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Biochimica et Biophysica Acta 1708 (2005) 91–101
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
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^{15}N-labeling to determine chlorophyll synthesis and degradation in *Synechocystis* sp. PCC 6803 strains lacking one or both photosystems

Dmitrii Vavilin[a,c], Daniel C. Brune[b,c], Wim Vermaas[a,c,*]

[a]*School of Life Sciences, Arizona State University, Box 874501, Tempe, AZ 85287-4501, USA*
[b]*Department of Chemistry and Biochemistry, Box 871604, USA*
[c]*Center for the Study of Early Events in Photosynthesis, Arizona State University, Tempe, Arizona 85287, USA*

Received 21 September 2004; received in revised form 21 December 2004; accepted 23 December 2004
Available online 12 January 2005

Abstract

Rates of chlorophyll synthesis and degradation were analyzed in *Synechocystis* sp. PCC 6803 wild type and mutants lacking one or both photosystems by labeling cells with $(^{15}NH_4)_2SO_4$ and $Na^{15}NO_3$. Pigments extracted from cells were separated by HPLC and incorporation of the ^{15}N label into porphyrins was subsequently examined by MALDI-TOF mass spectrometry. The life time (τ) of chlorophyll in wild-type *Synechocystis* grown at a light intensity of 100 μmol photons m^{-2} s^{-1} was determined to be about 300 h, much longer than the cell doubling time of about 14 h. Slow chlorophyll degradation (τ ~200–400 h) was also observed in Photosystem I-less and in Photosystem II-less *Synechocystis* mutants, whereas in a mutant lacking both Photosystem I and Photosystem II chlorophyll degradation was accelerated 4–5 fold (τ ~50 h). Chlorophyllide and pheophorbide were identified as intermediates of chlorophyll degradation in the Photosystem I-less/Photosystem II-less mutant. In comparison with the wild type, the chlorophyll synthesis rate was five-fold slower in the Photosystem I-less strain and about eight-fold slower in the strain lacking both photosystems, resulting in different chlorophyll levels in the various mutants. The results presented in this paper demonstrate the presence of a regulation that adjusts the rate of chlorophyll synthesis according to the needs of chlorophyll-binding polypeptides associated with the photosystems.

Keywords: Chlorophyll turnover; Mass spectrometry; Metabolic flux; Photosystem I; Photosystem II; Stable isotope labeling

1. Introduction

Photosynthesis relies on chlorophyll, which is used for light absorption and the transformation of light energy into chemical energy. However, chlorophyll and its biosynthesis precursors in excited state are potentially destructive and may cause severe damage to cellular constituents because of formation of singlet O_2. The destructive capacity of chlorophyll is greatly reduced by protein-mediated organization of chlorophylls and carotenoids in the photosynthetic apparatus. This pigment–protein organization facilitates the efficient transfer of excitations from the antenna to the reaction center, where the light energy is used for photochemical reactions, and efficient quenching of excitations that cannot be used for photochemistry. Free chlorophyll is toxic in the light and in the presence of O_2.

Cyanobacteria use phycobilisomes as the major peripheral light-harvesting apparatus, with phycobilins serving as light-absorbing chromophores. Both chlorophyll and phycobilins belong to the same family of tetrapyrroles formed by the condensation of eight molecules of aminolevulinic acid (for a review on the tetrapyrrole biosynthesis pathway see Ref. [1]). In oxygenic photosynthetic organisms amino-

Abbreviations: MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; OD_{730}, optical density at 730 nm; PS, photosystem; SCP, small Cab-like protein

* Corresponding author. School of Life Sciences and Center for the Study of Early Events in Photosynthesis, Arizona State University, Box 874501, Tempe, AZ 85287-4501, USA. Tel.: +1 480 965 3698; fax: +1 480 965 6899.
E-mail address: wim@asu.edu (W. Vermaas).

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doi:10.1016/j.bbabio.2004.12.011

levulenic acid is produced from glutamate, which enters the tetrapyrrole biosynthesis pathway via glutamyl–$tRNA_{Glu}$. Interestingly, the same glutamyl–$tRNA_{Glu}$ is also used for the synthesis of proteins.

The demand for chlorophyll may vary significantly depending on the species, growth conditions, and developmental stage of a photosynthetic organism [2–4]. This requires tight regulation of chlorophyll metabolism because the amount of this pigment in cells should be strictly coordinated with the number of chlorophyll-binding sites provided by photosynthesis-related proteins to minimize the amount of free chlorophyll. Results obtained with *Synechocystis* sp. PCC 6803 mutants that lack Photosystem (PS) II, PS I, or both photosystems provide an interesting example of coordinated accumulation of chlorophylls and chlorophyll-binding proteins, as in these mutants the chlorophyll content decreases proportionally with a decrease in the number of photosynthetic pigment-binding complexes [5], and no free chlorophyll in the membrane is apparent [5,6]. Thus far it is not clear whether this adjustment of chlorophyll concentration to the amount of chlorophyll-binding peptides is achieved by decelerated chlorophyll synthesis or by accelerated chlorophyll degradation.

Few data on chlorophyll stability in non-senescing in vivo systems are available. A 30% drop in chlorophyll concentration in *Synechocystis* sp. PCC 6803 within the first few hours of acclimation to high light intensity suggests that chlorophyll may degrade rather fast in this organism [7]. In another study, which employed a PS I-less/*chl*L^- mutant of *Synechocystis* (ChlL is a subunit of the light-independent protochlorophyllide reductase and therefore *chl*L^- mutants do not synthesize chlorophyll in the dark), the estimated half-life time of chlorophyll in darkness was about 3 to 4 days [8]. In algae, the reported half-life times of chlorophyll range from 30 min in *Chlorella* [9] to several hours in *Sceletonema costatum* [10], but may be of the order of many days in *Thalassiosira weissflogii* [11]. Slow chlorophyll degradation on the order of many days has been observed in leaves of higher plants as well [12]. In these studies radioactive labeling of chlorophyll with ^{14}C [9–11] or ^{3}H [12] has been used to determine rates of chlorophyll synthesis and degradation. Complex multistep protocols have been employed to isolate radiochemically pure pigments and to demonstrate incorporation of the label into the tetrapyrrole ring rather than in the phytol moiety [11,13].

In this paper, we describe a new method to monitor the rates of chlorophyll synthesis and degradation in photosynthetic organisms. This method is based on monitoring the incorporation of the stable isotope ^{15}N into chlorophyll. This approach has been used to evaluate the synthesis and degradation rates of chlorophyll in wild-type *Synechocystis* and in mutants lacking one or both of the photosystems.

2. Materials and methods

2.1. Strains and growth conditions

The construction of a PS I-less (*psa*AB^-) mutant of *Synechocystis* sp. PCC 6803 has been described by Boussiba and Vermaas [14], and the construction of PS II-less (*psb*B^-/*psb*DIC^-) and PS I-less/PS II-less (*psa*AB^-/*psb*B^-/*psb*DIC^-) mutants has been described by Xu et al. [8]. The strains were grown at 30 °C in liquid BG-11 medium [15] supplemented with *N*-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES)–NaOH buffer (pH 8.0) and 10 mM glucose, unless indicated otherwise. The light intensity varied from 4 to 100 μmol photons m^{-2} s^{-1}, as specified. Growth was monitored by measuring the optical density of cells in a 1 cm cuvette at 730 nm using a Shimadzu UV-160 spectrophotometer. One milliliter of a OD_{730}=0.2 culture of the wild-type and of the PS I-less, PS II-less and PS I-less/PS II-less strains contained $(2.6\pm0.8)\times10^7$, $(2.4\pm0.5)\times10^7$, $(2.7\pm0.7)\times10^7$, and $(3.4\pm0.6)\times10^7$ cells, respectively, as estimated using a cell counting chamber (Hausser Scientific, USA).

For ^{15}N-labeling experiments, cultures grown in BG-11 medium containing regular $NaNO_3$ as nitrogen source were diluted with approximately four volumes of fresh BG-11 medium without $NaNO_3$, to a final OD_{730} of 0.1 for the wild-type strain and PS II-less mutant, 0.2 for the PS I-less mutant or 0.4 for the PS I-less/PS II-less mutant. The cultures were then supplemented with a mixture of $(^{15}NH_4)_2SO_4$ and $Na^{15}NO_3$ to a final concentration of 2 mM and 9 mM, respectively. The ^{15}N-labeled compounds contained less than 2% of the ^{14}N isotope. The natural abundance of the ^{15}N isotope in unlabeled materials is less than 0.4%. Ammonia effectively suppresses the incorporation of the remaining $^{14}NO_3^-$ at the beginning of the experiment [16], whereas $^{15}NO_3^-$ was added to prevent nitrogen limitation later on, as the growth of some of the mutants was inhibited at concentrations of ammonia higher than 2 mM. The ^{15}N-labeled compounds were obtained from Cambridge Isotope Laboratories Inc. MA, USA.

2.2. Pigment extraction and HPLC analysis

To determine the incorporation of ^{15}N into chlorophyll, a 20 to 60 mL portion of the *Synechocystis* cell culture was harvested by centrifugation, chlorophyll in the pellet (<100 μL) was extracted with 2–3 mL of methanol and its concentration was

determined from the absorbance at 665 nm using an extinction coefficient for chlorophyll *a* in 100% methanol (71.43 mM^{-1} cm^{-1}) taken from [17]. Subsequently, the extract was dried under vacuum, the pigments were re-dissolved in 100 μL of methanol and then subjected to HPLC separation on an HP-1100 Chemstation with a Waters Spherisorb S5ODS2 (250 mm×4.0 mm) analytical column. The column was eluted with 100% methanol at a flow rate of 1.0 mL/min. The fraction containing chlorophyll *a* (R_t=4.3 min) was collected, dried under vacuum, and stored at −80 °C for mass spectroscopy analysis.

To analyze chlorophyllide and pheophorbide labeling in the PS I-less/PS II-less mutant of *Synechocystis*, 120 mL portions of cell culture were harvested by centrifugation, the pigments were extracted with up to 1.2 mL of methanol, and the resulting extract was injected into the HP-1100 chromatograph equipped with a 1.4 mL extension loop and a Waters Spherisorb S10ODS2 (250 mm×10 mm) Semi-Prep column. The column was eluted with 85% methanol containing 50 mM NH_4HCO_3 buffer at pH 7.8 (solvent A), methanol (solvent B), and acetone (solvent C) at a flow rate of 2.5 mL/min using the following gradient program: 0 to 1 min, 100% solvent A; 1 to 6 min, 85 to 100% of solvent B in A; 6 to10 min, 0 to 25% of solvent C in B; 10 to12 min, 25 to 60% of solvent C in B; 12 to 21 min, 60 to 100% of solvent C in B; and 21 to 25 min, 100% solvent C. The fractions containing chlorophyllide (R_t=8.5 min), pheophorbide (R_t=11.0 min), and chlorophyll (R_t=18.5 min) were collected, dried under vacuum, and stored at −80 °C until further use.

2.3. Mass spectroscopy

Positive ion mass spectra were obtained by matrix-assisted laser desorption/ionization delayed extraction time-of-flight (MALDI-TOF) mass spectrometry using a Voyager DE STR Biospectrometry Work Station (Foster City, CA). The spectrometer was operating in reflector mode using manufacturer-supplied default settings optimized for molecules with masses around 1300 Da. Chlorophyll was mixed with terthiophene, an electron transfer matrix introduced by McCarley and co-authors [18], dissolved in dichloromethane, and 2–5 μl of the mixture was dried on a stainless steel sample plate for analysis. The nitrogen laser excitation frequency was set at 3 Hz. The laser power was optimized to obtain a good signal-to-noise ratio after averaging 200–400 single-shot spectra.

2.4. Calculation of porphyrin isotopomer abundance

Each porphyrin molecule has four nitrogen atoms, of which zero, one, two, three, or all four atoms can be labeled with ^{15}N. Therefore, the mass spectrum of a porphyrin containing a mixture of ^{15}N and ^{14}N isotopes can be viewed as an overlay of five individual mass spectra, generated by the corresponding $^{15}N_0$, $^{15}N_1$, …$^{15}N_4$ group of molecules, in which the isotopes of all atoms except for nitrogen are present in natural abundance ($^{15}N_0$ corresponds to a $^{14}N_4$ molecule, or unlabeled porphyrin). In each group, the distribution of relative amplitudes of mass peaks $A_1 \ldots A_N$ spread at 1 Da (A_1 corresponds to the amplitude of the base peak) can be calculated from the molecular formula of the porphyrin and the natural abundance of C, O, H, and Mg isotopes. Thus, for chlorophyll ($C_{55}H_{72}N_4O_5Mg$), the amplitudes of the first six peaks $A_1 \ldots A_6$ are 1.000, 0.751, 0.420, 0.157, 0.041, and 0.008, respectively. For chlorophyllide ($C_{35}H_{34}N_4O_5Mg$), the amplitudes $A_1 \ldots A_6$ are 1.000, 0.522, 0.276, 0.080, 0.015, and 0.002, respectively (chlorophyllide ion was also an abundant chlorophyll fragmentation product generated upon MALDI-TOF; see Results). For pheophorbide ($C_{35}H_{36}N_4O_5$), the amplitudes $A_1 \ldots A_6$ are 1.000, 0.398, 0.088, 0.080, 0.014, and 0.002, respectively.

Upon ^{15}N-labeling, the distribution of the measured intensities of the first ten mass peaks $I_1 \ldots I_{10}$ of the porphyrin isolated from labeled cells can be described by the following set of linear equations:

$$\begin{aligned}
A_1{*}C\left({}^{15}N_0\right) &= I_1 \\
A_2{*}C\left({}^{15}N_0\right) + A_1{*}C\left({}^{15}N_1\right) &= I_2 \\
A_3{*}C\left({}^{15}N_0\right) + A_2{*}C\left({}^{15}N_1\right) + A_1{*}C\left({}^{15}N_2\right) &= I_3 \\
A_4{*}C\left({}^{15}N_0\right) + A_3{*}C\left({}^{15}N_1\right) + A_2{*}C\left({}^{15}N_2\right) + A_1{*}C\left({}^{15}N_3\right) &= I_4 \\
A_5{*}C\left({}^{15}N_0\right) + A_4{*}C\left({}^{15}N_1\right) + A_3{*}C\left({}^{15}N_2\right) + A_2{*}C\left({}^{15}N_3\right) + A_1{*}C\left({}^{15}N_4\right) &= I_5 \\
A_6{*}C\left({}^{15}N_0\right) + A_5{*}C\left({}^{15}N_1\right) + A_4{*}C\left({}^{15}N_2\right) + A_3{*}C\left({}^{15}N_3\right) + A_2{*}C\left({}^{15}N_4\right) &= I_6 \\
A_6{*}C\left({}^{15}N_1\right) + A_5{*}C\left({}^{15}N_2\right) + A_4{*}C\left({}^{15}N_3\right) + A_3{*}C\left({}^{15}N_4\right) &= I_7 \\
A_6{*}C\left({}^{15}N_2\right) + A_5{*}C\left({}^{15}N_3\right) + A_4{*}C\left({}^{15}N_4\right) &= I_8 \\
A_6{*}C\left({}^{15}N_3\right) + A_5{*}C\left({}^{15}N_4\right) &= I_9 \\
A_6{*}C\left({}^{15}N_4\right) &= I_{10}
\end{aligned} \tag{1}$$

where $C(^{15}N_0)$, $C(^{15}N_1)$, …,$C(^{15}N_4)$ are concentrations of the porphyrin isotopomers containing between zero and four ^{15}N-labeled atoms, respectively, and I_1 corresponds to m/z=592.2, 614.2 and 892.5 for pheophorbide, chlorophyllide, and chlorophyll, respectively. The sum of $C(^{15}N_0) \ldots C(^{15}N_4)$ equals the total concentration of the porphyrin in the sample. For the measured sets of $I_1 \ldots I_{10}$ the values of $C(^{15}N_0)$, $C(^{15}N_1)$, …,$C(^{15}N_4)$ were calculated by solving the overdetermined system of Eq. (1) using the MATLAB programming environment (MathWorks Inc., Natick, MA).

2.5. Protein and phycocyanin quantification

A 25 mL portion of a cell culture grown to OD_{730} ~0.5 was pelleted by centrifugation, resuspended in 1 mL of 50 mM Na-phosphate buffer (pH 7.2) containing 5 mM NaEDTA, and the concentrated cells were broken in a Bead Beater using 6 breaking cycles (45 s shaking with 0.1 mm glass beads followed by 2–5 min cooling on ice). Glass beads were precipitated by a 15 s centrifugation in a microcentrifuge at 1000 rpm and the supernatant was stored for protein and phycocyanin determination. The glass bead pellet was washed several times with small portions of the Na-phosphate buffer to extract the remaining proteins and the extracts were combined with the supernatant obtained earlier. Proteins, including phycobiliproteins, were precipitated from the supernatant by the addition of four volumes of ice-cold acetone followed by incubation of the samples for at least 2 h at −20 °C and a subsequent 20-min centrifugation in a microcentrifuge at 4 °C. The chlorophyll concentration in the resulting supernatant containing 80% acetone was determined photometrically using an extinction coefficient of 76.8 mM^{-1} cm^{-1} at 663.6 nm [17]. The protein pellet was suspended either in 1 mL of a 8 M urea/concentrated HCl (19:1 v/v) mixture to determine the phycocyanin content or in 200 μL of a mixture containing 25 mM Na-dodecyl sulfate, 8 M urea, 1.8% (v/v) Triton X-100, and 2 mM dithiotreitol to quantify proteins. The phycocyanin content was determined photometrically after a correction for light scattering [19] using an extinction coefficient of 35.4 mM^{-1} cm^{-1} at 660 nm [20]. The protein content was estimated using bicinchoninic acid protein assay reagents (Pierce Biotechnology, Inc., Rockford, IL) according to the manufacturer's instructions and using bovine serum albumin as a standard.

2.6. Glutamate quantification

To estimate the glutamate content in *Synechocystis* proteins, the total protein extract prepared as described above was dissolved in a small volume of 90% formic acid and then subjected to vapor-phase hydrolysis in the presence of 6 M HCl with 1% phenol as an antioxidant for 24 h at 105 °C. After hydrolysis, the samples were analyzed using an AminoQuant Analyzer (Hewlett-Packard) as described in more detail in [21]. In brief, a sample of the hydrolyzed amino acid mix containing approximately 100 ng amino acids was derivatized with *ortho*-phthalaldehyde and 9-fluorenylmethyl-chloroformate. Then the derivatized amino acids were separated by HPLC using a Hypersil AA-ODS 2.1×200 mm column (Agilent Technologies), and the quantity of amino acids was determined fluorometrically. Chromatographic conditions were as follows: solvent A, 20 mM sodium acetate (pH 7.2), 0.3% tetrahydrofuran, and 0.1% triethylamine; solvent B, 20 mM sodium acetate (pH 7.2), 40% methanol, and 40% acetonitrile; the derivatized amino acids were separated by a 17 min linear gradient from 0 to 60% solvent B at a flow rate of 0.45 mL/min. The total amount of 18 amino acids determined by this method (tryptophan and cysteine could not be detected, and histidine was incompletely resolved from glycine) comprised 81±7% of the mass of proteins determined by the bicinchoninic acid protein assay. During the acidic hydrolysis of proteins glutamine was converted to glutamate. Therefore, in order to calculate the glutamate content in the protein hydrolysate, the measured intensity of the glutamate/glutamine HPLC peak was multiplied by 0.48 considering that the genome of *Synechocystis* sp. PCC 6803 (http://www.kazusa.or.jp/cyano/Synechocystis/) contains 57,538 codons encoding glutamate and 62,641 codons encoding glutamine.

3. Results

3.1. Steady-state concentrations of chlorophyll, phycocyanin, and protein-associated glutamate

In Table 1 the concentrations of chlorophyll and phycocyanin, the two most abundant products of tetrapyrrole biosynthesis pathway in cyanobacteria, are compared with the amount of protein-associated glutamate in three strains of *Synechocystis* grown at a light intensity of 4 μmol photons m^{-2} s^{-1} in the presence of 10 mM glucose. Under these conditions the wild-type, PS I-less and PS I-less/PS II-less strains had very similar growth rates with doubling times of approximately 24 h. In agreement with earlier data [5], the chlorophyll content decreased by about a factor of 5 upon the deletion of PS I and by additional factor of 2.5 upon the deletion of both PS I and PS II, if the chlorophyll content was expressed on OD_{730} basis. The content of phycocyanin measured on OD_{730} basis was similar in wild-type and PS I-less cells, and decreased by a factor of two in the PS I-less/PS II-less strain. The difference in chlorophyll and phycocyanin content between the wild-type and PS I-less/PS II-less cells was slightly higher if the concentrations of pigments were expressed per amount of dry biomass or per total cell number (see Materials and methods for the relationship between OD_{730} and cell numbers in the various strains). The total amount of protein and the amount of glutamate obtained upon the hydrolysis of *Synechocystis* proteins was similar in all three strains, particularly when normalized to dry biomass or cell number.

3.2. Chlorophyll mass spectra

In MALDI-TOF mass spectral measurements, chlorophyll was ionized with the formation of two major types of ions peaking at *m*/*z* 892.5 and 614.2 (Fig. 1A). The heavier peak corresponds to the M^+ mass of chlorophyll,

Table 1
Steady-state concentrations of chlorophyll, phycocyanin, and protein-associated glutamate in different *Synechocystis* strains

Strain	Protein	Dry weight	Glutamate	Chlorophyll	Phycocyanin
	μg/(mL*OD_{730})		nmol/(mL*OD_{730})		
Wild type	90±10	240±15	30	2.75±0.15	0.90±0.09
PS II-less	n.d.	250±15	n.d.	2.20±0.10	n.d.
PS I-less	88±12	250±10	34	0.56±0.07	1.02±0.15
PS I-less/PS II-less	120±15	300±20	39	0.19±0.03	0.50±0.07

All strains were grown (photo)heterotrophically at a light intensity of 4 μmol photons m^{-2} s^{-1} in the presence of 10 mM glucose.
n.d. — not determined.

whereas the lighter peak is consistent with the M^+ mass of chlorophyllide, a product of chlorophyll fragmentation leading to the loss of the esterifying phytol. The relative intensity of the chlorophyllide peak varied from sample to sample and generally increased with increasing laser intensity. Minor peaks at *m*/*z* 915.5 and at 931.5 often observed in mass spectrograms are likely to originate from sodium and potassium adducts of chlorophyll, respectively. A small broad peak at *m*/*z* ~648–650 is an artifact of chlorophyll post-source decay; this peak completely disappeared when MALDI-TOF analysis was performed in the linear mode (data not shown).

When chlorophyll was isolated from *Synechocystis* cells grown in standard BG-11 medium, the measured molecular peak distributions for chlorophyll and chlorophyllide ions (Fig. 1B) agreed very well with the calculated isotopic patterns for these molecules (Fig. 1C), indicating that the ionization of chlorophyll in the presence of the matrix terthiophene resulted in the formation of only M^+ ions, and that quantification based on MALDI-TOF peak intensities was reliable.

The cultivation of *Synechocystis* in the media containing ^{15}N nitrate and ammonium salts significantly modified the mass spectra of chlorophyll molecules. Particularly prevalent in ^{15}N-labeled cultures were two peaks at *m*/*z*=618.4 and 896.5 (Fig. 2A), which reflected an increase in the amount of chlorophyll with all four nitrogen atoms substituted with the ^{15}N isotope (Fig. 2B). The calculation of the ^{15}N-labeling pattern of chlorophyll based on the measured molecular mass distributions of chlorophyll and chlorophyllide ions using Eq. (1) produced very similar results (Fig. 2B). Therefore, in further studies the ratios of different chlorophyll isotopomers in samples were calculated by averaging the results obtained from chlorophyll and chlorophyllide mass spectra. As the natural abundance of the ^{15}N isotope is very low (~0.36%), the probability of synthesizing a chlorophyll molecule containing one or more ^{15}N atoms in cells growing in the unlabeled media is about 0.014. Therefore, essentially all ^{15}N-labeled chlorophyll ($^{15}N_1 \ldots {}^{15}N_4$) accumulated after administering the ^{15}N label represents newly synthesized molecules.

Pheophorbide and chlorophyllide isolated from PS I-less/PS II-less *Synechocystis* cells grown in standard BG-11 media were ionized mostly with the formation of ions peaking at *m*/*z* 592.2 and 614.2, respectively, and the theoretical and experimentally measured molecular peak distributions for these types of molecules agreed very well (data not shown).

3.3. Chlorophyll synthesis and degradation in Synechocystis mutants

Fig. 3 shows the kinetics of chlorophyll labeling in different *Synechocystis* strains. The labeling was initiated by diluting the cultures with fresh BG-11 medium containing a mixture of $(^{15}NH_4)_2SO_4$ and $Na^{15}NO_3$ isotopes instead of unlabeled $NaNO_3$ that was present in the initial growth medium. Cyanobacterial nitrate–nitrite transporters and nitrate reductase activity are subject to rapid inhibition by ammonium (reviewed in Ref. [16]). Indeed, Fig. 3 shows that most chlorophyll molecules synthesized after applying the ^{15}N label had all four nitrogen atoms substituted with ^{15}N ([$^{15}N_4$]-chlorophyll). As compared to the rate of accumulation of [$^{15}N_4$]-chlorophyll, the rates of accumulation of [$^{15}N_3$], [$^{15}N_2$], and [$^{15}N_1$]-chlorophyll decreased with a decreasing number of ^{15}N-labeled atoms, and the amount of [$^{15}N_1$]-chlorophyll was generally less than 2% of the amount of [$^{15}N_4$]-chlorophyll. This trend suggests that the probability of the incorporation of ^{14}N into chlorophyll was small during cell growth in ^{15}N-containing medium. Therefore, after administering the ^{15}N label, the pool of [^{14}N]-glutamate, the precursor of chlorophyll, was rapidly replaced by [^{15}N]-glutamate in the cell and the relative concentration of [^{14}N]-glutamate remained low during the time course of the experiment.

Considering that the chance to synthesize an unlabeled chlorophyll molecule equals the relative abundance of [^{14}N]-glutamate in the labeled medium to the fourth power, synthesis of unlabeled chlorophyll after the addition of ^{15}N is negligible. For this reason, the rate of chlorophyll degradation can be determined from the rate of disappearance of the unlabeled chlorophyll from the culture. As shown in Fig. 3A, the concentration of unlabelled ($^{15}N_0$) chlorophyll exhibited only minor changes in the wild type upon growth at 100 μmol photons m^{-2} s^{-1}, indicating that chlorophyll degradation in this strain was slow, even at moderately high light intensity. Similarly slow chlorophyll degradation was

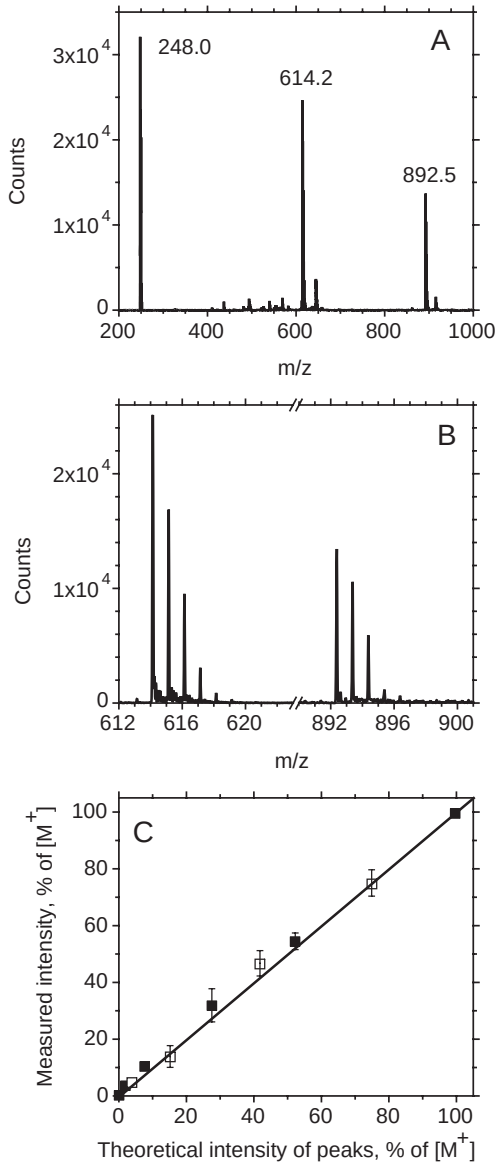

Fig. 1. MALDI mass spectrum of chlorophyll isolated from *Synechocystis* cells grown without added isotopes. (A) Wide scan showing peaks corresponding to chlorophyll ($C_{55}H_{72}N_4O_5Mg$; m/z=892.5), chlorophyllide ($C_{35}H_{34}N_4O_5Mg$, the product of chlorophyll fragmentation; m/z=612.2), and terthiophene used as a matrix (m/z=248.0). (B) Chlorophyll and chlorophyllide regions of the mass spectrum shown in panel A. The appearance of peaks at 1 mass unit intervals is due to the incorporation of naturally occurring isotopes. (C) Correlation between experimentally measured and calculated isotopic patterns for chlorophyll (□) and chlorophyllide (■). Intensities of the M^+ base peaks were taken as 100%.

observed in the PS I-less mutant upon growth at 4 μmol photons m^{-2} s^{-1}, which is close to the maximum light intensity that this strain can tolerate. In contrast, the degradation of chlorophyll was markedly accelerated in the PS I-less/PS II-less mutant grown at the same low light intensity (Fig. 3C).

Changes in the chlorophyll concentration C in exponentially growing *Synechocystis* culture can be expressed as

$$dC/dt = f^*m_o^*e^{\mu t} - k_d^*C, \quad (2)$$

where f is the fraction of chlorophyll relative to dry weight m, k_d is the rate constant of chlorophyll degradation, μ is the specific growth rate, and m_o is the dry weight of the culture at t=0. In Eq. (2), the first element on the right side ($f^*m_o^*e^{\mu t}$) describes chlorophyll synthesis including the increase in chlorophyll concentration in the culture due to exponential cell growth, and the second element (k_dC) describes chlorophyll degradation. The general solution of Eq. (2) is

$$C(t) = fm_oe^{\mu t}/(k_d + \mu) + B^*e^{-k_dt}, \quad (3)$$

where B is the integration constant determined by the initial value of C. As synthesis of unlabeled ($^{15}N_0$) chlorophyll was negligible after the addition of ^{15}N label (f=0), k_d can be determined from changes in unlabeled chlorophyll concentration (Table 2). Considering that the concentration of ^{15}N-labeled chlorophyll at t=0 is essentially zero, the kinetics of accumulation of chlorophyll molecules containing at least one ^{15}N atom is described by

$$C(t) = fm_o(e^{\mu t} - e^{-k_dt})/(k_d + \mu). \quad (4)$$

The values of f for different mutants can be determined by fitting Eq. (4) to the experimentally measured changes in

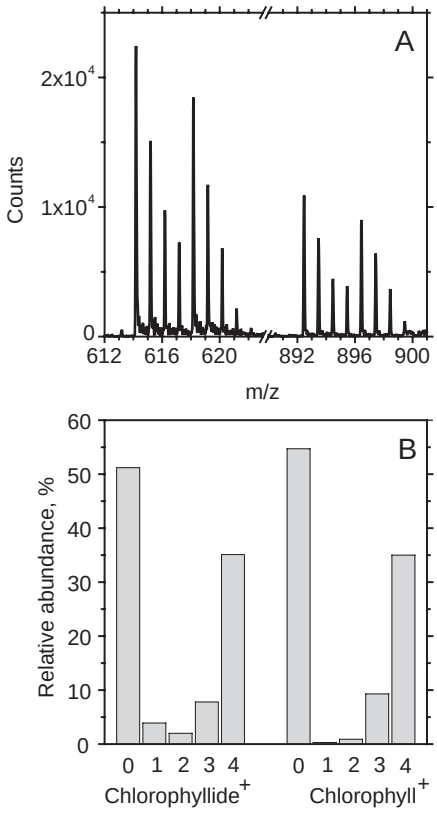

Fig. 2. MALDI mass spectra of chlorophyll(ide) after ^{15}N-labeling of cells. Chlorophyll was isolated from wild-type *Synechocystis* cells grown for 10 h in ^{15}N-enriched media at a light intensity of 40 μmol photons m^{-2} s^{-1}. (A) Mass spectra of chlorophyllide and chlorophyll regions. (B) Percentage of chlorophyll molecules containing various numbers of ^{15}N atoms, as calculated from chlorophyll and chlorophyllide molecular mass distributions (panel A) using Eq. (1).

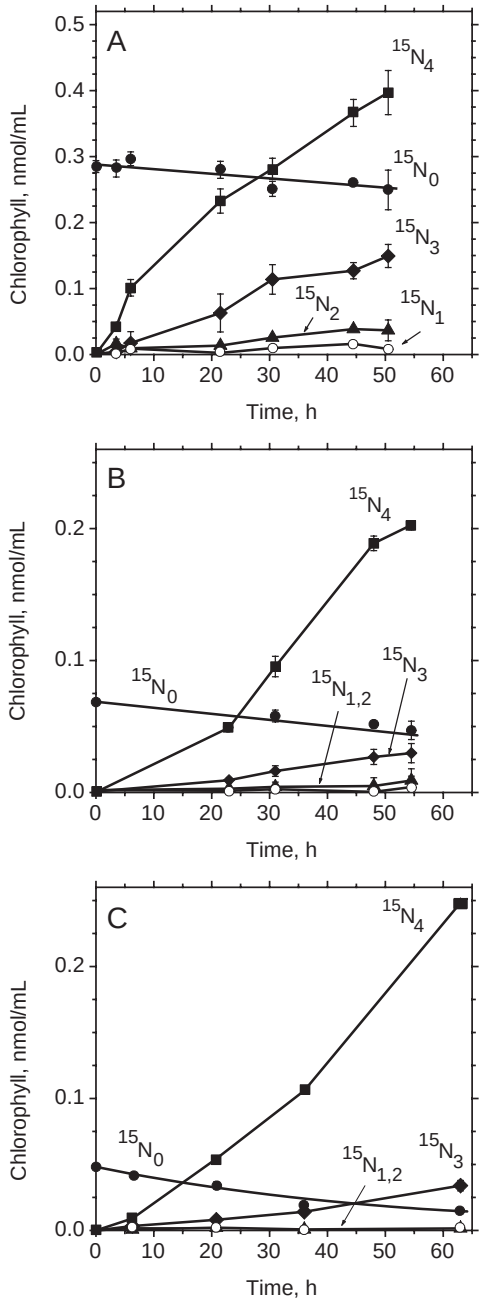

Fig. 3. Kinetics of chlorophyll labeling in different *Synechocystis* strains. Wild-type cells (A) were grown photoautotrophically at a light intensity of 100 μmol photons m^{-2} s^{-1}, and PS I-less (B) and PS I-less/PS II-less cells (C) were grown (photo)heterotrophically in the presence of 10 mM glucose and at a light intensity of 4 μmol photons m^{-2} s^{-1}. $(^{15}NH_4)_2SO_4$ and $Na^{15}NO_3$ were added to the growth medium at time t=0. Relative amounts of chlorophyll molecules containing different numbers of ^{15}N atoms were calculated from MALDI mass spectra according to Eq. (1). The changes in $[^{15}N_0]$-chlorophyll reflecting chlorophyll degradation were approximated with an exponential decay function. Under photoautotrophic conditions (panel A), cell growth gradually slowed down due to an increasing CO_2/HCO_3^- limitation in the growth medium; under (photo)heterotrophic growth conditions exponential growth was observed for a longer time, up to OD_{730} ~1.0.

the concentration of ^{15}N-labeled chlorophyll. However, considering that in the logarithmic growth phase $m(t)=m_o*e^{\mu t}$ and that the C/m ratio remains nearly constant during the exponential (photo)heterotrophic growth of the cultures (data not shown),

$$C/m = f/(k_d + \mu). \quad (5)$$

Eq. (5) links the values of steady-state (cellular) chlorophyll concentrations in growing cultures of *Synechocystis* strains with the rates of chlorophyll synthesis and degradation determined by the parameters f, μ, and k_d, thereby providing an alternative way of calculation of f as μ, k_d, and C/m are easily measured (Table 2).

The rates of chlorophyll synthesis and degradation in different mutants can be compared on the basis of their f and k_d values, respectively (formally f is equivalent to the first-order rate constant of chlorophyll biosynthesis). Eq. (5) shows the contribution of chlorophyll synthesis and degradation to the overall chlorophyll concentration in *Synechocystis* cells. The doubling time of all strains used in the experiments was similar (~24 h) when these strains were grown (photo)heterotrophically at low light intensity (4 μmol photons m^{-2} s^{-1}) in the presence of glucose, which corresponds to μ ~0.03 h^{-1}. In the wild-type, PS II-less and PS I-less cells $\mu \gg k_d$, indicating that chlorophyll degradation, which is much slower than cell growth, does not significantly affect the chlorophyll concentration in cells. Therefore, the nearly five-fold decrease in chlorophyll concentration in the PS I-less strain compared to the wild type should be attributed primarily to a decreased rate of chlorophyll synthesis. In the PS I-less/PS II-less mutant this rate is further attenuated. In this strain, in which k_d=0.020 h^{-1}, the chlorophyll level in the cell drops by 2.5-fold as compared to the PS I-less strain, whereas μ is similar to k_d (Table 2). Consequently, upon the deletion of both PS I and PS II, both chlorophyll degradation and chlorophyll biosynthesis are important factors in determining the chlorophyll level in cells.

Table 2
Chlorophyll synthesis and degradation rates in different *Synechocystis* strains

Strain	μ (h^{-1})	k_d (h^{-1})	f (h^{-1})
Wild type	$(26.8\pm1.5)\times10^{-3}$	$(3.1\pm0.5)\times10^{-3}$	0.30×10^{-3}
PS II-less	$(31.8\pm1.9)\times10^{-3}$	$(2.7\pm0.7)\times10^{-3}$	0.27×10^{-3}
PS I-less	$(32.7\pm2.9)\times10^{-3}$	$(4.6\pm0.5)\times10^{-3}$	0.07×10^{-3}
PS I-less/PS II-less	$(29.0\pm1.8)\times10^{-3}$	$(20.2\pm2.0)\times10^{-3}$	0.03×10^{-3}

The cultures were grown (photo)heterotrophically at a light intensity of 4 μmol photons m^{-2} s^{-1} in the presence of 10 mM glucose, and the specific growth rate μ of these cultures was determined by measuring changes in OD_{730}. The rate constants of chlorophyll degradation k_d were determined from changes in the concentration of unlabeled chlorophyll (see Fig. 3). The parameter f, the fraction of chlorophyll relative to dry weight, is related to the chlorophyll biosynthesis rate and was calculated according to Eq. (5).

3.4. Kinetics of chlorophyllide and pheophorbide labeling in the PS I-less/PS II-less strain

As has been shown earlier [8], PS I-less and PS I-less/PS II-less strains of *Synechocystis* accumulate a small amount of chlorophyllide. This raises the question whether this chlorophyllide will be converted to chlorophyll or is not used for chlorophyll synthesis and will be degraded. According to HPLC analysis of pigments in PS I-less/PS II less cells, pheophorbide (chlorophyllide that lost its Mg atom) was present in such cells as well. The absolute concentrations of chlorophyllide and pheophorbide in the PS I-less/PS II-less cells were in the range of 7 to 10 pmol/(mL*OD_{730}), which is high enough to perform isotopic analysis of these porphyrins by mass spectrometry. Fig. 4 compares the changes in the percentage of chlorophyll, chlorophyllide, and pheophorbide molecules containing various numbers of ^{15}N atoms in the course of ^{15}N-labeling of PS I-less/PS II-less *Synechocystis* cells. The ratios between different ^{15}N isotopomers were calculated from the measured molecular mass distributions of the corresponding porphyrins using Eq. (1). The results of this analysis show that the relative amounts of ^{15}N-labeled chlorophyllide, pheophorbide and chlorophyll increased over time with reasonably similar kinetics, implying that most chlorophyllide that is newly formed is converted to chlorophyll, and not degraded to pheophorbide.

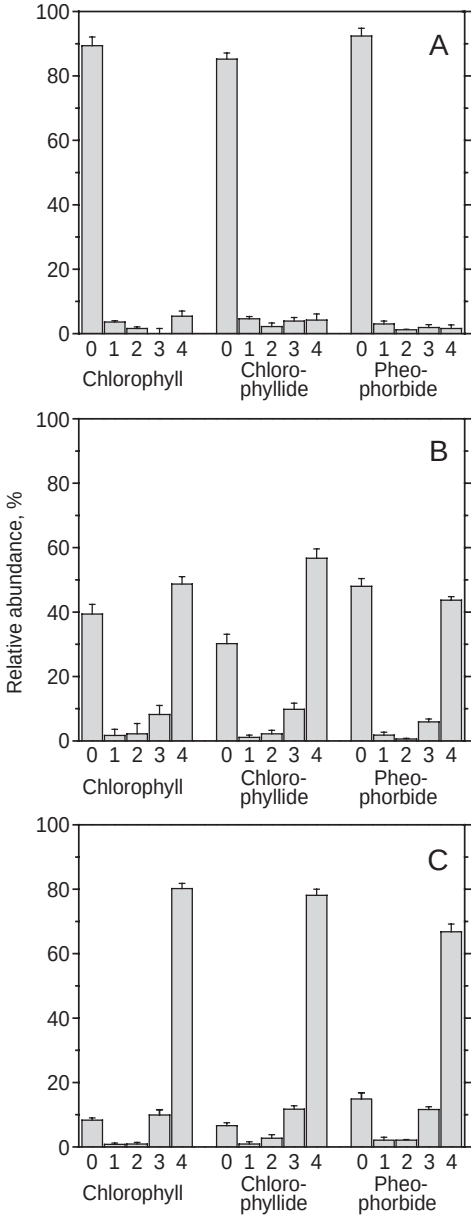

Fig. 4. Comparison of chlorophyll, chlorophyllide and pheophorbide labeling in the PS I-less/PS II-less *Synechocystis* strain. Relative amounts of these molecules with 0 to 4 ^{15}N atoms (shown as numbers under the bars) were calculated for cells collected after 4 (A), 21 (B), and 43 (C) h of growth in the presence of ^{15}N.

4. Discussion

In recent years, the analysis of metabolic fluxes using stable isotope labeling has become a powerful technique to study the function of biological systems. Quantitative analysis of metabolite flux in the tetrapyrrole pathway is important because of two reasons. First, synthesis and degradation rates of different tetrapyrrole-derived compounds may change significantly depending on the growth conditions, the developmental stage of the organism, etc. [1,3]. Secondly, synthesis of tetrapyrroles requires a significant amount of resources, especially in photosynthetic organisms. Chlorophyll and phycocyanin are the two most abundant tetrapyrrole derivatives in the cyanobacterium *Synechocystis* sp. PCC 6803; together their concentration in wild-type cells can be as high as 3.7 nmol/(mL*OD_{730}) (Table 1). As eight glutamate molecules are used for the synthesis of one molecule of tetrapyrrole, this corresponds to about 30 nmol/(mL*OD_{730}), which is close to the concentration of protein-associated glutamate (Table 1). This indicates that in cyanobacterial cells a large fraction of loaded $tRNA_{Glu}$ is used for tetrapyrrole biosynthesis rather than for protein biosynthesis.

4.1. Chlorophyll stability

In this paper we describe a new method to measure chlorophyll synthesis and degradation rates in *Synechocystis* cells on the basis of ^{15}N incorporation into the chlorophyll. The isotopic composition of chlorophyll was quantified using a combination of HPLC and MALDI-TOF mass spectrometry, and the concentration of [^{15}N]-chlorophyll isotopomers was calculated according to Eq. (1). Using this method we show that chlorophyll is very stable in actively growing wild-type *Synechocystis* cultures, as well as in mutants retaining at least one of the two photosystems (Fig. 3A, Table 2). Interestingly, the half-life time of the D1 protein of PS II in *Synechocystis* cells growing at a light

intensity of 100 μmol photons m^{-2} s^{-1} was reported to be about 2 h [22]. In another study, the half-life time of the D1 protein in wild-type *Synechocystis* cells grown at somewhat lower light intensity was estimated to be about 2 to 3 h and the half-lives of CP47, CP43, and D2 proteins were only a few times longer than that of D1 [23], which means that under these conditions PS II complexes are completely replaced by the new ones in less than a day. However, this turnover of PS II is not accompanied by chlorophyll degradation (Fig. 3A), suggesting that cells can effectively reuse earlier synthesized pigments for the assembly or reassembly of PS II centers.

Chlorophyll degradation was faster in the PS I-less/PS II-less mutant than in the other *Synechocystis* strains studied here (Fig. 3C and Table 2). Since no long-lived (3–5 ns) components of chlorophyll fluorescence were detected in PS I-less/PS II-less cells [6], the fluorescence of the small amount of chlorophyll retained in this strain is efficiently quenched. This suggests that chlorophylls are well-organized in this strain and are presumably bound to proteins. Potential candidates to bind chlorophyll in the PS I-less/PS II-less strain are *s*mall *C*ab-like *p*roteins (SCPs), which are predicted to have a single membrane-spanning region similar to the first and third transmembrane region of plant Cab (Lhc) proteins [24]. Unlike Cab/Lhc proteins in plants, SCP proteins in *Synechocystis* do not have significant light-harvesting function, but may have a role in the reuse of chlorophylls during the assembly/reassembly of the photosystems [8]. This function implies rather loose chlorophyll binding to the SCPs, which is consistent with the observed fast degradation of chlorophyll in the PS I-less/PS II-less strain. Part of the chlorophyll in this strain may also be transiently stabilized by the D1 and D2 proteins of PS II, which continue to be synthesized in the PS I-less/PS II-less cells, but do not accumulate in the absence of CP43 and CP47 proteins encoded by *psb*B and *psb*C genes that have been deleted in this strain [5].

4.2. Chlorophyll degradation intermediates

The accelerated turnover of chlorophyll observed in the PS I-less/PS II-less mutant raises the question of the mechanism of chlorophyll degradation in *Synechocystis* cells. In plants, massive chlorophyll breakdown occurring during leaf senescence involves a sequence of reactions including dephytylation of chlorophyll catalyzed by chlorophyllase, removal of the central magnesium by an unknown mechanism, oxidative opening of the chlorophyll macrocycle catalyzed by pheophorbide oxygenase leading to formation of the so-called red chlorophyll catabolite, and reduction of the C(1)C(20) double bond of the red chlorophyll catabolite by the corresponding reductase in a reaction yielding a blue-fluoresceing colorless compound [25]. Genes encoding chlorophyllase, pheophorbide oxygenase, and red chlorophyll catabolite reductase have been identified in many higher plants. Only one of these enzymes, pheophorbide reductase, can be recognized in cyanobacteria on the basis of sequence similarity [26]. HPLC analysis of pigment extracts from the PS I-less/PS II-less *Synechocystis* mutants showed the presence of a small amount of pheophorbide and chlorophyllide (3–5% of the amount of chlorophyll), which can be viewed as intermediate products of chlorophyll degradation in this strain. It should be noted that chlorophyllide lies at the crossroad of chlorophyll synthesis and degradation pathways. Therefore, chlorophyllide extracted from the cells can be represented by a mixture of newly-synthesized molecules formed from protochlorophyllide and molecules formed as a first step of chlorophyll degradation. Upon the addition of the ^{15}N label to the growth media nearly all newly-synthesized chlorophyllide molecules generated upon protochlorophyllide reduction should be labeled with ^{15}N. Considering the low chlorophyllide/chlorophyll ratio in the PS I-less/PS II-less cells the turnover rate of chlorophyllide should be very high in comparison to that of chlorophyll. This suggests that soon after the start of labeling nearly all chlorophyllide molecules in *Synechocystis* cells should contain ^{15}N if chlorophyll degradation does not contribute to the chlorophyllide pool. However, the replacement of unlabeled ($^{15}N_0$) chlorophyllide with the ^{15}N-labeled isotope in the PS I-less/PS II-less strain occurred rather slowly and followed the kinetics of disappearance of unlabeled chlorophyll (Fig. 4). On this basis we conclude that the majority of the chlorophyllide isolated from PS I-less/PS II-less cells originates from the degradation of the bulk of chlorophyll. As changes in the pheophorbide labeling pattern were similar to the changes in the labeling pattern of chlorophyllide and chlorophyll (Fig. 4), it is likely that pheophorbide represents the product of the second step of chlorophyll degradation in this mutant and is not formed from chlorophyllide that is synthesized but is not needed for chlorophyll synthesis.

Synechocystis cells can excrete water-soluble intermediates of the chlorophyll biosynthesis pathway when chlorophyll synthesis is inhibited [27]. However, we observed no accumulation of pheophorbide and chlorophyllide in the growth media of the PS I-less/PS II-less strain, suggesting that pheophorbide is rapidly degraded further, perhaps with the assistance of a putative pheophorbide reductase encoded by *slr*1747 [26].

4.3. Regulation of chlorophyll synthesis

Comparison of the *f* values reflecting the rates of chlorophyll accumulation in the different mutants (Table 2) suggests that chlorophyll synthesis rate decreases upon the deletion of photosystems, but alternative explanation might be that newly-synthesized chlorophyll is rapidly degraded and does not accumulate. However, as discussed earlier, the analysis of labeling pattern of chlorophyll degradation products precludes this alternate explanation for PS I-less/PS II-less cells. In the PS I-less mutant, the chlorophyll synthesis rate was 4 to 5 times higher (Table 2),

whereas cellular concentrations of chlorophyllide and pheophorbide were significantly lower [8] as compared to the PS I-less/PS II-less mutant. Therefore, it seems very unlikely that a substantial amount of newly-synthesized chlorophyll degrades in PS I-less cells as well. Consequently, we conclude that the chlorophyll synthesis rate in *Synechocystis* cells is tightly coordinated with the cell's need for chlorophyll.

Despite a nearly 5-fold reduction in chlorophyll content, the amount of phycocyanin in the PS I-less mutant was similar to that in the wild type. In the PS I-less/PS II-less mutant chlorophyll synthesis was 10-fold slower than in the wild type, as evidenced from *f* values (Table 2), whereas the amount of phycobilins was reduced by just a factor of 2 in this mutant (Table 1). Moreover, no substantial accumulation of Mg-containing precursors of chlorophyll was detected in the PS I-less and PS I-less/PS II-less mutants. Therefore, in these two strains chlorophyll biosynthesis is likely to be regulated at least at two sites: (a) at the level of Mg-chelatase and (b) before the branch point of heme and chlorophyll synthesis, probably at the level of glutamyl–tRNA reductase, which is generally considered to be the main regulatory enzyme in the tetrapyrrole biosynthesis pathway [1].

In conclusion, the ^{15}N isotope labeling technique is a powerful tool for the quantitative analysis of chlorophyll synthesis and degradation reactions. The results presented in this report demonstrate the existence of a mechanism that coordinates chlorophyll synthesis with the chlorophyll-binding needs of polypeptides in PS I and PS II. This coordination may be achieved by the (down)regulation of chlorophyll synthesis at the level of magnesium chelation by protoporphyrin IX and at the beginning of the tetrapyrrole biosynthesis pathway. The PS I-less and PS I-less/PS II-less *Synechocystis* mutants are good models to help understand the mechanisms of this regulation, as well as to identify the pathway(s) of chlorophyll degradation in cyanobacteria.

Acknowledgements

This work was funded by the US Department of Energy Grant DE-FG02-04ER15543.

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