Journal of Bioenergetics and Biomembranes, Vol. 18, No. 4, 1986

Immunological Characterization of Photosystem II Chlorophyll-Binding Proteins from the Cyanobacterium, *Aphanocapsa* 6714

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Received November 4, 1985; revised December 16, 1985

Abstract

Chlorophyll-binding proteins from the cyanobacterium *Aphanocapsa* 6714 were identified by immunoblotting procedures. Three chlorophyll-binding complexes, CPIII′, CPIIIa, and CPIIIb, were associated with PSII. CPIII′ likely serves as an antenna to PSII in *Aphanocapsa* since it could be removed from active PSII core preparations without loss of activity. The CPIII′ proteins cross-reacted to antibodies prepared against the maize PSII light-harvesting complex, LHC-II. The CPIIIa polypeptides cross-reacted to antibodies raised against the *Chlamydomonas* PSII chlorophyll-proteins 5 and 6, indicating that this complex contains the major chlorophyll-binding species of the cyanobacterial PSII core. Lastly, an antibody prepared against the cyanobacterial 36-kDa chlorophyll-binding protein [Pakrasi, H., Riethman, H., and Sherman, L. (1985). *Proc. Natl. Acad. Sci. USA* **82**, 6903–6907] recognized only the 36-kDa IIIb apoprotein, indicating that CPIIIb represents a distinct chlorophyll-protein complex.

Key Words: Chlorophyll-proteins; thylakoid; Photosystem II; cyanobacteria.

Introduction

Cyanobacteria are prokaryotes capable of oxygenic photosynthesis and thus provide a model system for understanding the organization and assembly of Photosystem I and Photosystem II. Comparison of thylakoid membrane fractions of higher plants and cyanobacteria has helped define the polypeptides playing important roles in photosynthesis. We are interested in determining the role of pigment-proteins in cyanobacterial PSII. To this end, we have probed *Aphanocapsa* 6714 PSII chlorophyll-proteins with antibodies

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0145-479X/86/0800-0285$05.00/0

raised against PSII components from heterologous species. *Aphanocapsa* 6714 is a heterotrophic, unicellular cyanobacterium whose thylakoid is well characterized (Bullerjahn *et al.*, 1985). This report demonstrates the efficacy of immunoblotting procedures to identify analogous thylakoid components between species.

Recent work in our laboratory has focused on two novel chlorophyll-binding complexes in cyanobacteria. PSII particles from both *Anacystis nidulans* R2 and *Aphanocapsa* 6714 are enriched in a 36-kDa chlorophyll-protein (Pakrasi *et al.*, 1985; Bullerjahn *et al.*, 1985). We demonstrate here that antisera raised against the *A. nidulans* 36-kDa polypeptide cross-reacts with the *Aphanocapsa* 36-kDa protein, proving that these components are analogous. Furthermore, we show that a 64-kDa PSII antenna protein that binds chlorophyll *a* is immunologically related to the protein which binds chlorophyll *a*/*b* in the light-harvesting complex (LHC-II) of higher plants. LHC-II serves primarily as an antenna which funnels excitation energy toward the PSII reaction center. The complex consists of an oligomer of subunits, each polypeptide having a molecular mass of approximately 25 kDa. LHC-II from various vascular plants and algae share antigenic determinants, indicating that the LHC subunits are highly conserved across large evolutionary distances (Thaler and Jay, 1985). This paper is the first to describe a cyanobacterial chlorophyll-protein which is likely functionally and structurally analogous to LHC-II.

Materials and Methods

Strain and Medium

Aphanocapsa 6714 *str*-5, a spontaneous mutant resistant to 25 μg/ml streptomycin, was employed throughout this study. Strain 6714 *str*-5 was grown in the medium described by Herdman *et al.* (1973) in 15-liter carboys under constant aeration and illumination (0.5 mW/cm^2).

Standard Methods

Extracts for chlorophyll protein (CP) polyacrylamide gels were prepared by first breaking *Aphanocapsa* cells [suspended in 50 mM morphilinoethanesulfonic acid (MES) at 1 mg/ml Chl] in a French pressure cell. The pressed material was centrifuged at 3,000 × *g* for 5 min to remove unbroken cells and envelope membranes. The supernatant fluid was centrifuged in a Beckman 60Ti rotor at 115,000 × *g* for 1 h to collect thylakoid membranes. The membrane pellet was resuspended to a Chl concentration of 350 μg/ml in 50 mM MES, pH 6.5. The protease inhibitors benzamidine, ε-amino

caproic acid, and phenyl methyl sulfonyl fluoride were included in all buffers at 1 mM final concentrations. Dodecyl-β-D-maltoside (Calbiochem) was added to the membranes to a final detergent : Chl ratio of 5 : 1. After centrifugation at 115,000 × *g* for 1 h, aliquots of the supernatant (termed the "maltoside extract") containing 10 μg Chl were subjected to electrophoresis according to Delepelaire and Chua (1979), except that dithiothreitol and LDS were omitted from the samples.

The polypeptides of chlorophyll-protein bands were resolved on denaturing LDS polyacrylamide gels (Guikema and Sherman, 1982) following solubilization of green bands excised from CP gels. Tetramethylbenzidine staining of cytochromes was performed as described previously (Guikema and Sherman, 1981). Protein blots of polyacrylamide gels were performed according to Towbin *et al.* (1979); antibody–antigen complexes were visualized by immunodecoration with peroxidase-conjugated goat anti-rabbit IgG followed by *o*-dianisidine treatment. Alternatively, primary antibody was stained with ^{35}S-labeled donkey anti-rabbit IgG (Amersham). Antibody against the maize light-harvesting chlorophyll-protein complex, LHC-II, was a gift from Dr. W. Taylor. Antibodies against *Chlamydomonas* components 5 and 6 were gifts of Dr. N.-H. Chua, and antibody to the 36-kDa PSII Chl-binding protein from *Anacystis nidulans* R2 was provided by H. Riethman. PSII activity measured as dichlorophenolindophenol photoreduction was performed under saturating light conditions essentially as described by Newman and Sherman (1978).

Results

Chlorophyll-Proteins of Aphanocapsa

Maltoside extract samples electrophoresed under nondenaturing conditions resolved three sets of bands, termed CPI to CPIII. Analyses of polypeptides and fluorescence emission at 77°K revealed that regions I and II are PSI-associated chlorophyll-protein complexes, and that the III region is comprised of CP bands associated with PSII and its antennae (Bullerjahn *et al.*, 1985). CPIII contains three components, III′, IIIa, and IIIb, which are fluorescent upon transillumination with long-wavelength (360 nm) ultraviolet light, whereas CPI and II are not UV-fluorescent (Fig. 1A). We demonstrated previously that CPIII′ contains a 64-kDa and a 54-kDa polypeptide and emits fluorescence at 684 nm, that Band IIIa contains several polypeptides in the 40–45 kDa range and emits fluorescence at 686 nm, and that CPIIIb fluoresces at 684 nm and is comprised solely of a 36-kDa polypeptide (Bullerjahn *et al.*, 1985). A PSII component in another

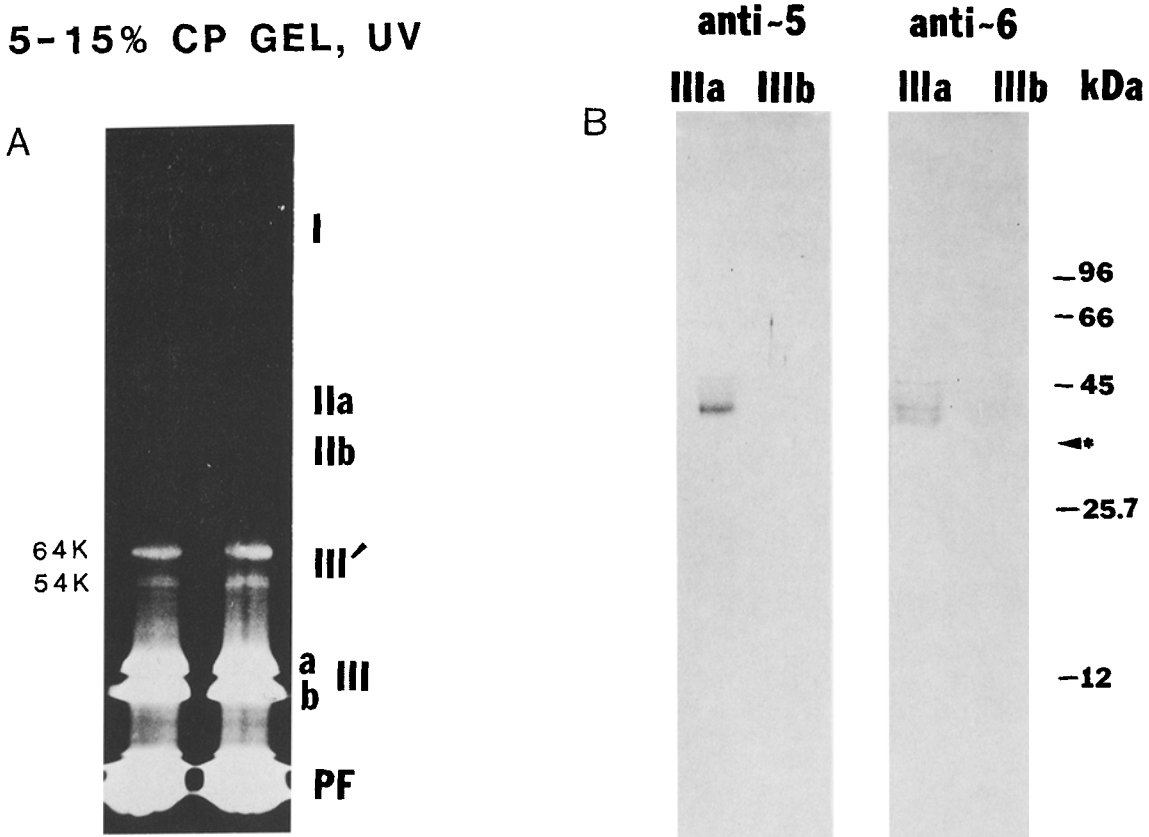

Fig. 1. Immunological analysis of CPIIIa. (A) PSII chlorophyll-proteins of *Aphanocapsa* resolved on a 5–15% polyacrylamide nondenaturing gel. Visualization of bands was achieved by transillumination of the gel with long-wavelength (360 nm) ultraviolet light. CPI and CPII: Nonfluorescing PSI complexes; PF, pigment front. (B) Complexes IIIa and IIIb were excised from nondenaturing gels and run onto 10–20% polyacrylamide gels under denaturing conditions. After electroblotting to nitrocellulose, the blots were probed with antibodies raised against the PSII chlorophyll-binding components 5 and 6 from *Chlamydomonas*. The electrophoretic mobility of the 36-kDa IIIb apoprotein is marked by the asterisk.

cyanobacterium, *A. nidulans* R2, also contains a CP complex with a 36-kDa apoprotein (Pakrasi *et al.*, 1985). III′ often consists of two green bands on CP gels; the upper band is enriched in the 64-kDa polypeptide, whereas the lower band is enriched in the 54-kDa protein. CPIII′ is most easily visualized during UV transillumination, because CPIII′ binds so little Chl that it is difficult to see as a green band. CPIII′ represents less than 10% of the Chl associated with PSII (Fig. 1A). The three CPIII complexes are present in an active PSII core fraction, although the III′ components are not present in stoichiometric amounts as judged by Coomassie staining. On the other hand, active PSII preparations always contained the IIIa or IIIb components.

A PSII core preparation containing the III′, IIIa, and IIIb components mediated electron transport from diphenylcarbizide (DPC) to dichlorophenolindophenol (DPIP) at rates of 143 μmol DPIP reduced/mg Chl h. After removal of the III′ components by filtration over Bio-Gel A0.5m, DPC to DPIP electron transport rates were not affected (150 μmol DPIP reduced/mg Chl h).

Immunoblotting Studies on CP Bands IIIa and IIIb

CP bands IIIa and IIIb were excised from nondenaturing gels and the green bands were placed in the wells of a denaturing LDS polyacrylamide gel. After electrophoresis, the proteins were electroblotted onto nitrocellulose. When these blots were probed with antibodies raised against the PSII chlorophyll-binding polypeptides 5 and 6 from *Chlamydomonas* (Delepelaire and Chua, 1979), only the proteins of CPIIIa cross-reacted. Each antibody identified several discrete bands in CPIIIa (Fig 1B); these patterns resembled the patterns of antibody staining seen when protein blots of whole thylakoid membranes were probed with anti-5 and anti-6 (Bullerjahn *et al.*, 1985). A polypeptide which migrates at 48 kDa after 0°C solubilization, but which migrates at 40 kDa after 70°C solubilization, is recognized by anti-5. Anti-6 cross-reacts to several bands of 40–45 kDa in thylakoids solubilized at 0 and 70°C (Bullerjahn *et al.*, 1985). We suggest that the bands showing cross-reactivity are different forms of the same polypeptide. Furthermore, the apoprotein of CPIIIb, the 36-kDa protein, was not recognized by these polyclonal antibodies (Fig. 1B). Blots of III′ material yielded no cross-reacting material to either anti-5 or anti-6 (data not shown). Conversely, an antibody prepared against the 36-kDa Chl-binding polypeptide from *A. nidulans* did not cross-react to proteins from CPIIIa or CPIII′, indicating that this component is immunologically distinct and probably not a different conformer of the IIIa or III′ proteins (Fig. 2).

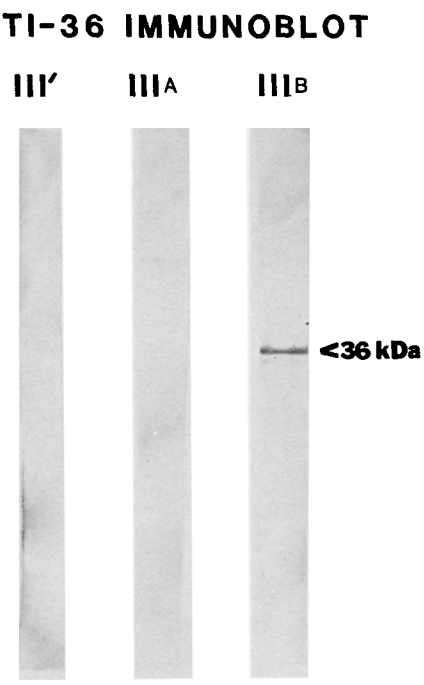

Fig. 2. Immunological identity of CPIIIb. CPIII′, IIIa, and IIIb were electrophoresed in denaturing gels, blotted to nitrocellulose, and probed with antibody to the 36-kDa cyanobacterial PSII Chl-binding component. The faint band below the band at 36 kDa corresponds to cytochrome *f*. Tetramethylbenzidine staining of the gel after blotting indicated the presence of cytochrome *f* which migrated at 34 kDa. The anti-36-kDa IgG was prepared against material containing a small amount of cytochrome *f*. The antibody recognized the cytochrome *f* contaminating the IIIb preparation. Coomassie staining of the post-blot gel revealed only two polypeptides: the major 36-kDa band and a minor band at 34 kDa.

Immunological Analysis of CPIII′

Maltoside extracts of *Aphanocapsa* thylakoids were run on a 5–15% nondenaturing polyacrylamide gel and electroblotted onto nitrocellulose. The abundant pigment-protein complexes CPII, CPIIIa, and CPIIb, all transferred as clearly visible green bands (Fig. 3A). The phycobiliproteins and the pigment front (PBS and PF) were also visible; CPIII′, which contained the least chlorophyll of the complexes, was a very faint band after transfer. Probing this blot with antibody to maize LHC-II revealed that the antibody recognized only the two green bands associated with the III′ complex (Fig. 3A). All other material visible in Fig. 3A are pigmented complexes (chlorophyll and phycobiliproteins) which transferred to nitrocellulose and

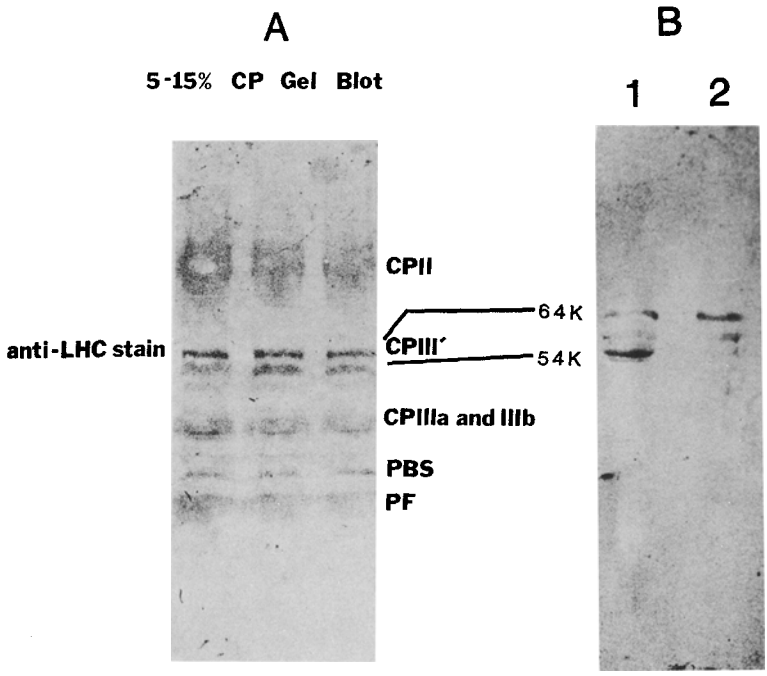

Fig. 3. Immunological analysis of CPIII′. (A) Maltoside extracts of *Aphanocapsa* thylakoids were run on a nondenaturing gel and electroblotted onto nitrocellulose. CPIII′, containing the least chlorophyll of the complexes, was barely visible after transfer. Probing this blot with antibody to maize LHC-II revealed that only the bands associated with the CPIII′ complex cross-reacted. (B) III′ bands were excised from nondenaturing gels and rerun on denaturing polyacrylamide gels. After blotting to nitrocellulose, the blot was probed with antibody against maize LHC-II. Detection of antibody–antigen complexes was achieved after immunodecoration with ^{35}S-labeled donkey anti-rabbit IgG. Lane 1: detection of the 64- and 54-kDa polypeptides in the lower CPIII′ band. Lane 2: detection of the 64-kDa protein in the upper CPIII′ band (see Fig. 1A).

did not stain with the secondary antibody. In addition, CPIII′ bands were excised from CP gels and subjected to electrophoresis. After blotting to nitrocellulose, the LHC-II antibody recognized the 64-kDa III′ apoprotein, as well as the 54-kDa polypeptide that was enriched in the lower III′ band (Fig. 3B). Control blots indicated that the LHC-II antibody was cross-reactive only to the light-harvesting chlorophyll components when maize thylakoids were probed. Furthermore, the antibody did not spuriously cross-react to the isolated 62-kDa PSI apoprotein, the major polypeptide in the 60–70 kDa molecular mass range which is a component of CPI and CPII (data not shown).

Discussion

In this study, we have used immunological techniques to identify the chlorophyll-binding proteins of *Aphanocapsa* 6714 that are associated with PSII. Band IIIa contains the analogous polypeptides to proteins 5 and 6 of *Chlamydomonas*; component 5 and its analogs in other species are believed to be the site of pheophytin binding, since the CP complex containing this polypeptide yields low-temperature fluorescence emission at 695 nm (Pakrasi *et al.*, 1985; Nakatani *et al.*, 1984). Analogs to component 6 likely comprise an antenna immediate to the PSII reaction center (Yamagishi and Katoh, 1985; Camm and Green, 1983). IIIa, therefore, consists of the major chlorophyll-proteins associated with the PSII core. Complexes similar to IIIa have been described previously in cyanobacteria (Guikema and Sherman, 1983) and in higher plants (Camm and Green, 1983). This paper documents the immunological identity of two novel Chl-binding species in cyanobacteria, CPIII′ and CPIIIb. Since neither anti-5 nor anti-6 cross-reacted to the IIIb apoprotein, we believe that this polypeptide is not a breakdown product of the IIIa components. Furthermore, we have shown that the 36-kDa IIIb apoprotein is analogous to the 36-kDa chlorophyll-protein from *A. nidulans*, so this polypeptide may represent a component common to cyanobacterial PSII. We are currently analyzing PSII particles to determine whether the IIIb protein plays a role in PSII photochemistry.

CPIII′ is another novel pigment-protein complex. The fluorescence emission spectrum of III′ at low temperature (λ_{max} = 684 nm) suggests that III′ is associated with PSII. Since PSII photochemistry does not depend on the presence of III′, this complex is not a PSII reaction center component. This fact points to an accessory role for III′; we suggest that this complex serves to form a small Chl antenna around PSII. The III′ complex contains a 64-kDa protein which cross-reacts to antibody directed against the PSII light-harvesting Chl *a*/*b* complex of maize. Therefore, LHC-II shares antigenic sites with a cyanobacterial Chl-binding complex which is likely functionally analogous. Since there are two III′ bands cross-reacting to the LHC antibody, we propose that the bands contain different forms of the same polypeptide. This raises the possibility that the genes encoding the III′ apoprotein and the LHC-II proteins arose from a common ancestral DNA sequence. Since the evidence is very strong that chloroplasts arose from a free-living, photosynthetic prokaryotic ancestor (Gray and Doolittle, 1982), it is attractive to postulate that the cyanobacterial antenna protein is an evolutionarily divergent form of LHC-II. Nevertheless, the primary structure of the 64-kDa polypeptide must be determined and compared to the LHC amino acid sequences to prove that the proteins are related.

The 64-kDa III′ polypeptide is probably the same as that described by Yamagishi and Katoh (1983) in PSII chlorophyll-protein complexes from *Synechococcus* sp. They reported a 66-kDa protein associated with the 47-kDa PSII core polypeptide and a 31-kDa protein. The authors stated that the 66-kDa protein was possibly an artifact due to dimerization of the 31-kDa protein. Our results indicate that the 64-kDa PSII-associated protein binds chlorophyll and is not a multimer of another polypeptide, since the polyclonal antibody did not identify other low-molecular-mass proteins. In addition, the antibody recognizing the 36-kDa IIIb component did not cross-react with the III′ polypeptides. The III′ polypeptide is probably a PSII component common to many cyanobacteria because the anti-LHC-II cross-reacted to a 65-kDa species in *A. nidulans* R2 (Riethman and Sherman, unpublished data).

Acknowledgments

We thank Jill Cunningham and Diane Nitta for excellent technical help, and N.-H. Chua, W. Taylor, and H. Riethman for supplying antibodies. This work was supported by grants from the National Institutes of Health (GM21827), the Department of Energy (DOE DEAC0281ER10899), and, in part, by the University of Missouri Institutional Biomedical Research Grant RR07053 from NIH. G. S. B. was an NIH Postdoctoral Fellow (DHHS-1-F32-GM10585-01).

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