Plant Physiology and Biochemistry 132 (2018) 524–534

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Response of the thylakoid proteome of *Synechocystis* sp. PCC 6803 to photohinibitory intensities of orange-red light

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ARTICLE INFO

Keywords:
Synechocystis
Thylakoid membranes
Photoinhibition
Quantitative proteomics
SWATH analysis

ABSTRACT

Photoautotrophic growth of *Synechocystis* sp. PCC 6803 in a flat-panel photobioreactor, run in turbidostat mode under increasing intensities of orange-red light (636 nm), showed a maximal growth rate (0.12 h^{-1}) at 300 $\mu mol_{photons}\ m^{-2}\ s^{-1}$, whereas first signs of photoinhibition were detected above 800 $\mu mol_{photons}\ m^{-2}\ s^{-1}$. To investigate the dynamic modulation of the thylakoid proteome in response to photoinhibitory light intensities, quantitative proteomics analyses by SWATH mass spectrometry were performed by comparing thylakoid membranes extracted from *Synechocystis* grown under low-intensity illumination (i.e. 50 $\mu mol_{photons}\ m^{-2}\ s^{-1}$) with samples isolated from cells subjected to photoinhibitory light regimes (800, 950 and 1460 $\mu mol_{photons}\ m^{-2}\ s^{-1}$). We identified and quantified 126 proteins with altered abundance in all three photoinhibitory illumination regimes.

These data reveal the strategies by which *Synechocystis* responds to photoinibitory growth irradiances of orange-red light. The accumulation of core proteins of Photosystem II and reduction of oxygen-evolving-complex subunits in photoinhibited cells revealed a different turnover and repair rates of the integral and extrinsic Photosystem II subunits with variation of light intensity. Furthermore, *Synechocystis* displayed a differentiated response to photoinhibitory regimes also regarding Photosystem I: the amount of PsaD, PsaE, PsaJ and PsaM subunits decreased, while there was an increased abundance of the PsaA, PsaB, Psak2 and PsaL proteins. Photoinhibition with 636 nm light also elicited an increased capacity for cyclic electron transport, a lowering of the amount of phycobilisomes and an increase of the orange carotenoid protein content, all presumably as a photoprotective mechanism against the generation of reactive oxygen species.

1. Introduction

Cyanobacteria are among the most ancient prokaryotic micro-organisms that appeared on Earth approximately 2.45–2.22 billion years ago. Their photosynthetic activity led to the accumulation of molecular oxygen in the atmosphere. *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) forms spherical cells and its thylakoid membranes enclose a luminal space, arranged parallel to the plasma membrane and occasionally converging toward it to form biogenesis centres (Heinz et al., 2016). Differently from their plant chloroplast counterparts, in cyanobacteria, the thylakoid membranes typically do not stack; rather they are uniformly sheet-like and densely packed with membrane proteins (Folea et al., 2008). The thylakoid membranes harbour the integral-membrane protein complexes of the photosynthetic electron transport chain, consisting of Photosystem (PS) II, Cytochrome b_6f (Cyt b_6f) and PSI, and also accommodate the ATP

Abbreviations: CEF, cyclic electron flow; DDT, Dithiothreitol; LEF, linear electron flow; MES, 2-(N-morpholino)ethanesulfonic acid; MS, mass spectrometry; NPQ, Non-Photochemical Quenching; OCP, orange carotenoid protein; PBR, photobioreactor; PBS, phycobilisome; PQ, plastoquinone; ROS, reactive oxygen species; SWATH, Sequential Window Acquisition of all THeoretical fragment ion spectra; TRIS, tris(hydroxymethyl)aminomethane

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https://doi.org/10.1016/j.plaphy.2018.10.002
Received 9 July 2018; Received in revised form 1 October 2018; Accepted 1 October 2018
Available online 05 October 2018

synthase (ATPase) and the majority of the respiratory electron transport complexes, amongst which are Complex I (NADH dehydrogenase I, NDH-1) and Complex II (succinate dehydrogenase, SDH). In the cyanobacterial thylakoid membrane system, there is no evidence of a specific lateral distribution of those complexes; indeed, a tight proximity exists, especially for the two photosystems (Folea et al., 2008). Nevertheless, compartmentalized modules and supercomplexes, responsible for the formation of specific spatially separated domains and interactions, similarly to the chloroplast thylakoids of higher plants, do exist (Agarwal et al., 2010; Casella et al., 2017). In cyanobacteria, the protein complexes more represented within thylakoids are PSII, which occurs as a dimer, and PSI that is normally trimeric, differently from its higher plant counterpart that exists in a monomeric state.

The most important component responsible for capturing sunlight and the subsequent transfer of excitation energy to the two PSs in cyanobacteria, is a supramolecular light harvesting system called phycobilisome (PBS). The PBS is a complex structure made up of phycobilins, water-soluble pigments strongly associated with proteins to form the brightly coloured phycobiliproteins, phycoerythrin (PE), phycocyanin (PC) and allophycocyanin (APC). Disks of phycobiliproteins, present as trimers and hexamers, are further arranged into cylinders by colourless linker proteins, and the cylinders are then assembled into PBS (Adir, 2005). Thanks to the phycobilin chromophores, the PBS can adsorb visible light in the region between 500 and 670 nm, thus widening the effective absorption range of Chlorophyll *a* (Chl *a*, with absorption peaks at 435 and 680 nm) that is present - in part also with an antenna function - in the PSs (Adir, 2005). The PBS interacts with the outer surface of the thylakoids and, although it transfers energy mainly to the PSII, there is evidence of its ability to channel energy directly also to PSI and to adjust its coupling to either PSII or PSI, depending on variation in illumination conditions (Rakhimberdieva et al., 2001).

The energy absorbed by PBSs is transferred to the reaction centres of PSs where the photochemical reactions take place. The two PSs are connected by low-molecular-weight hydrogen/electron carriers, the plastoquinone (PQ), which transfers electrons and protons from PSII to Cyt b_6f, and the plastocyanin and cytochrome c_6, which shuttle the electrons from Cyt b_6f to PSI. This linear electron flow (LEF) from PSII to PSI is accompanied by the formation of a proton gradient across the thylakoid membrane that is ultimately required to power ATP synthesis by the ATP synthase (Liu, 2016).

Prolonged intense irradiation strongly reduces the photochemical efficiency of all oxygen-evolving photosynthetic organisms. Photodamage of the oxygen evolving complex (OEC) in PSII is the first event occurring in this process, which leads to the formation of reactive oxygen species (ROS) that inactivate the photochemical reaction centre of PSII, mainly at the site of the D1 protein (Tyystjärvi, 2008). Moreover, an excess of electrons coming from LEF can also cause photodamage of PSI through the formation of ROS on its donor side (Shimakawa et al., 2016). Cyanobacteria, as all the oxyphototrophs, have developed mechanisms at different levels to defend themselves against photo-oxidative damage. Amongst these there is the Non-Photochemical Quenching (NPQ) process, that is used to dissipate excess absorbed energy in the form of heat (Latifi et al., 2009). In cyanobacteria NPQ can be achieved via photoactivation of the orange carotenoid protein (OCP) that then, in its active form, binds to the core of the PBS and functions as energy quencher, thereby allowing the conversion of excess excitation energy into heat (Rakhimberdieva et al., 2004). Another photoprotective mechanism relies on the association of the flavodiiron (Flv) proteins Flv2/Flv4 to PSII, where they act as an electron sink at the PSII acceptor side, and allow cells to keep the PQ pool relatively oxidized. In this way Flv2/Flv4 also play an important role in photoprotection of PSII against oxidative stress (Zhang et al., 2009). Upon high light exposure, presumably to prevent photodamage to PSI, cyanobacteria overexpress the Flv1/Flv3 proteins, which divert electrons released from water-splitting by PSII directly back to O_2 by forming H_2O without production of ROS in a Mehler-like reaction (Allahverdiyeva et al., 2011). Under high light conditions, also other enzymes, such as catalases, superoxide dismutases (SOD) and peroxidases, all involved in ROS inactivation, are increasingly expressed (Tichy and Vermaas, 1999). High light stress also promotes (increased) cyclic electron flow (CEF) around PSI, in which electrons are returned from the PSI acceptor side back to the PSI donor side, thus preventing PSI photoinhibition (Thomas et al., 2001). Since CEF, in contrast to LEF, does not involve PSII, it does not result in the generation of reducing power by extraction of electrons from water, but it only produces a proton motive force that can supply additional ATP required for CO_2 fixation. In cyanobacteria, several routes for CEF exist. In one route, ferredoxin, reduced on the PSI donor side, is oxidized by the PGR5 protein that transfers electrons to the PQ pool; this electron cycle is completed by moving the electrons back to PSI via Cyt b_6f (Mullineaux, 2014). Another route involves the thylakoid NDH-1 complex that participates in both respiratory and photosynthetic electron transport. In the latter case, reduced ferredoxin is oxidized by NDH-1, and the electrons are transferred to the PQ pool and, subsequently, to PSI through Cyt b_6f (Mullineaux, 2014).

In this work, we have investigated the effects of increasing intensities (from 50 to 1460 $\mu mol_{photons}\ m^{-2}\ s^{-1}$) of incident orange-red monochromatic light of 636 nm on the steady-state growth rate of *Synechocystis,* cultivated in turbidostat mode in a laboratory scale photobioreactor (PBR). This wavelength was chosen because it allows the highest growth rate of *Synechocystis* in continuous light (Van Alphen et al., 2018), which is important from an application perspective. The constant monitoring of the cellular growth rate, used as an indicator for the physiological state of the cells, allowed detection of the threshold of light intensity that triggered photoinhibition. *Synechocystis* proved to be rather resilient to high light stress by monochromatic light of 636 nm, experiencing a state of severe photoinhibition only at intensities above 800 μmol photons $m^{-2}s^{-1}$ (Cordara et al., 2018). From cells grown under photoinhibitory light intensities of 800, 950 and 1460 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ we isolated thylakoid membranes, on which then we performed an in-depth quantitative proteomic analysis, by using thylakoid membranes extracted from *Synechocystis* cells grown at 50 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ as the reference. For this analysis, we used SWATH-MS (Sequential Window Acquisition of all THeoretical ion spectra), an emerging label-free proteomic quantification approach that has been already used for accurate relative quantifications and large-scale identification of differentially abundant proteins in photosynthetic organisms such as plants (Albanese et al., 2018; Zhu et al., 2016) and green algae (Gao et al., 2016),however, to our knowledge, no study on *Synechocystis* sp. PCC 6803 proteomics was published until now. This analysis allowed us to depict the dynamics of the thylakoid membrane proteome upon exposure of *Synechocystis* cells to photoinhibitory intensities of orange-red light.

2. Material and methods

2.1. Pre-cultures

Pre-cultures of the glucose-tolerant wild-type *Synechocystis* sp. PCC6803 were prepared by growing cells in 25 mL flasks in BG11 medium supplemented with 10 mM $NaHCO_3$ (BG-11-PC), with a modified protocol as described in (Van Alphen and Hellingwerf, 2015). Pre-cultures were grown for 4 days at 30 °C in a shaking incubator at 120 rpm (New Brunswick Innova 44) under constant illumination with orange-red (632 nm) and blue (451 nm) light (10:1 photon ratio) at 30 $\mu mol_{photons}\ m^{-2}\ s^{-1}$, measured with a LI-250 quantum sensor (LI-COR).

2.2. Cell cultures

For studies of photoinhibition *Synechocystis* was grown in the commercial flat-panel PBR system FMT150.2/400 (Photon System Instruments), by inoculating 20 mL pre-culture in a final volume of