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Analysis of chlorophyll–protein complexes from the cyanobacterium *Cyanothece* sp. ATCC 51142 by non-denaturing gel electrophoresis

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Abstract

The unicellular diazotrophic cyanobacterium, *Cyanothece* sp. ATCC 51142 temporally separates N_2 fixation from photosynthesis. We are analyzing the mechanism by which photosynthesis is down-regulated so that O_2 evolution is minimized during N_2 fixation. Previous results suggested changes in photosynthesis that are mediated through the redox poise of the plastoquinone pool (a process involving state transitions, in which the redistribution of excitation energy between the two photosystems helps to optimize photosynthetic yield) and the oligomerization state of the photosystems. Our working hypothesis was that the regulation of photosynthesis involved changes in the oligomerization of the photosystems. To analyze this hypothesis, we utilized a low-ionic strength, non-denaturing gel electrophoresis system to study the Chl–protein complexes. We determined that PSI is mostly trimeric, whereas PSII appears mainly as monomers. We demonstrated that most of the Chl–protein complexes in *Cyanothece* sp. remained constant throughout the diurnal cycle, except for the transient accumulation of a Chl–protein complex (band C) which appeared only during the late light period. Based on the size of this complex, band C represents either an interaction of PSI and PSII or a PSII dimer. These results provide support for the dynamic nature of the photosystems with respect to the diurnal cycle.

Keywords: Photosystem; Thylakoid; Cyanobacteria; *Cyanothece* sp.; Green gel; Chlorophyll–protein

1. Introduction

Over the last decade, a great deal of progress has been made in the analysis of intact chlorophyll–protein (Chl–protein) complexes present in the thylakoid membranes of cyanobacteria [1,2] and plants [3–7]. The system of native, non-denaturing 'green gel' electrophoresis, which involves the solubilization and separation of Chl–protein complexes with virtually no release of non-covalently bound chlorophyll, permits the resolution of up to 20 Chl–protein complexes. It is highly probable that these green gel systems depict the complete organization of chlorophyll associated with protein in the thylakoid membranes. The ability to visualize the Chl–protein complexes allows for the analysis of various membrane isolation procedures, thylakoid protein composition, membrane organization, the comparison of membrane complex assembly under varying environmental con-

Abbreviations: LD, light/dark; LL, continuous light; DD, continuous dark; Chl, chlorophyll; Chl–protein, chlorophyll–protein; LHC, light-harvesting complexes; PQ, plastoquinone; DDG, dodecyl-glucoside; DM, decyl-maltoside

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ditions, and Chl–protein complex heterogeneity over time [8–11].

A number of authors, including Allen and Staehelin [3,4], Peter and Thornber [5,6] and Green and colleagues [12] have used these green gel techniques to develop a much more detailed view of Chl–protein complexes from plant and algal chloroplasts than had previously been accomplished. The native green gel systems have been utilized to analyze plant light-harvesting complexes (LHC) and their assembly during development, as well as the membrane organization of photosystem I (PSI) and photosystem II (PSII) in both plants and cyanobacteria [4,6,8]. Most of the bands detected with the green gel systems represented PSI or LHC, but PSII also migrated in a stable and consistent manner.

Cyanobacteria are photosynthetic organisms that perform oxygenic photosynthesis in a manner virtually identical to higher plants and algae, except for the difference in accessory pigments. Cyanobacteria lack chlorophyll B and the light-harvesting proteins, but possess phycobilisomes, which are light-harvesting complexes extrinsic to the thylakoid membrane. We have investigated the compositional organization of the Chl–protein complexes using previous gel procedures in other cyanobacteria, including *Synechococcus* sp. PCC 7942 [13,14], *Synechocystis* sp. PCC 6714 [15], and *Prochlorothrix hollandica* [16]. These studies provided fundamental information about the most stable PSI and PSII complexes in these cyanobacteria, as well as identifying a novel Chl–protein, CP43′. This complex is induced at high levels under certain environmental stress conditions, such as iron deficiency [13–14], and the green band corresponding to CP43′ was unequivocally detected on such gels. A similar protein has now been shown to be the Chl *a*/*b*-binding protein in prochlorophytes [12].

Some cyanobacteria have the ability to perform both O_2-evolving photosynthesis and to fix N_2 with the oxygen-sensitive enzyme nitrogenase. Mechanisms employed by cyanobacteria to protect nitrogenase from oxygen include the formation of heterocysts in filamentous cyanobacteria and temporal separation of O_2 evolution and N_2 fixation in unicellular strains [17,18]. We have been studying the diurnal metabolic rhythms and their regulation in the aerobic, unicellular, diazotrophic cyanobacterium *Cyanothece* sp. ATCC 51142 [19]. When grown in the absence of a fixed nitrogen source, a temporal regulation of N_2 fixation and O_2 evolution occurs in this organism with 24-h periodicities [20] that are characteristic of circadian rhythms [21]. When grown in 12-h light/12-h dark (LD)[1] in continuous light (LL), or in continuous dark heterotrophic (DD) conditions, *Cyanothece* sp. maintains a narrow peak of nitrogenase activity 4 h into the dark (or subjective dark) periods (± 2.5 h) [20,22,23].

The relationships of the daily metabolic rhythms between photosynthesis and N_2 fixation are most intriguing. We have investigated transcriptional and translational processes and determined that the *nifHDK* operon and the associated gene products are very tightly controlled [22,24]. Within every 24-h period, the nitrogenase components are transcribed, translated, active and then proteolytically degraded. At the same time, the photosynthetic mechanism is being continually modulated. The peak of photosynthesis activity occurs in the late light period under LD conditions such that photosynthesis peaks at L8, whereas nitrogenase activity peaks at D4 (at which time photosynthesis is at a minimum and respiration at a maximum). We have determined that many aspects of the photosynthetic mechanism are altered during this process. This includes transcription and translation of the major PSI and PSII gene products [22,24] and the lumenal extrinsic proteins involved with O_2 evolution (Tucker, Ondr and Sherman, unpublished observations). We have also determined that PSII displays a high level of heterogeneity, especially as the cells prepare for N_2 fixation. Most importantly, we find that the redox poise of the plastoquinone (PQ) pool appears to be a major controlling element of this regulation. Respiration supported N_2 fixation by oxidizing the carbohydrate granules that formed during photosynthesis; this process also reduced the PQ pool. This induced state 2 in PSII and lowered the capacity for O_2 evolution. We found that this state seemed to favor PSII monomers and PSI trimers. When the carbohydrate became exhausted at around 6 h into the dark period, this reduced respiratory electron flow which led to a more oxidized PQ pool which eventually led to state 1, which we concluded favored PSII dimers [25,26].

These results gave rise to hypotheses that involved the oligomerization state of PSI and PSII, as well as

the coupling of the phycobilisomes to PSII. Further tests of these hypotheses required an analysis of complexes in as close to their native state as possible. Thus, we report here on the optimization of a native green gel system for the separation of Chl–protein complexes in *Cyanothece* sp. ATCC 51142. These results demonstrated that there are a series of stable PSI and PSII Chl–protein complexes in this organism, including a high proportion of PSI trimers. We will also report on a Chl–protein band that is stable for only a portion of the diurnal cycle. The band is found during the late light period, a time that corresponds to high, but decreasing O_2-evolution activity. We will relate these findings and their importance to the regulation of photosynthesis in *Cyanothece* sp. ATCC 51142.

2. Materials and methods

2.1. Growth conditions, nitrogenase, and chlorophyll determination

Cyanothece sp. ATCC 51142 (formerly *Cyanothece* strain BH68K) was cultured as previously described [19,23] in ASP2 medium without $NaNO_3$. Cultures were grown in 15 l carboys, with air bubbling (flow rate of 18 f^3/h), at 30°C, under circular cool white fluorescent illumination of approximately 75–145 $\mu E/m^2/s$. For time course experiments, two 500-ml continuous-light-grown stationary-phase cultures were subcultured 12 h apart into duplicate 15-l carboys to a dilution of 10^6 cells/ml. This procedure permits 24 h experiments to be performed in 12 h. After subculturing, each carboy was illuminated for 12 h within each 24-h period. This procedure, cell counts, nitrogenase measurements, and chlorophyll determination have been described previously [20,25]. These experiments were performed under LD conditions.

2.2. Cell harvesting, breakage, and extract storage

Exponentially growing *Cyanothece* sp. cultures were harvested (500–7000 ml at $3–7\times10^6$ cells/ml), pelleted, washed, and broken using a protocol developed in *Cyanothece* sp. for the production of O_2-evolving extracts (Tucker and Sherman, unpublished observations). Harvested cells were washed twice in a wash buffer containing 50 mM MES pH 6.5 and 30 mM $CaCl_2$. Once harvested, the cells and subsequent cell extract were maintained on ice or at 4°C. The washed cells were resuspended in 10–20 ml of breakage buffer (50 mM MES pH 6.5, 20 mM $CaCl_2$, 0.8 M sorbitol, 1.0 M glycine betaine (Sigma), and 1 mM ε-amino-*n*-caproic acid). The cells were broken by two passages through a pre-cooled French pressure cell (model FA-073) at 1400 kg/cm^2 (136 MPa). Unbroken cells and large debris were removed by centrifugation ($8000\times g$ for 10 min) and the thylakoid membrane containing extract recovered. The chlorophyll concentration was determined and the cell extracts were stored at −80°C in 1–2-ml aliquots. It had been noted previously that frozen and stored membranes show no detectable change in the pattern of Chl–protein complexes [3].

Harvesting of LD time course samples was performed at 2-h intervals (500 ml per harvest) for a 24-h period. Bulk harvests (2000–7000 ml) were also performed at particular time points of interest in the *Cyanothece* sp. diurnal cycle. Cell sample breakage, and extract storage were always performed immediately following cell harvest.

2.3. Native green gel sample solubilization

The amount of *Cyanothece* sp. cell extract solubilized per lane was determined on a per chlorophyll basis by the number of lanes on the gel. Up to six sample lanes can be clearly distinguished on a non-denaturing PAGE gel. If more than 6 lanes are loaded onto a single gel, the bands become smeared with a less distinct band separation. For the best comparisons between lanes, the individual lanes were loaded with comparable Chl concentrations. In 4–6 lane gels, 50–75 μg total chlorophyll was added per lane. For 1–3 lane gels, 100–200 μg chlorophyll was added per lane. The exact optimal chlorophyll concentration per lane for a specific set of samples varied and was determined by trial and error. All samples were maintained at 4°C under dark conditions throughout solubilization and gel electrophoresis. Membranes were pelleted from the extracts ($130\,000\times g$ for 15 min), the supernatant was discarded, and the membrane pellet was solubilized in the 1.5-ml ultracentrifuge tube with the green gel

solubilization solution and a 1.5-ml Pellet Pestle (Kontes Glass, Vineland, NJ).

The optimal green gel solubilization solution for *Cyanothece* sp. contained 0.45% dodecyl-glucoside (DDG), 0.45% decyl-maltoside (DM), 0.1% lithium dodecyl sulfate (LDS), 10% glycerol, and 2 mM Tris maleate, pH 7.0. The solubilization buffer was added to the samples to obtain a non-ionic detergent to chlorophyll ratio of 20:1 [3]. Samples were solubilized on ice for 30 min and the insoluble material was pelletted at $130\,000 \times g$ for 30 min. The Chl–protein complex containing supernatant was carefully recovered and mixed with one or two drops of 80% glycerol. Samples were then loaded and ran on non-denaturing PAGE gels.

2.4. Non-denaturing chlorophyll–protein PAGE

Non-denaturing Chl–protein gels were prepared, buffered, and run by the method of Allen and Staehlen [3] as 8–12% gradient polyacrylamide gels with an acrylamide to bisacrylamide ratio of 100:1. All buffers and solutions were made fresh, stored at 4°C, and used within a 1-week period for the best and most consistent results. The chlorophyll–protein gels used were 180×125 mm (short gels) or 180×160 mm (tall gels) with a 3-mm gel thickness. The thickness of the gel allows for a higher chlorophyll load, permitting easier detection of faint green Chl–protein complex bands [3]. The Chl–protein gels were routinely electrophoresed at 7-mA constant current, in the dark, at 4°C, for 4–5 h (short gels) or 8–10 h (long gels). Electrophoresis was stopped when the fastest migrating carotenoid band reached the end of the resolving gel.

The softness of the stacking gel prohibited the use of gel combs for making lane-separating strips. A system was developed in which 1% high melting point (HMP) agar plugs were produced and used as lane separators at the surface of the stacking gel (Tracy Troyan, personal communication). The lane separating plugs were prepared by pouring 1% HMP agar between small glass plates separated by the 3 mm spacers used in gel preparation. After polymerization, a scalpel was used to cut the individual spacer plugs (approximately 5–8 mm wide and 15–30 mm long). Agar plugs can be stored in $1\times$ stacking buffer at 4°C for extended periods of time. A couple of layers of white labeling tape were added to the 3-mm gel spacers to slightly widen the plugs. This prevents the plugs from floating away from the stacking gel surface during gel loading.

2.5. Native green gel documentation and Chl–protein complex analysis

After gel electrophoresis, gels were photographed under visible and UV light with a Polaroid MP-4 Land Camera. Gels were then scanned in color with a Sharp JX-610 Scanner employing Adobe Photoshop 3.0 on a Power Macintosh computer. Data manipulation of the images was performed in Adobe Photoshop 3.0 to optimize the band clarity.

The determination of relative molecular mass (M_r) of the Chl–protein complexes was performed with non-denaturing markers in the range from 14.2 to 545 kDa (Sigma). The marker lanes (approximately 8–10 mm in width) were cut from the gel after gel documentation, stained with 0.2% Coomassie brilliant blue R-250 in 50% methanol, and de-stained (35.7% v/v methanol, 7.1% glacial acetic acid, and 1.4% v/v glycerol) for 1 h ($2–3\times$) at room temperature. The markers were then scanned in the same manner as the original gel. The marker lane and gel images were then spliced together with Adobe Photoshop 3.0 to allow marker comparison to the Chl–protein complexes.

Monomers and dimers of PSII, monomers and trimers of PSI, and a *psa*L deletion mutant of *Synechococcus* sp. PCC 7002 were used to identify specific photosystem oligomeric structures present in the *Cyanothece* sp. green gel banding pattern. The purified PSI and PSII oligomers were kind gifts of Dr. Mathias Rögner and Helena Kuhl [27–29]. PSI oligomers were isolated from *Synechocystis* sp. PCC 6803 and the purified PSII oligomers were from *Synechococcus elongatus*. The *psa*L gene product has been found to be necessary and required for PSI trimer formation in *Synechococcus* sp. PCC 7002 and *Synechocystis* sp. PCC 6803. The *psa*L deletion mutant of *Synechococcus* sp. PCC 7002 was a kind gift of Dr. Donald Bryant.

2.6. Western blot analysis of green gel bands

Western analysis of green gel bands was performed

to determine the protein composition of individual bands. Bands of interest were cut from green gels with a razor blade. These bands were placed into 1.5 ml Eppendorf tubes with an excess of 8×loading dye (120 mM Tris pH 6.8, 25% glycerol, 4% LDS, 10% βME, 0.01% bromophenol blue). Proteins were solubilized at 70°C for 10 min with rocking. After solubilization, the polyacrylamide strips containing the Chl–protein complexes were loaded onto 10–20% LDS polyacrylamide gels by gently pushing the gel fragments, between the gel plates, to the surface of the stacking gel. A 1–3-ml layer of 8×loading dye was added above the individual sample lanes (Harold Reithman, unpublished observation). Electrophoresis, Western transfer, and antibody application were performed as previously described [22].

Two-dimensional Western analysis was performed to compare protein profiles of all the Chl–protein complexes from an individual sample on a single gel and Western. Instead of cutting individual bands from a particular lane as just described, 1.5-mm longitudinal strips were cut from the entire sample lane (containing all of the bands of a particular extract). These long strips were solubilized together in a 13-ml sealable tube with 8×loading dye as described above. They were then layered, one on top of the other, onto 10–20% LDS polyacrylamide gels. Care was taken to insure that the individual Chl–protein bands in the strips lined up with one another. A layer of 8×loading dye was added above the strips, the gels were electrophoresed, the proteins were Western transferred, and antibodies were applied [22].

2.7. Antibody probes

Antibodies were diluted with 1×TBS (50 mM Tris pH 8.0, 150 mM NaCl) and 0.1% NaN_3. The following antibodies were employed: (1) anti-MSP (from Spinach, T. Kuwabara), (2) anti-D1 (from *Synechococcus vulcanus*, H. Koike), (3) anti-D2 (from *Synechococcus* sp. PCC 7942, S. Golden), (4) anti-PsbC (anti-43 from *Synechococcus* sp. PCC 7942, M. Ikeuchi and Y. Inoue), (5) anti-PsbB (anti-47 from *Chlamydomonas reinhardtii*, D. Sayre; or from spinach, T. Bricker and L. Sherman), (6) anti-PsaAB (anti-PSI from *Synechococcus* sp. PCC 7942, J. Guikema), (7) anti-PsaA (from *Synechocystis* sp. PCC 6803, P.R. Chitnis), (8) anti-PsaB (from *Synechocystis* sp. PCC 6803, P.R. Chitnis), (9) anti-PsaD (from *Synechococcus* sp. PCC 7002, D. Bryant), (10) anti-PsaE (from *Synechococcus* sp. PCC 7002, D. Bryant), and (11) anti-PsaL (from *Synechococcus* sp. PCC 7002, W.M. Schluchter).

3. Results and discussion

3.1. Solubilization, solution optimization and Chl–protein pattern in Cyanothece sp. ATCC 51142

The native green gel system resolved 10 Chl–protein complexes from *Cyanothece* sp., as well as a low molecular mass carotenoid band, with very little loss of free chlorophyll (Fig. 1). The band-labeling scheme employed for the *Cyanothece* sp. Chl–protein

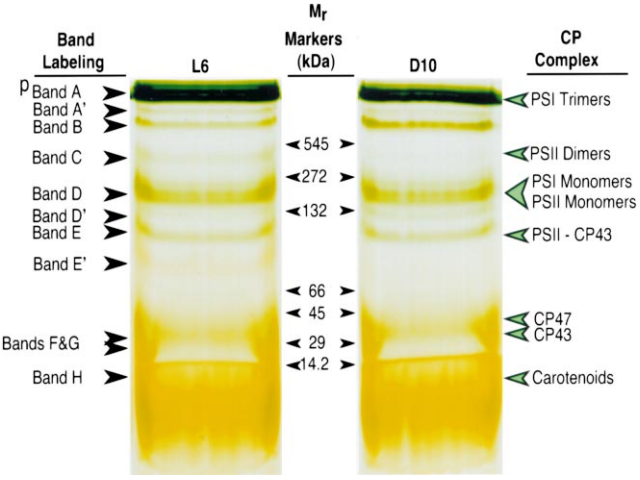

Fig. 1. Non-denaturing green gel analysis of the Chl–protein complexes in *Cyanothece* sp. ATCC 51142. Samples L6 and D10 were prepared from a *Cyanothece* sp. light/dark time course. The non-ionic detergents DDG (0.45%) and DM (0.45%) were used for sample solubilization. Electrophoresis of the 8–12% non-denaturing PAGE gel was for 8.0 h at 7-mA constant current. This extended period of electrophoresis permitted the visualization of bands A′, D′, and E′ which were not visible when the samples were electrophoresed for shorter (4 h) periods. Apparent molecular masses (M_r), displayed between the sample lanes, were determined with non-denaturing markers stained with Coomassie brilliant blue. Arrows on the left denote the band labeling scheme devised for *Cyanothece* sp. The green arrows to the right denote the proposed Chl–protein complexes present in the individual bands based on apparent molecular masses, comparison to isolated photosystem oligomers, and Western blot data (see also Figs. 2–4).

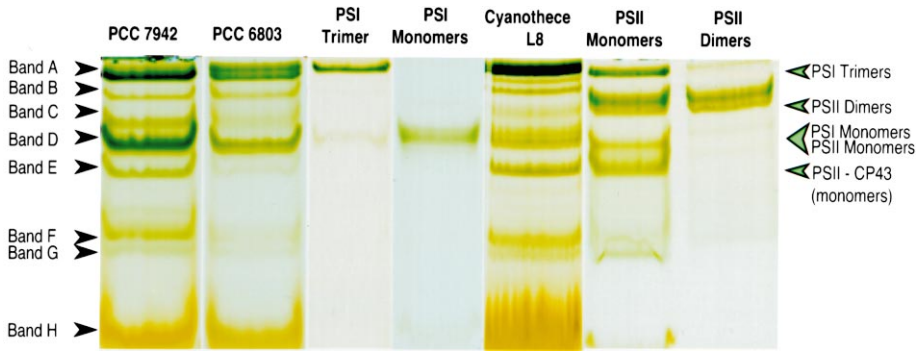

Fig. 2. Non-denaturing green gel analysis of Chl–protein complexes from cyanobacteria. The lanes contain, from left to right: sucrose gradient isolated thylakoids of *Synechococcus* sp. PCC 7942 and *Synechocystis* sp. PCC 6803, PSI trimers, PSI monomers, an extract of *Cyanothece* sp. (L8 from a LD time course), PSII monomers, and PSII dimers. The purified photosystem oligomers were kind gifts of Mathias Rögner and Helena Kuhl. The PSI oligomers were purified from *Synechocystis* sp. PCC 6803 and the PSII oligomers were from *Synechococcus elongatus*. The same solubilization solution was used for all sample lanes and was composed of the non-ionic detergents DDG (0.45%) and DM (0.45%). Individual 8–12% non-denaturing PAGE gels were electrophoresed for approximately 4.0 h at 7-mA constant current. After electrophoresis, visible images were produced for later Chl–protein band analysis. Lanes from the gel images were cut and spliced together with Adobe Photoshop 3.0 to aid in band comparison. Arrows to the right of the gel indicate the oligomerization state of the PSI and PSII oligomers. The arrows on the left show the band-labeling scheme for *Cyanothece* sp. From this analysis, it appears that the purified PSII monomer preparation also contained PSI oligomers and PSII dimers.

complex samples is identified at the left in Fig. 1. Bands A′, D′, and E′ were visible only after an extended electrophoresis of 8–12 h (Fig. 1), but all other labeled bands were separated and easily identifiable after 4 h of electrophoresis. The relative migration of the Coomassie-stained, non-denaturing molecular mass markers is noted between the green gel lanes in Fig. 1.

A variety of detergent combinations were employed during the optimization of the Chl–protein complex solubilization solution. A detergent mix containing 0.45% dodecyl-glucoside and 0.45% decyl-maltoside produced the most distinct *Cyanothece* sp. Chl–protein complex banding patterns under both visible light and UV fluorescence. It is noteworthy that a fresh solubilization solution produced a much more distinct banding pattern than a solubilization solution only 1 day old. It was important to remove the cell breakage buffer, which contained glycine betaine, prior to Chl–protein complex solubilization in order to prevent band smearing during non-denaturing PAGE.

3.2. Comparison of Chl–protein complexes from cyanobacteria and isolated photosystems

The Chl–protein complexes identified in *Cyanothece* sp. were compared to other cyanobacterial Chl–protein complexes, as well as to isolated photosystem oligomers. Fig. 2 is a composite of a series of green gels that were run under identical conditions (7-mA constant current for 4 h). The individual Chl–protein complex sample lanes were aligned by comparison of molecular weight markers and individual band migration. It is apparent from Fig. 2 that *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7942 have similar, but not identical, banding patterns to *Cyanothece* sp. This had been noted previously in our laboratory (Tracy Troyan, personal communication). A number of detergent combinations in the solubilization solutions were also tested with *Synechocystis* sp. PCC 6803, *Synechococcus* sp. PCC 7942, and for the photosystem oligomers as described above. For these samples, a solubilization solution containing 0.45% DDG and 0.45% DM was still found to produce the most distinct banding patterns. The photosystem oligomers (PSI monomers and trimers and PSII monomers and dimers) were solubilized directly and required no further purification on our part. Comparison of the photosystem oligomers to the *Cyanothece* sp. Chl–protein complex banding pattern aided in the identification of Chl–protein complex bands containing PSI and PSII oligomeric structures (Figs. 1 and 2).

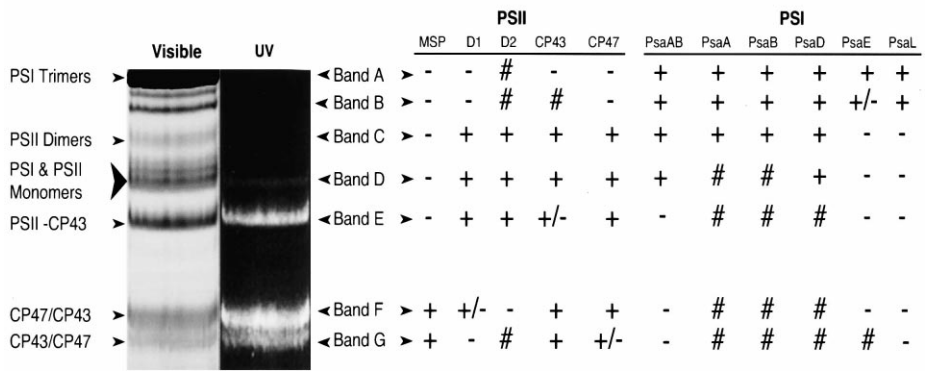

Fig. 3. The protein composition of seven major Chl–protein complexes from *Cyanothece* sp. ATCC 51142. The individual bands were resolved and excised from non-denaturing green gels. Chl–protein complexes from *Cyanothece* sp. were electrophoresed and solubilized as in Fig. 2. Individual bands (labeled A–G) were carefully cut from the non-denaturing gels, solubilized with a denaturing solubilization solution, and layered onto denaturing 10–20% LDS–PAGE gels. After protein electrophoresis and Western transfer, PSI and PSII antibodies were applied to the nitrocellulose membranes, which contained the separated proteins from the green gel bands. The antibodies used are indicated at the top of the figure. A non-denaturing green gel lane (shown with visible and UV images) is presented at the left of the figure for identification of the individual bands (bands A–G) and the determined Chl–protein complex composition. The presence and amount of the protein in the bands is designated as: +, protein present; −, protein absent; +/−, results inconclusive, antibody reaction light or variable; #, experiment not performed.

3.3. Western analysis of Cyanothece sp. Chl–protein complexes

Western analysis of the green gel bands was performed to determine the protein composition in the individual Chl–protein complexes of *Cyanothece* sp. (Fig. 3). Fig. 3 shows visible and UV images of a *Cyanothece* sp. green gel lane and the proteins present in the individual bands of the lane. The higher M_r Chl–protein complexes (bands A, B, C, and D) contain PSI proteins, whereas the mid to lower M_r Chl–protein complexes (bands C, D, E, F, G) are composed of PSII proteins (Fig. 3). The PSI and PSII antibodies used on the Western blots are identified above the figure. The antibodies PsaA and PsaB were developed against antigens of the individual proteins present in the PSI reaction center core heterodimer, whereas the antibody for PsaAB was raised against the complete PSI heterodimer core complex. PsaA and PsaB were used in conjunction with PsaAB to verify that both components of the PSI core heterodimer were present in the PSI containing Chl–protein bands. Chl–protein complexes containing PSII and the light-harvesting antenna of PSII, CP43 and CP47, fluoresce under UV light [30]. The PSI-containing Chl–protein complexes (bands A, B, C and D) do not fluoresce or fluoresce weakly due to the self-quenching nature of PSI. This is true even for bands C and D, which contained some PSII. The UV fluorescence visible in Fig. 3 emanated from bands E, F, and G, in agreement with the Western results which show only PSII proteins present in these Chl–protein complexes.

3.4. Summary of Chl–protein complex band analysis

The protein composition and proposed oligomeric structures of the individual Chl–protein complexes present on *Cyanothece* sp. green gels have been determined by: relative molecular mass (Fig. 1), comparison to purified photosystem oligomers (Fig. 2), analysis of Chl–protein complex UV fluorescence (Fig. 3), and by Western analysis of the individual Chl–protein complexes (Fig. 3). From the Western results, it is apparent that bands A and B appear to be composed entirely of PSI (Table 1). The high molecular weights of these bands (Fig. 1) and direct comparison to PSI trimers (Fig. 2) demonstrated that *Cyanothece* sp. band A is composed of PSI trimers. Band B appeared to be composed of PSI 'dimers', with the faster migration on the green gel possibly due to the loss of various proteins, including a PSI monomer. Band C contains both PSI and PSII proteins (Fig. 3 and Table 1) and migrated at the same rate as isolated PSII dimers (Fig. 2). The apparent molecular mass of band C, approximately 400 kDa

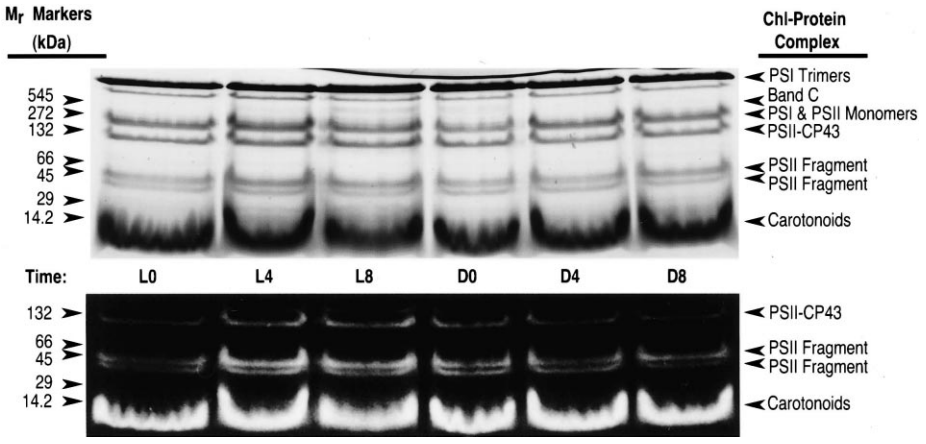

Fig. 4. Non-denaturing green gel analysis of *Cyanothece* sp. ATCC 51142 Chl–protein complexes from throughout the diurnal cycle. *Cyanothece* sp. cells were isolated at 2-h intervals over a 24-h period. Samples isolated every 4 h during the 24 h diurnal cycle were solubilized and loaded onto the non-denaturing PAGE gel. Visible (top) and UV (bottom) images of the green gel are presented. Electrophoresis and solubilization conditions were as in Fig. 2, and each sample contained 75 μg of chlorophyll. Apparent molecular masses, displayed on the left of the images, were determined with non-denaturing molecular weight markers stained with Coomassie brilliant blue. Arrows on the right denote the determined protein composition of the Chl–protein complex bands in *Cyanothece* sp. (see also Figs. 1–3).

(Fig. 1), is also consistent with the identification of a PSII dimer, since individual PSII complexes have been found to have a mass of approximately 250 kDa each [26]. The type of PSI structures present in band C have not been positively determined, but it likely consists of PSI monomers with some other PSI-associated proteins (PsaL is absent). Band D also contains both PSI and PSII proteins (Fig. 3), but the photosystems appear to be present in monomeric form. This was determined by comparison to the PSI and PSII oligomers (Fig. 2) and by comparison to the PsaL deletion mutant of *Synechococcus* sp. PCC 7002 which is unable to form PSI trimers (unpublished observations). Band E is composed of PSII proteins and may be PSII reaction center monomers stripped of some of the PSII-associated proteins (most complexes may lack CP43, although low levels of this protein were identified in band E on Westerns). Based on their apparent molecular weights on green gels, we believe that bands F and G are mainly composed of CP47 and CP43, respectively, although Westerns have identified other PSII proteins co-migrating with these bands. In the Chl–protein bands lacking self-quenching PSI, the PSII bands maintain their fluorescence on the green gels. We have found that fluorescent Chl–protein complexes begin to lose their fluorescence yield during extended electrophoresis (>5 h), even though green bands are still distinct and identifiable. The reason for this loss of fluorescence is currently unknown.

Table 1
Designation of *Cyanothece* sp. ATCC 51142 Chl–protein complexes from non-denaturing green gels

Band	M_r (kDa)	Chl–protein complex
A	1050	PSI trimers
B	680	PSI 'dimers' (-smaller PSI proteins)
C	390	PSII dimers+PSI monomers (-PsaL)
D	190	PSII monomers+PSI fragments (-PsaL)
E	115	PSII (-CP43)
F	35	PSII fragments (CP47/CP43)
G	29	PSII fragments (CP43/CP47)
H	13	Carotenoids

See Figs. 1–3 for original data used to develop this table.

This analysis was performed on many different gels from different time points (L6, L8, D0, D6 and D10) and these repetitions permitted some semi-quantitative conclusions. Band A, consisting of trimeric PSI, represented some 70–80% of the total Chl. At the times when it was present, band C represented $\sim$3% of the total Chl, and this level remained fairly constant during the times at which it was present (e.g. L6, L8, L10, and L12 (identified as D0)). The kinetics of band C formation and disappearance obviously occurred on a time scale that was too rapid to be studied on samples prepared every

2 h. Band D contained 8–10% of the total Chl and the levels of PSI and PSII in band D did not vary much during the diurnal cycle. Although band C was not present at all time periods, it was always detected in the late light period (L6–L12), which indicated that it represented a physiologically relevant Chl–protein complex which formed based on alterations in cellular physiology.

3.5. Analysis of Cyanothece sp. Chl–protein complex banding patterns during the diurnal cycle

Changes in the photosystem oligomerization states, which appear to be involved in regulating photosynthesis during the diurnal cycle, have been hypothesized in *Cyanothece* sp. based on 77K and PAM fluorescence results [26]. These changes in the oligomerization states of PSI and PSII have been related to the photosynthetic rhythm that occurs in *Cyanothece* sp. during its diurnal cycle, a process which appeared to be regulated by the redox poise of the PQ pool [26]. Therefore, it was important to determine if any change in the Chl–protein complex banding pattern occurred in *Cyanothece* sp. during its diurnal cycle and if this change could be related to the fluorescence and physiology data.

Visible and UV fluorescence images of Chl–protein complexes from a *Cyanothece* sp. LD time course are presented in Fig. 4. Cells were isolated and broken at 2-h intervals from 15-l cultures over a 24-h period. Samples isolated from every 4 h of the diurnal cycle were solubilized and loaded onto the non-denaturing green gel. By loading samples from 4-h intervals, we could identify changes in Chl–protein complex banding patterns during the entire *Cyanothece* sp. diurnal cycle on a single gel. All 12 samples were then analyzed on two gels that were run under as close to identical conditions as possible (data not shown).

Most of the Chl–protein complexes, with the exception of band C, appeared to remain constant throughout the diurnal cycle. Band C is composed of both PSI and PSII proteins (Fig. 3) and transiently appeared in the late light (from L6 to L8) and disappeared in the early dark (usually absent by D2) (Fig. 4). This time period coincided with the peak of O_2 evolution during the *Cyanothece* sp. 24-h diurnal cycle [25]. As indicated previously, band C migrates at a position consistent with the composition of a PSII dimer. UV illumination of this gel yielded fluorescence from bands D′ through G, but none from band C. This indicated that the PSII Chl in band C is unusual; this Chl may be in a form that quenches fluorescence or it is possible that the PSI present in band C quenches any PSII fluorescence. The UV fluorescence image in Fig. 4 ends immediately above the PSII–CP43 complex because no UV fluorescence was visible from the complexes above this band (data not shown). The transient appearance of band C was seen on multiple green gels and in samples from two different LD time course experiments. This transient appearance of band C may relate to the regulation of O_2 evolution through photosystem oligomerization or to PSII alterations in *Cyanothece* sp. during the late light period of the diurnal cycle

In summary, we have used non-denaturing gel electrophoresis to analyze the Chl–protein complexes from the thylakoids of the cyanobacterium *Cyanothece* sp. ATCC 51142. This system was a modification of that developed by Allen and Staehelin [3] and we conclude that the ten distinct Chl–protein complex bands observed in *Cyanothece* sp. closely resemble the in vivo pattern of Chl–protein complexes. Three of these bands (A′, D′ and E′) only become visible after extended gel electrophoresis and may represent degradation products of their slower migrating partners. The virtual lack of free chlorophyll at the migration front was used as an indication that all chlorophyll was still associated with the Chl–protein complexes.

It is evident that the bulk of PSI migrates as a trimer (greater than 80% of the total chlorophyll), whereas most of the PSII is found as monomers. The reproducibility of band A would lead us to suggest that PSI is mostly in the trimeric form in vivo, in agreement with Schluchter et al. [31]. PSI monomers run at about the level of band D and we detected no PSI proteins below this level. Thus, we conclude that PSI trimers are quite stable, and are representative of the in vivo state of oligomerization. Bands D and E are found throughout the life cycle of *Cyanothece* sp. and represent most of the PSII material. Our analysis indicates that the PSII in these bands are present as monomers, or as somewhat degraded monomers in band E, which mostly lack CP43. These are fragmented further into complexes enriched in CP47 and CP43 (bands F and G), which appear typical

of the faster-migrating Chl–protein complexes of plant and cyanobacterial thylakoids [2,5,7].

The enigmatic band C appeared transiently in the mid- to late-light phase (L6–L8) and disappeared by the beginning of the dark period. The time period around L8 coincided with the peak of O_2 evolution during the *Cyanothece* sp. diurnal cycle and at a time in which we concluded from fluorescence analysis that PSII would have a greater percentage of dimers [25]. Even for cells grown in the light, a period of declining O_2 evolution on a per Chl basis began at L8, and PSII was strongly down-regulated. The transient appearance of this Chl–protein complex could be due to new synthesis, enhanced stability, or the formation of different oligomeric interactions. New synthesis is the least likely of these possibilities, since L6 is near a minimum in transcriptional and translational activity [22,24]. The migration of the band was consistent with co-migration of PSII dimers and PSI monomers and evidence for both photosystems was determined by the use of antibodies (Fig. 3). The migration and composition was also consistent with a stable interaction between PSII and PSI monomers. Thus, we cannot conclusively demonstrate the oligomeric composition of band C, although it is evident that some change occurs about the time that PSII activity decreased. This time period of L6–L12 also coincided with a decrease in the stability of O_2 evolution and an increase in PSII heterogeneity [26]. The decreased stability of O_2 evolution involved instability of the Mn center, which may reflect altered interaction of the Mn-stabilizing protein (MSP) with the PSII reaction center. Through this dynamic process, which may involve additional conformational changes in the PSII proteins, interactions may be established that modifies the interaction of PSII with neighboring complexes. This may generate enhanced PSI–PSII or PSII–PSII interactions, which gives rise to band C. This is a useful working hypothesis that we will pursue in future experiments.

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