Fluorescence spectroscopy study of heterocyst differentiation in *Anabaena* PCC 7120 filaments

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Filamentous *Anabaena* PCC 7120 differentiates nitrogen-fixing specialized cells called heterocysts at regular intervals following removal of combined nitrogen from the medium. Phycobiliproteins are degraded during differentiation. Heterocyst differentiation was followed at the single cell level by using confocal fluorescence microscopy. The presence of an enhanced fluorescence emission peak from allophycocyanin (APC) indicates that the degradation of the phycobilisomes during nitrogen deprivation possibly initiates at the linker between APC and photosystem II in a bottom-to-top disassembly model. Furthermore, the fluorescence emission peak around 650 nm provides an advantageous marker to identify early candidates for differentiation.

**INTRODUCTION**

Filamentous nitrogen-fixing cyanobacteria, of which *Anabaena* PCC 7120 has become the archetypical example, differentiate nitrogen-fixing specialized cells called heterocysts at regular intervals along each filament in suitable medium. The length of the intervals is determined by the interplay of regulatory proteins: a transcription factor called HetR, two proteins that contain the amino acid sequence RGSGR (PatS and HetN), and other transcription factors (Flores & Herrero, 2010; Haselkorn, 1978, 2008). The PatS and HetN peptides prevent HetR from binding to DNA and destabilize the HetR protein (Huang et al., 2004; Risser & Callahan, 2009; Yoon & Golden, 1998). Under our laboratory conditions the vegetative cells between each heterocyst divide approximately every 16 h. During that time, a vegetative cell approximately halfway between two heterocysts differentiates into a new heterocyst. *Anabaena* PCC 7120 usually has about 5–10% heterocysts, but that fraction can be increased by overexpression of the *hetR* gene (Buikema & Haselkorn, 2001) or reduced by overexpression of the *patS* or *hetN* genes (Callahan & Buikema, 2001; Yoon & Golden, 2001).

Differentiation requires the destruction of many proteins, including the phycobiliproteins, parts of the photosystem (PS)II reaction centre, and ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO), ensuring that the heterocyst cannot generate oxygen and cannot fix carbon. Newly expressed proteins include enzymes for the synthesis of glycolipids and polysaccharides that form a double-layered envelope around the heterocyst, enzymes for reorganization of metabolism to produce reductants for nitrogen fixation, and the suite of enzymes and redox proteins for nitrogen fixation and assimilation (Fay, 1992; Golden & Yoon, 2003; Kumar et al., 2010). In all, HetR is responsible for the expression of some 1500 of the 7000 genes in the *Anabaena* PCC 7120 genome (Buikema & Haselkorn, 2001, 1991; Liang et al., 1992; Wolk, 1996).

The process of differentiation can be followed at the single cell level by using confocal fluorescence microscopy. Cyanobacteria harvest light energy across much of the visible spectrum using phycobiliproteins organized in cylindrical phycobilisomes (PBSs), which transfer energy principally to PSII reaction centres. The energy transfer can be monitored by measuring fluorescence emission from phycobiliproteins or chlorophyll (Chl) following excitation at a wavelength absorbed by phycobiliproteins (Glazer, 1985; Yoon & Golden, 1998). Any damage to the protein involved in energy transfer will result in alteration in the fluorescence emission spectra. By monitoring fluorescence emission, we can track single cell development.

**METHODS**

**Cell growth and induction.** *Anabaena* PCC 7120 cultures were grown in BG11 medium under room light (~9 μE m⁻² s⁻¹) and temperature (~24 °C). They were switched from BG11 to a BG11–0.75% agarose slab gel surface by being centrifuged and washed three times with BG11–medium.

**Microscopy and fluorescence spectroscopy.** The growth and fluorescence spectroscopy of filaments on the gel surface were monitored with a Leica SP5 Tandem Scanner Spectral 2-Photon confocal microscope with a Leica ×63 numerical aperture 0.9 water immersion lens. The fluorescence spectra were measured at a pinhole value of AU 1.3, meaning that the focus thickness along the z axis was...
about 4.5 μm. Samples were excited by the orange HeNe laser at 561 nm and the fluorescence emission spectrum was collected from 570 to 740 nm with 5.0 nm per step. Each sample was scanned 32 times and the mean of the fluorescence spectra is presented. The fluorescence intensity was recorded using arbitrary fluorescence units (au).

RESULTS

The absence of phycobiliprotein absorption in the heterocysts predicts that excitation at a wavelength absorbed by a phycobiliprotein will not yield emission from Chl in the heterocysts. This result is well known, and is shown for single cells in a differentiated *Anabaena* PCC 7120 filament in Fig. 1. The new and unexpected result is that after the differentiation begins, emission from the developing cell increases in intensity with a shift to shorter wavelength. The Chl (Chl a) emission intensity eventually drops until it is no longer visible. We interpret the short-lived increase in emission intensity to be due to release of the phycobilisomes from the PSII centres and the loosening of their structures, allowing some of the energy in phycobilisomes to contribute directly to fluorescence rather than be transferred to Chl (Gantt, 1981; Glazer, 1985). These differences allow us to use confocal microscopy of single cells to follow the evolution of a heterocyst by recording the fluorescence emission spectrum as a function of time after transfer of the cells from complete medium to one lacking combined nitrogen. Eventually the degradation of the pigments interrupts the energy transfer completely. As shown in Fig. 1, at stage II of heterocyst differentiation, the fluorescence emission has shifted to a shorter wavelength with increasing intensity. Watching the bright spots along the filament can directly identify early candidates for differentiation, as reported elsewhere (Toyoshima et al., 2010).

In Fig. 2, cell 1 is a mature heterocyst with low Chl fluorescence. Cells 2, 3, 6, 7, 8, 10, 11 and 12 are considered to be vegetative cells with similar fluorescence spectra. At around 645 nm, only one peak from phycocyanin (PC) is shown. Fluorescence emission from allophycocyanin (APC) is on the right shoulder of the PC peak. Compared with the fluorescence of the vegetative cells, cells 4, 5, 9 and 13 have a more resolved fluorescence emission peak at 650 nm, which we attribute to APC, due to decreased energy transfer from APC to Chl a (Glazer, 1985). Another possible contributor to this peak is C-phycocyanin (C-PC) (Gantt, 1981). These cells with the fluorescence peak at 650 nm were considered to be early candidates for heterocyst differentiation at stage I. The APC peak occurs almost at the initiation of heterocyst differentiation, much earlier than the candidates for differentiation become bright. About four vegetative cells separated these early candidates.

Cell 4 and cell 5 were descended from the same mother cell, and both show the appearance of the APC peak. Later, cell 5 has differentiated into a heterocyst, while cell 4 has reverted to a vegetative cell. We interpret these fates as resulting from early competition, mentioned earlier by others (Allard et al., 2007; Toyoshima, et al., 2010). Both cell 4 and cell 5 are induced to produce the early regulator PatS, which binds to HetR to prevent DNA binding and consequent differentiation (Allard et al., 2007; Yoon & Golden 2001). Here, we believe that cell 5 produces the PatS peptide, which is exported to cell 4, causing cell 4 to revert due to degradation of HetR, while cell 5 continues to differentiate due to the activity of HetR. Thus, the cell fates could be determined by a slight difference in the ability to

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**Fig. 1.** Fluorescence emission spectra of single *Anabaena* PCC 7120 cells in a filament. The fluorescence intensity is recorded as au. Four stages during heterocyst differentiation can be identified. Fluorescence excitation was at 561 nm.
export PatS. Alternatively, there might be stochastic differences in the amount of PatS made in the two cells.

With the same single cell tracking, we have been able to track the growth and differentiation of all individual Anabaena cells along the filament shown in Fig. 3 for the next 4 days. For example, note the development of cell 9 in Fig. 2. Its differentiation with the corresponding fluorescence during development is shown in Fig. 3. One day after the beginning of nitrogen deprivation, cell 9 began to show fluorescence emission at 650 nm. After 4 days, it had differentiated into a heterocyst. Using the appearance of the fluorescence peak at 650 nm as a measure of the candidates for differentiation, we found that except for a few cases, all early candidates with the fluorescence peak at 650 nm finally differentiated into heterocysts. Furthermore, no peak was seen at 650 nm in the vegetative cells. For example, five early candidates for heterocysts were identified by 650 nm fluorescence emission in the 52-cell filament shown as day 3 in Fig. 3. Their fluorescence spectra, together with those of five random vegetative cells in the same filament, are shown in Fig. 4. The fluorescence difference between the early heterocyst candidate and the vegetative cell is clearly demonstrated.

**DISCUSSION**

The key findings of this report can be summarized as follows: tracking Anabaena PCC 7120 cell evolution via single cell fluorescence reveals a peak at 650 nm from APC or from a possible combination of C-PC and APC. The peak at 650 nm appears very early during differentiation and can be used to identify early candidates for differentiation. The presence of this emission rise at 650 nm can be attributed to loss of energy transfer between APC and PSII. In Fig. 2, cell 4 and cell 5 were descended from the same parent cell, and both show the appearance of the APC peak. Cell 5 differentiated into a heterocyst, while cell 4 reverted to a vegetative cell. Fig. 2 also demonstrates that fluorescence emission from PC is constant and that from APC increases during the early stage of the differentiation. This means that...
PC remains on the rod and connected with APC, with energy transfer from PC to APC still efficient.

With these findings, it is possible to propose that the degradation of the phycobilisomes during nitrogen deprivation initiates at the linker between the APC and PSII. The linkers in the PBS of cyanobacteria and red algae play important roles in PBS assembly and energy transfer: one linker connects the rod with the PBS core, another linker holds the rod together, and another linker connects APC to PSII. The linker connecting the core of PBS with the thylakoid membrane and PSII is considered to be the major

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**Fig. 3.** (a) Images of the *Anabaena* PCC 7120 filament and differentiation of the individual cell (arrow); (b) fluorescence emission spectra of the individual cell (arrow) during differentiation. Fluorescence excitation was at 561 nm.
terminal energy emitter to PSII. During the initiation of PBS degradation, phosphatases first dephosphorylate the linkers. Linker dephosphorylation may act as a signal for protein degradation (Piven et al., 2005; Dines et al., 2008).

The appearance of the fluorescence emission peak at 650 nm possibly shows that the linker between the APC and PSII is cleaved at the start of cell differentiation. Consequently, energy transfer is blocked between APC and PSII and energy is released as fluorescence emission from APC. On the other hand, the linker connecting the rod and core is still intact and the energy from the PC absorption is still transferred efficiently to APC. In our fluorescence spectroscopy set-up, the sample excitation was at 561 nm, where PC has much stronger absorption than APC. If the linker between PC and APC was impaired, the fluorescence emission at 650 nm should decrease. These two factors contribute to the appearance of the APC fluorescence emission peak at 650 nm.

Earlier papers show a model in which PBS disassembly occurs with the loss of rod hexamers before degradation of the core, and from rod tips down toward the core (Dines et al., 2008 and references therein). But if degradation of PBS involves dephosphorylation and subsequent cleavage of the linkers from the bottom up, that would explain the appearance of the APC fluorescence emission peak at 650 nm at the beginning of cell differentiation.

Based on the observations and discussions above, the appearance of the APC fluorescence emission peak can be used as a sign of early candidacy for heterocyst differentiation along the Anabaena PCC 7120 filament. The early candidate is about four cells away from a mature heterocyst under our conditions. It is interesting to find that the fluorescence emission from PC, is constant and that the fluorescence emission from APC even increases during this early stage. This demonstrates that PC remains on the rod and that the energy transfer from PC to APC is still efficient.

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**REFERENCES**


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