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Balancing photosynthetic electron flow is critical for cyanobacterial acclimation to nitrogen limitation

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ABSTRACT

Nitrogen limitation forces photosynthetic organisms to reallocate available nitrogen to essential functions. At the same time, it increases the probability of photo-damage by limiting the rate of energy-demanding metabolic processes, downstream of the photosynthetic apparatus. Non-diazotrophic cyanobacteria cope with this situation by decreasing the size of their phycobilisome antenna and by modifying their photosynthetic apparatus. These changes can serve two purposes: to provide extra amino-acids and to decrease excitation pressure. We examined the effects of nitrogen limitation on the form and function of the photosynthetic apparatus. Our aim was to study which of the two demands serve as the driving force for the remodeling of the photosynthetic apparatus, under different growth conditions. We found that a drastic reduction in light intensity allowed cells to maintain a more functional photosynthetic apparatus: the phycobilisome antenna was bigger, the activity of both photosystems was higher and the levels of photosystem (PS) proteins were higher. Pre-acclimating cells to Mn limitation, under which the activity of both PSI and PSII is diminished, results in a very similar response. The rate of PSII photoinhibition, in nitrogen limited cells, was found to be directly related to the activity of the photosynthetic apparatus. These data indicate that, under our experimental conditions, photo-damage avoidance was the more prominent determinant during the acclimation process. The combinations of limiting factors tested here is by no means artificial. Similar scenarios can take place under environmental conditions and should be taken into account when estimating nutrient limitations in nature.

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1. Introduction

Photosynthetic autotrophs are self-reliant in regard to obtaining reduced carbon sources, needing little more than adequate illumination and carbon dioxide. A different aspect of autotrophy is the need for assimilation of essential nutrients directly from the environment. Nutrients are roughly divided into two groups: macronutrients, specifically, nitrogen, phosphorous, sulfur and potassium; and micronutrients like iron, manganese, copper and molybdenum. The bioavailability of nutrients varies and is dependent on different factors such as concentration and chemical speciation. In the aquatic domain the concentrations of nutrients are often limiting, even before taking their chemical state into account. In different parts of the world's aquatic habitats the bioavailability of nutrients such as N, P and Fe is extremely low. These nutrients are considered as major limiting factors in such areas [1,2]. Nutrient levels in aquatic environments fluctuate considerably over time and depth and are affected by numerous environmental phenomena such as Aeolian dust deposition, river runoff, sediment re-suspension, deep water mixing as well as sporadic local anthropogenic pollution events. In these environments, prokaryotic oxygen evolving photosynthetic organisms (cyanobacteria) account for approximately 30% of primary productivity [3]. Over the course of their evolution cyanobacteria developed acclimation mechanisms for dealing with fluctuating and often limiting nutrient regimes; reacting to nutrient shortages in a way that ensures their survival until the next enrichment event occurs.

Nitrogen is one of the most abundant elements on Earth. However, with the exception of diazotrophic bacteria, it is not bioavailable to microorganisms as N_2. As early as 1910, it was observed that non-diazotrophic cyanobacteria lose their typical blue-green hue and become yellow following transfer to nitrogen limiting conditions [4]. This effect was termed nitrogen bleaching and was found to be caused by the breakdown of phycocyanin (PC), a major antenna pigment in cyanobacteria. Research conducted in the 1960s concluded that when grown under nitrogen limiting conditions, the fresh water cyanobacterium *Anacystis nidulans* (Currently *Synechococcus elongatus*) stops growing and shows a substantial loss of PC, nucleic acids and proteins [4]. A reduction in chlorophyll levels was also observed under these conditions. These phenotypes could be reversed by the addition of nitrogen salts to the growth media [4]. The process of phycobilisome (PBS) breakdown and recovery following nitrogen

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supplementation, its kinetic properties and molecular control mechanisms have been subjected to intensive research conducted with different cyanobacterial strains [5–14]. The common feature reported in these studies was a massive, regulated breakdown of PBS on a time scale of a few hours to 48 h following nitrogen step-down. In these reports, the extent of chlorophyll degradation was variable. In certain cases it is reported that chlorophyll levels drop only slightly [4,7,11]. Some accounts, however, report a significant decrease in cellular chlorophyll levels under nitrogen starvation [6,10]. It was suggested that these discrepancies can be attributed to the different cyanobacteria strains analyzed and to a variation in the experimental setup, especially light intensities, CO_2 levels and even nitrogen step-down method [10].

These observations are supported by transcriptomic studies indicating that many cellular activities are down-regulated in the absence of nitrogen [11,15]. When faced with nitrogen limitation, cyanobacteria lower the rate of metabolic processes and change from an anabolic to a catabolic metabolic strategy, represented by the degradation of proteins and other cellular components [14].

Degradation of PBS was suggested to allow for the reallocation of nitrogen from this highly abundant protein to other critical pathways in the cell [16]. At the same time, nitrogen limitation grinds biosynthetic mechanisms to a halt, resulting in over reduction of photosynthetic electron carrier pools [17]. These conditions expose the photosynthetic apparatus to damage from reactive oxygen species. Decreasing PBS absorption cross-section alleviates this stress by reducing excitation pressure.

In this work we examined the response of photosynthetic apparatus to nitrogen limitation. In particular, we attempted to asses which of the stresses exerted on the photosynthetic apparatus during nitrogen limitations determines the changes in its function.

2. Methods

2.1. Cyanobacterial strains and culture conditions

Wild-type *Synechocystis sp.* Strain PCC 6803 cultures were grown in 120 ml of YBG11 medium [18] in acid washed 500 ml glass Erlenmeyer flasks. Cultures were maintained under constant shaking and illumination at 30 °C. Illumination was set at 60 μmol photons $m^{-2}s^{-1}$ (growth light – GL) except when low light conditions were applied (6 μmol photons $m^{-2}s^{-1}$– LL). In order to remove excess nitrogen, cultures were spun down at 4600 g for 5 min and then resuspended in YBG11 containing no nitrate (YBG11 N-). This was repeated once more and then equal aliquots were transferred to flasks containing either standard YBG11 (N+) or YBG11 (N−). Cell densities were counted with a hemacytometer. Mn limitation was achieved by removing it from the trace metal solution of YBG11. Mn levels in Mn− media were lower than 1.7 nM [19].

For the photo-damage measurements, the cells were transferred to N− conditions and grown for 48 h at GL and LL conditions. At that point the protein synthesis inhibitor chloramphenicol was added (25 μg ml^{-1}) and the cultures were transferred to GL conditions.

2.2. Oxygen evolution measurements

Synechocystis 6803 cells were spun down and re-suspended to a final chlorophyll concentration of 10 μg per ml. Oxygen evolution rates were measured using a Clark-type electrode (Pasco, Roseville, CA). Illumination was provided by a white LED array. The measurements were carried out at 30 °C. Oxygen evolution rates were corrected to the dark respiration rate.

2.3. Spectroscopic analysis

P_{700} photo-oxidation was measured in vivo in cultures brought to the same density i.e. constant cell number in all experiments. Where indicated, DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) or DBMIB (2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoqinone) were added to a final concentration of 10 μM. Measurements were performed using a Joliot-type spectrophotometer (JTS-10, Bio-Logic, Grenoble, France) as described previously [19]. A calibration curve confirmed that P_{700} photo-oxidation values are linearly correlated to chlorophyll concentration (supplemental Fig. 2). Phycobilisome absorption spectra were measured from soluble fractions extracted from broken cells, while chlorophyll concentrations were determined by methanol extractions of the membranal fraction and absorbance measurements at 665 nm (ε = 74.5 ml cm mg^{-1}). The soluble fraction was centrifuged at 24,000*g* for 30 min for sedimentation of small membrane vesicles. Fv/Fm measurements were carried out using an imaging PAM fluorometer (Heinz Walz GmbH, Effeltrich, Germany). The cells were dark adapted for 2 min prior to the measurements.

2.4. Protein analysis

For protein separation using BN-PAGE and SDS-PAGE techniques, *Synechocystis* 6803 cells were broken by rigorous bead beating. The resulting membrane fraction was collected by centrifugation [19]. Membranes were resuspended in a buffer containing 330 mM mannitol, 30 mM HEPES, 2 mM EDTA and 3 mM $MgCl_2$, pH 7.8. Linear 4.5–12% BN-PAGE was performed as described by Heinemeyer and co-workers [20] using the mild detergent *n*-dodecylmaltoside at a ratio of 0.03:1 (w/w) *n*-dodecylmaltoside:chlorophyll. SDS-PAGE was performed as described in [21]. Immunodetection was carried out using antibodies against PsbA, PsaA, PsbC and PsaD.

3. Results

Under nitrogen limiting conditions over-reduction of secondary electron transport carriers can occur. Low availability of terminal electron acceptors might lead to a mostly reduced plastoquinone (PQ) pool. A reduced PQ pool can, in turn, induce acceptor side PSII photoinhibition and the formation of harmful reactive oxygen species [22]. Therefore, the breakdown of PBS and other photosynthetic components may serve not only as a nitrogen reallocation mechanism but also as part of the cellular shift toward catabolic metabolism, providing protection against photoinhibition.

3.1. The light intensity dependence of cell density and pigment content in nitrogen limited cultures

In order to study the importance of light stress avoidance in the nitrogen limitation response we designed an experimental setup in which nitrogen limited *Synechocystis* 6803 were grown under very low light intensities (LL = 6 μmol photons $m^{-2}s^{-1}$). The kinetics of growth, PBS and chlorophyll breakdown, photosynthetic activity and protein content were compared to nitrogen limited cultures grown under growth light intensities (GL = 60 μmol photons $m^{-2}s^{-1}$). These light intensities are one to two orders of magnitude lower than the intensities required for saturation of electron transport rates (Supplemental Fig. 1).

Fig. 1 presents changes in cell density in N− and N+ cultures grown under low and growth light intensities. Nitrogen sufficient cell density was highly dependent on light intensities. Under N− conditions, the concentration of LL cultures was slightly, but not significantly, higher. As would be expected, under N− conditions light intensity and photosynthetic activity do not limit growth.

PBS and chlorophyll (Chl) content of the cells were also assessed in that experiment. Due to the variability of optical scattering which is dependent on cell size and morphology, we present the data in its raw form with no baseline manipulations (Fig. 2A). The peaks at 620 and at 678 nm arise from absorption by phycocyanin (PC) and Chl, respectively. The Chl peak was observable even after 49 h of nitrogen

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