Organization of pigment proteins in the photosystem II complex of the cyanobacterium *Anacystis nidulans* R2

(Chlorophyll proteins/fluorescence/phycobiliproteins/antibodies/photosynthesis)

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Communicated by E. R. Sears, June 19, 1985

**ABSTRACT** Two chlorophyll–protein complexes associated with photosystem II (PSII) of the cyanobacterium *Anacystis nidulans* R2 have been detected. The larger of the two complexes, CPV1-1, contained a 71-kDa and a 42-kDa protein. The 71-kDa protein was determined to be the anchor protein of the phycobilisomes (the light-harvesting complex of *A. nidulans* PSII), since it was recognized by an antibody raised against a similar protein from another cyanobacterium. The second complex, CPV1-4, contained a previously unobserved 36-kDa chlorophyll-binding protein. Additionally, two other PSII chlorophyll–protein bands were characterized. CPV1-2 contained a 52-kDa band that was recognized by an antibody raised against the presumptive PSII reaction center protein of *Chlamydomonas reinhardtii*. It gave rise to a fluorescence emission peak (77 K) at 695 nm, indicating that this chlorophyll–protein complex may harbor the reaction center of PSII. Finally, CPV1-3 was found to have a 45-kDa protein and to be immunologically related to the presumptive immediate-antenna protein of the *C. reinhardtii* PSII.

Photosynthetic *O*₂ evolution in cyanobacteria is mediated by a photosystem II complex (PSII) that is quite similar to that of higher plants. However, the major light-harvesting antennae of cyanobacterial PSII are phycobilisomes, pigment–protein complexes that are attached to the surface of the thylakoid membranes; thus, they are distinctly different from the integral membrane, light-harvesting chlorophyll a/b complexes of higher plant PSII. Most of the chlorophyll molecules inside cyanobacterial thylakoids are associated with photosystem I (PSI), whereas PSII has only a small percentage of the total number of chlorophyll.

The organization of chlorophyll-binding proteins of cyanobacterial PSII has not yet been examined in detail. Traditionally, the core complex and the light-harvesting structures of higher plant PSII have been examined by treatment of intact thylakoids with “neutral” detergents (e.g., digitonin) and non-denaturing polyacrylamide gel electrophoresis. This procedure permits the examination of individual “green” bands so that the association of chlorophyll molecules with one or more polypeptides can be determined (1). Guikema and Sherman (2) used this approach to identify six chlorophyll–protein bands (CPI to CPVI) in thylakoid preparations from the unicellular, nonthermophilic cyanobacterium, *Anacystis nidulans* R2. The largest complexes (CPI–CPV; 360, 250, 140, 100, and 75 kDa, respectively) are related to PSI, whereas only the smallest band (CPVI, 45 kDa) is associated with PSII. Yamagishi and Katoh (3, 4) used analogous techniques to isolate two different PSII-associated chlorophyll–protein bands from the thermophilic cyanobacterium *Synechococcus*. We have reexamined the organization of the PSII chlorophyll-binding proteins of *A. nidulans* R2. We have used the nonionic detergent, dodecyl β-D-maltoside, to solubilize thylakoid membranes of *A. nidulans* R2 and have detected four chlorophyll–protein complexes associated with PSII that migrate in the CPVI region. The complex with the highest apparent molecular weight, CPVI-1, contained a polypeptide that was immunologically identified as the anchor protein of phycobilisomes of the PSII core complex. Two chlorophyll–protein bands that were structurally similar to CPVI-1 and CPV-2 from higher plants (5) were also detected. Moreover, another chlorophyll–protein band (CPV-4) was observed in membrane preparations enriched in PSII. This band was particularly prominent in iron-deficient cells and contained a 36-kDa apoprotein. Structural similarities between different chlorophyll–protein bands from *A. nidulans* and higher plants were examined by analyzing immunological cross-reactivity of cyanobacterial polypeptides (on protein blots) with antibodies raised against chloroplast proteins. These analyses showed that there is a high degree of conservation of antigenic sites between proteins serving similar roles in PSII of cyanobacteria and higher plants. We will discuss the implications raised by the detection of a PSII chlorophyll-binding complex composed of 36-kDa apoprotein.

**MATERIALS AND METHODS**

**Materials.** Lithium dodecyl sulfate was obtained from Gallard Schlesinger (Carle Place, NY). Dodecyl β-D-maltoside was from Calbiochem–Behring. Materials for gel electrophoresis were purchased from Bio-Rad. Antibodies were kindly provided by: N.-H. Chua (Rockefeller University, New York, antibodies to proteins 5 and 6 of *Chlamydomonas reinhardtii* thylakoids); and E. Gantt (Smithsonian Research Center, Rockville, MD, antibody to the phycobilisome anchor polypeptide of *Nostoc* sp.).

**Strain.** The characteristics and growth conditions of *A. nidulans* R2 have been reported previously (6). Iron-deficient cultures were grown as described (7).

**Isolation and Solubilization of Thylakoid Membranes.** *A. nidulans* cells were harvested at late-logarithmic phase and suspended in buffer A (50 mM 2-(N-morpholino)ethanesulfonic acid, pH 6.5) with 1 mM each of benzamidine, e-aminoacaproic acid, and phenylmethylsulfonyl fluoride. All subsequent manipulations were carried out at 0–4°C. The cells were passed three times through a chilled French pressure cell (Aminoct) at 20,000 psi (1 psi = 6.895 kPa). Unbroken cells were sedimented by a low-speed (4000 × g, 5 min) centrifugation, and thylakoids were pelleted from the supernatant by a high-speed centrifugation (150,000 × g, 1 hr, Ti60 rotor, Beckman). The pelleted membranes were resus-

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**Abbreviation:** PS, photosystem.

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pended in buffer A to a chlorophyll concentration of 0.25 mg/ml (iron-deficient membranes) or 0.35 mg/ml (normal membranes). Dodecyl β-D-maltoside was added to a final detergent/chlorophyll ratio of 10:1 (wt/wt), and the suspension was incubated on ice for 15 min and then centrifuged as before. The resulting supernatant (henceforth called "maltoside extract") was highly enriched in PSII relative to PSI.

Analysis of Chlorophyll–Protein Complexes. The chlorophyll-binding proteins were analyzed on non-denaturing gradient acrylamide gels as previously described (2, 8). Gels were maintained at 4–6°C throughout the electrophoresis procedure, and the upper buffer contained 0.1% LiDodSO4 and 1 mM EDTA. Thylakoid membrane samples from both regular and iron-deficient cells were solubilized in 1.5% LiDodSO4 just before loading, whereas maltoside extracts were either loaded directly (normal cells) or after addition of 0.5% LiDodSO4 just prior to loading (iron-deficient cells). Electrophoresis was carried out in the dark for about 1 hr at 2 W constant power (until green material stacked) and then for 1–2 hr more at 3.5 W to resolve the CPVI region. Gels were photographed in visible light through a blue filter to record chlorophyll-containing bands and then through an orange filter during transillumination with 365 nm UV light to detect fluorescent bands.

The polypeptide composition of each chlorophyll–protein band was analyzed by slicing the green bands out of the non-denaturing gel and re-electrophoresing the excised band on a denaturing gel. The slices were heated at 70°C for 10 min in the presence of 20–40 μl of 6% LiDodSO4 (vol/vol)/6% 2-mercaptoethanol (vol/vol)/5% sucrose (wt/vol) and 10 mM Tricine, pH 7.5/plus 1 mM each of the three protease inhibitors mentioned above. The gels were subsequently stained with Coomassie blue or silver (for higher sensitivity) as described (9). Polypeptides separated by LiDodSO4/PAGE were electrophoretically transferred onto nitrocellulose and immunostained following the procedure of Towbin et al. (10). Chlorophyll concentrations were determined by the method in (11).

RESULTS

Chlorophyll–Protein Complexes of A. nidulans R2. A. nidulans R2 membranes solubilized with 1.5% LiDodSO4 (wt/vol) were resolved into six chlorophyll–protein complex-
and 8). It should be noted that the chlorophyll–protein complexes of PSI fluoresced weakly, whereas all of the PSII complexes were highly fluorescent (12).

**Fluorescence Emission Spectra (77 K) of Chlorophyll-Protein Bands.** Excitation of chlorophyll molecules in intact *A. nidulans* cells frozen at 77 K gives rise to three emission peaks at 686, 696, and 716 nm (hereafter referred to as F686, F696, and F716). The former two (F686 and F696) emenate from PSII, whereas F716 emanates from PSI (2, 13). The emission spectra at 77 K of the chlorophyll–protein bands were determined by excising acrylamide gel pieces that contained the chlorophyll–protein complex and exciting the chlorophyll with light at 435 nm. CPVI-3 gave rise to F685, whereas CPVI-2 emitted F695 (Fig. 2). Fluorescence emission at 695 nm has previously been associated with the presumptive PSI reaction center of spinach thylakoids, CP47 (5), and a similar band, CP2-b, from a thermophilic cyanobacterium (4). As noticed by Yamagishi and Katoh (3, 4), the thermophilic nature of their experimental organism made the chlorophyll–protein complexes very stable. In comparison, CPVI-2 from *A. nidulans* was extremely labile. Inclusion of LiDODSO4 in the solubilization buffer tended to shift the CPVI-2 emission peak to 685 nm. Moreover, to obtain F695, the gel fragments containing this band had to be maintained at or near 0°C, and the fluorescence emission spectra had to be obtained very soon after electrophoresis was completed. Emission of F695 from CP47 has been attributed to the presence of phycocyanin and has been used to indicate the probable presence of the PSI reaction center in this chlorophyll-binding protein (5). In addition to CPVI-3, CPVI-1 and CPVI-4 emitted F685 only.

**Apoproteins of the PSII Chlorophyll-Protein Bands.** The polypeptide composition of the discrete chlorophyll–protein bands from maltoside extracts is shown in Fig. 3. During the course of these studies, we have analyzed the individual green bands after initial separation on 5–15%, 10–15%, and 12–15% acrylamide gradient gels. The results were always identical, indicating that the proteins discussed below are intrinsic components of each complex and are not due to comigrating contaminants (see ref. 14). Thus, we will present only the patterns from green bands that were originally isolated from 10–15% green (non-denaturing) gels and then re-run on 10–20% denaturing gels. Since silver staining rarely detected other protein components, the Coomassie-stained gels are presented as representative.

CPVI-1 contained two polypeptides, 71 and 42 kDa (Fig. 3, lane 1). Comparison of the protein profiles of unfractionated membranes and isolated phycobilisomes from *A. nidulans* (9) suggested that the 71-kDa polypeptide was possibly the anchor protein that connects the phycobilisomes to the membrane-bound PSI complexes (see below). CPVI-2 was somewhat more complex, giving rise to a major band at ~50 kDa (Fig. 3, lane 3) that was often resolved as a series of discrete bands between 44 and 52 kDa. A 48-kDa protein has often been observed as a major constituent of the PSI complex in *A. nidulans* (2, 9) and enriched levels of a 52-kDa protein have been observed in an O2-evolving PSI preparation from *A. nidulans* (9). Significantly, CPVI-2 from membranes of iron-deficient cells appeared to contain the 52-kDa polypeptide exclusively (lane 6), but very little of the 48-kDa protein could be detected after silver staining.

The apoprotein of CPVI-3 (lane 4) was a 45-kDa polypeptide. Another chlorophyll–protein band migrating in the same region, CPVI-4, was always observed, but was particularly prominent in membranes from iron-deficient cells (see Fig. 1). The apoprotein of CPVI-4 was a 36-kDa protein (Fig. 3, lane 7). Thus, on the basis of this difference in their respective apoproteins, CPVI-3 and CPVI-4 were considered to be different chlorophyll–protein bands.

**Functional Identity of Proteins by Immunodecoration with Heterologous Antibodies.** Since the functional proteins in thylakoid membranes from a large number of photosynthetic organisms have been shown to be antigenically similar (15), the apoproteins of the different chlorophyll–protein bands were identified immunologically. Proteins in gels similar to those in Fig. 3 were electrophoretically transferred onto nitrocellulose filters and then immunoblotted with different antibodies. The 71-kDa polypeptide from phycobilisomes and from CPVI-1 (from either regular or iron-deficient cells) was recognized by an antibody raised against the phycobilisome anchor polypeptide of *Nostoc* sp. (Fig. 4, lanes 1–3). E. Gantt (personal communication) has demonstrated that anchor proteins from many strains of cyanobacteria cross-react with this antibody. On the basis of this information, we have identified the 71-kDa protein in CPVI-1 as the phycobilisome anchor protein of *A. nidulans* R2. The 42-kDa protein of CPVI-1 (see Fig. 3, lane 1) was not recognized by this antibody, and thus, was not obviously a breakdown product of the 71-kDa protein.

The complexity of the CPVI-2 polypeptide pattern was resolved by this procedure, using antibody prepared against *C. reinhardtii* protein 5, one of the two chlorophyll-binding proteins of the chloroplast PSI core complex (8, 16). Both the 48- and the 52-kDa polypeptides were recognized by this antibody (Fig. 4, lanes 4–6). Essentially the same pattern was obtained with maltoside extracts (lane 4), or with CPVI-2 from normal (lane 5), and from iron-deficient cells (lane 6). The data presented here showed that the 52-kDa polypeptide is antigenically related to the 48-kDa protein. We believe that these two closely migrating polypeptides represent the same apoprotein with different degrees of association with chlorophyll molecules. Moreover, the high degree of cross-reactivity of the antibody for the 52-kDa protein indicates that it is the apoprotein of CPVI-2.

The 45-kDa apoprotein of CPVI-3 was recognized by an antibody prepared against the polypeptide 6 (8, 16) of *Chlamydomonas* PSII (Fig. 4, lanes 7–9). We have previously shown that protein 6 antibody recognizes the 45-kDa apoprotein of CP43, a chlorophyll–protein associated with the PSII core complex of spinach (12). In analogy with the higher plant system, we suggest that CPVI-3 serves as an antenna for the PSII reaction center of *A. nidulans* R2. Anti-protein 5 antibody did not recognize the 45-kDa protein, and anti-protein 6 antibody did not cross-react with the 52-kDa and 48-kDa proteins. Moreover, the 42-kDa polypep-
tide of CPVI-1 was not recognized by either anti-protein 5 or anti-protein 6 antisera. Therefore, the 42-kDa polypeptide was distinct from the chlorophyll-binding apoproteins of CPVI-2 and CPVI-3. Additionally, the 36-kDa polypeptide from CPVI-4 was not recognized by any of the three antibodies used in this study. These results make it seem likely that PSII of A. nidulans R2 is comprised of four distinct chlorophyll-binding proteins.

**DISCUSSION**

In this study, we have examined the chlorophyll-protein complexes that are associated with PSII of A. nidulans R2. The use of dodecyl maltoside, an effective but mild detergent, allowed us to analyze four such complexes, CPVI-1 to CPVI-4 (Fig. 1). Among these, most of the chlorophyll was normally associated with CPVI-2 and CPVI-3, which contained apoproteins of 52 and 45 kDa, respectively. Yamagishi and Katoh (3, 4) have recently shown that PSII of the thermophilic cyanobacterium, *Synechococcus* sp., contains two complexes, CP2-b and CP2-c. The slower migrating species, CP2-b, contains four proteins and mediates primary PSII photochemistry. It also has a 77 K fluorescence emission peak at 694 nm. CP2-b is readily converted into simpler CP2-a and CP2-d bands, both of which have only a 47-kDa component (3). CP2-2 in our studies may actually be identical to CP2-a or CP2-d. By virtue of its fluorescence emission peak (77 K) at 695 nm, CP2-2 appears to be similar to spinach CPa-1 (5). This report thus verifies the existence of a 695 nm emission peak in a chlorophyll-protein band from a nonthermophilic cyanobacterium.

CPVI-3 was similar to CP2-c from *Synechococcus* sp. (4). Since CP2-c consists of a 40-kDa polypeptide, which is probably analogous to the 45-kDa apoprotein of CPVI-3, and both CP2-c and CPVI-3 fluoresce at 685 nm, Yamagishi and Katoh (4) have suggested that CP2-c may serve as an immediate antenna to the reaction center of PSII. We have detected immunological cross-reactivity between the 45-kDa apoprotein of CPVI-3 and the apoprotein of CPa-2 from spinach, also believed to serve as an immediate antenna of the PSII reaction center (17). Based on these data, it is likely that CPVI-3 is an antenna to the reaction center of PSII.

A major finding of this study was two more chlorophyll-protein bands associated with PSII, CPVI-1 and CPVI-4. The use of heterologous antibodies for the identification of the apoproteins of different chlorophyll-protein complexes was extremely helpful in assessing the functional significance of CPVI-1 (Fig. 4). This band contained the 71-kDa anchor polypeptide that connects the phycobilisomes to the PSII complex. Redlinger and Gantt (18) have shown that the anchor polypeptide from a red alga had bound chlorophyll

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**Fig. 3.** Protein components of the *A. nidulans* R2 chlorophyll-protein complexes. The individual complexes (CPVI-1 to CPVI-4) were excised from gels similar to those in Fig. 1. A single slice was placed in a well of a 10–20% gradient gel, and electrophoresis was performed. Lanes: 1, CPVI-1; 2, CPVI-2; 3, CPVI-3; 4, CPVI-2 from iron-deficient cells; 5, CPVI-4; 2 and 5, molecular size standards: bovine serum albumin (67 kDa), ovalbumin (45 kDa), chymotrypsin (25 kDa), and cytochrome c (12 kDa).

**Fig. 4.** Immunological blots with rabbit antibodies raised against chloroplast membrane proteins showing cross-reactivity to the apoproteins of PSII chlorophyll-protein complexes from *A. nidulans*. Electrophoresis was performed as in Fig. 3. After electroblotting, filters were probed with antibody prepared against the following proteins: lanes 1–3, phycobilisome anchor polypeptide from *Nostoc*; lanes 4–6, protein 5 from *C. reinhardtii*; and lanes 7–9, protein 6 from *C. reinhardtii*. Samples: lane 1, purified phycobilisomes from *A. nidulans*; lane 2, CPVI-1 from regular cells; lane 3, CPVI-1 from iron-deficient cells; lane 4, maltoside extract; lane 5, CPVI-2, regular cells; lane 6, CPVI-2, iron-deficient cells; lane 7, maltoside extract; lane 8, CPVI-3, regular cells; lane 9, CPVI-3, iron-deficient cells.
when isolated from the thylakoid membranes. We have identified the presence of a 42-kDa polypeptide in the CPVI-1 complex, which was not antigenically related to the 52- or 45-kDa apoprotein of CPVI-2 and CPVI-3. Moreover, our studies have indicated that the 42-kDa polypeptide is an integral membrane protein (15) and is not part of the extrinsic phycobilisome. Recent studies from other laboratories have shown that phycobilisomes are functionally as well as structurally connected to the PSI complex in the thylakoid membranes of cyanobacteria (19–21). The 42-kDa protein may act as the protein inside the membranes that directly binds the 71-kDa polypeptide. It is important to note that we have never observed a chlorophyll–protein band containing only the 42-kDa polypeptide. Hence, it is not possible at this stage to determine whether this protein binds chlorophyll molecules and thus acts as an intermediate transducer of harvested light energy between the phycobilisome and PSI.

Another chlorophyll–protein band, CPVI-4, was also observed during this study. This band (36-kDa apoprotein) is present in cells grown under normal conditions but becomes the major chlorophyll–protein complex in membranes from iron-deficient cyanobacteria (Fig. 1, lane 7). Under iron-deficient conditions, the cells are perfectly viable; however, most of their phycobilisomes disappear (6), and there is a major alteration in the organization of the chlorophyll within the thylakoid membrane. One possible interpretation is that CPVI-4 acts as an intermediate antenna complex for PSI and increases in quantity significantly as the level of phycobilisomes diminishes.

However, it is still not possible to rule out the involvement of the 36-kDa chlorophyll-binding protein in the primary PSI photochemistry. Nakatani et al. (5) and Camm and Green (17) have attempted to correlate primary PSI activity with the complex CP47 by using fluorescence and electron transport assays, respectively. Although our fluorescence results for CPVI-2 are in agreement with Nakatani et al. (5), we have not been able to demonstrate PSI electron transport activity (diphenylcarbazine to dichlororindophenol) on a chlorophyll–protein band containing only the 52-kDa protein (unpublished observations). Similar results have been reported by de Vitry et al. (22). They obtained a PSI reaction center complex from C. reinhardtii that required the presence of a 1:1 molar ratio of protein 5 (50 kDa) and protein 6 (47 kDa) for complete PSI photochemistry. Their active complex also contained the intrinsic membrane protein D2 (32 kDa), although D2 appeared to be present in nonstoichiometric quantities. The D2 polypeptide is currently of interest because of the structural homology between the gene sequences of D2 and the M subunit of the reaction center from photosynthetic bacteria (23). This has led to the suggestion that D2 may be involved in quinone binding (23); however, the analogy has been extended to indicate that the D2 protein may contain the PSI reaction center (24). The finding of a 36-kDa chlorophyll-binding protein may be important in this regard. We have isolated fractions enriched in PSI from maltoside extracts after centrifugation on sucrose gradients. These PSI core preparations still contain relatively large amounts of CPVI-4 and the 36-kDa polypeptides, in addition to the 45- and 52-kDa proteins (unpublished observations). Furthermore, PSI core preparations from Aphanocapsa sp. also contain very high levels of 36-kDa chlorophyll-binding protein (unpublished observations). These results indicate that the 36-kDa chlorophyll-binding protein may be a functionally important component of the PSI reaction center.

We greatly appreciate the expert technical assistance provided by Ms. Jill Cunningham. We also extend our sincere gratitude to N.-H. Chua and E. Gantt for providing the antibodies used in this study. This work was supported by Grant GM21827 from the National Institutes of Health and by University of Missouri Institutional Biomedical Research Grant RR07053 from the National Institutes of Health. H.C.R. was a National Institutes of Health Predoctoral Trainee (DHHS 5 T32 GM07494).