase). An interesting exception was the D subunit of Mg-chelatase, which exhibited an opposite mode of regulation with the highest concentration reached under LL. Moreover, only at this light intensity was a large portion of the D subunit of Mg-chelatase protein bound to the membranes.

Although these data suggested that the heme/Chl biosynthetic pathway was upregulated after 24 h of acclimation to increased irradiance, the Chl content per cell started to fall quickly after the light shift and the process finished in 16 h (Fig. 1B). Therefore, the changes in enzyme levels were checked after 2 and 4 h after the shift to HL2 and most of the monitored proteins started to accumulate or at least showed little variation (Fig. 5B). A characteristic feature of Chl biosynthesis up-regulation was an enhanced association of Mg-chelatase subunits H and I with membranes, which was visible after 4 h at HL2 (Fig. 5B). In contrast to the other monitored enzymes, the amount of geranylgeranyl reductase underwent a rapid and significant decline in 2 h after the shift to HL2 and then recovered only very slowly (Fig. 5).

As enzyme levels could not necessarily reflect changes in metabolic flow through the pathway, accumulation of Chl precursors was also quantified. To preserve steady-state levels of precursors, which are probably transient and quickly change during cell handling, we developed a very sensitive method based on HPLC equipped with two fluorescence detectors (see “Material and Methods”). This enabled us to detect very low abundant intermediates of the Chl biosynthesis pathway in extracts from 2-mL cell cultures prepared just in several minutes (Fig. 6A). Employing this method, we observed that the growth at increased irradiance induced an accumulation

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**Figure 2.** Transmission electron microscopy of the Synechocystis cells. Representative stained ultrathin sections of cells grown photoautotrophically for 24 h under ML (A) and HL2 (B); black arrows indicate glycogen granules.

**Figure 3.** Accumulation of PSI and PSII in Synechocystis cells acclimated to different light intensities. A, Total amount of PSI and PSII detected using specific antibodies against PSII subunits D1 and D2 and PSI subunit PsaD. Membrane proteins corresponding to 100 μL of cells at OD750 = 1.0 were loaded per lane, separated by SDS-PAGE, and blotted. B, Separation of monomers and oligomers of PSI and PSII using CN-PAGE. After separation, the gel was scanned in transmittance mode (Transmit) using a LAS 4000 imager (Fuji), and to better visualize PSI complexes, Chl fluorescence in gel (Chl fluor) was detected after excitation by blue light using the same equipment. Proteins were loaded as in A. Designation of complexes: PSI(1) and PSI(3), Monomer and trimer of PSI, respectively; PSI(1) and PSI(2), monomer and dimer of PSII, respectively.
of Mg-protoporphyrin monomethyl ester and protochlorophyllide, whereas pools of other Chl precursors appeared to be quite stable (Fig. 6B). Finally, to assess the capacity of the whole tetrapyrrole pathway to produce Chl, we followed incorporation of [14C]Glu into Chl molecules. Briefly, cells were acclimated to tested light conditions for 24 h and then supplemented with 180 μM [14C]Glu for 30 min. Chl (as well as geranylgeranyl-Chl) was immediately extracted by an excess of methanol and converted to Mg-chlorin as described in "Material and Methods." The solution containing Mg-chlorin was separated on a silica thin-layer chromatography (TLC) plate and exposed to an x-ray film. We found that the incorporation of [14C]Glu into Chl is much faster at both HL1 and HL2 than at ML; in contrast, the labeling at LL was very weak (Fig. 6C; see Supplemental Fig. S5 for a color version). Together, these data suggest that the acclimation of the Synechocystis cells to HL results in a great up-regulation of Chl biosynthesis despite the total relative decline in the cellular level of Chl.

Distribution of a Newly Synthesized Chl into Chl-Proteins and Complexes

Formation of an early precursor ALA is accepted as a critical check point controlling the metabolic flow through the whole tetrapyrrole pathway (Czarnecki and Grimm, 2012). Labeling of Chl by [14C]Glu then ensures that the total capacity of the pathway can be assessed involving the ALA barrier as well as all other regulatory steps located down the pathway (Supplemental Fig. S1). Moreover, as Glu is also used for protein synthesis, radiolabeling with [14C]Glu enabled us to assess the approximate portions of Glu incorporated into the proteins and Chl. Membrane proteins were isolated from HL2 cells, separated by two-dimensional CN/SDS-PAGE and exposed to an x-ray film. Chl migrates on the SDS gel faster than proteins and can be easily distinguished as spots on the bottom of the gel, below the edge of proteins, designated by the arrowheads in Figure 7. We found that the majority of the metabolized [14C]Glu was used for the synthesis of Chl. Moreover, even at HL2, most of the labeled Chl was directed to the PSI trimer (Fig. 7A). On the protein side, the strongest radioactive signal was localized in the D1 protein and much less in other PSII subunits; higher molecular mass labeled proteins were probably core subunits of PSI and ATP synthase (Fig. 7A).

To trace the fate of Chl in ML- and HL2-acclimated cells more precisely, we labeled Chl using [14C]ALA, and this allowed us to detect new Chl directly in the protein complexes visualized by CN-PAGE. After a 30-min pulse, the Chl labeling in PSII was apparently enhanced in HL2. However, flux of the newly made Chl into the trimeric PSI dominated under both conditions with additional enhancement at HL2 when compared with ML (Fig. 7B). This result contrasted to the very weak [35S] labeling of PSI trimer when compared with PSII monomer/dimer and, intriguingly, also to PSI monomer (Fig. 4A), suggesting a specific channeling of labeled Chl into the PSI trimer. However, one has to be careful to assess Chl distribution just according to intensity of Chl labeling in individual PSI/PSII complexes. One PSI trimer contains 288 Chl molecules (Jordan et al., 2001), which is about 4 times more than the sum of Chls and pheophytins in a PSII dimer (Umena et al., 2011) and about 8 times more than number of Chls and pheophytins in a PSII monomer. We used the same methodology as for the [35S] labeling to quantify the distribution of labeled Chl in PSI and PSII complexes in cells acclimated to HL2 and labeled by [14C]ALA (Table II). As expected, the majority (58%) of Chl was located in trimeric PSI, while only 18% in PSI monomer and 24% in PSII complexes. To take into account different protein/Chl ratios in each complex, we calculated what portion of labeled...
Chlorophyll would theoretically be present in each PSI/PSII complex in the case that the protein labeling corresponds to Chl labeling and new Chls are evenly distributed to individual complexes. Less than 10% of labeled Chl should be bound to PSI trimers due to the very weak synthesis of this complex (Table II). The fact that 6 times more of the new Chl is bound to PSI trimer than expected from protein labeling demonstrates a crucial role of a precise distribution of de novo and recycled Chl in the biogenesis of photosystems.

**DISCUSSION**

Growth of cyanobacteria under high irradiance induces a fast decrease in harvesting capacity by reducing the number and size of phycobilisomes and abundance of PSI in the thylakoid membranes. However, the total excitation pressure is not the only factor controlling changes in the harvesting capacity. Although an adjustment of PSI and phycobilisome content is characteristic for light acclimation, a regulation of antenna size appears to be initiated by any redox imbalance of the electron transport chain (Wallner et al., 2012), and the actual abundance/ratio of photosynthetic complexes then results from the combined effects of light intensity and quality, temperature, or nutrient availability (Murakami and Fujita, 1993; Murakami et al., 1997; Miskiewicz et al., 2002). Light acclimation can be viewed as a set of regulatory events activated to restore a redox equilibrium in the cell. Indeed, a defect in the mechanism balancing the excitation of PSI and PSII, like the one caused by the pmgA mutation, generates a detrimental redox poise (Sonoike et al., 2001) and presumably locks the mutant cell in a perpetual unsuccessful effort to achieve the redox equilibrium.

The cytochrome b$_6$f complex was shown to serve as a redox sensor, triggering acclimatory machinery in cyanobacteria (Murakami and Fujita, 1993), though it remains unknown how this signal is transduced and processed in the cell. Undoubtedly, the tetrapyrrrole pathway is an important target of this regulation. In cyanobacteria, the main flow of tetrapyrrrole biosynthesis intermediates is directed to the synthesis of Chl and phycobilins that serve as the main light-harvesting pigments bound to the PSII-associated phycobilisome antenna. During light acclimation, Chl and intermediates of its biosynthesis deserve special care due to their phototoxic nature and high concentration of Chl in the cell. A tight coordination between synthesis of Chl and Chl apoproteins is expected to be crucial for the viability of the photosynthetic cell as formation of a pool of unquenched Chl or its intermediates would generate harmful reactive oxygen species.

The PSI trimer is the site where the majority of Chl is placed in the cyanobacterial cell, and as we show in this work, this complex is almost an exclusive sink for de novo Chl (Fig. 7B). For the reasons described above, a relatively fast down-regulation of the PSI level during the acclimation to HL has to be reflected by a regulation of Chl biosynthesis or/and Chl trafficking in the cell. A relation between the regulation of the PSI level and Chl biosynthesis is likely to be even more intimate since de novo Chl appears to be a rate-limiting factor for the translation of the PSI core subunits (Eichacker et al., 1996). It is expected that the PSI level is controlled via the regulation of Chl synthesis; thereby, the Chl limitation is the key factor causing the...
decrease in PSI level at HL (Fujita et al., 1990; Muramatsu et al., 2009). The proposed mechanism is based on an assumption that a surplus of PsaA/B proteins is continuously translated but finally degraded due to a shortage in Chl. Although it seems to be a waste of energy, it might also provide a crucial function to ensure that there is always a place where Chl molecules can be safely incorporated even when synthesized in excess. Moreover, it is obvious from Figure 2, that Synechocystis cells are packed with glycogen when grown at HL2; thus, some energy can be easily sacrificed for a tight control over Chl-(protein) biosynthesis.

Although there is compelling evidence that both the transcription rate and stability of the psaA/B transcript are carefully regulated during light acclimation (Hihara et al., 2001; Herranen et al., 2005), the abundance of the psaA/B transcript does not appear to limit PSI synthesis consistently with the proposed regulatory role of Chl. In an elegant experiment Muramatsu et al. (2009) demonstrated that a Synechocystis strain engineered to have a constantly upregulated psaA/B transcript was still able to reduce the PSI level under HL.

Results so far published on an interplay between the Chl biosynthesis and the PSI/PSII stoichiometry concern a time period spanning up to 16 h after the shift to...
Quantification of proteins and Chl labeling in individual PSI and PSII complexes

The same Synechocystis cell culture acclimated to HL2 was labeled separately by \( ^{[15}S\text{Met}/\text{Cys} \) mixture or by \( ^{14}\text{C}\text{ALA} \) for 30 min and isolated cell membranes separated as a dilution series by CN-PAGE (see Figs. 4 and 7; Supplemental Fig. S6). After exposure of gels in a phosphor imager, bands of individual complexes were quantified using ImageQuant TL 7.0 software (GE Healthcare).

Complex | Protein Labeling \(^{[15}\text{S}\text{Met+Cys}\) | Normalized Labeling \(^{[15}\text{S}\text{Met+Cys}\) | Chl Labeling \(^{14}\text{C}\text{ALA}\) | Normalized Labeling \(^{14}\text{C}\text{ALA}\) | Relative Values
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<tr>
<td>PSI trimer</td>
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<td>(0.21 \times 10^6)</td>
<td>26.3</td>
<td>(29.44 \times 10^6)</td>
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<td>PSI monomer</td>
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<td>(5.78 \times 10^6)</td>
<td>17.5</td>
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aAbsolute values of protein and Chl labeling in PSI and PSII complexes obtained using ImageQuant software and a calibration curve for each complex (Supplemental Fig. S6). b\(^{[15}\text{S}\text{Met+Cys}\) labeling normalized to a total number of Met and Cys in D1, D2, CP43, and CP47 core proteins for PSII complexes and PsaA and PsaB core proteins for PSI. Although synthesis rates of individual PSI core proteins are different (see Fig. 5), all four proteins contain a similar number of Met and Cys and then their different syntheses can be neglected. cActual synthesis of PSI/PSII complexes expressed in relative values. dExpected Chl labeling simulates a situation when labeled Chls are evenly distributed to individual complexes synthesized in rates determined by \(^{[15}\text{S}\text{Met+Cys}\) labeling. Calculation is based on known number of Chl molecules bound to each complex (see text).
Synthesis of Chlorophyll-Binding Proteins under High Light

binding proteins called HLIPs that are almost absent under LL intensities but very quickly accumulate upon exposure of cells to various stress conditions, including HL (He et al., 2001; see also below).

Based on our data, we propose that the synthesis of PSII subunits does not rely on the availability of the de novo Chl to such an extent as the trimeric PSI (Fig. 7B); thus, the synthesis of the most light-sensitive D1 subunit can be accelerated even when the availability of de novo Chl is limited during the initial phase of the HL acclimation. After this initial emergency phase, the photoprotective mechanisms start to work and in 6 h of the HL2 acclimation the $F_r/F_m$ returns to the value observed under ML (Supplemental Fig. S4). At that particular moment, cells were already growing rapidly (J. Kopecka and R. Sobotka, unpublished data); thus, the rate of Chl formation has to be boosted to prevent an excessive loss of the thylakoid membranes. The adjustment of the PSI level and the level of phycochlorisomes in proceeding approximately 10 h is probably based on a sophisticated regulatory network seeking for a balance among the redox state, Chl availability, growth rate, antenna size, and probably a number of other parameters. The two different phases of the HL acclimation are in line with the phenotype of the $pmgA$ mutant; there is no change in the PSI level during the first phase, but in the second phase the PSI accumulates more than it should be (Hihara et al., 1998), indicating that due to the $pmgA$ mutation, more Chl than necessary is directed to PSI.

Once the new equilibrium under HL is achieved, the levels of photosystems and phycochlorisomes remain constant when the culture is kept at a similar (low) optical density that prevents shading (Fig. 1B; 16 and 24 h). As discussed above, the HL2-acclimated cells have to up-regulate Chl production to support fast growth. Labeling using $[14C]$Glu or $[14C]$ALA demonstrated that the main sink for the newly synthesized Chl is the trimeric PSI, a stable, Chl-rich building block of the thylakoid membranes. On the other hand, our study confirmed enhanced synthesis of PSII subunits in comparison with PSI proteins at all studied irradiances (Fig. 4). The HL sensitivity of the PSII complex to the light-induced damage necessitates a higher turnover rate of its protein subunits (especially the D1 protein) than subunits of PSI (Yao et al., 2012b). A significant disproportion between strong protein labeling versus weak Chl labeling in PSII complexes shows that the resynthesis of PSII Chl-proteins largely occurs at the expense of the recycled Chl previously released from degraded Chl-proteins. PSII complexes are completely replaced by new ones in less than a day, but the lifetime of total Chl in Synechocystis cells is much longer (see above; Vavilin et al., 2005; Yao et al., 2012a). Moreover, Xu et al. (2004) suggested that the preexisting Chl molecules in a periphery of PSI could be released and redistributed for PSII biosynthesis in the etiulating cyanobacterial cells. The exact mechanism of the Chl recycling process is not known; nevertheless, it seems to include a dissociation of the phytol chain and the chlorophyllide ring (Vavilin and Vermaas, 2007) upon PSII protein degradation and their subsequent reassociation before or during their reuse in the biogenesis of new PSII Chl-proteins (Vavilin et al., 2005; Vavilin and Vermaas, 2007). Nonetheless, despite the apparent importance of the Chl recycling, there should always be a certain input of newly synthesized Chl that replaces the lost/degraded Chl released from PSII and that also maintains sufficient Chl quantity for the enhanced de novo/repair-related PSII synthesis under the increased irradiance. The labeling of pigments showed that the input of the newly synthesized Chl into the PSI complex is enhanced after the HL2 acclimation (Fig. 7B).

The input of the de novo Chl into PSI synthesis seems to be largely independent on the main flow of the tetrapyrrole intermediates directed to PSI trimer and might represent a separate branch of the pathway. We speculate that Chl biosynthesis occurs in Chl biosynthesis centers, which contain some common general components, such as Chl biosynthesis enzymes, but may differ in regulatory proteins that are specific for individual Chl proteins (Komenda et al., 2012). In this regard, it is intriguing that the monomeric PSI is synthesized faster than the trimeric PSI but contains much less labeled Chl (Figs. 4A and 7B; Table II). This indicates that cyanobacteria possess a portion of PSI serving perhaps a specific function, which is not assembled into a trimer, has a faster turnover, and is synthesized utilizing recycled Chl. Such separated biogenesis of PSI complexes would be consistent with our idea of protein/complex-specific Chl biosynthesis centers in the cell. The small membrane proteins called HLIPs (also named small chlorophyll $a/b$ binding proteins [SCPs]) possessing the conserved Chl $a/b$ binding motif are promising candidates for factors controlling a precise distribution of Chl into apoproteins. HLIPs seem to play a crucial role in Chl recycling. In Synechocystis HLIP-less mutants, the half-life of Chl molecules is not affected under LL; however, under HL2, Chl is degraded much faster in the mutant than in the wild type (Vavilin et al., 2007). HLIPs also somehow modulate early steps of the Chl biosynthesis (Xu et al., 2002; Yao et al., 2012a) and physically interact with PSI assembly intermediates. Furthermore, cyanobacterial and plastidic ferrochelatase enzymes are fused at the C terminus with a HLIP protein forming the so-called CAB domain. A deletion of this domain in Synechocystis has no effect on the ferrochelatase activity or stability, but the resulting mutant accumulates significantly higher Chl level under HL (Sobotka et al., 2011).

In higher plants, particularly in matured leaves, the regulation of the harvesting capacity and the PSI/PSII ratio has to differ from cyanobacteria as plants do not contain phycochlorisomes and the dilution of the thylakoid membranes by a slowly dividing chloroplast is minimal compared with fast-growing cyanobacteria. Interestingly, Chl labeling in Arabidopsis ($Arabidopsis thaliana$) was found to be accelerated after a long-term acclimation
to HL (Beisel et al., 2010), which implies that light up-regulates the Chl formation in both plants and cyanobacteria. However, in another radiolabeling experiment carried out on HL-exposed rye (Secale cereale) chloroplasts, de novo Chl was localized predominantly in PSII, much less in PSI or in light-harvesting antennas (Feierabend and Dehne, 1996). Thus, opposed to cyanobacteria, most of the Chl produced in plant chloroplasts is probably used to support the PSI repair cycle since the need for synthesis of the new PSI complexes is very limited. Nonetheless, since the tetrapyrrole biosynthesis and the structure of the photosynthetic apparatus is so well conserved in both cyanobacteria and chloroplasts, we expect that, in principle, molecular mechanisms harmonizing the synthesis of Chl and photosystem subunits are shared generally by all oxygenic phototrophs.

**MATERIALS AND METHODS**

**Growth Conditions**

A nonmottled, Glo-tolerant strain of Synechocystis (Synechocystis sp. PCC 6803; Williams, 1988) obtained from the laboratory of Peter J. Nixon (Imperial College, London) was grown photoautotrophically in BG11 medium (Rippka et al., 1979). Sixty milliliters of a liquid culture was grown at 28°C in a rotating 250-mL Erlenmeyer flask in a growing chamber under continuous illumination of 40 μE m⁻² s⁻¹ (ML). For described experiments, cells grown at ML were diluted to OD₇₅₀ = 0.25 and shifted to 10 μE m⁻² s⁻¹ (LL), diluted to OD₇₅₀ = 0.1 and shifted to 150 μE m⁻² s⁻¹ (HL1), or diluted to OD₇₅₀ = 0.06 and shifted to 300 μE m⁻² s⁻¹ (HL2). After 24 h, cells were harvested in an exponential growth phase (OD₇₅₀ = approximately 0.3). In all experiments,illumination was provided by cool-white fluorescent tubes (Osram).

**Absorption Spectra and Determination of Chl Content**

Absorption spectra of whole cells were measured at room temperature with a UV-3000 spectrophotometer (Shimadzu). Chl was extracted from cell pellets using 20 mM TES to a 10-mL glass tubes for 30 min at 30°C at the same light conditions as acclimated (2 mL, OD₇₅₀ = approximately 0.3) with 100% (v/v) methanol, and its concentration was measured spectrophotometrically according to Porra et al. (1989).

**Radioactive Labeling of Proteins and Preparations of Cell Membranes**

Cells (50 μg of Chl) in an exponential growth phase (OD₇₅₀ = approximately 0.3) were harvested by centrifugation, washed, and resuspended in fresh BG11 with 20 mM MES to a final volume 475 μL. The cell suspension was shaken in 10-mL glass tubes for 30 min at 30°C at the same light conditions as acclimated before for 24 h. [³⁵S]Met/Cys mixture (>1,000 Ci/mmol; Amersham Biosciences) was then added with the final activity of 500 μCi mL⁻¹, and illumination was continued for another 30 min. After this time period, cells were immediately frozen in liquid nitrogen. To prepare cyanobacterial membranes and cytosolic products, harvested cells were washed, resuspended in the thylakoid buffer containing 25 mM MES/NaOH, pH 6.5, 5 mM CaCl₂, 10 mM MgCl₂, and 20% glycerol, and broken using glass beads. The broken cells were pelleted (20,000g, 15 min). The supernatant represented the soluble fraction, while the sediment was resuspended in the excess volume of the thylakoid buffer, pelleted, and resuspended again in the thylakoid buffer to obtain the membrane fraction.

**Electrophoresis and Immunoblotting**

Analysis of membrane proteins under native conditions was performed by CN-PAGE as described by Wittig and Schägger (2008). The isolated membranes were resuspended in 25 mM MES/NaOH, pH 6.5, containing 5 mM CaCl₂, 10 mM MgCl₂, and 20% glycerol. The membranes were then solubilized in 1% n-dodecyl-β-maltoside and analyzed at 4°C in a 4% to 14% or 4% to 12% polyacrylamide gel. Individual proteins in membrane complexes were resolved in the second dimension by SDS-PAGE in a 12% to 20% linear gradient polyacrylamide gel containing 7 M urea (Sobotka et al., 2008). One-dimensional SDS-PAGE and immunodetection was carried out as described by Sobotka et al. (2008). The primary antibodies against subunits of Mg-chelatase and against Mg-protoporphyrin methyltransferase, light-dependent protolchorphyllide oxidoreductase, and geranylgeranyldiol reductase were kindly provided by Prof. C. Neil Hunter (University of Sheffield), ferrochelatase and Gnd4 antibodies were kindly provided by Prof. Annegret Wilde (Justus-Liebig University, Giessen), and antibody against the plant homolog of the cyclase component Sl11214 was purchased from Agrisera.

**Electron Microscopy**

A small volume of the Synechocystis cells acclimated to ML and HL was concentrated in a scaled 200-μL tip by centrifugation at 5,000 rpm for 5 min. The obtained cell pellets were loaded into a specimen carrier (200-μm deep; Leica), pretreated with 3% lactic in chloroform, and then ultra-rapidly frozen in a high-pressure freezer (Em Pact2; Leica). Samples were freeze-substituted in a solution of 1% tannic acid and 0.5% glutaraldehyde in acetone using an automatic freeze substitution unit (AFS, EM; Leica) as follows: 72 h at −85°C, three times rinsed with acetone for 1 h followed by a change of solution to 1% osmium/acetone, 4 h at −85°C, 5 h warming up to −25°C, 12 h at −25°C, and 6 h warming up to 4°C. Samples were then rinsed for 5 min with medium carriers and rinsed three times in acetone at room temperature. Resin infiltration was done stepwise with 20%, 25%, 33%, 50%, 66%, and 80% steps with low viscosity Spurr’s resin in acetone (SPI Supplies) for 3 h each. After infiltration with 100% resin overnight, the samples were polymerized for 48 h at 60°C. Ultrathin sections were cut on a ultramicrotome (UCT; Leica), collected on copper grids with a formvar coating, and stained with uranyl acetate for 5 min followed by lead citrate for 1 min. Sections were examined in a transmission electron microscope (JEOL 1010) equipped with a Mega View III camera (SIS).

**Chl Radiolabeling, Extraction, and Detection Using TLC**

The procedure of labeling Chl was identical to the protein labeling except that 180 μl of [³⁵C]Clu or [³⁴C]ALA (specific activity approximately 55 mCi/mmol; American Radiolabeled Chemicals) was used instead of [³⁵S]Met/Cys. Chl was extracted from pelleted cells using 750 μL of methanol/0.2% NH₄OH (v/v), cell debris pellets by centrifugation, and supernatant collected. This step was repeated and supernatants combined, and NaCl was added to the final concentration of 100 mM. This solution was mixed with 400 μL of hexane and the upper phase taken. The hexane extraction was repeated three times, combined, and completely evaporated in a SpeedVac (Eppendorf). The pellet was resuspended in 0.3% KOH/methanol (v/v) and incubated at room temperature for 15 min to convert Chl to phytol-less Mic chlorophyll. The suspension was then washed three times with 200 μL of hexane, concentrated by evaporation to 50 μL, and washed with 100 μL of petroleum ether (boiling range 45°C to 60°C). The solution of Mgchlorophyll was dried by evaporation in a SpeedVac, resuspended in 30 μL of methanol/chloroform (1:1, v/v), and loaded on a TLC plate (SIL G-25; Macherey-Nagel). The mobile phase used was methanol/10 ml Na₂HPO₄ pH 6.8 (3:1, v/v); the dried TLC plate was exposed to an x-ray film for 5 d.

**Quantification of Chl Precursors**

For quantitative determination of Chl precursors in the cells, 2 mL of culture at OD₇₅₀= 0.35 was harvested. Pigments were extracted with 100 μL of 70% methanol, the sample was centrifuged, and the supernatant containing the extracted pigments was collected. The pellet was then extracted again using 100 μL of 80% methanol. Supernatants were combined and immediately separated by HPLC (Agilent 1100) on a reverse-phase column (Nova-Pak, C18, 4-mm particle size, 3.9 × 150 mm; Waters) using 30% methanol in 1 M ammonium acetate, pH 6.7, and 100% methanol as solvents A and B, respectively. Porphytrins were eluted with a linear gradient of the solvent B (65%–74% in 20 min) at a flow rate of 0.9 mL min⁻¹ at 40°C. Porphytrins were detected by two fluorescence detectors. The first fluorescence detector was set to 435/675 nm (excitation/emission wavelengths) for 0 to 11 min, 435/640 nm for 11 to 14 min, and 400/640 nm from 14 to 25 min, and the second fluorescence detector was set at 416/595 nm throughout the experiment. For retention times of individual precursors, see Figure 5A.

**Supplemental Data**

The following materials are available in the online version of this article.

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Supplemental Figure S1. A scheme of the tetrapyrrole biosynthetic pathway in cyanobacteria.

Supplemental Figure S2. Levels of major carotenoids and chlorophyll determined in Synechocystis cells acclimated to LL, ML, and HL2.

Supplemental Figure S3. Whole-cell spectra of Synechocystis wild-type cells grown for 24 h at HL2 (300 μE m⁻² s⁻¹) and at 600 μE m⁻² s⁻¹.

Supplemental Figure S4. Time course of changes in Fm/Fm’ ratios following shift of Synechocystis cells from ML (40 μE m⁻² s⁻¹) to HL2 (300 μE m⁻² s⁻¹).

Supplemental Figure S5. Color dilution of [14C]-labeled Mg-chlorin separated on a TLC plate.

Supplemental Figure S6. A dilution series of [35S]-labeled membrane separations by CN-electrophoresis and a calibration curve used for quantification of labeled PSII(2) complex.

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