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Excitation energy transfer to Photosystem I in filaments and heterocysts of *Nostoc punctiforme*

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ABSTRACT

Cyanobacteria adapt to varying light conditions by controlling the amount of excitation energy to the photosystems. On the minute time scale this leads to redirection of the excitation energy, usually referred to as state transitions, which involves movement of the phycobilisomes. We have studied short-term light adaptation in isolated heterocysts and intact filaments from the cyanobacterium *Nostoc punctiforme* ATCC 29133. In *N. punctiforme* vegetative cells differentiate into heterocysts where nitrogen fixation takes place. Photosystem II is inactivated in the heterocysts, and the abundancy of Photosystem I is increased relative to the vegetative cells. To study light-induced changes in energy transfer to Photosystem I, pre-illumination was made to dark adapted isolated heterocysts. Illumination wavelengths were chosen to excite Photosystem I (708 nm) or phycobilisomes (560 nm) specifically. In heterocysts that were pre-illuminated at 708 nm, fluorescence from the phycobilisome terminal emitter was observed in the 77 K emission spectrum. However, illumination with 560 nm light caused quenching of the emission from the terminal emitter, with a simultaneous increase in the emission at 750 nm, indicating that the 560 nm pre-illumination caused trimerization of Photosystem I. Excitation spectra showed that 560 nm pre-illumination led to an increase in excitation transfer from the phycobilisomes to trimeric Photosystem I. Illumination at 708 nm did not lead to increased energy transfer from the phycobilisome to Photosystem I compared to dark adapted samples. The measurements were repeated using intact filaments containing vegetative cells, and found to give very similar results as the heterocysts. This demonstrates that molecular events leading to increased excitation energy transfer to Photosystem I, including trimerization, are independent of Photosystem II activity.

1. Introduction

Nostoc punctiforme sp. ATCC 29133 (henceforth referred to as *N. punctiforme*) is a filamentous cyanobacterium with the capacity for nitrogen fixation when other sources of nitrogen are depleted [1]. As a response to nitrogen starvation, 5 to 10% of the cells in a filament differentiate into heterocysts where nitrogen fixation takes place. Extensive modifications on a physiological level create a microoxic environment in the heterocyst, to protect the nitrogenase from inhibition by molecular oxygen. One important difference from vegetative cells is that Photosystem II (PSII) is inactivated in heterocysts, leading to alterations in the electron transport chain [1–5]. Heterocysts are supplemented with carbohydrates by the neighboring vegetative cells, supplying the reducing equivalents for the nitrogenase reaction [5–8]. Due to the large ATP demand of nitrogen fixation, the heterocysts carry out cyclic photophosphorylation around Photosystem I (PSI). The rate of nitrogen fixation is several times higher under illumination than in the dark [9–12]. In addition, the relative amount of PSI is several times higher in the heterocysts than in the vegetative cells [13,14]. Thus, it can be concluded that PSI-driven cyclic electron transfer is important for heterocyst function.

Non-diazotrophic cyanobacteria enter chlorosis after prolonged nitrogen depletion, as phycobilisomes are degraded and used as a provisional source of nitrogen [15,16]. Heterocystous cyanobacteria also modify their phycobilisomes during nitrogen deprivation. Heterocysts show an altered visible absorption spectrum due to changes in the amount of phycobilisome proteins [7,17–19]. After the onset of nitrogen fixation however, the phycobilisome levels return to normal in the vegetative cells, while in the heterocysts the relative amount of phycobiliproteins is lower [13,15]. The lower fluorescence intensity which is a characteristic of heterocysts, is partly a result of changes in the phycobilisomes, and partly due to a lower relative amount of PSII centers on a total protein basis [13,14].

The phycobilisome is the main light harvesting complex, absorbing within a wide range between ca 550 and 675 nm [20–24]. It is a large protein complex, which is associated with the membrane surface at

Abbreviations: APC, allophycocyanin; ATP, adenosine triphosphate; Chl(s), Chlorophyll(s); EDTA, ethylene diamine tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; OCP, orange carotenoid protein; PMSF, phenylmethylsulphonyl fluoride; PSI, Photosystem I; PSII, Photosystem II

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the stromal side of the thylakoid membrane. Generally, the phycobilisome is composed by a protein "core" made of two to five cylindrical allophycocyanin (APC) complexes, located close to the membrane surface. Radiating from the core are several phycobiliprotein "rods", where the most common phycobiliproteins are phycoerythrin and phycocyanin. Within the APC core two main components absorb at longer wavelengths than the rods; the phycobiliproteins ApcD (or allophycocyanin B), and ApcE, also referred to as the core-membrane linker, L_{CM}, for its role as a linker polypeptide between the phycobilisome and PSII [24,25]. ApcE carries a phycobilin molecule that has an absorption maximum at ca 674 nm and emission at ca 680–683 nm [26]. This is suggested to be the energy conduit to the photosynthetic reaction center, and is therefore sometimes called the terminal emitter of the phycobilisome [27–29].

Cyanobacteria have evolved the capacity to redistribute the amount of excitation energy that is transferred to each photosystem, if light becomes a limiting factor [30–33]. This reorganization of membrane components under different light conditions is often referred to as state transitions. While the mechanisms for this process are still unclear, movement of the light harvesting antenna from one photosystem to the other is understood to be the main result of state transitions [34–37]. In state 1, the phycobilisomes are preferentially coupled to PSII so that excitation is directed to PSII. A transition from state 1 to state 2 will occur when cells are illuminated with light that excites PSII to the point that it is excessive. The phycobilisomes, and to some extent the reaction centers, will then rearrange to redistribute the excitation energy to PSI [36–39]. Conversely, a transition from state 2 to state 1 occurs when the cells are illuminated by e.g. far-red light that is preferentially absorbed by PSI, so that the energy will be redirected to PSII. The redox status of the plastoquinone pool has been suggested to play an important part in triggering state transitions, so that transition to state 2 takes place when the plastoquinone pool becomes reduced, and transition to state 1 occurs when the plastoquinone pool becomes oxidized [31,35,40,41]. However, despite decades of research of state transitions in cyanobacteria, it remains unresolved how changes in the plastoquinone redox state is linked to the association or disassociation of phycobilisomes to the reaction centers.

The association of the phycobilisomes to the photosynthetic reaction centers is also not well understood. Mutagenesis studies have suggested several of the APC core proteins as being necessary for energy transfer to the reaction centers, e.g. the ApcD, E, and F proteins. However, it is difficult to single out any particular component as the anchor for the phycobilisome [42]. It is also not clear if the structural association of phycobilisomes to PSI is in any way similar to their association to PSII. In cyanobacteria trimerization of PSI complexes has been shown to be important for association and efficient energy transfer of phycobilisomes to PSI [43–46]. It has also been suggested that the formation of PSI trimers would function as a way to increase the absorption cross-section of PSI under low light conditions [47–49]. In summary, PSI–phycobilisome associations are a significant part of the antenna–reaction center interactions, and may therefore be of vital importance for PSI function in the PSII-deficient heterocysts.

Surprisingly little is known about the electron transport pathways in the heterocysts, and many discoveries remain to be made regarding e.g. the function of cyclic electron transfer, and how the activity of PSI is regulated. Recently we published proteomic and spectroscopic studies on the thylakoid membranes from vegetative cells [50] and isolated heterocysts [14] from *N. punctiforme*. We concluded that the heterocyst thylakoid membrane is highly enriched in Photosystem I (PSI) and that the overall composition of protein complexes is consistent with a specialization in cyclic photophosphorylation. Contrary to expected, we also showed that the heterocysts from *N. punctiforme* contain intact PSII complexes. Although no water splitting activity was detected, light-induced electron transfer in PSII was measured in vitro [14]. Despite the smaller amount of phycobiliproteins found in heterocysts, we also showed that excitation energy transfer from the phycobilisomes to PSI takes place in heterocysts of *N. punctiforme* [14].

In the present study we have addressed the question if energy transfer to PSI in the heterocysts, is regulated by any type of short-term light adaptation mechanism similar to in the vegetative cells. Specific interactions between the phycobilisome and PSI may be difficult to study in cyanobacterial cells, since the presence of PSII leads to state transitions that mask the antenna–reaction center interactions that only involve PSI. The heterocyst is a natural cyanobacterial cell devoid of PSII activity, making it possible to study antenna–PSI interactions separately. Here we demonstrate that the regulation of excitation transfer to PSI occurs in similar ways in isolated heterocysts and in vegetative cells. We propose a mechanism for distribution of excitation energy which is independent of PSII activity, and discuss the modified phycobilisome present in heterocysts as more suited for energy transfer to PSI.

2. Materials and methods

2.1. Culture conditions and isolation of heterocysts

Filaments of *N. punctiforme* were cultivated under nitrogen fixing conditions in $BG11_0$ media: 1.5 l batch cultures at a total volume of 6 l were supplied with air enriched with ca 2% CO_2, stirred continuously and illuminated with a light intensity of 30 μE m^{-2} s^{-1}. Heterocysts were isolated from the culture after 7 days of growth, as described in detail in [14]. The Chl-*a* concentration was measured spectrophotometrically after extraction with 90% methanol, and calculated by using an extinction coefficient of 78.74 l g^{-1} cm^{-1} at 665 nm [51].

2.2. Isolation of phycobilisomes from intact filaments

Pure phycobilisomes were obtained using the rapid procedure for isolation described in [52] with the following modifications: filaments of *N. punctiforme* were resuspended in buffer solution A (1 M NaH_2PO_4/K_2HPO_4 ($NaKPO_4$) at pH 7.5, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM benzamidine). The cells were subjected to three pressurization/de-pressurization cycles in a Parr Cell Disruption Bomb (model 4639, Parr Instrument Company), in a N_2 atmosphere at 150 bar. 1% (v/v) Triton X-100 was added to the broken cell suspension and the mixture was let stand at room temperature for 30 min in the dark. Membranes and cell wall debris were precipitated by centrifugation at 50,000 × *g* for 30 min and 10 °C, leaving phycobilisomes in the supernatant. The phycobilisomes were precipitated from the supernatant by centrifuging at 72,000 × *g* for 2 h, at 10 °C. The phycobilisomes were then resuspended in buffer solution A and used immediately for fluorescence spectroscopy measurements. That the suspension containing the phycobilisomes was completely free of Chl-*a*, was verified by recording an absorption spectrum (not shown).

2.3. Illumination protocol and fluorescence spectroscopy

Separate samples of intact filaments and isolated heterocysts respectively, were resuspended in $BG11_0$ and 30% (v/v) glycerol was added as a glassing agent. The final sample concentration was 5 μg Chl-*a* ml^{-1}. The cells were kept in the dark at all times from harvest, until pre-illumination. The samples were then illuminated for 5 min each at ca 20 °C. Illumination was provided with a 150 W slide projector lamp equipped with narrow band pass filters for the following wavelength regions: blue light was achieved by using a filter with peak transmittance at 480 nm giving a light intensity at the sample of 24 μE m^{-2} s^{-1}; green light was achieved by using a band pass filter with peak transmittance at 560 nm in combination with a neutral density filter to yield a light intensity at the sample of

33 μE m^{-2} s^{-1}; red light was achieved by using a band pass filter with peak transmittance at 708 nm, giving a final light intensity of 13 μE m^{-2} s^{-1}. All band pass filters had a bandwidth of ±6 nm at 50% of the maximum transmittance and were purchased from Oriel Corporation, Stratford, Connecticut, USA. Illumination was immediately followed by freezing of the sample in liquid N_2 in the dark.

Fluorescence emission and excitation spectra from illuminated samples of filaments and heterocyst cells were recorded at 77 K on a Spex Fluoromax 2 spectrometer (HORIBA Jobin Yvon, Longjumeau, France), slit widths of 5 nm were used.

2.4. Electron paramagnetic resonance spectroscopy

EPR spectra were recorded with a Bruker X-Band E500-ELEXYS spectrometer (Bruker GmbH, Germany). Room temperature measurements were made using a rectangular 4102 standard cavity with an optical port. EPR spectra of thylakoids from intact filaments or isolated heterocysts were recorded after suspending the thylakoids in buffer solution B (50 mM HEPES/NaOH, pH 7.2, 0.4 M sucrose, 10 mM NaCl, 10 mM EDTA), to a concentration of ca 0.5 mg Chl-*a* ml^{-1}. For recording the P_{700}^{+} radical signal the sample was illuminated continuously while recording the EPR spectrum. Illumination was made through the optical port in the EPR cavity, using narrow band pass filters as described above. Spectrometer configuration: microwave frequency 9.77 GHz; modulation frequency 100 kHz; modulation amplitude 3 G; and microwave power 10 mW.

3. Results

3.1. Light-induced changes in fluorescence emission at 77 K

To investigate the effect of different light regimes on the phycobilisome coupling to PSI, we illuminated dark adapted heterocysts or intact filaments of *N. punctiforme* prior to fluorescence measurements. The filaments consist to over 90% of vegetative cells, and measurements made on intact filaments are therefore representative of vegetative cells. All samples were illuminated for 5 min at 20 °C by either red light (708 nm) or green light (560 nm). Fluorescence emission and excitation spectra of the pre-illuminated samples were recorded at 77 K. Fluorescence emission spectra from intact filaments of *N. punctiforme* are shown in Fig. 1A. Excitation at 570 nm (phycobilisome-specific excitation) resulted in strong fluorescence emission from the phycobilisomes, where the 645 nm band originates from phycocyanin, and the shoulder at 652 nm corresponds to the highest energy emitting pigments in the allophycocyanin (APC) core [21]. The spectrum also displayed a peak at ca 680 nm, arising from a superimposition of emission bands from the Chl-*a* binding proteins in PSII (emitting at 685 nm) and the terminal (lower energy) emitters of the APC core (emitting at 680 nm). A broad fluorescence band associated with emission from PSI Chl-*a* in PSI with maximum around 730 nm [53] was also observed, indicating energy transfer from the phycobilisomes to the PSI reaction centers.

The spectrum drawn with a dotted line in Fig. 1A was recorded after the filaments had been pre-illuminated with red light prior to freezing at 77 K. This spectrum was indistinguishable from a spectrum recorded from dark adapted filaments (not shown). After the filaments had been pre-illuminated with green light, the fluorescence emission spectrum showed significant changes: the emission intensity with a peak at 680 nm became markedly reduced. The visible peak was only ca 30–40% compared to the intensity of the peak in the red pre-illuminated sample (Fig. 1A, solid line). In addition, the peak maximum was red-shifted by 4 nm. This indicates that the emission with maximum at 680 nm, originating from the terminal emitter of the phycobilisome, was quenched. Instead the remaining emission peak (at 684 nm) originated mostly from Chl-*a* in the PSII antenna.

In the sample pre-illuminated with green light, we also observed the appearance of a new shoulder at ca 750 nm. Studies in *Spirulina* (*Arthrospira*) *platensis* have shown that fluorescence emission from the longest wavelength emitting Chl-*a* molecules of the PSI core antenna is increased at 760 nm after trimerization of PSI [44,54–56]. Monomeric PSI does not display the long-wavelength fluorescence band at 760 nm in *S. platensis*. The trimerization and appearance of the 760 nm fluorescence occur in *Spirulina* cells after illumination with phycobilisome-specific light under equivalent experimental conditions as in our experiments. Thus, we tentatively assign the band at 750 nm in *N. punctiforme* to emission from PSI in the trimeric form.

Fluorescence emission spectra from isolated heterocysts are shown in Fig. 1B. In heterocysts, emission from the phycobilisomes was observed at 645 and 652 nm. This was similar to the spectra from the intact filaments, but the emission intensity was lower in the heterocysts. The peak at 680 nm from the terminal emitter on the

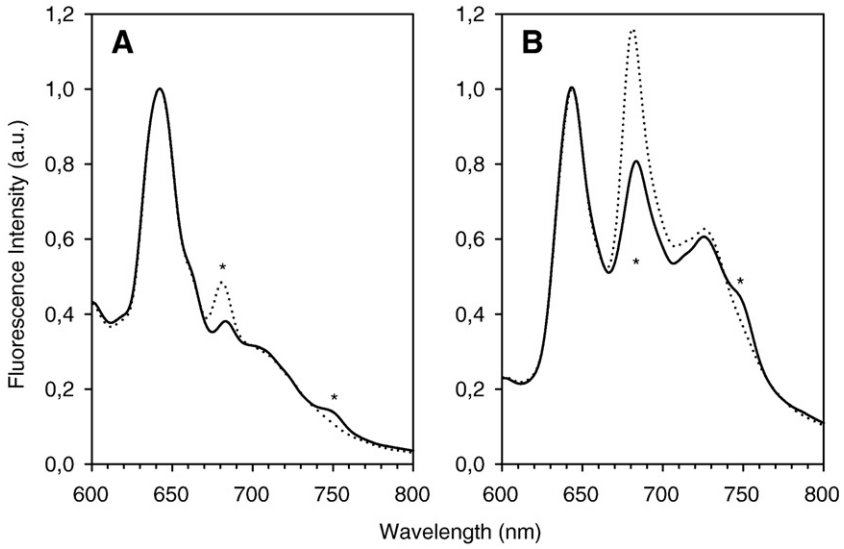

Fig. 1. Fluorescence emission spectra recorded at 77 K with $\lambda_{exc} = 570$ nm, from intact filaments (A) and heterocysts (B) of *Nostoc punctiforme*. Spectra were recorded after samples had been pre-illuminated at room temperature with either red light (···) or green light (—) (see text for details). Green pre-illumination led to a decrease in emission intensity at 680 nm in both filaments and heterocysts, and to the appearance of emission from trimeric PSI at 750 nm (differences marked with asterisks). The spectra were normalized at the emission peak at 645 nm.

other hand, was of similar magnitude in the heterocysts (Fig. 1B) compared to in the intact filaments (Fig. 1A). We have previously shown that vegetative cells from *N. punctiforme* have an approximate PSI to PSII ratio of 4:1 [14]. In heterocysts we observed a fourfold increase in PSI centers whereas the relative PSII abundance was unchanged in heterocysts compared to intact filaments, resulting in a much higher PSI:PSII ratio in the heterocysts [13]. Thus, it is unlikely that the emission at 680 nm in heterocysts has a significant contribution from PSII. Instead, the peak more likely originates almost completely from the terminal emitter in the APC core of the phycobilisome. An interesting observation is that the cells seem to be in state 1 in the dark, since there the emission spectrum was almost identical to that after pre-illumination by red light. Cyanobacteria are known to transition to state 2 in the dark, which leads to a lower emission from the phycobilisomes. We have no explanation for the cells seemingly being in state 1 in the dark, one possibility might be that the metabolism under nitrogen fixing conditions leads to a lower reduction level of the plastoquinone pool.

The isolated heterocysts were subjected to the same illumination protocol as the filaments. Fig. 1B shows spectra from excitation at 570 nm in samples pre-illuminated with red and green light respectively (Fig. 1B, dotted and solid lines). Similar differences between the 77 K emission spectra were observed in the heterocysts as in the filaments: the heterocysts that had been pre-illuminated with green light, displayed a significantly lower emission peak, less than 50%, at 680 nm than the red pre-illuminated heterocysts. However, the emission peak that remained at 680 nm was not red-shifted after green light pre-illumination, as in the spectrum from the filaments, supporting the conclusion that there was little or no contribution from PSII in the 680 nm emission peak from heterocysts. In addition, and similar to the intact filaments, we also observed the appearance of a shoulder at 750 nm from trimeric PSI (Fig. 1B).

To summarize, we observed two effects in the emission spectrum after pre-illumination of intact filaments and isolated heterocysts with green light: 1) the emission at 680 nm was quenched; and 2) emission from trimerized PSI appeared at 750 nm. Both these effects were observed after illumination at wavelengths that are absorbed by the phycobilisomes, usually resulting in the transition from state 1 to state 2. However, the conventional picture of state transitions cannot be envisioned in the heterocysts. Heterocysts show no water splitting activity from PSII, the PSI:PSII ratio is much higher than in vegetative cells and the linear electron transport pathway is not functional in heterocysts. Yet, our observations were very similar to what was observed in the filaments. We observed quenching of the phycobilisome fluorescence at 680 nm also in the heterocysts. We also observed trimerization of PSI in the heterocysts, induced the same way as in the intact filaments. Therefore, we conclude that light-induced quenching of phycobilisomes as well as trimerization of PSI can occur also in heterocysts, independently of PSII activity.

3.2. Energy transfer from phycobilisomes and trimerization of Photosystem I

Since the quenching of phycobilisome emission occurred simultaneously with increased PSI emission, we hypothesized that this result might be explained by an increased energy transfer efficiency from the phycobilisomes to trimeric PSI. It has been demonstrated earlier that energy transfer from phycobilisomes to PSI is more efficient to the trimeric forms of PSI rather than monomeric PSI complexes [44,45]. To investigate this possibility, we recorded excitation spectra from intact filaments and heterocysts after pre-illumination with either red or green light. Excitation spectra were taken both at 728 nm, at the emission maximum for PSI, and at 750 nm where emission from trimeric PSI was observed. Excitation spectra were recorded for both intact filaments (Fig. 2A) and isolated heterocysts (Fig. 2B). A peak at 440 nm which is a signature of Chl-*a* excitation was visible in all spectra. The small peaks at 470 and 510 nm represent carotenoid excitation [53]. Peaks at 550, 570, and 620 nm depict excitation by different parts of the phycobilisome, and the peak at 674 nm arises both by excitation of the terminal emitters of the APC core, and by Chl-*a*.

In the samples that were pre-illuminated by red light there was virtually no difference, after normalization, in the excitation spectra recorded at 728 and 750 nm respectively (Fig. 2, dotted line). After pre-illumination by green light, the region between 550 and 650 nm showed increased intensity, indicating that energy transfer from the phycobilisomes to PSI was more efficient (Fig. 2, solid and dashed lines). In addition, the spectra recorded at 750 nm (Fig. 2, dashed lines), showed that the relative increase in excitation in the 550–650 region was higher than that observed at 728 nm. In other words, while pre-illumination with green light increased the energy transfer from phycobilisomes to PSI overall, more energy was transferred to trimeric PSI relative to monomeric PSI (Fig. 2, dashed lines).

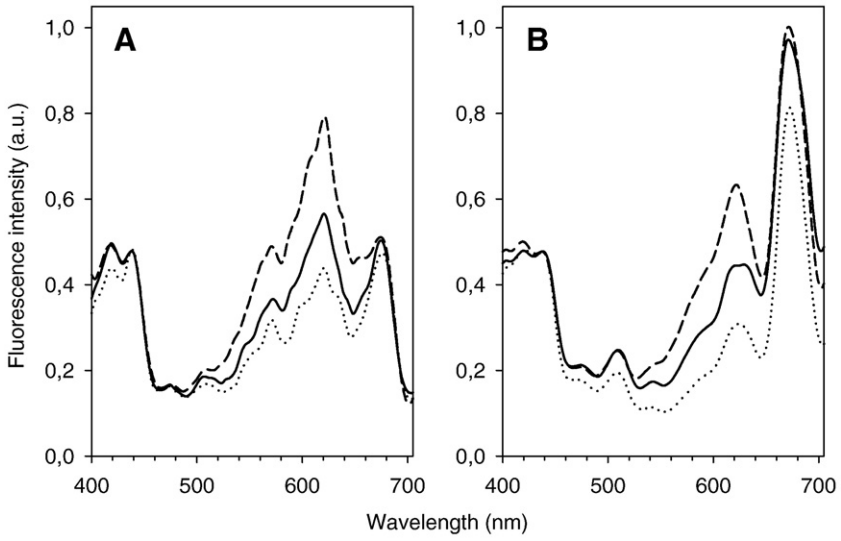

Fig. 2. Excitation spectra from intact filaments (A) and heterocysts (B). Dotted lines (· · ·) depict emission in samples that were pre-illuminated with red light. Solid and dashed lines show emission from samples that were pre-illuminated with green light and collected at 728 nm (—) and 750 nm (– – –) respectively. The spectra were normalized at the Chl-*a* excitation peak at 440 nm. (In samples pre-illuminated with red light, the emission spectrum collected at $\lambda_{em} = 750$ was close to identical, after normalization, to the spectrum collected at $\lambda_{em} = 728$ nm, and has therefore been omitted for clarity.)

In the excitation spectra from heterocysts (Fig. 2B) the overall intensity in the phycobilisome region, between ca 520 nm and 640 nm, was decreased compared to that in the intact filaments (Fig. 2A) due to lower abundancy of phycobiliproteins after heterocyst differentiation (see below). Nevertheless, the intensity of these bands increased significantly after green illumination, indicating that an increased efficiency of energy transfer from phycobilisomes to PSI occurred also in the heterocysts.

We conclude that pre-illumination by green light led to increased excitation transfer from the terminal emitter to trimeric PSI complexes, in both filaments and heterocysts. A similar observation was made in *S. platensis* where a major part of the energy transfer was taking place from phycobilisomes to trimeric PSI complexes [44]. Trimerization of PSI has also been found to be required for stable state transitions in *Synechococcus* sp. PCC 7942 [57,58]. We observed the same changes occurring after green pre-illumination in both intact filaments and isolated heterocysts, indicating that these light-induced events occur independently of PSII and linear electron transfer.

Our interpretation of these results is that the phycobilisomes couple to PSI trimers that form as a response to green light illumination. Furthermore, this coupling is less efficient in the dark or after red light pre-illumination when PSI is monomeric. To rule out the possibility that PSI may have been in the trimeric form already prior to illumination, but invisible in the fluorescence spectrum due to quenching of the F_{750} band, we used EPR spectroscopy. Quenching of the fluorescence from PSI trimers has been found to occur when P_{700} is oxidized [59,60]. Thus, for the longest wavelength Chl-*a* fluorescence to be observed at 750 nm, PSI must be in its trimeric form and with P_{700} reduced. EPR spectra recorded in the dark and under illumination, showed that P_{700} was reduced in dark adapted samples of either filaments or isolated heterocysts (Fig. 3). During illumination at room temperature by either red or green light, we observed the appearance of the P_{700}^{+} radical signal (Fig. 3). After the light was switched off, the P_{700}^{+} radical quickly decayed. Therefore, P_{700} was reduced in the samples prior to the fluorescence measurements. We conclude that the appearance of the 750 nm band after green illumination was due to formation of trimers, and that PSI was in the monomeric form prior to illumination.

The mobility of phycobilisomes has been suggested to be important for state transitions in cyanobacteria [43,45,57]. 1 M glycinebetaine has previously been used to lock the phycobilisomes into their position, rendering them unable to diffuse on the membrane surface [46,58,61]. When we added 1 M glycinebetaine to filaments and heterocysts, and allowed them to incubate in the dark for either 5 min or 1 h before illumination, we could still observe the same spectral changes after illumination as described above (not shown). Thus, quenching of the 680 nm emission did seemingly not require movement of the phycobilisomes. A possible explanation for this could be that the phycobilisomes were already associated with PSI reaction centers, but that the increase in energy transfer happened only after trimerization.

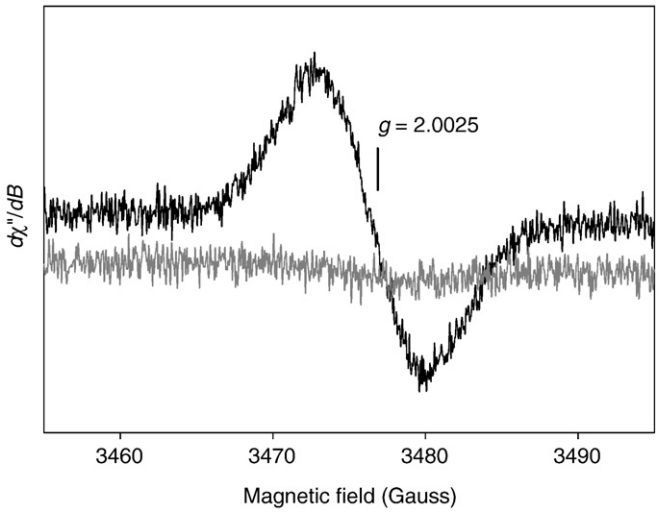

Fig. 3. The light-induced P_{700}^{+} radical (black trace) from PSI in heterocysts, measured by EPR spectroscopy at room temperature under continuous illumination. Illumination of the heterocysts was done in the EPR cavity while recording the spectrum, using a projector lamp equipped with a narrow band pass filters with maximum transmittance at 708 nm. Illumination at 560 nm produced a nearly identical spectrum (not shown). The grey trace was recorded in the dark, demonstrating that P_{700} was reduced. Spectrometer configurations: see Materials and methods section.

In recent years, it has been found that dissipation of excess excitation energy in cyanobacteria may be accomplished by a small, orange carotenoid binding protein (OCP) [62–64]. The OCP acts as a blue-green photoreceptor, and by direct structural interaction with the phycobilisome it effectively quenches excitation in the phycobilisome in a process known as non-photochemical quenching (NPQ). The chromophore carotenoid of the OCP has a broad absorbance spectrum from ca 400 to ca 600 nm, with a maximum at 480 nm in the relaxed state. Illumination of cyanobacterial cells with broad-spectrum blue-green light, leads to a conformational change in the OCP which allegedly increases the coupling of the chromophore to the phycobilisome terminal emitter, leading to energy transfer to the carotenoid [64].

To test if the OCP might be responsible for the observed quenching of F_{680} in our study, we illuminated intact filaments as well as isolated heterocysts with narrow band blue light with a maximum at 480 nm. The emission spectra after blue pre-illumination showed a similar response as in the green illuminated samples, with a slightly decreased emission at 680 nm from the terminal emitter. However, the fluorescence was only quenched to a very small degree, after blue illumination, compared to that observed after green illumination. Furthermore, the emission at 750 nm was barely elevated after blue illumination, and did not compare to the increase observed after green illumination. In summary, the spectrum was to a large degree the same as in the dark adapted samples. Since the absorbance of the OCP is much larger at 480 nm than at 560 nm, the blue illumination would have had a much stronger effect on the quenching and trimerization, if the OCP had been responsible for the results. Therefore, under our illuminating conditions, OCP does not seem to contribute to the quenching of the 680 nm fluorescence, nor to the trimerization of PSI. It is more likely that the quenching is due to direct energy transfer to PSI, as indicated by the excitation spectra (Fig. 2).

From the results presented here, we suggest that trimer formation and phycobilisome coupling to PSI are induced by illumination with green, i.e. phycobilisome-specific, light in both isolated heterocysts and intact filaments. This demonstrates that they can take place completely independently of PSII activity.

3.3. Spectral characterization of isolated phycobilisomes

In isolated phycobilisomes from filaments, the fluorescence emission spectra at room temperature and 77 K, showed major bands at 674 and 680 nm originating from the longest wavelength emitting phycobilins in the phycobilisome core (Fig. 4) [65]. The excitation spectra collected at 680 nm showed peaks at 570 nm from phycoerythrin and 620 nm from phycocyanin (Fig. 4B), that match the corresponding peaks in the excitation spectrum from the filaments (Fig. 2A). This confirms that the fluorescence emission at ca 680 nm has a large contribution from the phycobilisome terminal emitter.

4. Discussion

4.1. Energy transfer from the phycobilisome to Photosystem I

In cyanobacteria, state transitions are thought to promote the redistribution of excitation energy from the phycobilisomes between

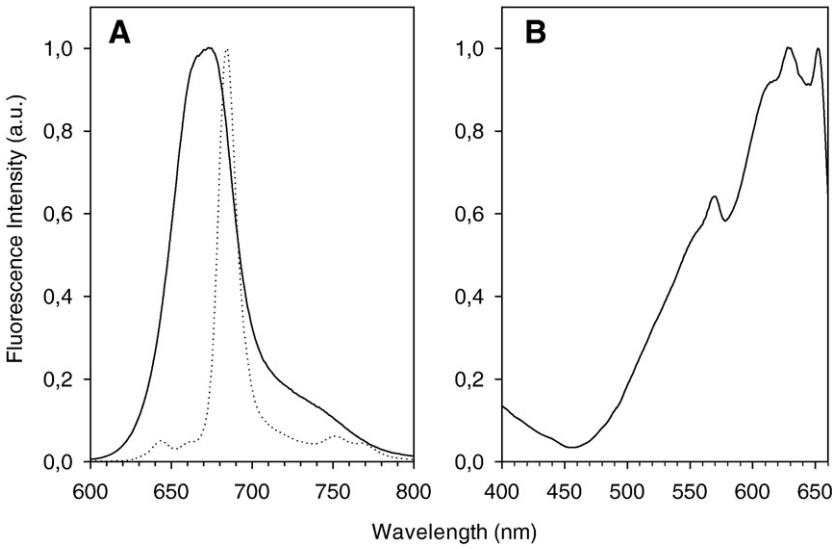

Fig. 4. Spectral properties of isolated phycobilisomes from vegetative cells. A, Emission fluorescence spectra of isolated PBS when excited at 570 nm recorded at room temperature (solid line) and 77 K (dotted line). Spectra were normalized at the maximum intensity peaks. B, Excitation spectrum at 77 K when the fluorescence is collected at the 680 nm emission band of the terminal emitter.

PSII and PSI, in response to changing light conditions [30–37]. One effect of a transition from state 1 to state 2 is the decoupling of phycobilisomes from PSII and increased energy transfer to PSI [38,39]. Experimentally, this can be observed as a decrease in fluorescence emission from the 680–695 nm region. Poising cyanobacterial cells in state 2 can be made by illumination with light in the absorption region of the phycobilisomes, i.e. green to yellow light, for 5–10 min. The common rationalization for the transition to state 2 after this treatment is that the phycobilisomes, originally coupled mainly to PSII, absorbs the light and triggers some sort of "signal" to distribute some of the energy to PSI. Several investigations have indicated that state transitions depend on light-induced changes in the redox status of the plastoquinone pool, and/or a change in the transmembrane proton gradient [31,35,40,41].

In this study, when either isolated heterocysts or intact filaments were pre-illuminated with green light we observed quenching of the fluorescence at 680 nm, originating mainly from the terminal emitter in the APC core of the phycobilisome. Concomitantly we observed trimerization of PSI. The observed spectral changes are similar to a transition to state 2, but the effects should have nothing to do with excitation of PSII since PSII is inactivated in the heterocysts. Instead, we suggest that these light-induced events occur completely independent of linear electron transport.

The relative increase in excitation transfer from the phycobilisome, was higher at the far-red emitting Chl-*a* molecules in the PSI trimers (observed at 750 nm) than at the "normal" emission maximum from PSI (728 nm), suggesting that a greater relative increase in energy transfer was largely taking place to trimeric PSI. Since the formation of the PSI trimers was observed in the same samples, we propose that these two events are connected. In mutant studies of *Synechococcus* sp. PCC 7002 unable to form PSI trimers, light-induced state transitions could still occur, but changes in antenna coupling were faster than in the wild type [45,66]. This was suggested to be due to the increased mobility of the PSI complexes, which decreased the coupling of the phycobilisomes to PSI. Any interactions between the phycobilisomes and PSI would be quickly reversed due to the lack of stabilization of the formed antenna–reaction center complex, in the absence of trimerization. In our study the trimerization seems strongly connected to increased energy transfer, and we therefore suggest that trimerization is a prerequisite for phycobilisome coupling and energy transfer to PSI. In addition, this was observed in both intact filaments and isolated heterocysts suggesting that trimerization of PSI is induced completely independent of PSII.

It is hard to speculate about what molecular events are causing the trimerization, but our results imply that it requires pre-illumination by wavelengths that are predominantly absorbed by the phycobilisomes. In previous studies in *S. platensis*, a similar light-induced trimerization of PSI was observed. The authors conjectured that the reaction was not dependent on wavelength, but simply depending on "light on–light off" [48,67]. It was hypothesized that the formation of PSI trimers is driven by local light-induced proton gradients. When we illuminated heterocysts by either red or green light in the EPR experiment shown in Fig. 3, the signal amplitude of the P_{700}^{+} radical was independent of the illumination wavelength. Therefore, the yield of P_{700}^{+} was the same at 708 nm and at 560 nm. It is reasonable to assume that any light-induced changes in the redox state of the plastoquinone pool, or formation of proton gradients by this treatment, also were independent of wavelength in our experiments. However, the trimerization of PSI and quenching of phycobilisome fluorescence was clearly wavelength dependent. This indicates that proton or electron transport may not be responsible for the effect. Instead, our results might be explained by light sensing by the phycobilisome.

Since the formation of P_{700}^{+} was independent of the wavelength of the illumination, in contrast to the trimerization and phycobilisome coupling, we propose the following: light absorbance by the phycobilisome could lead to an alteration in the protein structure, thereby changing the phycobilisome coupling to PSI. The change in the structural coupling of the phycobilisome to PSI could then facilitate trimer formation. The association between the phycobilisome and PSI is stabilized by trimerization and energy transfer is increased. This type of mechanism, only depending on the structural changes in the phycobilisome, could explain why trimerization only occurs after green illumination.

Heterocysts do not possess water oxidation, and "state transitions" in the classical sense of redistribution of energy transfer between PSI and PSII therefore lose their meaning [3,14,68]. Heterocysts must perform cyclic, photosynthetic electron transfer to provide enough ATP for the nitrogenase reaction. It has been shown that nitrogen fixation is several times more efficient under illumination than in the dark [9–12]. Heterocysts are also capable of keeping stable ATP concentrations under varying light intensities and other factors affecting the plastoquinone redox state [69]. A mechanism that regulates the

amount of excitation energy transferred to PSI would provide a way for the heterocyst to respond to the quality of light by coupling or decoupling the phycobilisome to PSI, thereby supporting PSI function in the heterocyst.

In a recent study of mutants in *Synechococcus* sp. PCC 7002, the APC core protein ApcD was found to be essential for state transitions, and increase the energy transfer to PSI under state 2 conditions [70]. The increase in energy transfer to PSI in state 2, was lower in a PSII-less mutant. However, heterocysts are naturally PSII-less cyanobacterial cells, in contrast to mutants. The light-induced events that we observe thus resemble a state transition "half reaction" in several respects. Our conclusion that PSI-centered events during state transitions are independent of PSII, is therefore strongly supported.

We can then summarize our model for short-term light adaptation in heterocysts: by illuminating the cells with red light, the phycobilisomes are weakly associated to PSI in a state resembling state 1. Prolonged illumination by phycobilisome-specific light leads to an increased affinity of the phycobilisome to bind to PSI, which facilitates trimerization and energy transfer from the phycobilisome to PSI (leading to "state 2"). We have thereby found a relationship between PSI trimer formation and phycobilisome function that has not been clarified earlier. Since the observations were similar in intact filaments, we hypothesize that the changes in PSI oligomerization and antenna coupling may be independent of PSII activity also in the vegetative cells. This distinction is normally difficult to make in cyanobacterial cells due to the presence and activity of PSII, but by studying the heterocysts it is possible to discern some of the PSI-centered mechanisms independently.

Table 1
Quantification of phycobilisome proteins in heterocysts of *Nostoc punctiforme*. Abundance ratios below 1.0 depict lower abundancy in heterocysts than in filaments. It can be noted that the terminal emitter-protein ApcE was found in higher relative abundance than most other phycobiliproteins. Data compiled from [13].

Locus tag	Gene name	Abundance ratio[a]	Comment
Allophycocyanin			
Npun_R4842	*apcA*	0.14 ± 0.03	Phycobiliprotein
Npun_F5388	*apcB*	0.30 ± 0.01	Phycobiliprotein
Npun_R4840	*apcC*	0.35 ± 0.02	Linker polypeptide
Npun_F0878	*apcD*[b]	0.14 ± 0.02	Phycobiliprotein
Npun_R4843	*apcE*	0.45 ± 0.02	Terminal emitter
Npun_R4841	*apcB*	0.20 ± 0.02	Phycobiliprotein
Phycocyanin			
Npun_F5290	*pcyA*	0.12 ± 0.02	Phycobiliprotein
Npun_F5289	*pcyB*	0.15 ± 0.02	Phycobiliprotein
Npun_F5292	*cpcC*	0.28 ± 0.01	Linker polypeptide
Npun_F5291	*cpcC2*	0.57 ± 0.01	Linker polypeptide
Npun_F5293	*cpcD*	0.34 ± 0.04	Linker polypeptide
Npun_F5295	*cpcF*[b]	0.40 ± 0.02	Phycocyanobilin lyase
Npun_F3811	*cpcG1*[b]	0.25 ± 0.01	Linker polypeptide
Phycoerythrin			
Npun_R3806	*cpeA*	0.15 ± 0.04	Phycobiliprotein
Npun_R3807	*cpeB*	0.11 ± 0.02	Phycobiliprotein

[a] The abundance ratio is defined as the relative abundance of each protein between isolated heterocysts and intact filaments. See [13] for details.
[b] Due to incomplete annotation, the protein identity was found by comparison to *Synechococcus* sp. PCC 7002.

4.2. The function of heterocyst phycobilisome

The excitation spectra in Fig. 3, as well as proteomic results [13], indicate that phycobilisome degradation during heterocyst differentiation does not affect all phycobilisome components alike. The excitation spectra in isolated heterocysts showed a decreased intensity in the entire region between 550 and 650 nm, compared to that of the filaments (Figs. 2A, B). However, the spectra also indicated that excitation at 570 nm was much less prominent than excitation at 620 nm in the heterocysts. We therefore conclude that phycoerythrin, which is located at the tip of the rods and absorbing the shortest wavelengths, has diminished to a greater extent than phycocyanin. We therefore propose that the phycobilisomes in the heterocysts are modified compared to in the vegetative cells, so that efficient energy transfer to PSI is maintained. Phycobiliprotein composition is known to be regulated during long-term light adaptation, and a similar regulation most likely takes place during heterocyst differentiation.

This conclusion is supported by proteomic studies of isolated heterocysts, where a number of phycobilisome related proteins were identified and quantified by iTRAQ [13]. We have made a compilation of the quantification data, presented in Table 1. The phycoerythrin proteins were found to be the most down-regulated in heterocysts, and were on average 7.5 times less abundant in the heterocysts than in the intact filaments. The subunits related to phycocyanin showed varying relative abundancies and were on average 3.5 times less abundant in heterocysts. The APC core proteins also showed significant variations in relative abundance, indicating that the subunit stoichiometry of the core is altered in the heterocysts. Interestingly, the terminal emitter ApcE protein was found to be the least down-regulated of the phycobiliproteins in heterocysts, being only 2.2 times less abundant than in the vegetative cells (Table 1).

The data suggest that the phycobilisomes in heterocysts are composed of an APC core and phycocyanin rods, with altered stoichiometry in the subunit composition. These modifications would be optimal for light absorption at longer wavelengths and for energy transfer to PSI. Mutant studies in *Synechocystis* sp. PCC 6803 have indicated that energy transfer to PSI also might occur via the phycocyanin rods directly, in a modified, coreless antenna [21,65,67,71,72]. We have previously suggested that phycocyanin might transfer energy to PSI in a similar way in *N. punctiforme* [14]. Here we have investigated the excitation spectra in heterocysts in more detail (Fig. 2B), and we propose that a different type of altered phycobilisome dominates in heterocysts. The excitation spectra in the heterocysts showed a decreased intensity in the phycocyanin and phycoerythrin bands, and enhanced intensity at 674 nm, compared to filaments. Chl-*a* excitation is the main contributor to the 674 nm peak, but also the terminal emitter (ApcE) from the phycobilisome contributes to excitation at this wavelength. Taken together with the changes in the abundancy of the phycobiliproteins in the heterocysts, these results suggest that phycobilisomes are specifically altered, rather than unspecifically degraded, during heterocyst differentiation, and that energy transfer in the heterocysts takes place from the phycobilisome, via the terminal emitter, and into PSI.

4.3. A model for the events leading to energy transfer to PSI

Fig. 5 shows a schematic representation of a model that illustrates our results and might be applied to both heterocysts (upper panel) that have a modified phycobilisome composition, and to filaments (lower panel), with normal phycobilisomes. To the left in each panel is the situation after pre-illumination by red light: PSI is primarily monomeric and the phycobilisomes are decoupled or only slightly associated to PSI. Since red illumination can be efficiently harvested by the PSI reaction center antenna, the phycobilisome is not required for optimizing light harvesting. In the heterocysts, the decoupled antenna emits fluorescence from its terminal emitter when the phycobilisome is excited. To the right in each panel the second situation is depicted, which is induced by phycobilisome-specific (green light) illumination. It is characterized by the formation of trimeric PSI and the coupling of the phycobilisome to the PSI trimers. When the phycobilisomes are excited the energy will be transferred to PSI and the fluorescence from the terminal emitter will be quenched. It is important to keep in mind that we have not attempted to investigate all aspects of the normal state transitions in the filaments, and the

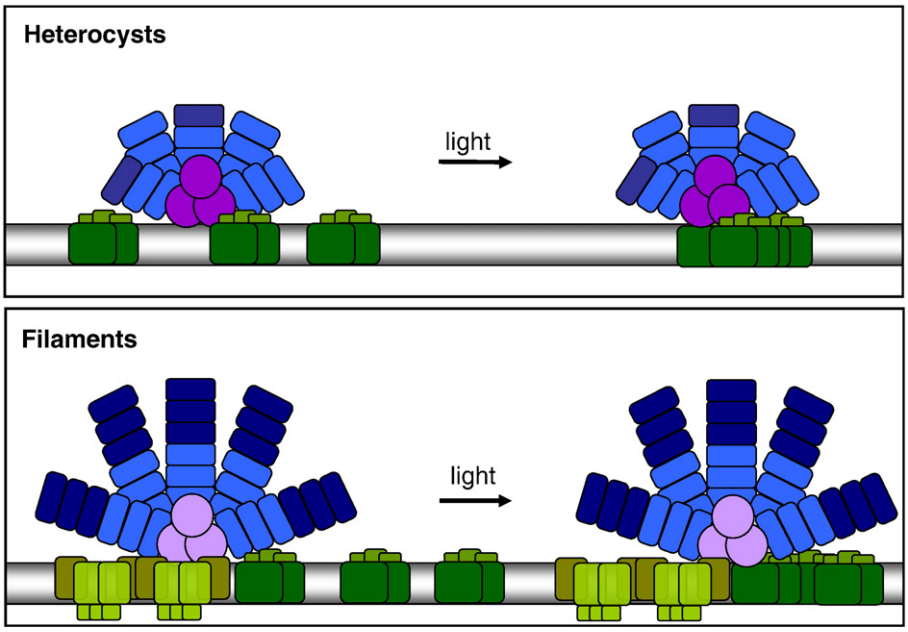

Fig. 5. Graphic representation of the effect of phycobilisome-specific illumination. The upper panel shows the heterocyst thylakoid membrane with reaction center complexes, and modified phycobilisomes compared to the vegetative cells. In the upper panel only the PSI reaction centers are shown, while both PSI and PSII are depicted in the lower panel. The vegetative cells (lower panel) have a typical phycobilisome antenna, while the heterocyst phycobilisomes have shorter rods and an altered allophycocyanin core. Phycobilisome-specific illumination by a green–orange light, causes a change in the phycobilisome which facilitates the trimerization of PSI and the energetic coupling of the phycobilisome. See text for details.

model in the lower panel is by no means a full account of current ideas about state transitions. We want to point out, that the same PSI-centered events seem to take place in both vegetative cells and heterocysts, and that this may shed some light on the molecular mechanisms behind some of the light-induced changes that take place during state transitions.

5. Conclusions

We have shown that in both filaments and isolated heterocysts, illumination with 560 nm light at medium intensity causes quenching of the fluorescence emission from the phycobilisomes, simultaneously with trimerization of PSI. A similar illumination regime using 480 nm light did not cause the same changes, demonstrating that the OCP is not involved in the quenching mechanism. Excitation spectra showed that 560 nm illumination increased the energy transfer from the phycobilisomes to the trimeric PSI reaction centers. Since the effect is clearly observable in heterocysts, which naturally do not posses PSII water splitting activity, both the trimerization of PSI and increased energy transfer from phycobilisomes are independent of linear electron transfer. Thus, our results suggest that the light-induced events taking place in heterocysts might be dependent only on light sensing.

The observed effects of illumination are analogous to state transitions that normally occur in cyanobacterial cells. We therefore propose that some of the molecular events we observed are also taking place during normal state transitions. Furthermore, these events are independent of the function of PSII.

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