The Proteome and Lipidome of *Synechocystis* sp. PCC 6803 Cells Grown under Light-Activated Heterotrophic Conditions*

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Cyanobacteria are photoautotrophic prokaryotes with a plant-like photosynthetic machinery. Because of their short generation times, the ease of their genetic manipulation, and the limited size of their genome and proteome, cyanobacteria are popular model organisms for photosynthetic research. Although the principal mechanisms of photosynthesis are well-known, much less is known about the biogenesis of the thylakoid membrane, hosting the components of the photosynthetic, and respiratory electron transport chain in cyanobacteria. Here we present a detailed proteome analysis of the important model and host organism *Synechocystis* sp. PCC 6803 under light-activated heterotrophic growth conditions. Because of the mechanistic importance and severe changes in thylakoid membrane morphology under light-activated heterotrophic growth conditions, a focus was put on the analysis of the membrane proteome, which was supported by a targeted lipidome analysis. In total, 1528 proteins (24.5% membrane integral) were identified in our analysis. For 641 of these proteins quantitative information was obtained by spectral counting. Prominent changes were observed for proteins associated with oxidative stress response and protein folding. Because of the heterotrophic growth conditions, also proteins involved in carbon metabolism and C/N-balance were severely affected. Although intracellular thylakoid membranes were significantly reduced, only minor changes were observed in their protein composition. The increased proportion of the membrane-stabilizing sulfoquinovosyl diacyl lipids found in the lipidome analysis, as well as the increased content of lipids with more saturated acyl chains, are clear indications for a coordinated synthesis of proteins and lipids, resulting in stabilization of intracellular thylakoid membranes under stress conditions. *Molecular & Cellular Proteomics* 14: 10.1074/mcp.M114.042382, 572–584, 2015.

Cyanobacteria are a widespread group of photoautotrophic organisms, which significantly contribute to global carbon fixation. Cyanobacteria and plant chloroplasts share a common ancestor, and thus cyanobacteria have a plant-like photosynthetic metabolism (1, 2). Consequently, they are established model organisms for studies, aiming to elucidate photosynthetic mechanisms. Both, chloroplasts and cyanobacteria, have two internal membrane systems, that is, the inner envelope and the cytoplasmic membrane (CM) in chloroplasts or cyanobacteria, respectively, as well as the thylakoid membrane (TM) system, which harbors the complexes of the photosynthetic electron transfer chain (3, 4). The photosynthetic electron transfer chain typically consists of the three membrane integral protein complexes: photosystem I (PS I), photosystem II (PS II), and the cytochrome b_{6f} complex, as well as of the soluble electron carriers plastoquinone and plastocyanin (5, 6). In the end, reduction equivalents are produced, which are used for CO_{2}-fixation (7). However, besides the ability to grow photoautotrophically, some cyanobacteria are also capable to grow photoheterotrophically, where they use reduced organic compounds as carbon source, or even completely heterotrophically by using reduced organic compounds as carbon and energy source (8). The well-characterized cyanobacterium *Synechocystis* sp. PCC 6803 (9) can grow in darkness under light-activated heterotrophic growth (LAHG) conditions by using glucose as carbon and energy source (10). Enhanced sugar catabolism in LAHG cultures is, for example, reflected by increased activities of enzymes involved in sugar catabolism, such as glucokinase and pyruvate kinase (11). The effects of

1 The abbreviations used are: CM, cytoplasmic membrane; DGDG, Digalactosyldiacylglycerol; LAHG, Light-activated heterotrophic growth; MGDG, Monogalactosyldiacylglycerol; NSAF, Normalized spectral abundance factor; PG, Phosphatidylglycerol; PS I, Photosystem I; PS II, Photosystem II; ROS, reactive oxygen species; SQDG, Sulfoquinovosyldiacylglycerol; SRM, selected reaction monitoring; TM, thylakoid membrane.
LAHG conditions on the abundance of soluble *Synechocystis* proteins have been analyzed previously, although only 23 proteins with a significantly altered expression level (LAHG versus autotrophic growth) have been described. This study has e.g. indicated that under LAHG conditions glucose is mainly degraded by the oxidative pentose phosphate (OPP) pathway (12). The histidine kinase 8 (Hik8) as well as the sigma factor E (SigE), regulating the expression of sugar-degrading genes, were shown to be essential for LAHG (13, 14).

Although readjustments of the cellular energy metabolism are important, the impact on the cellular membrane architecture is more striking. The ability of *Synechocystis* to grow under LAHG conditions has been used recently to analyze TM formation within cyanobacterial cells (15). Although dark-adapted *Synechocystis* cells have no active PS II complex, complete photosynthetic activity is regained within 24 h after transferring dark-adapted cells into the light. Furthermore, reappearance of photosynthetic electron transfer processes is coupled to the formation of internal TMs. However, it is essentially still completely enigmatic how the formation of internal TM is controlled, although some proteins have been suggested to be involved. These proteins include the vesicle inducing protein in plastids 1 (Vipp1), DnaK proteins, a prohibitin-like protein, as well as the YidC protein, a membrane protein integrase (16–19). Nevertheless, although some proteins have been suggested to be more directly involved in TM formation, the stability of the TM is also globally affected indirectly by pathways, which control the biogenesis of lipids and/or cofactors, and mutants defective in synthesis of chlorophyll or of the membrane lipid phosphatidylglycerol (PG) have severely reduced TM systems (20, 21).

In the present work, we combined prefractioning of *Synechocystis* cellular membranes with a global proteome and lipidome analysis, to shift the analytical focus toward the rearrangement of the internal thylakoid membrane system observed in *Synechocystis* cells under LAHG conditions, with a significantly larger coverage of the proteome than in former studies. Furthermore, also the effect on *Synechocystis* lipids was analyzed in a targeted mass spectrometric approach, revealing significant adjustment of fatty acid saturation in response to the LAHG conditions.

**EXPERIMENTAL PROCEDURES**

**Growth Condition**—A glucose-tolerant *Synechocystis* sp. PCC 6803 wild-type strain was cultivated photo-mixotrophically in liquid BG11 media (1) supplemented with 10 mm glucose. Erlenmeyer flasks were incubated at 30 °C on an orbital shaker (150 rpm) under fluorescent white light at a light intensity of 30 μmol photons m−2 s−1. For LAHG cultures, light-grown cells were diluted to an OD750 of about 0.2 and grown in the dark in BG11 media supplemented with 60 mm glucose in Erlenmeyer flasks on an orbital shaker at 150 rpm. Cultures were illuminated with white light (10 μmol photons m−2 s−1) for 15 min per day (10). When LAHG cultures had reached an OD750 of one or above, cultures were diluted to OD750 = 0.2 in fresh medium. LAHG cultures were grown in the dark for at least 2 weeks, during which cultures were diluted at least five times in fresh medium.

**LC-MS Data Analysis**—The LC-MS/MS data were analyzed using the Sequest algorithm (27) implemented in the proteome discoverer 1.3 software (Thermo Scientific). For peptide identification, spectra were matched to a database derived from the genome sequence of *Synechocystis* (28, 29), containing 3661 protein sequences. Tryptic was selected as the specified enzyme, and maximal two missed cleavages were permitted. The precursor ion mass tolerance was set to 10 ppm and the fragment ion mass tolerance to 0.8 Da. Percolator, implemented in proteome discoverer 1.3 was used to estimate confidence of peptide identification and peptides with false discovery rates bigger than 5% (q-value > 0.05) were discarded (30, 31).

Mean and standard deviation were calculated for all technical and biological replicates. Proteins were quantified using spectral counting (32) and normalized spectral abundance factors (NSAF) (33, 34) when at least eight peptides per protein were identified. The NSAF takes into account the protein length (L), which has an impact on the number of spectral counts (Spc) per protein. In the first step, the
The cytosolic and total membrane protein fractions of three independent Synechocystis cultures grown under photonitrophic conditions in the light, and of four independent LAHG cultures, were isolated, digested with trypsin and analyzed in an untargeted mass spectrometric approach, using at least three technical replicates per biological replicate. Prior to trypptic digest, protein samples were purified and concentrated by focusing the protein samples in an SDS gel as one single, focused band per sample between the stacking and separating gel to remove particulate matter and matrix contaminants (Fig. 2A, supplemental Fig. S1). Following LC-MS
Analysis, individual peptides were identified using the Sequest algorithm (27), and filter criteria were set to achieve a false positive rate of less than 5%. In total, the analysis resulted in the identification of 1528 proteins (supplemental Table S1). With 374 identified membrane integral proteins, a proportion of 24.5% was obtained, which is close to the 25% of membrane proteins predicted to be encoded in the *Synechocystis* genome (28). The distribution of mass and pI, as well as mass and hydrophobicity of the identified proteins indicated no apparent bias, when compared with the complete set of proteins annotated in the *Synechocystis* genome.

For relative quantification, spectral counting (32) and the concept of normalized spectral abundance factors (33, 34) were applied. The coefficient of variation (CV) declined strongly with the number of identified peptides per protein, as it is generally observed for MS-based label free quantification approaches (Fig. 3A). For quantifications based on eight or more identified peptides per protein, the CV declined to about

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**Fig. 2.** *Proteome analysis of Synechocystis cells grown under LAHG and mixotrophic conditions.* A, Workflow of the proteome analysis. After cultivation (i) and breakage by glass beads, membrane and soluble protein fraction are obtained by ultracentrifugation (ii). For analysis, proteins are concentrated and separated from lipids and particles by in-gel focusing (iii). Samples are loaded on a SDS gel. Electrophoresis is stopped when migration front reaches interface between stacking and separating gel. The focusing process is followed by a prestained protein marker. For each sample, all proteins are focused as a single band at the stacking/separating gel interface (supplemental Fig. S1). These bands are subjected to a tryptic in-gel digest and LC-MS/MS analysis. B, Number of proteins identified for both culture conditions. C, Mass, isoelectric point and hydrophobicity of the proteins identified during the analysis and all proteins annotated in the *Synechocystis* genome.
30% and did not decrease further with rising number of identified peptides. Requiring a minimum of eight identified peptides per protein as a threshold, 641 proteins were finally quantified in our analysis (supplemental Table S2). The correlation between the p value and the fold-change between LAHG and mixotrophic growth conditions is shown in a volcano plot in Fig. 3B and 3C for the cytosolic and membrane fraction, respectively. Based on a t test significance level of 95% and at least twofold regulation, 261 proteins have altered cellular concentrations, with 188 proteins being more and 73 proteins being less abundant under LAHG conditions (supplemental Table S2).

Metabolic Pathways Associated with High Glucose and C/N-balance—On the level of individual proteins (Table I), the glycogen synthase GlgA (Sll1393 and Sll0945) was one of the
### Table I

Selected proteins quantified in the membrane fractions of mixotrophic and LAHG cultures (see supplemental Table S2 for complete list of all quantified proteins)

| DBID* | Name | Mixotrophic %b | LAHG %b | Fold changec |
|-------|------|----------------|---------|--------------|
| sll1393 | Glycogen starch synthase G IgA | n.d. | 0.079 ± 0.019 | — |
| sll1045 | Glycogen synthase G IgA | 0.002 ± 0.004 | 0.028 ± 0.009 | 13.4 |
| sll1585 | 1.4-alpha-glucan branching enzyme gi gB | 0.009 ± 0.004 | 0.073 ± 0.026 | 8.1 |
| slr1176 | Glucose-1-phosphate dehydrogenase | 0.015 ± 0.007 | 0.060 ± 0.031 | 4.0 |
| sll0726 | Phosphoglucosaminate | 0.006 ± 0.010 | 0.053 ± 0.016 | 9.3 |
| sll1334 | Phosphoglucosamine synthase | 0.001 ± 0.002 | 0.004 ± 0.007 | 4.1 |
| sll1356 | Glycogen phosphorylase | 0.109 ± 0.020 | 0.024 ± 0.015 | 0.2 |
| sal0707 | Nitrogen regulatory protein P-II glnB | 0.378 ± 0.171 | 0.756 ± 0.150 | 2.0 |
| slr1423 | Global nitrogen regulator NtcA | n.d. | 0.025 ± 0.013 | — |
| sll1450 | Nitrate/nitrite transport system NrtA | 0.093 ± 0.014 | 0.181 ± 0.010 | 2.0 |
| sll1452 | Nitrate/nitrite transport system NtcA | n.d. | 0.023 ± 0.009 | — |
| sll1453 | Nitrate/nitrite transport system NrtD | n.d. | 0.049 ± 0.021 | — |
| slr0447 | ABC-type urea transport system UrtA | 0.299 ± 0.061 | 0.603 ± 0.029 | 2.0 |
| sll0374 | Urea transporter UrtE | n.d. | 0.023 ± 4.94E-05 | — |
| slr1993 | PEA-specific beta-ketothiolase phaA | n.d. | 0.016 ± 0.002 | — |

Proteins involved in photosynthetic and respiratory electron transport

| sll1867 | Photosystem II D1 protein PsbA | 0.201 ± 0.094 | 0.060 ± 0.001 | 0.3 |
| slr0906 | Photosystem II core light harvesting protein PsbB | 0.881 ± 0.113 | 0.140 ± 0.018 | 0.2 |
| sll0051 | Photosystem II CP43 protein PsbC | 0.290 ± 0.046 | 0.194 ± 0.002 | 0.7 |
| sll1084 | Photosystem II reaction center D2 protein PsbD | 0.182 ± 0.025 | 0.093 ± 0.011 | 0.5 |
| ssl2598 | Photosystem II PsbH protein PsbH | 0.252 ± 0.025 | 0.241 ± 0.014 | 1.0 |
| smr0007 | Photosystem II Psbl protein PsbL | 0.305 ± 0.013 | 0.125 ± 0.047 | 0.4 |
| slr1418 | Photosystem II oxygen-evolving complex 23K protein PsbP | 0.013 ± 0.001 | 0.006 ± 0.012 | 0.5 |
| sll1194 | Photosystem II 12 kDa extrinsic protein PsbU | 0.094 ± 0.081 | 0.029 ± 0.012 | 0.3 |
| sll0258 | Cytochrome c550 PsbV | 0.316 ± 0.094 | 0.035 ± 0.007 | 0.1 |

Metabolic pathways associated with high glucose and C/N-balance

| DBID* | Name | Mixotrophic %b | LAHG %b | Fold changec |
|-------|------|----------------|---------|--------------|
| sll1398 | Photosystem II reaction center 13 kDa protein Psb28 | 0.072 ± 0.006 | 0.085 ± 0.007 | 1.2 |
| slr2048 | PratA | 0.005 ± 0.011 | 0.003 ± 0.004 | 0.6 |
| sll0933 | PAM68 | 0.037 ± 0.006 | 0.043 ± 0.006 | 1.2 |
| slr1645 | Photosystem II 11 kDa protein Psb27 | 0.271 ± 0.093 | 0.126 ± 0.009 | 0.5 |
| sll1414 | Psb29/Thz1 | n.d. | 0.027 ± 0.004 | — |

Membrane translocation

| DBID* | Name | Mixotrophic %b | LAHG %b | Fold changec |
|-------|------|----------------|---------|--------------|
| sll0616 | Preprotein translocase SecA | 0.013 ± 0.004 | 0.003 ± 0.003 | 0.3 |
| sll1814 | Preprotein translocase SecY | 0.022 ± 0.006 | 0.003 ± 0.005 | 0.1 |
| sll3335 | Preprotein translocase SecE | 0.150 ± 0.047 | 0.059 ± 0.002 | 0.4 |
| slr1471 | YidC | 0.043 ± 0.004 | 0.015 ± 3.88E-05 | 0.4 |
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Table I—continued

| DBID  | Name                        | Mixotrophic %  | LAHG %   | Fold change |
|-------|-----------------------------|----------------|----------|-------------|
|       |                             | b              | b        |             |
| sII1987 | Catalase                  | 0.001 ± 0.001  | 0.052 ± 0.015 | 66.3       |
| sII0623 | Thioredoxin (TrxA)        | 0.087 ± 0.064  | 0.201 ± 0.056 | 2.32       |
| sII0276 | 60kD chaperonin GroEL1    | 0.165 ± 0.073  | 0.764 ± 0.043 | 4.64       |
| sII0416 | 60 kDa chaperonin GroEL2   | 0.054 ± 0.035  | 0.224 ± 0.022 | 4.14       |
| sII0058 | DnaK1 (Hsp70)             | n.d.           | 0.022 ± 0.008 | —          |
| sII0170 | DnaK2 (Hsp70)             | 0.048 ± 0.013  | 0.084 ± 0.037 | 2.0        |
| sII0086 | Similar to DnaK protein   | 0.034 ± 0.004  | 0.004 ± 0.003 | 0.1        |

a Identification number assigned during genome sequencing (28).

b Values represent normalized spectral abundance factors (34) averaged over three (mixotrophic) and four (LAHG) biological replicates and the corresponding standard deviation.

c Ratio of the normalized spectral abundance factors for LAHG and mixotrophic fractions.

proteins, which was most strongly enriched under LAHG conditions. Proteins involved in glycogen synthesis were in general more abundant under LAHG conditions. Remarkably, because of the applied ultra-centrifugation conditions, all proteins involved in glycogen turnover were predominantly found in the membrane fraction. Besides the glycogen synthase GlgA, the glycogen branching enzymes GlgB, the glucose activating ADP glucose pyrophosphorylase GlgC, as well as the phosphoglucomutase (SII0726 and SII1334), which provides glucose-1-phosphate for glycogen synthesis (40), were enriched under LAHG conditions. Remarkably, the phosphoglucomutase (SII0726 and SII1334), which provides glucose-1-phosphate for glycogen synthesis (40), was increased by factors of 4–13 in their relative abundance (Table I). In contrast, the abundance of the glycogen phosphorylase GlgP (SII1356), which is involved in glycogen degradation (41), was fourfold decreased under LAHG conditions. Thus, these changes might indicate a shift toward glycogen synthesis and storage under LAHG conditions. However, in Synechocystis the activity of enzymes involved in glycogen synthesis is controlled by photosynthesis via the ferredoxin/thioredoxin system and glycogen is synthesized exclusively in the light (42, 43). In line with this and despite the apparent increase in enzyme abundance, we did not observe enrichment of glycogen granules in the EM micrographs of the dark-grown Synechocystis cells (Fig. 1).

Although marked differences were also observed for proteins involved in the glucose energy metabolism, these differences were self-evidently smaller than the differences observed before, when LAHG cells were compared with Synechocystis cultures grown in complete absence of glucose (12). Several proteins involved in glucose metabolism did not pass the thresholds for quantification. However, as listed in the supplemental Table S1, the cellular content of several glycolytic enzymes was significantly increased under LAHG conditions, most likely caused by the fact that glucose is the only energy source in the dark.

Furthermore, enzymes involved in the NADPH-producing steps of the oxidative pentose phosphate pathway were also significantly increased under LAHG conditions, likely reflecting the need for producing NADPH by this pathway in the dark. The general importance of the oxidative pentose phosphate pathway as a major route of sugar catabolism under heterotrophic growth conditions has been discussed before (44–46). In line with this, the cellular concentration of enzymes involved in the subsequent steps of this pathway remained unaltered when compared with photomixotrophically grown Synechocystis cells. Together, the observed changes of proteins involved in the glucose energy metabolism highlight the need of LAHG cells to utilize glucose as the solely available energy source. The increased need for energy production provided by glycolysis is e.g. further supported by the observation that also the relative abundance of enzymes of the TCA cycle and the upstream pyruvate dehydrogenase reaction were increased. Expression of glucose catabolite genes in Synechocystis is controlled by SigE and the PamA proteins (13), and, in line with this, the PamA protein (SII0985) was only detected under LAHG conditions. The final products of the glycolysis and the subsequent oxidative TCA cycle is 2-oxoglutarate (2-OG), as cyanobacteria do not contain a 2-OG dehydrogenase (47). 2-OG is subsequently used for ammonium fixation, resulting in formation of glutamate. Thus, the 2-OG level directly links the TCA cycle (and the preceding glucose metabolism) to N-assimilation (47). 2-OG is a regulatory metabolite, coordinating the carbon and nitrogen metabolism in cyanobacteria (2). It appears to be likely that this signal transduces the inherent C/N imbalance, caused by the high glucose concentration in the culture media. Normally, 2-OG accumulates under N-starvation, resulting in activation of signaling proteins and in increased expression of proteins involved in N-fixation (48). Two bypasses, the 2-OGDH shunt (47) and the GABA shunt (49), have been suggested recently to close the TCA cycle and to counteract 2-OG accumulation. Although the 2-OG dehydrogenase, an essential component of the 2-OGDH shunt, was not detected under photomixotrophic conditions, all enzymes involved in the GABA shunt were detected, and the N-acetylornithine aminotransferase (SII1022) was significantly increased in LAHG cultures. Increased abundance of proteins involved in N-metabolism was observed although the growth conditions are not N-limiting, most likely because of the increased metabolic flow through...
the glycolysis and TCA cycle and increased production of the regulatory metabolite 2-OG. In fact, PamA functionally interacts with the PII sensor protein, a protein highly conserved in prokaryotes and plant chloroplasts (50), which was also significantly more abundant under LAHG conditions. PII appears to monitor the cellular C- as well as the N-status by binding 2-OG in a cooperative manner together with ATP, and furthermore, phosphorylation might additionally trigger the PII sensory function (51). As interaction of PamA with PII controls expression of genes involved in sugar catabolism (13), this observation nicely rationalizes the observed increased expression of proteins involved in glucose metabolism (compare above). However, PII integrates and simultaneously regulates the energy, carbon, and nitrogen metabolism in cyanobacteria (52). PII interacts with the nitrogen regulator PipX (Ssl0105) as well as with the global nitrogen regulator NtcA (Sll1423) (40). NtcA was among the proteins most strongly increased in their abundance under LAHG conditions. Typically, PipX, which was detected exclusively under LAHG conditions, interacts with NtcA under N-limited growth conditions and controls expression of PII as well as of the glutamine synthetase (GlnN) to facilitate efficient acclimation of cyanobacteria to conditions of nitrogen limitation (53). Although the growth conditions were not N-limiting per se, the N-starvation response was most likely activated in Synechocystis because of access C supply (60 mM glucose in growth medium), possibly sensed as C/N imbalance. In line with an undesignated activation of N-starvation signaling, also the nitrate transporter subunits NrtA, C, and D (Sll1450, Sll1452, and Sll1453), as well as the urea transporter subunits UrtA and UrtE (Sll0447 and Sll0374) were more abundantly expressed in LAHG cells. An overload of the TCA cycle, potentially resulting in accumulation of 2-OG, is also indicated by the observation that LAHG cells produce and accumulate polyhydroxalkanoates (PHA). Production of PHAs enables the cell to store excess acetyl-CoA (provided by glycolysis and PDH reaction). Thus far, the best studied PHA poly-3-hydroxybutyrate is produced in three consecutive steps from acetyl-coenzyme A, and the PHA-specific beta-ketothiolase (phaA), which catalyzes the first step in PHA synthesis (53), was identified only in LAHG cells.

Together, these observed changes in the abundance of enzymes involved in carbon or nitrogen metabolism reflect the metabolic situation that Synechocystis uses solely glucose as an energy source under LAHG conditions. Proteins Involved in Photosynthetic and Respiratory Electron Transport—Alterations of the TM structure are apparent during LAHG conditions. However, although the amount of intracellular membranes was severely reduced (Fig. 1), the changes in protein composition appeared to be minor. Among the protein complexes involved in electron transport in the TMs, photosystem II (PS II) showed the most prominent changes. On average, the 12 quantified subunits of the active PS II were reduced in the dark to ~50% of the level observed in photomixotrophically grown cells (Table I). However, while the amount of the large PS II core subunits (D1, D2, and CP47) was decreased, the amounts of the small subunits and of components relevant for the initial assembly (16, 54) of the protein complex (PsbE, PsbH, PsbY, Psb28, PAM68, and YCF48) remained rather constant and were not reduced during LAHG conditions. This could be perceived as a strategy to keep metabolic costs for protein synthesis and quenching of chlorophyll low, while maintaining the competence to assemble active PS II and adapt to photosynthetic growth conditions quickly. The differential regulation of assembly factors and large subunits was most apparent for the CP47 subunit. The abundance of this subunit was reduced by a factor of five under LAHG conditions, whereas the assembly factors Psb28 (Sll1398) and PAM68 (Sll01933) binding unassembled CP47 (55, 56) as well as the small PS II subunits PsbH and PsbY forming an assembly intermediate with CP47 remained at the levels of light adapted cells. In agreement with this, a similar correlation is observed for D1, PsbE, and the assembly factors Ycf48 (57). Also, Psb29 (Sll1414), a protein involved in early steps of PS II complex assembly (58), was exclusively found in LAHG cells. In contrast, the amount of Psb27, a component mainly involved in the PS II repair cycle (59–61) was decreased twofold in line with the dark growth conditions and the obvious absence of light-stress. In addition to the small subunits and assembly factors involved in the early steps of PS II assembly, proteins involved in chlorophyll supply to PS II, such as the POR and Sll1694 (62), were identified exclusively under LAHG conditions or had also an increased abundance. Thus, while having an inactive PS II (15), Synechocystis cells appear to be prepared to rapidly assemble functional PS II complexes in order to adopt a photoautotrophic lifestyle. However, as dark-adapted Synechocystis cells have no functional PS II and are thus light-sensitive, the potential risk of excitation energy transfer from the light-harvesting phycobilisomes to the photosystems is suppressed by a strong overexpression of the orange carotenoid protein (OCP and Sll1963), a protein, which is typically induced under high-light (63). Its abundance was increased in the LAHG membrane fractions by a factor of 11. An increased OCP accumulation in Synechocystis cells grown under heterotrophic versus photoautotrophic conditions has been reported in a preceding proteome analysis (12), with an OCP accumulation that apparently was also not light-induced. An increased OCP abundance has also been observed in a Synechocystis mutant deficient in both photosystems (64), as well as in the Synechocystis PAL mutant, which is devoid of phycobilisomes (65). Combined, these observations indicate a general rise in abundance of OCP under condition of redox imbalance, which is additionally supported by an increased expression during acclimation to low CO2 growth conditions (66).

The quantity of pigment binding subunits of the light-harvesting phycobilisomes, which are the most abundant proteins in cyanobacteria, had not significantly changed, in line with recent observations (15). However, the abundance of linker
proteins $L_{R}^{33}$ (Sll1580), $L_{R}^{30}$ (Sil1579), and $L_{RC}$ (Sll1471) were dramatically reduced, indicating that the phycobilisomes might not be properly assembled. In line with this assumption, an increased fluorescence emission, caused by uncoupled phycobilisome proteins, was recently observed in LAHG-grown Synechocystis cells upon phycobilisome excitation (15).

The quantitative analysis covered all large subunits of the active PS I complex (Table I). For none of these subunits a significant change was observed, which is in line with the recent observation that LAHG cells have fully functional PS I centers (15). Only the PS I assembly factor BtpA (Sll0634) (67) was increased 18-fold under LAHG conditions, whereas the content of other quantified PS I assembly factors, such as Ycf3 (67) or Ycf37 (68), was not altered. Similarly to PS I, also no significant changes in the amount of cytchrome $b_{6}f$ complex, NADH dehydrogenase, or ATP synthase subunits, all well covered in our analysis, were detected.

Together, while Synechocystis cells cannot use photosynthetic electron transfer reactions under LAHG conditions, we only observed significant changes of proteins somehow linked to PS II, whereas the relative abundance of subunits of other protein complexes involved in energy transfer has not changed. This is in line with former studies, showing decreased PS II activity and full PS I activity in dark-adapted Synechocystis cells (15).

Translation and Membrane Translocation—Ribosomal proteins were significantly less abundant under LAHG conditions. From 50 ribosomal proteins quantified in the analysis, 24 subunits (19 of these in the membrane fraction) showed less than 50% of the abundance detected in cells cultivated under photomixotrophic conditions. This might reflect the significantly reduced growth rate of Synechocystis cells grown under LAHG conditions (10). In bacteria and chloroplasts, unfolded proteins are translocated across a membrane (cytoplasmic or thylakoid membrane) via the Sec pathway (69, 70), whereas folded proteins are translocated via the Tat pathway (69–72). The amount of the Sec translocase subunits SecA (Sll0616), SecY (Sll1814), and SecE (Sll3335) was significantly reduced under LAHG conditions. Furthermore, besides the Sec translocase, the YidC protein (named Alb3 in chloroplasts) mediates integration of membrane integral proteins in bacteria and TM (73). As observed for Sec translocase subunits, also the relative amount of YidC was reduced in LAHG cells. As these two proteins are important for the biogenesis of TM (Vipp1, Sll1580), hydrogen peroxide ($H_{2}O_{2}$), or hydroxyl radicals (OH$^{-}$). The cellular concentration of the superoxide dismutase SodB (Sir1515) has recently been reported to increase during LAHG (38, 76). Here we found that several oxidative stress related proteins, such as superoxide dismutase (Sir1516), catalase (Sir1987), thioredoxin (Str0623), peroxiredoxin (Sil0755), and glutathione peroxidase (gpx2, Sir1992) rose in their abundance under LAHG conditions in the soluble or membrane fraction, respectively (Table I). Also the heat shock protein HtpG, which is important for acclimation to oxidative stress in cyanobacteria (77), was found to be up-regulated, in line with previous observations (76). Furthermore, several other chaperones involved in a general stress response, were more abundant in LAHG cells (Table I). This again reflects that the metabolic changes associated with LAHG conditions imply cellular stress for the cyanobacterial cells. Although the cellular concentration of the Hsp60 proteins GroEL-1 and GroEL-2 did not change when proteomes of cells grown under photomixotrophic versus phototrophic conditions were compared (12), increased abundances of both proteins in dark-adapted cells have also been described in previous studies (38, 76). Noteworthy, although DnaK2 (Sir0170) is the major Hsp70 protein in Synechocystis (19, 78), this protein was not significantly overrepresented in LAHG cells, whereas DnaK1, a nonessential Hsp70 protein with a still enigmatic function (19), was found to be severely overrepresented. Furthermore, the thus far completely uncharacterized DnaK-like chaperone Sir0086 was one of the most significantly down-regulated proteins identified in our proteomic screen. This might indicate a specific function of this protein in the light.

Proteins Suggested being Involved in TM Biogenesis—In the past, some proteins have been suggested to be of special importance for the biogenesis of TMs in cyanobacteria. These proteins include the vesicle inducing protein in plastids 1 (Vipp1, Sll0617), the DnaK chaperone DnaK3 (Sll1933), the YidC/Alb3 protein, as well as the prohibitin-like protein Sir1768 (16–19). Noteworthy, the exact mode of action of any of these proteins still is completely mysterious. The proteins Vipp1 and DnaK3 did not pass the quality control, and thus we did not obtain reliable information on their cellular content. In case of the YidC/Alb3 protein, we observed a reduced cellular level in LAHG cells, as described above. However, reduction of the Synechocystis TM system upon depletion of YidC/Alb3 (74) was caused indirectly, as insertion of proteins into the TM was generally impaired in the generated mutant strain. Thus,
the function of YidC/Alb3 is only indirectly linked to TM biogenesis. In contrast to the gene encoding YidC, the slr1768 gene was completely deleted in *Synechocystis* and the mutant strain displayed defects in TM formation. Because of this, it was concluded that the prohibitin-like protein has an important role in TM formation and/or maintenance (18). In the present analysis, we indeed found that the membrane attached Slr1768 protein was significantly more abundant in the membrane fraction under LAHG conditions, but below the detection limit in the light adapted cultures. Although thylakoid biogenesis certainly depends on multiple factors, it appears as if the dark-adapted *Synechocystis* cells store Slr1768 to immediately generate TMs in the light.

**The *Synechocystis* Lipidome under LAHG Conditions**—In addition to the proteome analysis, we also analyzed changes in the lipidome of LAHG cells. From three independent light-grown cultures and four independent LAHG-grown cultures, lipids were extracted as described in the methods section (Fig. 4). Because of the lower complexity of the lipidome in contrast to the *Synechocystis* proteome, a targeted approach, using selected reaction monitoring, was performed. MGDG and DGDG were analyzed in a positive mode, SQDG and PG in a negative mode, as described (37).

After addition of lipid standards, the absolute concentrations of the lipid classes were quantified (Fig. 5A). In comparison to photoautotrophic grown cells under photomixotrophical conditions, the proportion of MGDG (72%) was significantly increased, whereas the amounts of DGDG (12%) and PG (4%) were reduced (79, 80). The SQDG content (11%) was not affected by the mixotrophic growth conditions. In LAHG cells, the amount of SQDG was increased as compared with cells grown under photomixotrophic conditions (Fig. 5A), whereas the proportions of MGDG, DGDG, and PG remained constant within the experimental error. This is perfectly in line with the observation that the cellular content of the SQDG biosynthesis protein SqqB (Slr1020) was up-regulated in the dark (Table I). As the TMs were severely reduced in LAHG cells, the impact of CM lipids on the totally detected lipid composition was increased. However, in recent analyzes of cyanobacterial membrane lipids, no significant differences have been observed between these two internal membrane systems (81). The major constituent of cyanobacterial membranes (as well as of chloroplast TMs) is MGDG, a nonbilayer forming lipid, which tends to form inverse hexagonal lipid phases, rather than lamellar structures (82). TMs have been shown to only exist because of the presence of membrane integral proteins, which together with the lipids form a membranous structure (83). Thus, when the amount of membrane integrated proteins, such as the photosystems, is reduced, there is a need for bilayer stabilization, e.g. by increasing the content of bilayer forming lipids. SQDG is a bilayer forming lipid, and thus the increased relative abundance of SQDG might simply reflect the need for bilayer stabilizing membrane lipids.

![Fig. 5. Absolute amount of lipid classes A, and relative amount of molecular lipid species of all four lipid classes, B–E, in mixotrophic and LAHG cultures. Prominent lipid species of B; MGDG, C; DGDG, D; SQDG, and E; PG, quantified by SRM. Dark gray bars: mixotrophic cultures, light gray bars: LAHG cultures. Error bars display the standard deviation based on three (mixotrophic) or four (LAHG) biological replicates.](image-url)
For a more detailed analysis, the individual fatty acid species of all lipid classes were quantified, and a general shift toward more saturated fatty acids was observed (Fig. 5B–5E). The fatty acids of MGDG and DGDG lipids were shifted from unsaturated 34:4 and 34:3 species toward more saturated 34:2 and 34:1 ones in the dark (Fig. 5B and C). In case of SQDG, a shift from 34:2 to 34:1 and 34:0 (Fig. 5D) was observed, and for PG a shift from 34:3 to 34:1 and 34:0 was observed under LAHG conditions (Fig. 5E). In total, the cyanobacterial membranes appear to become less fluid, as more saturated lipid species were more abundant under LAHG conditions. However, increased lipid packing in the acyl-chain region again stabilizes the lipid bilayer structure, which appears to be advantageous under LAHG conditions.

Together, the changed lipid composition of LAHG cells might be a consequence of the reduction of TM proteins and protein complexes, which normally stabilize the membranous structure.

**Final Remarks**—Significant effort has been dedicated to characterization of Synechocystis cells cultivated under LAHG conditions, which are characterized by high glucose concentrations in the media and besides short (15 min) illumination periods per day the absence of light during the cultivation. These— for photosynthetic organisms—highly artificial conditions result in severe changes in the cellular membrane structure. Although in the past proteome studies concentrated on the carbon metabolism, cellular membranes were in the focus of proteome and lipidome analysis in this work. Next to a significant influence on proteins involved in C/N-balance and ROS homeostasis, our analysis confirmed that despite reduction of cellular TM content, changes to the photosynthetic machinery are mainly limited to a reduction of PS II complexes (15).

As the TM integrated proteins stabilize the integrity of the TMs and TM structures only exist because of a delicate interplay of proteins and lipids (82, 83), the rearrangement of the TM compositions eventually results in membrane destabilization. Such destabilization might be compensated by the cells via moderate alterations of the membrane lipid composition. Indeed, lipids with increased bilayer stabilizing properties are found under LAHG conditions. Although several proteins have been suggested in the past to be involved in biogenesis of TMs, we expected to see an impact on the cellular content of these proteins when Synechocystis cells were grown in the dark. However, solely the prohibitin-like protein Slr1768 is more prominently expressed under LAHG conditions. Although the function of this protein is still unresolved, our observation together with the results presented in (18) strongly indicate a prominent role of this protein in TM assembly in Synechocystis.

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This article contains supplemental Fig. S1 and Tables S1 to S4.
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