Plant Physiol. (1997) 115: 991–1000

Temporal Changes in State Transitions and Photosystem Organization in the Unicellular, Diazotrophic Cyanobacterium *Cyanothece* sp. ATCC 51142[1]

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The unicellular cyanobacterium *Cyanothece* sp. ATCC 51142, grown under alternating 12-h light/12-h dark conditions, temporally separated N_2 fixation from photosynthesis. The regulation of photosynthesis was studied using fluorescence spectra and kinetics to determine changes in state transitions and photosystem organization. The redox poise of the plastoquinone (PQ) pool appeared to be central to this regulation. Respiration supported N_2 fixation by oxidizing carbohydrate granules, but reduced the PQ pool. This induced state 2 photosystem II monomers and lowered the capacity for O_2 evolution. State 2 favored photosystem I trimers and cyclic electron transport, which could stimulate N_2 fixation; the stimulation suggested an ATP limitation to N_2 and CO_2 fixation. The exhaustion of carbohydrate granules at around 6 h in the dark resulted in reduced respiratory electron flow, which led to a more oxidized PQ pool and produced a sharp transition from state 2 to state 1. This transient state 1 returned to state 2 in the remaining hours of darkness. In the light phase, photosystem II dimerization correlated with increased phycobilisome coupling to photosystem II (state 1) and increased rates of O_2 evolution. However, dark adaptation did not guarantee state 2 and left photosystem I centers in a mostly monomeric state at certain times.

O_2 is inhibitory to the enzyme nitrogenase, which catalyzes N_2 fixation (Fay and Cox, 1967; Fay, 1992). Thus, organisms that carry out oxygenic photosynthesis in N_2-poor environments are challenged by the need for both processes. A successful strategy of some filamentous cyanobacteria (heterocystous cyanobacteria) is to segregate the processes of photosynthesis and N_2 fixation in different cells, which then share the products (Wolk et al., 1994). Some other cyanobacteria are capable of carrying out both processes in the same cell but at different times, which is a control process termed temporal regulation (Mitsui et al., 1986; Grobbelaar et al., 1987).

The unicellular cyanobacterium *Cyanothece* sp. ATCC 51142, whether grown under alternating LD or in continuous light, fixed N_2 with a peak at approximately D4 in the dark or subjective-dark phases (Reddy et al., 1993; Schneegurt et al., 1994; Colón-López et al., 1997). The carbohydrate produced by photosynthesis was stored as glycogen in large granules that formed between the thylakoid membranes. The number of granules reached a peak toward the end of the photosynthetic period (i.e. near L8) and declined precipitously during the course of N_2 fixation (Schneegurt et al., 1994). These results (Schneegurt et al., 1994), as well as high respiration rates during N_2 fixation (Colón-López et al., 1997), suggest that the carbohydrate in the granules was used as a substrate for respiration. Thus, respiration acted to provide energy for N_2 fixation as well as to protect nitrogenase by consuming O_2 in the cell. Exposing the cells to light during the N_2 assay stimulated N_2 fixation (Reddy et al., 1993), which suggests that photosynthetic reaction centers could provide energy for N_2 fixation without inactivating it with O_2. Such experiments suggest that the metabolic activities in *Cyanothece* sp. ATCC 51142 are temporally regulated.

Respiration should influence photosynthesis in cyanobacteria, because the respiratory and photosynthetic electron transport chains intersect at the level of the PQ pool (Hirano et al., 1980). NADH reduces the PQ pool via a PQ-NAD(P)H oxidoreductase (Mi et al., 1992, 1995), and reduced PQ is oxidized by a Cyt oxidase (Vermaas et al., 1994). The balance between these two reactions, and therefore the supply of NADH and O_2 and the activity of the enzymes allowing the oxidation and reduction of the PQ pool, determined the redox poise of the PQ pool in the dark. This process affected photosynthesis because PSI centers require a certain redox poise of the PQ pool to perform cyclic electron flow efficiently (Bendall and Manasse, 1995). The redox poise of the PQ pool is used as a signal for the short-term regulation of photosynthesis through state transitions (Mullineaux and Allen, 1990). In addition, PSII centers become closed to photochemistry in the presence of reduced primary electron-accepting PQ of PSII, the quantity of which is a function of the redox state of the PQ pool (Diner, 1977). The acceptor side appeared to be a significant factor in the regulation mechanism of PSII in *Plectonema boryanum* grown under diazotrophic conditions (Misra and Desai, 1993).

Photosynthesis influences respiration in a reciprocal fashion. PSI activity inhibits terminal oxidase activity by stealing electrons, a process also known as the Kok effect that is well-studied in higher plants and green algae (Pel-

[1] This work was supported by U.S. Department of Agriculture grant no. 93-37306-9238 and Department of Energy grant no. DE-FG02-89ER14028A to L.A.S.

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Abbreviations: DX, X h of darkness; LD, 12-h light/12-h dark cycle; LX, X h of light; PAM, pulse-amplitude modulated; PQ, plastoquinone.

tier et al., 1987). In this manner, the oxidation of the PQ pool by PSI centers under flashing light produces respiratory O_2 transients (Boichenko et al., 1993; Meunier et al., 1995a), and could reopen PSII centers that were closed because of respiratory activity. In addition, PSI cyclic electron flow reduces the cellular need for respiration by generating ATP.

The so-called state transitions were first detected in cyanobacteria by Murata (1969), who noted the effect of light quality on the relative activity of PSI and PSII. Preferential excitation of PSI caused an increase in energy transfer to PSII and a small decrease in energy transfer to PSI, whereas preferential excitation of PSII or incubation in darkness reversed this effect. A model for state transitions in cyanobacteria has been developed by Rögner and colleagues (Kruip et al., 1994; Rögner et al., 1996; Bald et al., 1996), who have also invoked the oligomeric state of PSI and PSII in the overall mechanism. In this model, state 1 (which favors linear electron flow from O_2 production to CO_2 fixation) had a dimeric PSII and a monomeric PSI. Under these circumstances, the phycobilisomes are primarily attached to PSII. State 2 (which favors cyclic electron flow) had trimeric PSI complexes and monomeric PSII. In this case, the phycobilisomes could more readily attach to PSI. State transitions are known to be a major source of nonphotochemical quenching of fluorescence in cyanobacteria (Campbell and Öquist, 1996). This relationship of oligomerization of the photosystems and state transitions will represent an important component of the present study.

In this paper we will discuss the changes in photosynthesis that occur during growth of *Cyanothece* sp. ATCC 51142 in alternating DL under N_2-fixing conditions. The cellular capacity for O_2 evolution was low during N_2 fixation, even when samples were assayed under saturating light. We will present a correlation between the intensity of the 77 K fluorescence yield at 720 nm (the fluorescence yield of PSI) and the monomerization of PSI centers, and propose a similar relationship between the fluorescence intensities at 695 and 685 nm (under chlorophyll excitation at 435 nm) and the dimerization of PSII centers. The relationship of photosystem oligomerization to phycobilisome attachment was compared by measurement of fluorescence spectra after excitation at 580 nm. The results suggest that phycobilisome coupling to PSII correlated with PSII center dimerization, whereas PSI trimerization could occur either concurrently or independently. Our results also suggest that many of these short-term adaptations are controlled at the level of the PQ redox poise. These results will be used to develop a more comprehensive model of the physiological changes that occur in *Cyanothece* sp. ATCC 51142 under N_2-fixing conditions.

MATERIALS AND METHODS

Growth Conditions, Nitrogenase, and Chlorophyll Determination

Cyanothece sp. ATCC 51142 (formerly strain BH68) was grown as previously described in ASP2 medium without $NaNO_3$, with shaking at 100 or 125 rpm, at 30°C, under cool-white fluorescent illumination of approximately 50 μE m^{-2} s^{-1} (Reddy et al., 1993). Continuous-light-grown, stationary-phase cultures (2×10^7 to 4×10^7 cells mL^{-1}) were subcultured by dilution to a concentration of 10^6 cells mL^{-1}. Duplicate flasks were subcultured 12 h apart, using the same stock culture, to permit 24-h experiments to be performed in 12 h. After dilution, each flask was illuminated for 12 h. This protocol and the determination of cell number were done as described previously. Nitrogenase activity was measured as the reduction of acetylene to ethylene by the nitrogenase enzyme (Schneegurt et al., 1994). For the experiment shown in Figure 2, four identical samples were assayed at each time point, with two samples incubated in the dark and two in the light. One sample in the light and one in the dark were treated with 10 μM DCMU during the incubation.

The chlorophyll concentration was determined with Macintosh freeware "Chlorophyll" (available at http://bilbo.bio.purdue.edu/~pmeunier/download.html). Light scattering by whole cells usually superimposes a sloping baseline that distorts the measurements, so light scattering was increased by adding Magic Tape No. 810 (3M) to the light path before and after the cuvette of a single-beam spectrophotometer (model DU-7, Beckman). The added light scattering by whole cells became negligible compared with that introduced by the tape. The baseline was recorded without the cells so that the actual measurement was corrected for light scattering. We found the Magic Tape to be a better light scatterer for this purpose than various frosted filters and diffusing plates. This yielded a fast and reproducible assay that was linear from $A^A = 0.01$ up to about $A = 0.5$ optical units of the most absorbed peaks (around 620 and 678 nm), and that was within 10% of chlorophyll determinations performed with 80% acetone extraction. We used a combination of the cyanobacterial method of Arnon et al. (1974) and the relative method of Beale and Chen (1983). Although the Arnon method worked well with whole cells or thylakoid preparations, it produced negative estimates of phycobilisome contents in purified PSI and PSII preparations. The Beale and Chen method, although limited to relative measurements, produced better results with purified preparations. By using both the Arnon and the Beale and Chen methods on thylakoid preparations, the chlorophyll concentrations of the purified extracts could be estimated. Acetone extraction and chlorophyll estimation by the method of Porra et al. (1989) gave similar results.

Fluorescence Measurements

Fluorescence emission spectra were measured with an SLM 8000 fluorometer (Spectronics, Rochester, NY) using between 2 and 5 μg chlorophyll mL^{-1} (the chlorophyll concentrations of all of the centrifuged samples throughout one experiment had a relative SD of 10%). The cells were rapidly mixed with prealiquoted 20% (final) glycerol or potassium glycerophosphoric acid in Eppendorf tubes and placed inside cylindrical cuvettes (2 mm i.d.) with a syringe and cannula, and plunged into liquid nitrogen; from mixing to freezing took less than 5 s. Polyacrylamide and

glycerol gels were found to affect phycobilisome attachment (Bruce and Biggins, 1985). However, phosphoglyceric acid and glycerol appeared to be fully interchangeable under our experimental conditions (quick mixing and freezing of whole cells). We have run complete experiments with one or the other and discerned no difference that could be ascribed to the freezing media. The isolated PSI reaction centers were frozen with phosphoglyceric acid. The monochromators were set at a 2-nm bandwidth, except that the last slit of the excitation double monochromator was set at 4 nm to facilitate the positioning of the sample cuvette. Fluorescein (sodium salt) was added as an internal standard (0.5 μM final concentration) and excited at 435 nm. *Cyanothece* sp. ATCC 51142 samples were centrifuged at room temperature for 10 min at 8000*g* and resuspended in fresh growth medium. Fluorescence emission spectra were routinely normalized to the fluorescein intensity peak around 500 nm, giving a reproducibility within 10% on different experiments. This peak intensity was almost independent of pH, in contrast to the peak around 530 nm that appears in the presence of glycerol. A further normalization to the chlorophyll concentration was necessary for centrifuged samples and when comparing samples during the growth of a culture. The spectra were not corrected for spectral sensitivity. The PAM fluorescence was measured using the fluorometer ED-101 cuvette kit (Heinz Walz, Effeltrich, Germany), with the optional 590-nm excitation light (model L-590 102), and by adding a small magnet and stirrer to keep the sample agitated. In the experiments reported here, the actinic light was the original red light-emitting diode that came with the PAM instrument, and had an intensity of 68 $\mu E\ m^{-2}\ s^{-1}$.

O_2 Evolution–Clark Electrode

Steady-state O_2 production measurements were performed with a Clark electrode (model 5331, Yellow Springs Instruments, Yellow Springs, OH), with an amplifier (model 5300) in a cuvette thermostatted at 30°C. Actinic light was provided by a fiber-optic illuminator and filtered by a CS 2-63 red filter to minimize photoinhibition, and by a heat filter. The signal was digitized and a linear regression was applied to appropriate parts of the signal by using the LabView program (National Instruments, Austin, TX) on a Macintosh computer. The rate of O_2 evolution was estimated from the net rate of O_2 production in the light by subtracting the combined respiration and Rubisco-dependent O_2 uptake, which were measured after turning off the actinic light. The cell suspension was supplemented with 10 mM $NaHCO_3$ (final) before the experiment. Substituted quinones, when used, were prepared in ethanol; assay concentrations were 400 μM quinone and 1% ethanol. Under these conditions, significant stimulations of O_2 production could be obtained in the cyanobacteria *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7942.

Preparation of PSI and PSII Monomers and Trimers

The purified photosystem particles were provided by H. Kuhl and M. Rögner (University of Münster, Germany). Pure monomeric and trimeric PSI complexes from *Synechocystis* sp. PCC 6803 were isolated as described (Rögner et al., 1990; Kruip et al., 1994; Turconi et al., 1996). PSII dimer preparations and PSII monomer-enriched preparations were prepared from *Synechococcus elongatus* and frozen in 60 mM $MgSO_4$, 20 mM Mes, pH 6.5, 10 mM $CaCl_2$, 10 mM $MgCl_2$, and 0.03% β-dodecylmaltoside. The isolation procedure will be described in more detail with the publication of the PSII experimental results.

RESULTS

Rates of O_2 Evolution and N_2 Fixation

The metabolic periodicities of N_2 fixation, O_2 evolution, and respiration for LD-grown cells are outlined in Figure 1. N_2 fixation peaked at D4 (4 h into the dark phase of growth), as previously observed (Reddy et al., 1993; Schneegurt et al., 1994; Colón-López et al., 1997). O_2 evolution also cycled with a 24-h periodicity, and the minima

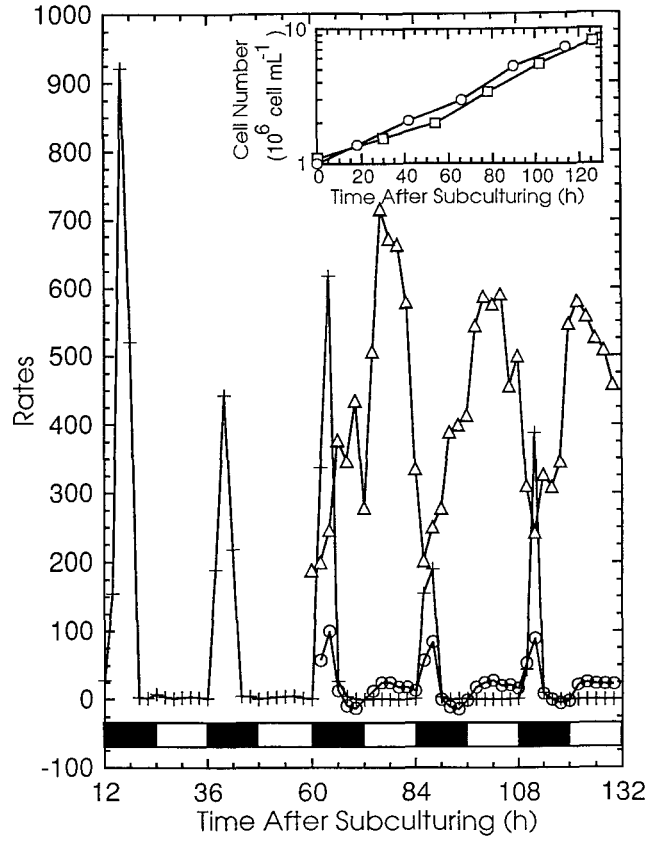

Figure 1. Rhythms of N_2 fixation, photosynthesis, and respiration in *Cyanothece* sp. ATCC 51142 grown under a diurnal regime of 12-h light/12-h dark. Metabolic activities were assayed every 2 h for 3 to 5 consecutive days. For convenience, the metabolic rates are plotted on the same scale with nitrogenase activity (maximum rate = 185 nmol N_2 fixed 10^8 cells^{-1} h^{-1}) multiplied by 5. Solid and open bars represent dark and light growth periods, respectively. +, Nitrogenase; △, photosynthesis (maximum rate = 715 μmol O_2 mg^{-1} chlorophyll h^{-1}); ○, respiration (maximum rate = 100 μmol O_2 mg^{-1} chlorophyll h^{-1}). Inset, Growth curves of the duplicate cultures (500 mL) used during this experiment.

and maxima of O_2 production occurred around D4 to D6 and L4 to L6, respectively. The peak of respiration was coincident with the peak of N_2 fixation (D4), and there was a lower level of respiration in the light. Because $NaHCO_3$ was the final electron acceptor, the O_2 evolution rates represented whole-chain electron transport rates. Attempts to use various artificial quinones as electron acceptors of PSII resulted in an inhibition of PSII activity in *Cyanothece* sp. ATCC 51142 that varied in percentage and extent throughout the 24-h cycle, whereas the same quinones at the same concentration significantly stimulated PSII activity in the nondiazotrophic cyanobacterium *Synechocystis* sp. PCC 6803 (Meunier et al., 1995a, 1995b). This phenomenon has not been explained completely, although we have noticed a constant inhibition in mutants of *Synechocystis* sp. PCC 6803 that were missing PSII extrinsic proteins (P.C. Meunier and L.A. Sherman, unpublished data).

We are interested in understanding the variable properties of PSII and photosynthesis and the mechanism by which they are regulated. One important question concerned the contribution of photosynthesis to the energetic requirements for nitrogenase activity. Previous results indicated that nitrogenase activity was stimulated when assayed in the light, compared with the usual dark assay (Reddy et al., 1993), and we wished to determine the role of PSI and PSII in this effect. We repeated this experiment in the presence and absence of 10 μM DCMU (which inhibited PSII function) and the stimulation of nitrogenase activity by light remained unchanged at a 44% increase (total N_2 fixed) compared with the dark (Fig. 2). From these experiments, we conclude that N_2 fixation assayed in the light benefited from ATP supplied by PSI cyclic electron transport, did not require a functional PSII, and was uninhibited by the low PSII activity at those times. Nonetheless, about 70% of the maximum N_2-fixation rate and 70% of the N_2 fixed under illumination could be attained by energy supplied solely from respiration. These results are compatible

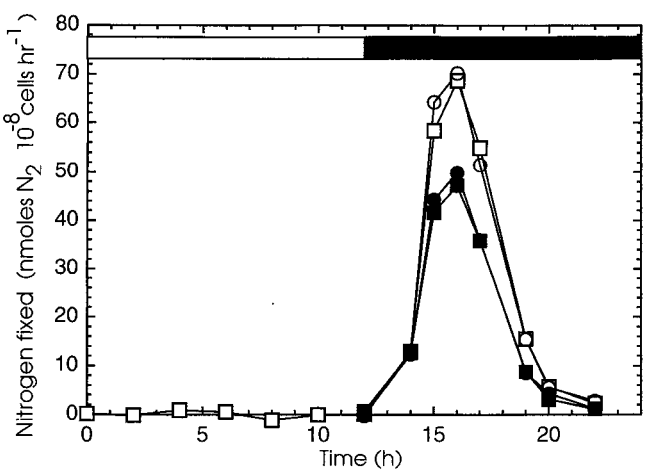

Figure 2. Effect of DCMU on N_2 fixation rates in *Cyanothece* sp. ATCC 51142 assayed in the dark (■, ●) and under growth light intensities (50 μE m^{-2}; □, ○). DCMU (10 μM final concentration; the ethanol was diluted to a final concentration of 1%) was added before the cells in the assay tubes (●, ○). The rates were averaged over 2 d.

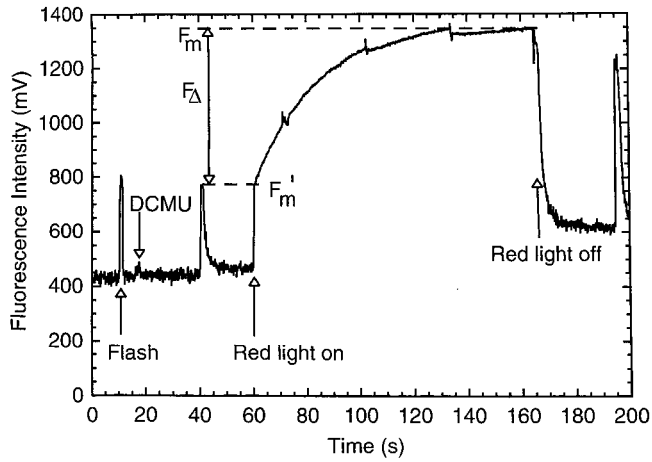

Figure 3. Fluorescence intensity changes in the presence of DCMU and an actinic red light in *Cyanothece* sp. ATCC 51142 cells at D2. The red actinic light was at the maximum setting on the PAM fluorometer (intensity 11). Arrows mark the addition of 10 μM DCMU (1:100 dilution resulting in 1% ethanol concentration), the saturating flashes used to measure F_m (given at 30-s intervals), and when the actinic light was turned on and off.

with the observation of a PSII-independent component to N_2 fixation in *Plectonema boryanum* (Misra and Tuli, 1994).

State Transitions

An important aspect of Figure 1 is that photosynthesis began declining in the light and photosynthetic capacity began increasing in the dark. This suggests that the regulation of photosynthesis was not directly dependent on light control. We measured numerous photosynthetic parameters, including fluorescence kinetics and 77 K fluorescence spectra, throughout the 24-h period, to better define the changes in photosynthesis. We discovered that the fluorescence characteristics of *Cyanothece* sp. ATCC 51142, growing under N_2-fixing conditions, were complex and dynamic throughout the day. The dynamic nature of this phenomenon was verified by repeating the fluorescence experiments on more than five separate cultures and achieving similar results. Such dynamic alterations can be attributable to long-term regulation (e.g. biosynthesis leading to changes in photosystem stoichiometry) or to short-term regulation (e.g. state transitions). We will interpret the fluorescence results as being indicative of short-term changes, such as state transitions and oligomerization of the photosystems. These characteristics permitted us to develop a hypothesis for the photosynthetic apparatus, which indicated that it is under constant flux and is ever-adapting to the energetic needs of the cell.

We induced state transitions in *Cyanothece* sp. ATCC 51142 by adding 10 μM DCMU and by a red-light illumination (the actinic light of the PAM fluorometer). Figure 3 is an example of the PAM measurement using cells at the D2 growth phase. The 77 K fluorescence of these cells is then depicted in Figure 4. The fluorescence intensity under the saturating flashes fired every 30 s measured F_m', which is the fluorescence yield in the absence of photochemical

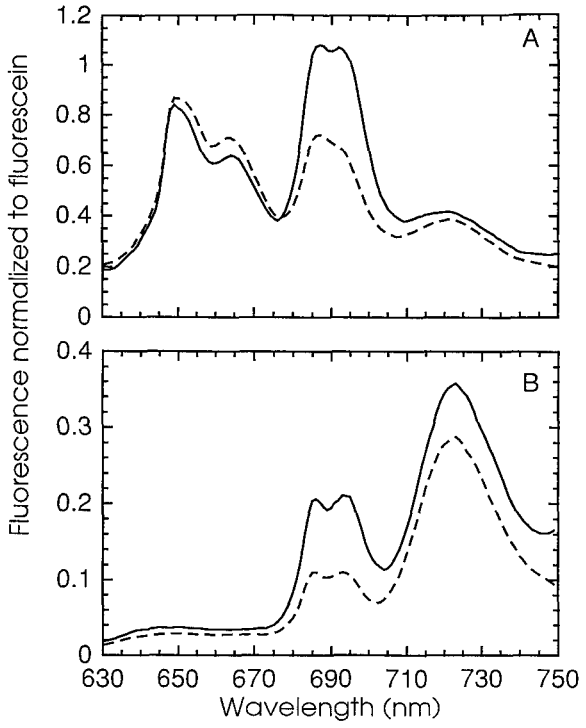

Figure 4. Fluorescence emission spectra at 77 K of *Cyanothece* sp. ATCC 51142 cells at D2, before (dashed line) and after (solid line) the PAM treatment (protocol as in Fig. 3, but with different samples). The excitation wavelengths were 580 nm (A) and 435 nm (B). Samples were prepared (mixed) in the 4-mL cuvette under the actinic red light provided by the PAM fluorometer, and then transferred quickly to the cylindrical cuvette used for 77 K measurements and frozen.

quenching. The maximal fluorescence yield, F_m, is attained in the absence of both photochemical and nonphotochemical quenching. In an attempt to reach F_m, DCMU was added after 15 to 20 s, and the actinic red light was turned on 60 s after the beginning of the experiment and remained on for 100 s (Fig. 3). Because PSII activity was inhibited by DCMU, PSI oxidized the PQ pool during illumination by the actinic red light. As a result, an important source of nonphotochemical quenching was removed and the fluorescence yield increased by about 70%, from 769 to 1312 mV, as indicated by the difference, F_Δ (Fig. 3). This nonphotochemical quenching was not relieved by the addition of carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone, indicating that energetic quenching was negligible in *Cyanothece* sp. ATCC 51142, as previously reported for other cyanobacteria (Campbell and Öquist, 1996). Because the relief of nonphotochemical quenching produced by this treatment was accompanied by large changes in phycobilisome coupling (see below), we conclude that 44% of the fluorescence was quenched by state 2 in the dark, and we obtained a transition to state 1 in about 100 s for cells at D2.

The 77 K fluorescence emission spectra of cells at the D2 growth phase are shown in Figure 4. Phycobilisomes were excited by 580-nm light, which is a low-absorbance region for chlorophyll (Fig. 4A). The peaks around 650 and 665 nm are associated with phycobilisome pigments (including phycocyanin and allophycocyanin), the peak at 685 nm comes from both the phycobilisome terminal emitter (allophycocyanin B) and the small chlorophyll antenna of PSII (CP43), the peak around 695 nm is emitted by P680 and CP47 of functional PSII reaction centers, and the peak around 720 nm comes from PSI (Bruce and Biggins, 1985; Bruce et al., 1985; Golitsyn et al., 1995). The measured fluorescence intensities are a function of the concentration of emitters, their quantum yield of fluorescence at 77 K, and the number of excitons captured and transmitted to them (coupling to the excited pigments). The spectra were corrected for the relative sample concentration by using fluorescein as an internal standard. The increased fluorescence at 685 and 695 nm under excitation at 580 nm after the DCMU and red-light treatment (Fig. 4A, solid line) indicated a better energetic coupling of phycobilisomes to PSII; this was strong evidence for a transition to state 1.

The transition to state 1 resulted in increased fluorescence yields at 685, 695, and 720 nm under chlorophyll excitation at 435 nm (Fig. 4B, solid line). We now have the tools to study this phenomenon, which relates to the organization of the photosystem complexes in the membrane. Thus, we compared *Cyanothece* sp. ATCC 51142 spectra with the fluorescence emission spectra of preparations of monomeric and trimeric PSI complexes from *Synechocystis* sp. PCC 6803 (provided by M. Rögner and H. Kuhl), measured with fluorescein as an internal standard and at the same chlorophyll concentration (Fig. 5). The fluorescence spectra demonstrated that PSI monomers fluoresced at 1.75 times the intensity of PSI trimers under excitation at 435 nm. Based on these results, we interpreted the increase in the fluorescence yield at 720 nm in whole cells of *Cyanothece* sp. ATCC 51142 (Fig. 4B) as reflecting a monomerization of PSI.

The comparison of 77 K spectra under excitation at 435 nm of PSII dimer-enriched and monomer-enriched preparations revealed that PSII monomers had a lower fluorescence yield, especially at 695 nm (P.C. Meunier, H. Kuhl, M. Rögner, and L. Sherman, unpublished observations).

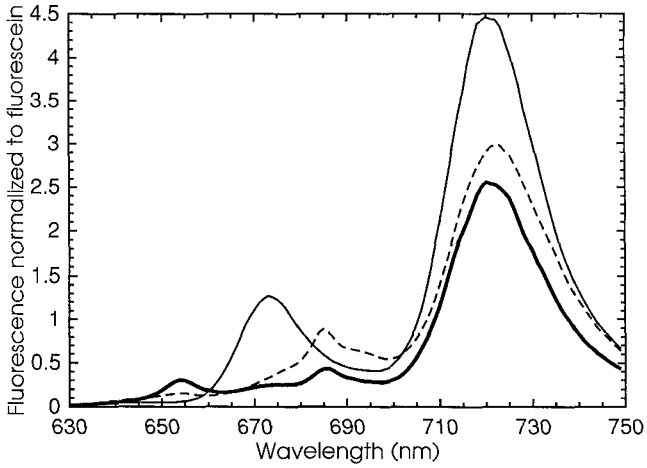

Figure 5. Fluorescence emission spectra at 77 K of PSI trimers (thick solid line), monomers (solid line), and thylakoid preparations (dashed line) of *Synechococcus* sp. PCC 6803. The excitation wavelength was 435 nm.

This suggested that the lower fluorescence intensity at 695 nm under excitation at 435 nm might be correlated with PSII monomers. Therefore, all of the fluorescence results indicated that the transition to state 1 in *Cyanothece* sp. ATCC 51142 reflected an increase in phycobilisome coupling to PSII, the dimerization of PSII centers, and the monomerization of PSI reaction centers.

Similar state 1 transitions were induced in cultures growing in the light near the peak of photosynthesis (L5), as shown in Figure 6. Phycobilisome coupling to PSII centers increased somewhat after red-light treatment (Fig. 6A, solid line), indicating a transition from a partial state 2 to state 1. However, fluorescence excitation at 435 nm showed no increase at 720 nm, thus suggesting that there was no change in the oligomerization state of PSI (Fig. 6B). Nevertheless, fluorescence emission at 685 and 695 nm increased, which suggests that some PSII became dimerized. This experiment suggests that, in *Cyanothece* sp. ATCC 51142, phycobilisome coupling and oligomerization changes do not always relax to a full state 2 in the dark. The PAM data for L5, similar to those shown in Figure 3, also showed a smaller state transition (data not shown); note that in repeat experiments, this occurred around L2 to L4 because the exact timing of the physiological state was variable. It also demonstrated that phycobilisome coupling to PSII and PSII oligomerization changes can occur without PSI oligomerization changes.

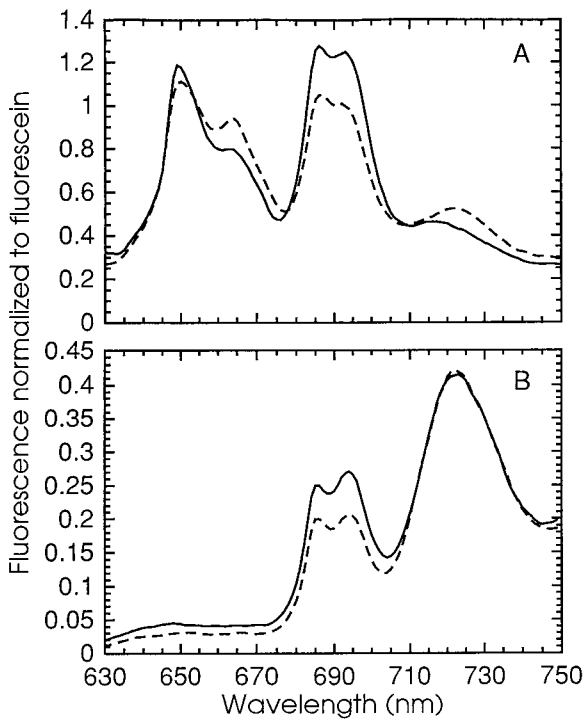

Figure 6. Fluorescence emission spectra at 77 K of *Cyanothece* sp. ATCC 51142 cells at L5, before (dashed line) and after (solid line) the PAM treatment (performed as in Fig. 3). The excitation wavelengths were 580 nm (A) and 435 nm (B).

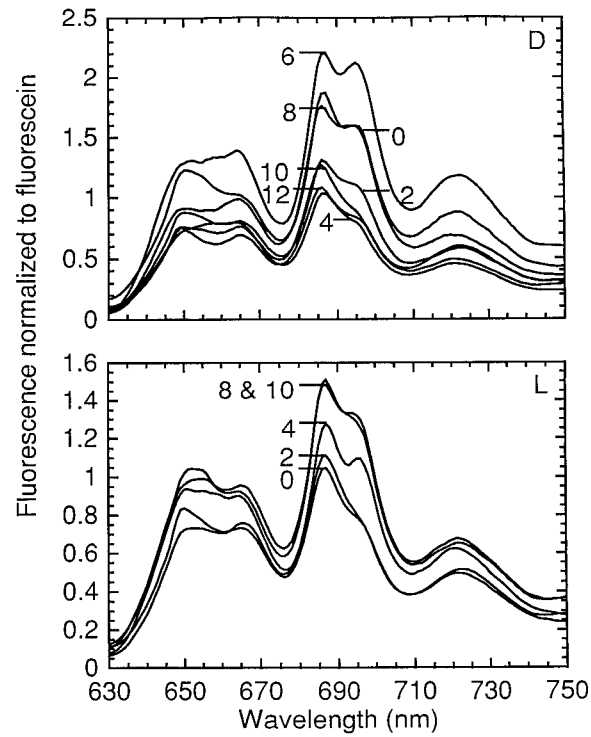

Figure 7. Fluorescence emission spectra at 77 K of *Cyanothece* sp. ATCC 51142 cells during the alternating 12-h light (L) and dark (D) periods. The numbers identify the time in the light or dark cycle when samples were obtained for measurement. The excitation wavelength was 580 nm. Certain samples were omitted for clarity.

State Transitions during the *Cyanothece* sp. ATCC 51142 Diurnal Cycle

The fluorescence spectra at 77 K of LD-grown *Cyanothece* sp. ATCC 51142 cells were measured every 2 h with excitation at 435 and 580 nm, after a 15-min dark adaptation for centrifugation and sample preparation (Figs. 7 and 8, respectively). Because fluorescein was used as an internal standard, the fluorescence intensities represented the fluorescence yields of the different pigment-protein complexes. Figure 7 relates the different fluorescence spectra after excitation by 580-nm illumination. At D0, the fluorescence intensities at 685 and 695 nm were indicative of an intermediate configuration that was mostly state 1. These intensities decreased by approximately a factor of 2 between D0 and D4, which was indicative of a predominantly state 2 population at D4. In addition, the fluorescence at 695 nm decreased proportionately more at D4 than the intensity at 685 nm, suggesting more PSII monomers.

At D6, fluorescence emitted at 695 and 685 nm after excitation at 580 nm increased to a level higher than at D0, indicating a transition to state 1 and an oxidation of the PQ pool (Fig. 7). The intensity at 695 nm relative to 685 nm increased greatly, reflecting an increase in PSII dimers consistent with state 1 (Fig. 7). Thereafter, the system relaxed to the state 2 preferentially observed in other cyanobacterial species under conditions of darkness (Fork and Satoh, 1983). At D6, respiration was in a rapidly decreasing flux (Fig. 1); the data suggest a temporary imbalance be-

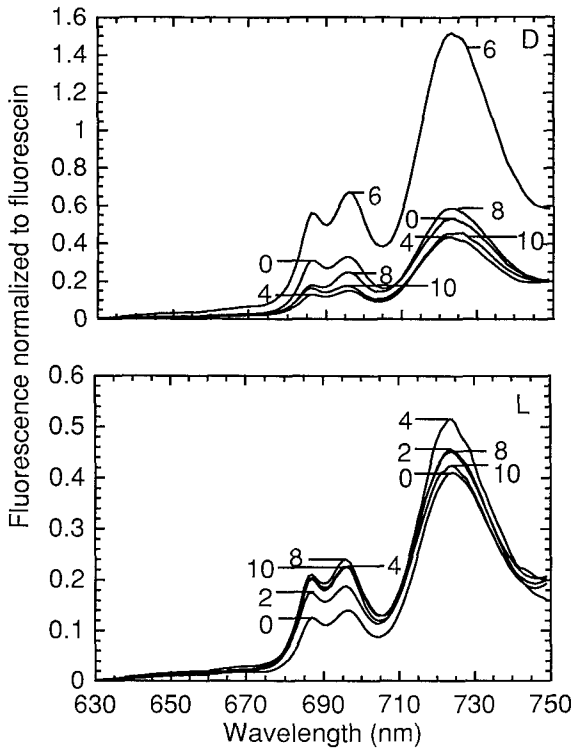

Figure 8. Fluorescence emission spectra at 77 K of *Cyanothece* sp. ATCC 51142 cells during the alternating 12-h light (L) and dark (D) periods. The numbers indicate the time in the light or dark cycle when samples were obtained for measurement. The excitation wavelength was 435 nm. Certain samples were omitted for clarity.

tween the respiratory oxidation and reduction reactions of the PQ pool, which was later corrected in the dark. These phenomena underline the importance of respiratory electron flow in establishing the redox level of the PQ pool in the dark.

At L0, the fluorescence emissions at 695 and 685 nm indicated an intermediate configuration that was mostly state 2 (Fig. 7). During the first 6 to 8 h of the light phase, the fluorescence intensities increased and indicated a progressive change toward state 1. The cells were in a mixed state (as pertains to phycobilisome coupling) at L8, L10, and L12 (the D0 sample), but there seemed to be a shift toward state 2, as indicated by an increase in PSI trimers (see below).

The fluorescence emission spectra in *Cyanothece* sp. ATCC 51142 of the same samples as in Figure 7, but excited at 435 nm, are shown in Figure 8. The fluorescence intensity at 720 nm increased slightly from L0 to L4, indicating that more PSI centers were in the monomeric form at L4 (Fig. 8). Later in the day, the fluorescence intensity at 720 nm decreased and the fluorescence at L10 returned to a level comparable to that at L0, which indicated the formation of PSI trimers. However, the fluorescence intensities at 685 and 695 nm increased from L0 through L8, which suggests that PSII remained mainly in a dimeric state. From D0 to D4, the fluorescence intensity at 720 nm remained fairly constant and low, indicating that PSI centers were mostly in the trimeric form (Fig. 8). Meanwhile, the fluorescence intensities at 685 and 695 nm suggested that PSII progressively formed monomers, and at D4 it was mainly in a monomeric state. However, at D6 the fluorescence intensities abruptly increased by more than a factor of 2, indicating the formation of PSI monomers (extremely high fluorescence emission at 720) and PSII dimers (high F685 and F695). The fluorescence intensity decreased for D8 cells and even further for D10, indicating the formation of PSI trimers and PSII monomers. This observation has been repeated five times with different cultures, and this fluorescence increase was always present immediately after the peak of N_2 fixation. Yet, in the five repetitions of this experiment, the increase in phycobilisome coupling to PSII was not always simultaneous with the formation of PSI monomers, which suggests that these events did not occur at the same rate.

We also obtained 77 K spectra on samples that were rapidly frozen (i.e. no centrifugation) at each time point and immediately monitored for fluorescence. Such measurements required the use of wider slit widths in the excitation and detection monochromators, which resulted in broader spectra. In this manner, the spectra better reflected the states of the cells under the growth conditions, with less than a 15-s interval between the harvesting and the freezing. Importantly, the results were close to those obtained with centrifugation at room temperature and were indistinguishable from the summary given in Table I (P.C. Meunier and L.A. Sherman, unpublished observations). Further studies are needed to address and understand this apparent dark stability.

Our findings are summarized in Table I. From L0 to L6, PSII centers formed dimers, PSII activity increased, phycobilisome excitation indicated a transition closer to state 1, and PSI centers formed monomers. In the late light period (L8 to L12), phycobilisomes remained well coupled to PSII, and PSII remained in a mainly dimeric state. However, whole-chain electron transport rates declined and PSI centers formed more trimers, thus favoring cyclic electron flow. In the early dark period there was a transition of PSII centers to state 2 with the formation of PSII monomers, more PSI trimers, and a further decline in whole-chain electron transport rates. A transitory state formed at D6, which was immediately after the peak of N_2 fixation and the consumption of the glycogen granules. At this time, PSI formed monomers and PSII formed dimers with strong phycobilisome coupling to PSII, indicating an abrupt transition to state 1. After this time point in the dark, PSI centers reverted to trimers, PSII to monomers, and phycobilisome coupling indicated a state 2. Thus, our findings demonstrated that the state transitions indicated by phycobilisome attachment changes can operate independently of the oligomerization state of PSI centers, but were synchronous with PSII oligomerization changes.

DISCUSSION

The unicellular, diazotrophic cyanobacterium *Cyanothece* sp. ATCC 51142 regulates photosynthesis to facilitate the cellular need for N_2 fixation. Thus, photosynthesis provides reducing power and energy at the appropriate time, but the

Table I. *Summary of the state transitions and oligomerization changes observed in Cyanothece sp. ATCC 51142*

Time Period	PSI (λ_{EX} = 435 nm)[a]	PSII (λ_{EX} = 435 nm)	Phycobilisome Coupling (λ_{EX} = 580 nm)	State
Light				
L0 to L6	Trimers → monomers	Activating; monomers → dimers	Low → high	State 2 → state 1
L8 to L12	Monomers → trimers	Mostly dimers	High	Mostly state 1
Dark				
D0 to D3	Mostly trimers	Inactivating; more monomers	Decreasing	Shifting to state 2
D4 (N_2 fixation)	Trimers	Minimal activity; monomers	Low	State 2
D6	Monomers	Dimers	High	State 1
D8 to D12	Monomers → trimers	Dimers → monomers	High → low	State 1 → state 2

[a] λ_{EX}, Excitation energy.

production of O_2 by linear electron transport must also be controlled. In this work we have described a short-term regulation that involves the oligomerization of the photosystems, corresponding state transitions, and, presumably, the redox poise of the PQ pool. The down-regulation of PSII began in the light and the up-regulation began in the dark. In general, the phenomena correlated with the presumed redox state of the PQ pool rather than with the presence or absence of light for growth. This was in agreement with the similar metabolic periodicities observed in light/dark and continuous light conditions (Schneegurt et al., 1994; Colón-López et al., 1997). These results portray a dynamic structure/function relationship of the pigment-protein complexes in this cyanobacterium and provide an important physiological basis for state transitions.

Transitions to state 1 in *Cyanothece* sp. ATCC 51142 consisted of the dimerization of PSII centers, phycobilisome coupling to PSII, and the monomerization of PSI reaction centers. Transitions to state 2 consisted of the uncoupling of phycobilisomes from PSII, monomerized PSII centers, and trimerized PSI centers. However, throughout the LD, partial changes in the oligomerization states of the PSI and PSII reaction centers were not always in the same direction, whereas there appeared to be a constant correlation between PSII dimerization and phycobilisome coupling to PSII. This suggested that there were additional constraints (e.g. ATP levels) to PSI oligomerization compared with PSII oligomerization.

The dichotomy between reaction center oligomerization changes was most apparent in the late part of the day, when PSI centers were undergoing trimerization and PSII centers remained dimers. According to the current model of state transitions, PSI trimerization favors cyclic electron flow for the generation of additional ATP (Bald et al., 1996). This suggested that PSI was converted into a form favoring cyclic electron flow from L8 through D4 to support the cellular need for ATP (for CO_2 fixation and N_2 fixation).

There were several reasons for the lower assay rates of O_2 evolution in the dark phase of LD growth. Because O_2 evolution was measured with $NaHCO_3$ as the electron acceptor, the observed rates were indicative of CO_2 fixation rates. Because PSII activity requires CO_2 fixation as an electron sink (not considering artificial acceptors), and CO_2 fixation requires ATP, sustained PSII activity requires ATP. The depletion of ATP levels by N_2 fixation would inhibit CO_2 fixation, and thus PSII activity. Indeed, the stimulation of nitrogenase activity by PSI cyclic electron transport (Fig. 2) suggested that N_2 fixation was partially rate-limited by the available ATP. Thus, the inhibition of CO_2 fixation would inhibit the reoxidation of NADPH and the PQ pool, and PSII activity would reduce the PQ pool until O_2 evolution was inhibited.

The PQ pool was strongly reduced around D4, as indicated by the increased amount of state 2, because of the intense respiratory electron flow necessary to support nitrogenase activity (see Fig. 1). The reduction of the PQ pool is known to result in a closure of PSII centers and a blockage of PSII activity, and to accelerate S-state deactivations in the water-splitting mechanism (Diner, 1977; Meunier and Popovic, 1990). The stimulation of PSI activity by red light during the O_2 evolution measurements at D4 did not necessarily result in a net oxidation of the PQ pool by linear electron transport, because (as outlined above) the cells were ATP limited, and thus CO_2 fixation could not function as an efficient electron sink.

The depressed status of the O_2 evolution mechanism was confirmed by a lowering of the fluorescence at 695 nm compared with that at 685 nm under phycobilisome excitation (580 nm). The change in 695-nm fluorescence represented a key finding, because mutants with a limitation on the oxidizing side of PSII had lower fluorescence than the wild type at 695 nm compared with 685 nm (Burnap and Sherman, 1991). Moreover, in state 2, the energy captured by phycobilisomes is directed away from PSII. We also observed what we interpret as a monomerization of PSII centers at that time. This combination of factors suggested that, during N_2 fixation, PSII developed a configuration in which the centers were mostly inactive. Thus, O_2 evolution was depressed during N_2 fixation by a concurrent combination of factors.

The period around D6 deserves special mention. This represented the period immediately after the peak of N_2 fixation and the time when virtually all of the glycogen granules had been exhausted (Schneegurt et al., 1994). The nitrogenase proteins were targeted for degradation (Colón-López et al., 1997), respiration was declining (Fig. 1), and the cyanophycin granules were increasing in size and number (Schneegurt et al., 1997). The fluorescence spectral changes were impressive and highly reproducible, indicating a full transition to state 1. The intensity at 695 nm relative to 685 nm increased greatly, which suggested restorative changes on the oxidizing side of PSII as well as a

dimerization of the reaction centers. Nevertheless, O_2 evolution assayed in saturating light remained low at that time, suggesting a limitation to PSII activity other than one related to the oxidizing side of PSII, e.g. a limitation by the available ATP or a down-regulation in the Calvin cycle. Thus, the cell was under significant metabolic flux and may have had a low energy charge (i.e. a depletion in ATP levels). The physiology of this time period is an interesting area of future research.

We observed state transitions in the dark phase in *Cyanothece* sp. ATCC 51142 that relate to presumed changes in the balance between the oxidation of NADH and PQ by the respiratory electron transport chain. Dark adaptation was not a guarantee of a return to state 2. This was demonstrated by the lack of significant difference between the spectra of rapidly frozen and dark-adapted samples, and the presence of state 1 or PSI monomers after dark adaptation (see Fig. 6). This is not a conclusion limited to *Cyanothece* sp. ATCC 51142. In *Synechococcus* sp. PCC 6301, an attempt to induce state 2 by dark adaptation or by PSII illumination resulted in lowered F685 and F695, but higher F720 (Salehian and Bruce, 1992). This suggests that PSI centers remained in mostly monomeric form in that particular physiological state of *Synechococcus* sp. PCC 6301, leading to a condition in which state 2 could not be reached.

It has been shown that a shift between the two forms of the PSII reaction center protein D1 induces a shift in state transitions (Campbell et al., 1996). In *Cyanothece* sp. ATCC 51142, shifts in the forms of the D1 protein have been observed throughout the diurnal cycle (M.S. Colón-López and L.A. Sherman, unpublished data). After dark adaptation, any redox effect of these different protein forms should vanish. We believe that the persistence of state 1 or PSI monomers in darkness, and the negligible effect of dark adaptation at certain times, is evidence that redox signals may not be the only controlling factors of state transitions. Thus, cells may be poised toward state 1 or state 2 by other components of their physiological state. The dynamic nature of *Cyanothece* sp. ATCC 51142 may provide an excellent system with which to better understand the organization of pigment-protein complexes in cyanobacteria, and the role of the different protein forms.

The higher fluorescence yield of PSI monomers compared with trimers that we observed cannot be explained on the basis of interactions between antennae of different PSI, since such interactions have been ruled out (Shubin et al., 1995). There seemed to be no differences in fluorescence excitation energy transfer and primary charge separation yield between monomers and trimers (Turconi et al., 1996). However, a quenching process at long wavelengths (760 nm) that involves $P700^+$ was detected in trimers and was absent in monomers (Shubin et al., 1992, 1995). However, the spectra were normalized to the 726-nm peak because the samples did not have an internal standard (Shubin et al., 1992), and the possible quenching of 726-nm fluorescence by $P700^+$ in trimers was not investigated.

The results demonstrated that organizational alterations of the pigment-protein complexes occurred throughout the 24-h period. Because we only made measurements every 2 h, we must assume that we were witnessing snapshots throughout a continuum. For this reason, we only indicated in Table I that the cells were in a predominantly state 1 or state 2 condition. Similarly, the oligomerization of PSI or PSII was rarely 100% oligomers or monomers and the fluorescence spectra were indicative of such mixed populations. In general, we feel that the principles developed by Rögner and colleagues (Bald et al., 1996; Rögner et al., 1996) represent an excellent unifying model for the organization of pigment-protein complexes in cyanobacteria. However, the complexities demanded by diazotrophy in the unicellular *Cyanothece* sp. ATCC 51142 present an additional layer to the fundamental process.

In conclusion, the fluorescence intensity at 720 nm was correlated to the monomerization of PSI centers, whereas the intensities at 685 and 695 nm were correlated to the dimerization of PSII centers. Phycobilisomes became coupled to PSII in synchrony with the dimerization of PSII centers; in addition, high PSII activity appeared to be correlated with the presence of PSII dimers. However, phycobilisome coupling to PSII appeared to be independent of PSI reaction center oligomerization changes. There were progressive changes in phycobilisome attachment and PSI monomerization throughout the day, but not always toward the same state. Thus, the photosystems were regulated only in part in response to the redox state of the PQ pool brought about by variable respiratory activity in the dark and by photosynthetic electron transport in the light. In addition, we observed a transition to state 1 during growth in darkness (after the peak of nitrogenase activity) that produced a monomerization of PSI centers and maximal energy transfer from phycobilisomes to PSII. Thus, there were large changes in phycobilisome association and energy distribution, as well as changes in the oligomerization of reaction centers, in response to metabolic events (e.g. the exhaustion of carbohydrate granules) and the energetic requirements of the cell.

ACKNOWLEDGMENTS

We are grateful to Matthias Rögner and Helena Kuhl (University of Münster) for the gift of PSI monomers and trimers, and to Don Tucker for supplying some of the samples used in this study.

Received May 20, 1997; accepted July 18, 1997.

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