Transformation of Thylakoid Membranes during Differentiation from Vegetative Cell into Heterocyst Visualized by Microscopic Spectral Imaging

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Some filamentous cyanobacteria carry out oxygenic photosynthesis in vegetative cells and nitrogen fixation in specialized cells known as heterocysts. Thylakoid membranes in vegetative cells contain photosystem I (PSI) and PSII, while those in heterocysts contain predominantly PSI. Therefore, the thylakoid membranes change drastically when differentiating from a vegetative cell into a heterocyst. The dynamics of these changes have not been sufficiently characterized in situ. Here, we used time-lapse fluorescence microspectroscopy to analyze cells of *Anabaena variabilis* under nitrogen deprivation at approximately 295 K. PSI degraded simultaneously with allophycocyanin, which forms the core of the light-harvesting phycobilisome. The other phycobilisome subunits that absorbed shorter wavelengths persisted for a few tens of hours in the heterocysts. The whole-thylakoid average concentration of PSI was similar in heterocysts and nearby vegetative cells. PSI was best quantified by selective excitation at a physiological temperature (approximately 295 K) under 785-nm continuous-wave laser irradiation, and detection of higher energy shifted fluorescence around 730 nm. Polar distribution of thylakoid membranes in the heterocyst was confirmed by PSI-rich fluorescence imaging. The findings and methodology used in this work increased our understanding of how photosynthetic molecular machinery is transformed to adapt to different nutrient environments and provided details of the energetic requirements for diazotrophic growth.

The most essential pigment-protein complexes for oxygenic photosynthesis are PSI and PSII, which are embedded in the thylakoid membranes of chloroplasts and cyanobacteria. Cooperation between PSI and PSII achieves light-driven noncyclic electron transport from the oxidative splitting of water to the reduction of ferredoxin and is accompanied by the generation of a proton gradient for ATP synthesis. Phycobilisomes (PBS), another pigment-protein complex, are attached to the stromal side of the thylakoid membrane in cyanobacteria and red algae; they work as light-harvesting antennae to transfer electronic excitation energy mainly to PSI and, in some cases, to PSI (Gantt 1994). The integration of these pigment-protein complexes changes in response to light conditions, nutrient status, and developmental stage (Fujita et al., 1994; Grossman et al., 1994; Wolk et al., 1994).

Some cyanobacteria, including *Anabaena variabilis*, are able to grow diazotrophically using the nitrogen-fixing enzyme nitrogenase. Because nitrogenase is sensitive to oxygen, oxygenic photosynthesis is not readily compatible with diazotrophic growth. When this filamentous cyanobacterium is grown under fixed nitrogen-deficient conditions, approximately 1 in 10 to 20 vegetative cells differentiates into a heterocyst, in which oxygenic photosynthesis is suppressed and nitrogenase becomes operative (Haselkorn, 1978; Wolk et al., 1994). The other vegetative cells continue oxygenic photosynthesis. The differentiation of heterocysts from chains of vegetative cells has been studied extensively (Golden and Yoon, 2003; Toyoshima et al., 2010). The abundances of PSII and PBS decrease during the transition. PSI appears to persist in the heterocyst to produce ATP by cyclic electron transport, because nitrogen fixation demands a large amount of ATP (Wolk et al., 1994). However, the mechanisms by which PBS and PSII are degraded during heterocyst differentiation remain unclear, and whether the amount of PSI per cell changes is unknown.

The PBS of *A. variabilis* contain three types of phycobiliproteins, pigment-protein complexes with distinct absorption and fluorescence spectra. The core PBS contains allophycocyanin (APC), which absorbs around 654 nm (Ying and Xie, 1998); the core is most closely connected to PSI. More peripherally in the PBS, the so-called rod contains phycoerythrocyanin (PEC) and phycocyanin (PC), which absorb maximally around 575 and 604 to 620 nm, respectively (Switalski and Sauer, 1984; Zhang et al., 1998). Photon energy is absorbed by PEC, then transferred...
downhill through PC and APC and finally to PSI. The structure of PBS is probably optimized not only for efficient energy transfer to PSII and/or PSI but also for transformation and/or degradation under various nutrient conditions. However, the order in which these subunits degrade during heterocyst differentiation remains unknown. One strategy to address this question is to isolate heterocysts at several stages during differentiation and quantify their proteomes via mass spectrometry. However, such isolation procedures work well only when there is a good understanding of the properties of cells at different stages. Ideally, noninvasive methods should be used to understand changes in the integrity of PSII and PBS in intact cells in filaments.

In principle, time-lapse microscopic observations can clarify the process of differentiation from a vegetative cell into a mature heterocyst. Spectral microscopy is an ideal tool to analyze physiological state and/or amounts of pigment-protein complexes under various conditions. Acquiring microscopic fluorescence spectra of individual cells is a natural extension of laser scanning confocal fluorescence microscopy, which has been applied to several types of cyanobacterial cells, including heterocysts (Peterson et al., 1981; Ying et al., 2002; Wolf and Schüssler, 2005; Kumazaki et al., 2007; Vermaas et al., 2008; Sukenik et al., 2009; Bordowitz and Montgomery, 2010; Collins et al., 2012, Sugiyama and Itoh, 2012). Microscopic fluorescence spectra reflect the concentration of pigment-protein complexes and the energy transfer dynamics between photosynthetic pigments. However, to date, there have been no thorough time-lapse investigations of the fluorescence spectra of heterocysts and vegetative cells during the differentiation process.

In this study, we investigated the dynamic changes in thylakoid membranes of _A. variabilis_ during heterocyst differentiation. Our unique microscopic system can acquire fluorescence spectra from an entire linearly illuminated region with about 2-nm wavelength resolution in a single exposure (Kumazaki et al., 2007). Heterocyst formation was induced by transferring filament under OPE qualitatively differed from that under two- and contiguous vegetative cells. Pigment PSI in individual heterocysts compared with its parental et al., 2010, 2011). We used this technique to quantify PSI in individual heterocysts compared with its parental proteomes via mass spectrometry. However, such isolation procedures work well only when there is a good understanding of the properties of cells at different stages. Ideally, noninvasive methods should be used to understand changes in the integrity of PSII and PBS in intact cells in filaments.

RESULTS

Time-Lapse Fluorescence Spectral Imaging by TPE and Transmission Spectral Imaging

A time course of fluorescence images of a single filament under 808-nm TPE at five selected fluorescence regions was generated with our microscopic fluorescence spectrometer (Fig. 1). The five regions were F615 (600–630 nm), F643 (630–655 nm), F662 (650–670 nm), F685 (670–700 nm), and F715 (700–730 nm), which approximately corresponded to PEC, PC, APC, PSI, and PSI fluorescence regions, respectively, as confirmed by our spectral analyses (Supplemental Fig. S1). After 2 h of nitrogen deprivation, all cells had similar shapes in the bright-field images and similar intensities in the fluorescence images. After 24 h of nitrogen deprivation, the terminal cell in the filament appeared to be larger than the other cells (Fig. 1; T620, T680, and T730), a typical feature of heterocysts. The terminal cell also fluoresced more weakly than the other cells, especially at F662, F685, and F716, although the signal at F643 was comparable to that of the other cells. From 24 to 96 h, all fluorescence signals of the terminal cell gradually decreased to zero, except at F715, where it retained a faint signal.

Bright-field microscopy images with monochromatic illumination reflect absorption by photosynthetic pigments at the selected wavelengths of 615 to 625 nm (Fig. 1; T620), 675 to 685 nm (T680), and 725 to 735 nm (T730). At T620, the terminal cell became more transparent (brighter) over time than the nearest cell, indicating a decrease in abundance of phycobilin molecules due to degradation of the PBS complex, as reported previously (Grossman et al., 1994; Wolk et al., 1994). The difference in transmission between the terminal cell and its neighbor was less obvious at T680 than at T620; at 96 h, the darkness of the terminal cell was similar to that of the nearest vegetative cell, indicating the presence of chlorophylls. Thus, the T620 and T680 images indicated a substantial decrease in PBS in the terminal heterocyst, but the amount of chlorophyll
was also a small increase in intensity at around 685 nm. The fluorescence images were taken at wavelength regions 600 to 630 nm (F615), 630 to 655 nm (F643), 655 to 670 nm (F662), 670 to 700 nm (F685), and 700 to 730 nm (F715). Bright-field images with monochromatic illumination were taken at 615 to 625 nm (T620), 675 to 685 nm (T680), and 725 to 735 nm (T730).

Each gray-scale fluorescence image was scaled by its maximum and minimum intensities except in the bottom row (at 96 h), which was scaled by 40% of the maximum intensity to visualize weakly fluorescent heterocysts. White and black arrows indicate terminal cells. Bar = 10 μm.

The fluorescence spectra of heterocyst cells clearly changed over time, while there were only small changes in those of vegetative cells (Fig. 3). At 2 h, two major peaks, at 663 ± 1.5 and 684 ± 1.5 nm, were observed in both preheterocysts and vegetative cells. In the heterocyst at 24 h, the overall intensity of the fluorescence decreased, and the main peak was located around 648 nm, which was clearly blue shifted compared with the peak at 2 h (Fig. 3A). Between 24 and 97 h, there was a large decay at wavelengths less than 700 nm, while the fluorescence intensity remained nearly constant around 730 nm. In vegetative cells, the main decay in intensity was at around 620 nm, and there were minor decays at wavelengths less than 660 nm (Fig. 3, B and C). There was also a small increase in intensity at around 685 nm.

**Time-Lapse Fluorescence Spectral Imaging by NIR OPE**

The fluorescence images obtained under 785-nm OPE (Fig. 4) differed in several respects from those obtained under 808-nm TPE (Fig. 1). From the earliest (2 h) to the latest (75 h) observation time, the fluorescence intensities in the F615 and F643 regions were too weak for detailed analyses of individual cells. These weak intensities were explained by preferential excitation of relatively long-wavelength-absorbing pigments (Hasegawa et al., 2010, 2011). The F715 intensities of heterocyst cells remained comparable to those of vegetative cells up to 72 h. The F662 and F685 intensities decayed in the heterocyst cell, but the rates of decay were slower than those observed under TPE (Fig. 1). These relatively slow decay rates of APC and PSII correlated with the delayed change in cell shape observed in the bright-field images (T620, T680, and T730 in Fig. 4). The terminal cell was already larger than the nearby vegetative cells at 24 h (Fig. 1; T620, T680, and T730), while differences in cell size and shape first became visible at 48 h when fluorescence images were obtained with 785-nm OPE (Fig. 4). The different timings of heterocyst formation did not appear to be due to different excitation laser conditions but to factors not yet completely controlled (e.g. extracellular residual nitrogen and/or intracellular stock of fixed nitrogen), as will be addressed below with examples.

Overall, the microscopic fluorescence spectra of heterocysts under 785-nm OPE were very similar to those of adjacent vegetative cells (Fig. 5, A and B). There were small but reproducible differences between the heterocyst and vegetative cells in the wavelength region around 650 to 690 nm, while the spectral shapes around 715 to 755 nm were nearly identical (Fig. 5C). These fluorescence spectra were also very similar to those of heterocyst cells under 808-nm TPE (Fig. 5C). Notably, the spectra containing PSI fluorescence as the dominant contribution were reminiscent of the fluorescence spectrum at 77 K of A. variabilis vegetative cells under nitrogen-sufficient growth conditions (Peterson et al., 1981; Mannan and Pakrasi, 1993), which showed...
peaks at 666, 685, 695, and 735 nm (Fig. 5D). The fluorescence spectra measured at 295 K appeared to reflect spectral broadening of the low-temperature spectrum.

Spectral Decomposition of Single-Cell Fluorescence Spectra

We obtained 1,625 fluorescence spectra from individual cells at various stages of heterocyst differentiation using two different excitation methods (785-nm OPE or 808-nm TPE). Simultaneous analysis of all these spectra by singular value decomposition (SVD) yielded five component spectra (Supplemental Figs. S1 and S2; Supplemental Materials and Methods S1). The five components, PEC, PC, APC, PSII, and PSI, were sufficient and necessary to fit all raw single-cell spectra, including different stages of heterocyst differentiation (Fig. 6). Analyses of spectral decomposition yielded time-dependent amplitudes of the five spectral components (Figs. 7 and 8). Under 808-nm TPE (Fig. 7), the APC and PSII components were very similar to each other in heterocyst dynamics (Supplemental Text S2); compared with the other components in heterocyst and vegetative cells, both decayed quickly and completely to zero in developing heterocysts but remained largely unchanged in neighboring vegetative cells. The PC components in heterocyst cells increased transiently before finally decaying to a lower value than the original intensity, while vegetative cells showed a
The PEC component in heterocysts decayed to below the original intensity, but the decay rate was much slower than that of APC. In vegetative cells, the PEC component decreased rapidly in the first 40 h and remained at approximately 10% to 15% of the value recorded at 2 h. The PSI component in heterocysts remained comparable to, or slightly higher than, the original value at 2 h, while it decreased slightly in vegetative cells.

The response times of the fluorescence spectra to nitrogen deprivation were similar or well synchronized among different filaments in the same glass-bottomed dish, but an independent experiment on filaments in nominally identical culture conditions showed a temporal delay (Supplemental Fig. S3; compare with Fig. 7 or its normalized version, Supplemental Fig. S4). Aside from the delay in responses, the relative timing of changes in the different spectral components was similar between the two independent experiments. For instance, in both data sets, the peak in PC fluorescence intensity was recorded when the APC and PSI fluorences first reached about 20% to 30% of their original intensities.

In the decomposition data obtained under 785-nm OPE (Fig. 8; Supplemental Fig. S5), PSI fluorescence was the dominant component, and its intensity remained essentially unchanged in both heterocysts and vegetative cells. The APC component had decayed to zero at approximately 72 h in the heterocysts, but the PSI component in heterocysts decayed more slowly. This difference in decay rate should be carefully interpreted, as discussed below.

Time-lapse analyses of identical filaments undergoing heterocyst differentiation (Figs. 7 and 8; Supplemental Figs. S3–S5) were obtained by illuminating several identical filaments with lasers at different time points; data at a single time point consisted of lateral scans (xy scans) at three different focal depths (z sections). Therefore, the cumulative effects of photochemical damage may have affected heterocyst differentiation. To examine such artifacts, several glass-bottomed dishes containing A. variabilis filaments were prepared from the same cell suspension at a time defined as 0 h. Several filaments in one of the dishes were microspectroscopically analyzed once at a single time point after fixed-nitrogen starvation began. The time-dependent intensities of the spectral components in these experiments (Supplemental Figs. S6 and S7) were nearly identical to those in the rigorous time-lapse experiments (Figs. 7 and 8; Supplemental Figs. S3–S5). These data indicated that repeated laser illumination had little effect on heterocyst formation and that differentiation of A. variabilis filaments was well synchronized even among different glass-bottomed dishes under these experimental conditions. We also sometimes observed cell divisions in filaments that were repeatedly analyzed by spectral imaging at 10- to 24-h intervals. Therefore, cellular viability appeared to have been preserved under these experimental conditions.

Heterocyst and Vegetative Cells under Long-Term Diazotrophic Growth Conditions

We obtained microscopic spectral features of A. variabilis filaments grown under diazotrophic conditions for longer than 10 d in a shaken flask. The filaments were transferred to a glass-bottomed dish just before microscopic experiments (Fig. 2D; Table I; Supplemental Figs. S8 and S9). PSI fluorescence intensity remained largely identical between the heterocyst and vegetative cells in the same filaments under both 785-nm OPE and 808-nm TPE; the PSI signal observed under 785-nm OPE was particularly stable (Supplemental Fig. S9E). PEC intensity was also very low in both heterocyst and vegetative cells (Supplemental Figs. S8A and S9A). At 2 h in the time-lapse measurements under 808-nm TPE, relative peak intensities of PEC in both the heterocysts and vegetative cells averaged 10% to 20% of the APC component in vegetative cells (Table I; Fig. 7; Supplemental Figs. S3 and S4), but on average, less than 1% of relative peak intensity was detected in both heterocyst and vegetative cells in cultures grown diazotrophically for more than 10 d. These findings indicated that PEC was stably present only when exogenous fixed nitrogen was supplied, at least under these growth conditions. Some heterocysts appeared to contain PC, APC, and PSI components during long-term diazotrophic growth (Supplemental

Figure 4. Time-lapse microspectroscopic images of A. variabilis filaments, including a terminal cell differentiating into a heterocyst, under 785-nm OPE. Panels are as described in Figure 1. Bar = 10 μm.
However, because we could not determine the age of heterocysts in the long-term diazotrophic culture, some of them may have been immature and therefore still contained detectable amounts of PC, APC, and/or PSII.

Intracellular Distribution of Thylakoid Membranes in the Heterocyst

There was a nonhomogenous distribution of PSI in the fluorescence images of both intercalary and terminal heterocysts under 785-nm OPE (Fig. 9; Supplemental Fig. S10). PSI fluorescence inside the heterocysts often showed a gradient parallel to the filament axis, as reported previously from an electron microscopy study (Maldener and Muro-Pastor, 2010). The intercalary heterocysts often showed bright PSI fluorescence peaks near their connections to adjacent vegetative cells, while their central regions were relatively dark. This sharp within-cell fluorescence contrast was not observed in

Figure 5. Time-lapse observations of microscopic fluorescence spectra of single heterocysts and neighboring vegetative cells of *A. variabilis* under 785-nm OPE. A and B, Spectra are averages of seven individual cells from separate filaments, including the one shown in Figure 4, at equivalent times and filament positions. A, Heterocysts (Het.). B, Single vegetative cells adjacent to heterocysts (Veg. 1). C, Comparison of three single-cell fluorescence spectra rich in PSI. Het., TPE represents averaged spectra of heterocysts under 808-nm TPE; Het., OPE represents averaged spectra of heterocysts under 785-nm OPE; and Veg., OPE represents averaged spectra of single vegetative cells under 785-nm OPE. These three spectra were selected for their smaller contributions from PSII and PBS compared with other spectra obtained under equivalent conditions. D, Fluorescence spectrum of vegetative cells of *A. variabilis* grown photoautotrophically and measured at 77 K (from Mannan and Pakrasi, 1993). a.u., Arbitrary units.

Figs. S7 and S8). However, because we could not determine the age of heterocysts in the long-term diazotrophic culture, some of them may have been immature and therefore still contained detectable amounts of PC, APC, and/or PSII.

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Figure 6. Spectral decomposition of microscopic fluorescence spectra in Figure 3. Experimental spectra are shown with black circles, whereas spectral components are shown as follows: PEC is a blue solid line (peak at 620 nm), PC is green (closed squares; peak at 646 nm), APC is magenta (peak at 661 nm), PSII is brown (white circles; peak at 686 nm), and PSI is a black broken line (peak around 730 nm). Simulated spectra are shown with black solid lines. For details, see text and Supplemental Materials and Methods S1. a.u., Arbitrary units; Het., heterocyst; Veg. 1, vegetative cell adjacent to the heterocyst.
vegetative cells under 785-nm OPE. Vegetative cells had relatively low-fluorescence regions near their centers because thylakoid membranes are localized at the periphery of the cytoplasm (Maldener and Muro-Pastor, 2010), as confirmed by the central dark regions of vegetative cells under 808-nm TPE (Fig. 1). The spatial resolution under 785-nm OPE (only slit confocal effect) was worse than under 808-nm TPE (slit confocal effect and suppressed out-of-focus excitation), especially in depth (Hasegawa et al., 2010). Thus, the central regions of vegetative cells (Fig. 9, B and C) appeared to fluoresce strongly because of the peripheral thylakoid membranes below and above the true three-dimensional center of the cells.

DISCUSSION

Different Dynamic Changes of PBS Subunits and PSII between Heterocyst and Vegetative Cells

The following descriptions of PBS subunits and PSII, particularly PEC and PC, focus mainly on fluorescence data obtained under 808-nm TPE. In the time-lapse microscopic fluorescence spectra, the decomposition patterns clearly indicated that the decay of APC fluorescence was highly synchronized with that of PSII (Fig. 7; Supplemental Figs. S3, S4, and S6). Although PEC, PC, and APC are subunits of the PBS, PC and PEC fluorescences decayed much later than that of APC during heterocyst formation. In fact, the PC and PEC fluorescences rose and then decayed at around the time the fluorescence intensities of APC and PSII reached 20% to 30% of their original values. In one experiment, the decay in PSII and APC fluorescence amplitudes did not start within the first approximately 15 h (Supplemental Fig. S3), which is later than in an equivalent experiment (Fig. 7; Supplemental Fig. S6). The difference may reflect residual fixed nitrogen in the culture solution and/or within cells. However, the relative timing of changes among the five fluorescence components was well preserved among independent experiments.

Greater pigment fluorescence can generally be attributed to increased concentrations and/or a decrease of nonradiative decay processes, including energy transfers. The decrease in absorbance around 600 to 640 nm, where PC subunits absorb, in heterocysts was largely monotonic (Fig. 2A; Ducret et al., 1996); therefore, the transient increase in PC fluorescence is best explained by a decrease in energy transfer.

Figure 7. Time dependence of five fluorescence components obtained from seven filaments with heterocysts of A. variabilis in the same glass-bottomed dish under 808-nm TPE. Average amplitudes (solid lines) are shown without normalization. Error bars show ±s.d. Broken lines show maximum and minimum values. a.u., Arbitrary units; Het., heterocyst; Veg. 1, vegetative cell adjacent to heterocyst.

Figure 8. Time dependence of five fluorescence components obtained from eight filaments with heterocysts of A. variabilis in the same glass-bottomed dish under 785-nm OPE. Average amplitudes (solid lines) are shown without normalization. Error bars show ±s.d. Broken lines show maximum and minimum values. a.u., Arbitrary units; Het., heterocyst; Veg. 1, vegetative cell adjacent to heterocyst.
efficiency from PC to APC. The energy acceptor, APC, was more rapidly lost from the original PBS complex than were the PC and PEC energy donors. The fast and synchronous degradations of APC and PSII seemed to be physiologically critical to suppress energy transfer to PSII and consequent oxygen evolution, which is essential for rapid nitrogenase functionality. The synchronous behavior of APC and PSII observed here was consistent with the recently reported statistical correlation analysis of fluorescence spectra of Nostoc punctiforme cells, including heterocysts, at 40 K based on an excitation wavelength of 532 nm (Sugiura and Itoh, 2012). In that analysis, the stoichiometric ratio between APC and PSII (APC/PSII) was more stable than the ratios of other pairs of fluorescence components, although no time-lapse measurement was performed and APC/PSII was more scattered and lower on average in heterocysts than in vegetative cells. Notably, PSII fluorescence appeared to decay more slowly during heterocyst differentiation than that of APC under 785-nm OPE (Fig. 8; Supplemental Fig. S5). Direct excitation of PSII by 780-nm OPE was reported (Thapper et al., 2009). Previously, we showed that excitation of PSII in chloroplasts was detectable under 785-nm OPE in plant mesophyll cells (maize) and a green alga (P. kessleri; Hasegawa et al., 2010, 2011). In those studies, the contribution of PSII to total fluorescence was more substantial than that observed in this work on vegetative cells of A. variabilis. Thus, direct excitation of PSII and/or indirect excitation of PSII through PSI (uphill spillover) at physiological temperatures is probably observable and larger than the excitation of APC under 785-nm OPE. Therefore, 785-nm OPE, and not 808-nm TPE, appeared to indicate that very small but detectable amounts of PSII remained for a longer time in heterocysts.

Several studies have reported that remaining PBS in heterocysts work as light-harvesting antennae for PSI (Peterson et al., 1981; Cardona et al., 2009). The relatively long lifetime of the rod part of PBS (PEC and PC) compared with the core (APC) observed in heterocysts in this work was consistent with the previous finding that heterocysts in Anabaena sp. 7119 accumulate PBS without APC (Yamanaka and Glazer, 1983). Two types of PBS with different linker proteins (CpcG1 and CpcG2) were found in the unicellular cyanobacterium Synechocystis sp. PCC 6803 (Kondo et al., 2007). One was a normal PBS consisting of a PC rod and APC core (CpcG1-PBS); the other was an abnormal PBS lacking APC (CpcG2-PBS) that seemed to transfer excitation energy preferentially to PSI (Kondo et al., 2007).

### Table 1. Peak amplitudes of fluorescence components in heterocysts and adjacent vegetative cells of A. variabilis

<table>
<thead>
<tr>
<th>Excitation Mode</th>
<th>Cell Type</th>
<th>Time after Fixed-Nitrogen Deprivation</th>
<th>PEC</th>
<th>PC</th>
<th>APC</th>
<th>PSII</th>
<th>PSI</th>
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<tr>
<td>TPE (808 nm, 0.2 picoseconds, 76 MHz)</td>
<td>Heterocyst</td>
<td>2 h</td>
<td>0.20</td>
<td>0.14</td>
<td>1.01</td>
<td>0.90</td>
<td>0.15</td>
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<tr>
<td></td>
<td></td>
<td>96 h</td>
<td>0.007</td>
<td>0.034</td>
<td>0.006</td>
<td>0.015</td>
<td>0.18</td>
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<td></td>
<td></td>
<td>&gt;10 d</td>
<td>0.003</td>
<td>0.012</td>
<td>0.08</td>
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<td>0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 h</td>
<td>0.21</td>
<td>0.135</td>
<td>1c</td>
<td>0.96</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>97 h</td>
<td>0.015</td>
<td>0.023</td>
<td>1c</td>
<td>1.23</td>
<td>0.10</td>
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<tr>
<td></td>
<td></td>
<td>&gt;10 d</td>
<td>0.003</td>
<td>0.098</td>
<td>1c</td>
<td>0.52</td>
<td>0.12</td>
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<tr>
<td>OPE (785 nm, CW)</td>
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<td>2 h</td>
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<td>n.d.</td>
<td>0.99</td>
<td>0.80</td>
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<td>n.d.</td>
<td>0.1</td>
<td>14.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 h</td>
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<td>1c</td>
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<td>1c</td>
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<td>n.d.</td>
<td>1c</td>
<td>1.10</td>
<td>14.5</td>
</tr>
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* Average values from Supplemental Figure S4, derived from Figure 7.  
† Average values from Supplemental Figure S8. All amplitudes were divided by that of APC of the vegetative cell adjacent to the heterocyst in a filament.  
‡ Average values from Supplemental Figure S9. Under 785-nm OPE, normalized amplitudes below 0.1 were designated not detected (n.d.). Note that the absolute signal intensity of APC of the vegetative cell adjacent to the heterocyst under 785-nm OPE was about 2 orders of magnitude smaller than that under 808-nm TPE; therefore, a value of 0.1 under 785-nm OPE was comparable to 0.001 under TPE.

Figure 9. Heterocyst of A. variabilis on a glass-bottomed dish showing polar distribution of PSI-rich fluorescence after 96 h of nitrogen deprivation. A, Bright-field image using a conventional spectrum of illumination without a narrow band-path filter. B, Fluorescence image obtained under OPE (CW of 785 nm) laser irradiation. The integrated fluorescence wavelength range was 700 to 753 nm. C, Fluorescence intensity (Flu. Int.) profile of the data in B calculated along the line indicated by the arrowheads. a.u., Arbitrary units.
rod part of PBS in *A. variabilis* heterocysts analyzed in this work may be similar to CpsG2-PBS in *Synechocystis* sp. PCC 6803. Given the very low-level fluorescence of PEC in heterocysts relative to that of PC under long-term diazotrophic conditions (more than 10 d; Table I), the PSI light-harvesting antennae in heterocysts seemed to contain mainly PC and a reduced content of PEC compared with PBS rods in fixed-nitrogen-supplemented culture. Some decay of PC and PEC fluorescence over a few tens of hours in heterocysts may be caused not only by proteolytic degradation but also by newly formed energy transfer coupling to PSI.

Cardona et al. (2008) reported that the vegetative cell adjacent to the heterocyst showed transient differences in fluorescence properties compared with other vegetative cells in *N. punctiforme*. Our spectral decomposition results, however, showed no reproducible differences in fluorescence between the nearest and second-nearest vegetative cells to heterocysts (Supplemental Figs. S3–S7). The difference between our results and theirs may be due to species differences and/or our lower sampling frequency (only once or twice per day).

The effects of nitrogen starvation on PBS in non-diazotrophic cyanobacteria were previously studied in *Synechococcus* sp. PCC 6301 (Yamanaka and Glazer, 1980) and sp. PCC 7942 (Collier and Grossman, 1992). In those studies, PBS degradation began with the trimming of peripheral PC rods, accompanied by degradation of the APC core. The monotonic decrease of PEC in vegetative cells of *A. variabilis* was similar to peripheral rod trimming (Fig. 7; Supplemental Figs. S3, S4, and S6). There was no recovery of PEC fluorescence in vegetative cells even after the heterocyst began supplying fixed nitrogen after approximately 24 h. On the other hand, PC and/or APC in PBS seemed to increase in vegetative cells after approximately 24 h, as evidenced by increases in absorbance in the 600- to 650-nm region (Fig. 2, B and C). PC fluorescence in vegetative cells decreased more slowly than that of PEC, and in some cases it even increased slightly (Supplemental Fig. S6). Notably, fluorescence intensities of APC and PSII were somehow maintained in vegetative cells, even 60 to 96 h after the onset of nitrogen deprivation diminished almost all PEC (Fig. 7; Supplemental Figs. S3, S4, and S6). Thus, some minor portion of PEC may have been originally uncoupled with PSI in terms of energy transfer, and degradation of this highly fluorescent PEC was one of the earliest responses to nitrogen starvation, allowing rapid recycling of amino acids from the pigment-protein complexes.

All our microscopic measurements were performed at 295 ± 2 K (approximately 22°C), lower than the previously reported optimum temperature for *Anabaena* spp. growth of around 28°C to 30°C (Wang et al., 2007). Despite the temperature difference, the appearance of a transient minimum of microscopic absorption at 24 h (Fig. 2) appeared to match the changes in PC and chlorophyll contents per cell 24 h after nitrogen deprivation reported in cells at approximately 303 K (Ehir and Ohmori, 2006). The timing of the transient minimum indicated that degradation of PBS and PSII apparently stopped at around 24 h, and nitrogen supplied by the heterocysts enabled vegetative cells to increase photosynthetic pigments. PBS fluorescence in heterocysts increased after fixed-nitrogen deprivation, and its intensity peaked at around 20 h in *Anabaena* spp. at 32°C (Toyoshima et al., 2010). This timing was very similar to the PC fluorescence peak (approximately 24 h) in our time-lapse measurements under TPE (Fig. 7), which was acquired from the same filaments as analyzed for microscopic absorption spectra (Fig. 2). Given these comparisons of the transient features of thylakoid membranes, there appeared to be no significant differences in heterocyst differentiation dynamics between 22°C and 28°C to 32°C. The timing of fluorescence changes was similar among all our data sets when the lag phase before the start of APC fluorescence decay was taken into account (Supplemental Fig. S3).

Many studies have examined the timing and spatial patterns of gene expression after the onset of nitrogen deprivation in heterocyst-forming cyanobacteria (Flores and Herrero, 2010; Kumar et al., 2010; Maldener and Muro-Pastor, 2010; Christman et al., 2011; Flaherty et al., 2011). For example, heterocyst differentiation was critically controlled by two regulator proteins, HetR and NtcA, and their encoding genes were strongly expressed in certain spatially separated cells that ultimately became heterocysts (Black et al., 1993; Olmedo-Verd et al., 2006). Our laboratory is currently investigating the timing and spatial relationships between the transformation of thylakoid membranes unveiled in this work and the expression of genes related to the transition to diazotrophic growth conditions.

### Number and Oligomeric Status of PSI and PSII in Single Cells

The fluorescence intensities shown as spectra and amplitudes in our figures were averaged on a per-pixel basis across whole-cytoplasm areas enclosed by contour lines of fluorescent regions. This means that average per-pixel fluorescence signals were equivalent to averages over whole thylakoids in single cells. The most direct estimate of PSI concentration in individual cells, at least in our work, was given by the microscopic fluorescence spectra under 785-nm OPE. There was no substantial difference in the whole-thylakoid PSI concentration between heterocysts and adjacent vegetative cells from the initiation of heterocyst differentiation to heterocyst maturity (Figs. 4, 5, A and B, and 8; Supplemental Figs. S5 and S7). Because of the decrease in PSII and PBS in heterocysts, the relative abundance of PSI should inevitably increase. This prediction was confirmed by a recent mass spectrometry-based proteomic study on filaments of *A. variabilis* (Ow et al., 2009). In that study, the relative abundance of PSI was 3.3-fold higher on average in heterocyst cells than in vegetative cells. If proteomic analysis is conducted on samples with an adjusted concentration of total
proteins, the original absolute concentrations of proteins in intact single cells may be difficult to estimate. Our work is unique in that the concentration of PSI per thylakoid in single cells was directly compared between heterocysts and their parent or adjacent vegetative cells. Our method of quantifying PSI involved little interference from possible energy transfer between PBS and PSI, because of the direct excitation of PSI by the CW NIR laser and the relatively small uphill energy transfer from PSI to the other pigment-protein complexes.

The total quantity of PSI in single cells was estimated by integrating the fluorescence between 700 and 753 nm under 785-nm OPE across the whole cell (Supplemental Text S3). We estimated that the average quantity of PSI per cell was comparable between intercalary heterocysts and single vegetative cells but that a single terminal heterocyst contained approximately 30% less PSI than a single vegetative cell (Supplemental Table S2). These estimates contradicted the results of a previous study on Anabaena cylindrica, in which the quantity of PSI was 1.5 times higher in heterocysts than in vegetative cells (Alberte et al., 1980).

In the cells of another heterocystous cyanobacterium, N. punctiforme, the PSI complex was reported to be in equilibrium between the monomer and trimer, which had fluorescence peaks around 728 and 750 nm, respectively, at 77 K (Cardona and Magnuson, 2010). However, another study proposed that this spectral change was attributable to equilibria among the monomer, dimer, and tetramer based on the phylogenetic similarity between Anabaena sp. PCC 7120 and N. punctiforme (Watanabe et al., 2011). A change in the oligomerization status of the PSI complex was accompanied by changes in absorption and fluorescence spectra of the long-wavelength-absorbing spectral forms, some of which were detectable at approximately 295 K (Karapetyan et al., 2006). We previously demonstrated that the oligomeric status of chlorophylls with various fluorescence peaks could be sensitively detected under 785-nm OPE (Hasegawa et al., 2011). Thus, the oligomeric status of PSI probably remained largely unchanged in heterocysts and vegetative cells during the adaptation to nitrogen deprivation, because there was an almost constant spectral shape in the wavelength region between 700 and 755 nm (Fig. 5).

The absolute concentration of chlorophyll a was derived from absorbance values of the microscopic absorption spectra (Fig. 2; Supplemental Table S2). We estimated average chlorophyll quantities on a whole-cell basis, although there was clearly an uneven distribution of thylakoid membranes in the plastids of both heterocysts and vegetative cells (Figs. 1 and 9B; Supplemental Fig. S10B). The value for $A_{680}$ of mature heterocyst was approximately 70% of that of vegetative cells when A. variabilis filaments were grown diazotrophically for longer than 10 d (Fig. 2D). The decline in absorbance was primarily attributed to a decrease in chlorophyll concentration in single cells, because even the longest wavelength-absorbing subunit of PBS, APC, showed only 5% to 7% of absorbance compared with the peak at around 650 nm (Grabowski and Gantt, 1978; Murakami et al., 1981; Rolinski et al., 1999; MacColl et al., 2003). Based on these absorbance values and an estimate of the optical path length (Supplemental Text S4), the total amounts of chlorophyll in the terminal and intercalary heterocysts were estimated to be 50% and 71%, respectively, of that in a vegetative cell (Supplemental Table S2). The mass of chlorophyll per cell was estimated as 0.12, 0.17, and 0.23 picograms (pg) in terminal heterocysts, intercalary heterocysts, and vegetative cells, respectively (Supplemental Table S2). These values were largely consistent with those obtained by whole-culture pigment analysis and cell counts of A. cylindrica (Alberte et al., 1980; 0.09 pg in heterocysts, 0.17 pg in vegetative cells).

Because all chlorophyll a remaining in heterocysts is primarily attributable to PSI, the absolute amount of PSI can be estimated in single heterocyst cells (Supplemental Table S2). The different numbers of PSI units between single terminal (0.88 x 10$^6$) and single intercalary (1.2 x 10$^6$) heterocysts seemed to be due not to the density of PSI on thylakoid membranes but to the different extents of fluorescent area of PSI (Supplemental Table S1), which can sometimes be visualized by the unipolar and bipolar distribution of PSI in terminal and intercalary heterocysts, respectively (Fig. 9B; Supplemental Fig. S10B). The difference may reflect the number of vegetative cells to which fixed nitrogen must be transported.

If the decrease in chlorophyll during heterocyst differentiation is attributed only to the complete disappearance of PSI, and if the relatively constant concentration and oligomeric status of PSI are taken into account, the stoichiometric ratio between PSII and PSI in a vegetative cell can be estimated. Based on a simple model and parameters (Supplemental Table S2), the PSII-to-PSI ratio on a monomer basis (PSII/PSI) in vegetative cells was estimated as 1.0. This ratio was either consistent with or slightly larger than previously reported values for A. variabilis (PSII/PSI = 0.7–1.4 [Kawamura et al., 1979]; approximately 0.3–0.6 [Fujita et al., 1985]), Synechocystis sp. PCC 6714 (approximately 0.5–0.7; Murakami et al., 1997), Synechocystis sp. PCC 6803 (approximately 0.5–0.8; Hihara et al., 1998), and N. punctiforme (approximately 0.26; Cardona et al., 2007). These relatively low estimates for PSII/PSI were based on biochemical treatments and analyses of whole-cell suspensions. A recent microspectroscopic study on Nostoc sp. KU001 showed that the absorbance at around 680 nm in the heterocyst was about 48% of that in a vegetative cell (Sugiura and Itoh, 2012). This value would yield an even greater PSII/PSI ratio than ours if the amount of PSI were similar between heterocysts and vegetative cells in that species. Further examination of the reliability of microscopic absorption spectroscopy to quantify photosynthetic pigments in living cells will be useful. Verification should include comparisons with other methods, other samples, and various experimental conditions.
CONCLUSION

We characterized the differentiation of A. variabilis vegetative cells into heterocysts and the adaptation dynamics of vegetative cells to diazotrophic growth conditions at approximately 295 K using time-lapse microspectroscopy. During heterocyst differentiation, APC and PSII showed a highly synchronous decay with a constant stoichiometric ratio within approximately 30 h. In all cells, including vegetative ones, PEC and PC decayed during the transition from nitrogen-replete to nitrogen-deficient conditions before the heterocysts became operative. These decays slowed or ceased after heterocyst function began. The transient heterocysts became operative. These decays slowed or could be fully satisfied by the more rapid decays of APC and PSII than of PC and PEC. The decomposition of microspectroscopic fluorescence spectra into PEC, PC, APC, PSI, and PS components was greatly assisted by the highly pure PSI spectrum obtained at approximately 295 K under 785-nm OPE. That spectrum had very little interference due to energy transfers from other shorter wavelength-absorbing pigment-protein complexes. Selective excitation of PSI was useful to quantify and wavelength-absorbing pigment-protein complexes. This combination of microscopic absorption and fluorescence spectral data obtained under 785-nm OPE in cells grown photoautotrophically in normal BG-11 medium (Stanier et al., 1971) for 10 to 20 d at 29°C, which corresponded to the mid-exponential growth phase under these conditions. The photosynthetic photon flux density at the sample position was 35 ± 5 μW m⁻² s⁻¹. PSII components was greatly assisted by the largely unchanged oligomeric status of the PSI complex. The constant spectral shape of PSI fluorescence under 785-nm OPE during differentiation was best explained by the largely unchanged oligomeric status of the PSI complex. This combination of microscopic absorption and fluorescence spectral data obtained under 785-nm OPE is a promising method to quantify PSI and PSII in individual intact living cells.

MATERIALS AND METHODS

Strain and Growth Conditions

Anabaena variabilis (strain NIES-2095) cells were purchased from the microbial culture collection at the National Institute of Environmental Studies in Tsukuba, Japan. Before fixed-nitrogen deprivation, filaments were grown photautotrophically in normal BG-11 medium (Stanier et al., 1971) for 10 to 20 d at 29°C, which corresponded to the mid-exponential growth phase under these conditions. The photosynthetic photon flux density at the sample position was 35 ± 5 μW m⁻² s⁻¹. Cells were grown under a 15-h-light/9-h-dark photoperiod. Nitrogen-free BG-11 medium was prepared by removing NaNO₃ and replacing ferric ammonium citrate with ferric citrate in the BG-11 formulation. The nitrogen deprivation conditions were as follows: an aliquot of the cell suspension, typically 1 cm³, was centrifuged for 30 s at 1.8 × 10⁶ g (the supernatant (greater than 0.97 cm³) was removed), and the pellet was diluted with nitrogen-free BG-11 medium to a volume of 1.0 cm³. This process was repeated twice to ensure nitrogen deprivation. The final suspension was transferred to a glass-bottomed dish (D111505; Matsunami). The upper surface of the glass was coated with poly-L-Lys before the suspension of A. variabilis filaments was added to immobilize cells. A grid pattern on the lower glass surface was used to locate and examine several identical filaments at 12- to 24-h intervals for up to 60 to 96 h after the start of nitrogen starvation.

We conducted two types of time-lapse microscopic observations. First, several identical filaments in the same glass-bottomed dish were inoculated from an original culture suspension and kept continuously on the microscope stage (time-lapse experiment). In this experiment, identical filaments were laser illuminated several times, and we could not rule out the possibility that illumination affected the physiological changes. Second, multiple glass-bottomed dishes were simultaneously inoculated from an original culture suspension. Each dish was used once to analyze several filaments at a single observation time (single-time experiment). Except when being microscopically analyzed, these dishes were continuously illuminated by white fluorescent light (photosynthetic active radiation of approximately 15 μmol photons m⁻² s⁻¹).

Microscopic Spectroscopy and Analysis

Details of the microspectroscopy systems have been described previously (Kumazaki et al., 2007; Hasegawa et al., 2011). The system is based on an inverted microscope with an oil-immersion microscope objective (UPLSAPO, 100XO, numerical aperture = 1.40; Olympus). For microscopic fluorescence spectral measurements, the average laser power at the sample position was 35 ± 5 μW m⁻² s⁻¹. In the case of OPE of mainly PSI with a CW laser centered at 808 nm, the average laser power at the sample position was 30 ± 2 μW. The excitation laser was resonantly scanned at 7.9 kHz along a linear region of approximately 20 μm. Microscopic fluorescence spectra were recorded with an EMCCD camera attached to an imaging polychromator. We used an exposure time of 0.5 s per array of pixels with a length of approximately 20 μm. The microscopic absorption spectrum was given by the ratio in a bright-field image between regions with and without a filament of cells in the region of interest. Monochromatic illumination (centered at 600, 610, 620, 640, 650, 660, 671, 680, 690, 700, and 730 nm, with full-width at half-maximum of 10 nm) was automatically changed by a filter wheel on a halogen lamp (Hasegawa et al., 2011). Offset absorbance at a given wavelength was the difference in apparent absorbance between the wavelength and 730 nm. Fluorescence and absorption spectral imaging were performed at three z positions, with intervals of 0.6 μm. All microscopic measurements were conducted in a laboratory at 295 ± 2 K.

Analyses of microscopic data, including SVD of many spectra of individual cells, were performed using a set of in-house programs in Mathematica version 5.2 (Wolfram Research). Single-cell fluorescence spectra were averages of single-pixel spectra in the intracellular regions enclosed by contour lines along the cell periphery given by a human-assisted averaging program, which also gave the areas of fluorescent regions in individual cells. Single-cell absorption spectra were calculated as described previously (Hasegawa et al., 2011). Because of focus position drift, some z-section data that were clearly far from the central height of cells were excluded from averaging.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Fluorescence spectra of PEC, PC, APC, PSI, and PEC.

Supplemental Figure S2. Five spectra derived by singular value decomposition of 1,635 single-cell spectra.

Supplemental Figure S3. Time dependence of relative peak amplitudes of five normalized spectral components in six A. variabilis filaments, including heterocysts and adjacent vegetative cells.

Supplemental Figure S4. Normalized amplitudes of data shown in Figure 7.

Supplemental Figure S5. Time dependence of fluorescence components obtained from eight A. variabilis filaments with heterocysts in the same glass-bottomed dish under 808-nm TPE.

Supplemental Figure S6. Time dependence of fluorescence components in single-time experiments under 808-nm TPE.

Supplemental Figure S7. Time dependence of fluorescence components under 785-nm OPE.

Supplemental Figure S8. Cell position dependence of relative spectral amplitudes of fluorescence components under 808-nm TPE in cells grown diazotrophically for longer than 10 d.

Supplemental Figure S9. Cell position dependence of relative spectral amplitudes of fluorescence components under 785-nm OPE in cells grown diazotrophically for longer than 10 d.
LITERATURE CITED


Mannan RM, Pakrasi HB (1993) Dark heterotrophic growth conditions result in an increase in the content of photosystem II units in the filamentous cyanobacterium Anabaena variabilis ATCC 29413. Plant Physiol 103: 971–977

observed with the cyanophyte Synechocystis PCC 6714. Plant Cell Physiol 38: 392–397


Thylakoid Dynamics in Heterocyst Differentiation

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