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Genetically engineered mutant of the cyanobacterium *Synechocystis* 6803 lacks the photosystem II chlorophyll-binding protein CP-47

(photosynthesis/cartridge mutagenesis/reaction center/chlorophyll fluorescence)

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ABSTRACT **CP-47 is absent in a genetically engineered mutant of cyanobacterium *Synechocystis* 6803, in which the *psbB* gene [encoding the chlorophyll-binding photosystem II (PSII) protein CP-47] was interrupted. Another chlorophyll-binding PSII protein, CP-43, is present in the mutant, and functionally inactive PSII-enriched particles can be isolated from mutant thylakoids. We interpret these data as indicating that the PSII core complex of the mutant still assembles in the absence of CP-47. The mutant lacks a 77 K fluorescence emission maximum at 695 nm, suggesting that the PSII reaction center is not functional. The absence of primary photochemistry was indicated by EPR and optical measurements: no chlorophyll triplet originating from charge recombination between $P680^+$ and $Pheo^-$ was observed in the mutant, and there were no flash-induced absorption changes at 820 nm attributable to chlorophyll P680 oxidation. These observations lead us to conclude that CP-47 plays an essential role in the activity of the PSII reaction center.**

Photosystem II (PSII) is a pigment–protein complex in the thylakoid membrane consisting of at least five integral membrane proteins [CP-47, CP-43, the 32- to 34-kDa proteins D1 (the "herbicide-binding protein") and D2, and cytochrome *b*-559] and several extrinsic proteins (1, 2). Cofactors, serving in photosynthetic electron transport from water to plastoquinone, are associated with these proteins. However, in many cases it has not been established as to which protein or protein domain a certain cofactor is bound. In particular, it is not yet clear which protein(s) binds the reaction center chlorophyll P680.

The following evidence suggests that the chlorophyll-binding protein CP-47 harbors P680. (*i*) Nakatani *et al.* (3) showed chlorophyll bleaching upon illumination of CP-47, which was attributed to photooxidation of P680. However, this bleaching was largely irreversible and might have been caused by bleaching of chlorophylls other than P680. (*ii*) Yamagishi and Katoh (4, 5) have isolated a PSII particle enriched in CP-47 that is depleted in D1 and in D2 and missing CP-43, the other chlorophyll-binding protein of PSII. This particle shows light-induced reduction of Q_A, the primary electron-accepting quinone in PSII; from this, these authors concluded that CP-47 harbors the PSII reaction center.

It has also been suggested that D1 and D2 might harbor P680 (6–8), because of the regional amino acid homology between the reaction center proteins L and M from purple photosynthetic bacteria and the D1 and D2 proteins. The amino acid sequence in the region of the L and M proteins to which the reaction center bacteriochlorophylls are bound can also be found in an analogous location in the D1 and D2 proteins.

To obtain more information on the role of CP-47 (and thereby possibly to localize P680), a well-defined mutant of the cyanobacterium *Synechocystis* 6803 was constructed. In this mutant (9) the only change was an interruption of the *psbB* gene (encoding CP-47) by a piece of DNA containing the gene for aminoglycoside-3′-phosphotransferase, conferring kanamycin resistance. This mutant is no longer capable of PSII electron transport (9). In this paper, the PSII properties of this mutant are described.

MATERIALS AND METHODS

The characteristics and growth conditions of *Synechocystis* 6803 have been reported (9, 10). The *psbB* mutant was propagated as the wild type, but 5 mM glucose, kanamycin at 20 μg/ml, and 20 μM atrazine were added to all growth media.

Isolation and Solubilization of Thylakoid Membranes. Cells were harvested and resuspended at high concentration into a buffer containing 25 mM Mes·NaOH, pH 6.0/50 mM $CaCl_2$/0.2 M sorbitol/1 mM phenylmethylsulfonyl fluoride. All subsequent manipulations were carried out at 0–6°C. The cells were passed three times through a chilled French pressure cell (Aminco) at 20,000 psi (138 MPa). More than 90% of the cells were broken after this treatment. The thylakoids and the remainder of intact cells were pelleted by centrifugation (4000 × *g*, 5 min) and washed several times in the resuspension buffer to remove phycobilisomes. To prepare PSII-enriched thylakoid fragments, the membranes were resuspended in the same buffer to 1 mg of chlorophyll per ml, and dodecyl-β-D-maltoside (Calbiochem) was added to a final detergent/chlorophyll ratio of 2.5:1 (wt/wt). The suspension was incubated for 30 min at 0°C and subsequently centrifuged at 40,000 × *g* for 20 min. The supernatant was loaded on a 10–30% sucrose gradient in 25 mM Mes·NaOH, pH 6.0/1 mM phenylmethylsulfonyl fluoride and centrifuged at 40,000 rpm for 3 hr using a Beckman Ti45 rotor. The upper part of the major green band located ≈1/4 of a tube length from the top of the gradient was collected, and then concentrated and washed in a resuspension buffer using an Amicon filter. This fraction was enriched in PSII and virtually lacked photosystem I.

Protein Analysis. The procedure of $NaDodSO_4$/polyacrylamide gel electrophoresis has been described (11). Samples were heated at 70°C for 5 min before loading the gel. Protein blotting onto nitrocellulose and incubation with antibody was as described (12). ^{125}I-labeled protein A (Amersham) was used for immunodecoration.

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Abbreviation: PSII, photosystem II.

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Functional Analysis. Fluorescence induction measurements were carried out (13); however, a 695-nm interference filter (10-nm bandwidth; Corion, Holliston, MA) was put in front of the photodiode. Fluorescence emission spectra (77 K) were measured (14) using a 435-nm excitation wavelength. The low-temperature EPR equipment was described (15). Flash-induced absorption changes at 820 nm were measured (16).

RESULTS

Protein Composition of the Mutant. In the construction of the mutant, the "kanamycin resistance cartridge" was inserted almost at the end of the *psbB* gene, 178 base pairs from the 3′ end and 1342 base pairs from the 5′ end (9). The first question to be asked was whether in the mutant a modified *psbB* gene product (consisting of most of the CP-47 protein and amino acids encoded by the kanamycin resistance insert) was present in the thylakoid membrane. Since the large complexity of membrane proteins in cyanobacteria does not allow an unequivocal answer from analysis of a stained protein gel, a protein gel from thylakoids and PSII-enriched particles from wild-type and mutant cyanobacteria was blotted onto nitrocellulose; the blot was subsequently probed with polyclonal antibodies raised against *Chlamydomonas* CP-47 (the antibodies were a kind gift from N.-H. Chua). The results (Fig. 1) show that in the mutant no CP-47-like protein is incorporated in the membrane. A longer exposure of the probed blot also does not show any bands that are specifically labeled in the mutant lanes (data not shown). Therefore, we conclude that no gene product of modified *psbB* is incorporated into the membrane. It remains to be established whether the protein is synthesized but not incorporated into the membrane or whether the mRNA transcribed from the mutant *psbB* gene is unstable. However, preliminary evidence indicates that the soluble protein fraction from the mutant does not contain a quantity of CP-47 protein that can be detected with the CP-47 antibody, suggesting a rapid breakdown of either the *psbB* mRNA or the modified CP-47 protein in the mutant.

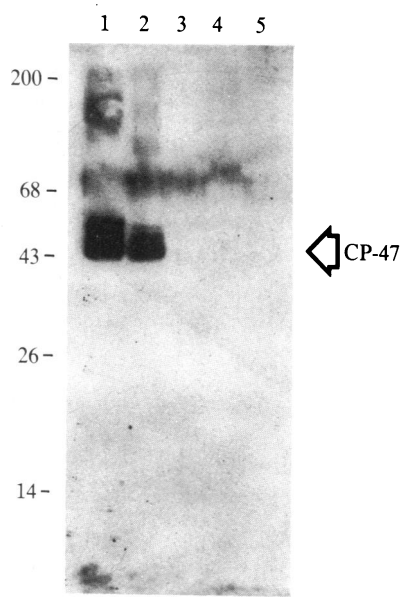

FIG. 1. Immunoblot probed with antibodies against *Chlamydomonas* CP-47. Lanes 1 and 2, proteins from wild-type thylakoids and PSII-enriched particles. Lanes 4 and 5, proteins from CP-47 mutant thylakoids and PSII particles. Lane 3, protein standards; the corresponding sizes in kDa are indicated to the left. Each lane with thylakoid proteins (lanes 1 and 4) had 5 μg of chlorophyll. Each lane with PSII-enriched particles (lanes 2 and 5) had 0.5 μg of chlorophyll.

A complete loss of a "CP-47-like" protein in thylakoid membranes from the mutant might indicate that PSII does not assemble in the absence of intact CP-47 protein. Thus another chlorophyll-binding protein from PSII, CP-43, was assayed in thylakoids and PSII-enriched particles from wild type and mutant (Fig. 2). CP-43 was found in mutant thylakoid membranes, implying that at least part of the PSII core complex is still present in the membrane when CP-47 is absent. Therefore, it is implausible that loss of CP-47 leads to loss of incorporation of PSII components into the thylakoid membrane. This indicates that the effects of the *psbB* gene interruption on PSII activity are likely to be related directly to CP-47. These effects may be caused either by disappearance of the cofactors originally bound to CP-47 or by structural changes in the remaining PSII core complex due to the loss of CP-47.

Fluorescence Emission at 77 K. Two chlorophyll fluorescence emission maxima at 77 K are known to be related to PSII. One emission peak is near 685 nm and is usually thought to represent antenna chlorophyll. The other maximum is at ≈695 nm (often seen as a shoulder on the 685 nm peak). The fluorescence emission spectrum of PSII-enriched particles from wild type and mutant are shown in Fig. 3. It is clear that the 695-nm peak is absent in the mutant, whereas the 685-nm peak has not shifted its position. These data fit well with the observation that isolated CP-47 fluoresces at 695 nm (17). Based on chromophore orientation studies, it has been suggested that the fluorescence at 695 nm originates from pheophytin *a*, the primary electron acceptor of PSII (18). If this is true, then the loss of CP-47 should result in a loss of primary photochemistry. This implication was probed, and the results are in the next section.

The Site of Electron Transport Inhibition in the Mutant. It has been shown that the mutant lacking CP-47 does not grow photosynthetically and does not evolve oxygen in the presence of an electron acceptor (9). However, in that study the site at which electron transport was inhibited was not pinpointed.

To determine where electron transport was inhibited in the

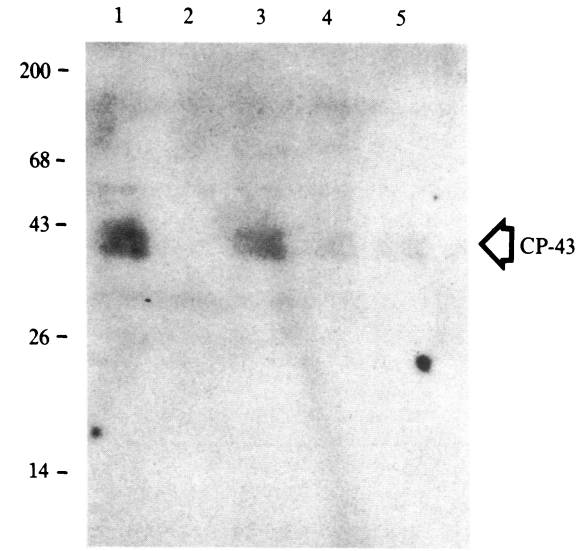

FIG. 2. Immunoblot probed with antibodies against *Chlamydomonas* CP-43. Lane 1, wild-type thylakoids. Lane 2, thylakoids from a mutant in which part of the gene encoding CP-43 has been deleted (J.G.K.W., unpublished results). This mutant served to check that the protein recognized primarily by the antibody indeed is CP-43. Lane 3, thylakoids from the CP-47 mutant. Lane 4, wild-type PSII-enriched particles. Lane 5, PSII-enriched particles from the CP-47 mutant. Lanes 1–3: 5 μg of chlorophyll. Lanes 4 and 5: 0.5 μg of chlorophyll.

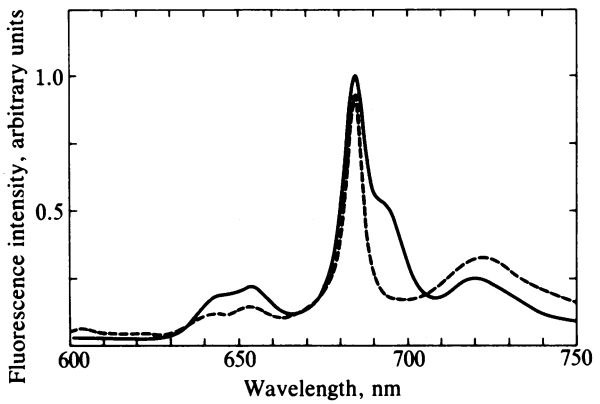

FIG. 3. Fluorescence emission spectrum of PSII-enriched wild-type (——) and CP-47 mutant (——) particles at 77 K in the presence of 50% (vol/vol) glycerol. Excitation wavelength, 435 nm; excitation bandwidth, 8 nm; emission bandwidth, 2 nm.

absence of CP-47, fluorescence induction in PSII-enriched particles from wild type and mutant were compared (Fig. 4) using hydroxylamine as artificial electron donor. Whereas the wild-type particles showed variable fluorescence, none was detected in the mutant, indicating that the site of inhibition was between the hydroxylamine electron-donation site and Q_A.

For a more precise investigation of the inhibition site in the absence of CP-47, the spin-polarized chlorophyll triplet was measured by ESR at 4 K in strong light in the presence of dithionite (19, 20). The results are shown in Fig. 5. Whereas wild-type PSII-enriched particles yielded a well-defined chlorophyll triplet signal (peaks at approximately 300, 308, 352, and 360 mT), with the spin–polarization pattern characteristic of its origin from charge recombination between $P680^+$ and $Pheo^-$, no signal could be detected in mutant PSII-enriched particles. The signals present in the light at ≈345 and 350 mT in both the wild type and the mutant are due to reduced iron–sulfur centers in photosystem I, which is present as a contaminant. The absence of triplets indicates that there is no PSII charge separation in the absence of CP-47.

This was confirmed by absorption change measurements at 820 nm on the μs time scale (Fig. 6). These measurements monitor formation of oxidized chlorophyll ($P680^+$, absorbing at 820 nm) followed by reduction. The wild-type particles displayed normal P680 oxidation and subsequent reduction. However, the mutant did not show any flash-induced P680 oxidation; the small, noninstantaneous rise in 820-nm absorption in the mutant is presumably unrelated to $P680^+$ formation.

Assuming the absorption coefficient of $P680^+$ at 820 nm to be 4200 $M^{-1}\cdot cm^{-1}$ (21), the amplitude of the ΔA_{820} signal in

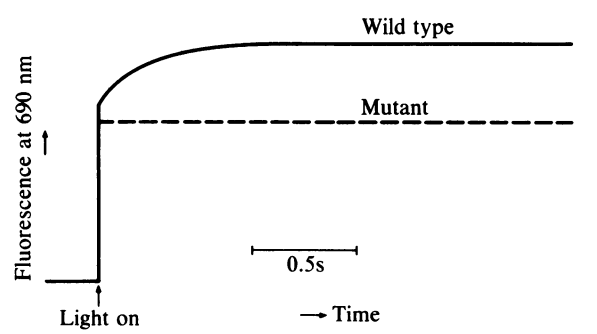

FIG. 4. Room temperature fluorescence induction curve of PSII-enriched wild-type (——) and mutant (——) particles, measured at 690 nm (10 nm bandwidth). The reaction mixture contained chlorophyll at 5 μg/ml in 25 mM tricine·NaOH, pH 7.6/0.2 M sorbitol/25 mM $CaCl_2$/5 mM hydroxylamine.

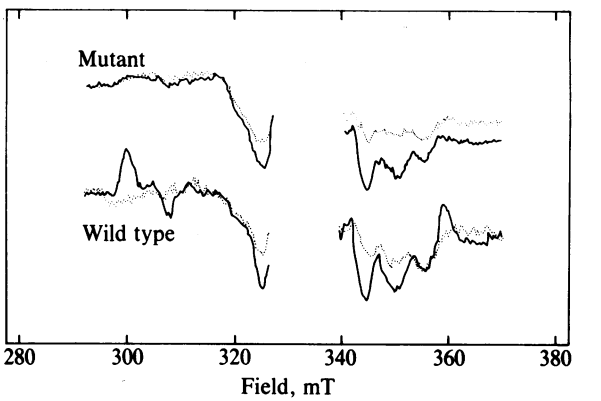

FIG. 5. EPR spectra of the light-induced chlorophyll triplet signal in wild-type (*Lower*) and mutant (*Upper*) PSII-enriched particles in the presence of dithionite. Dotted line, in darkness; solid line, during strong illumination. Instrument settings: temperature, 3.6 K; microwave power, 63 μW (35 dB attenuation from 200 mW); frequency, 9.44 GHz; modulation amplitude, 32 G; gain, 1×10^6.

the wild type corresponds to one P680 turning over on the μs time scale per ≈350 chlorophyll molecules. This is comparable to what was observed in oxygen-evolving PSII particles from *Synechococcus* sp. (21). The relatively low P680/chlorophyll molar ratio may indicate either a fast phase in $P680^+$ reduction (21) that is below the response time (μs) of the instrument used or an inactivation of primary charge separation in part of the PSII reaction centers due to the preparation procedure used.

The results presented above could be explained by the assumption that CP-47 regulates primary charge separation. However, the absence of CP-47 also affects herbicide binding, assumed to occur at the D1 protein at or near the binding site of the secondary quinone Q_B. The CP-47 mutant appears to lack significant specific binding of the PSII herbicides atrazine, diuron, and ioxynil (data not shown). This suggests that CP-47 may have an effect on a wide range of PSII

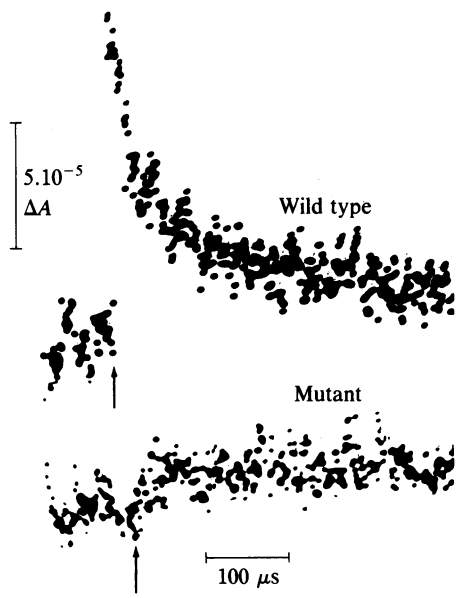

FIG. 6. Flash-induced absorption changes measured at 820 nm in PSII-enriched particles from wild type (*Upper*) and CP-47 mutant (*Lower*) in 25 mM Mes·NaOH, pH 6.0/50 mM $CaCl_2$/0.2 M sorbitol. The chlorophyll concentration was 10 $\mu g\cdot ml^{-1}$. The laser flash was given at the moment indicated by the arrows. Each trace is the average of eight measurements.

functions, or at least that its absence disturbs the organization of the PSII complex.

DISCUSSION

The interruption of the *psbB* gene by the kanamycin resistance cartridge leads to a complete loss of the CP-47 protein from the membrane, even though the 5′ 87% of the gene is unmodified. Even without CP-47, at least part of the PSII complex appears to be present in the membrane. CP-43 is detectable in PSII-enriched preparations (Fig. 2), and the chlorophyll fluorescence peak is still around 685 nm (Fig. 3), indicating that the pigment–protein interaction in CP-43 is normal. In this respect, the effects of the absence of CP-47 appear to be less severe than those of the absence of D1 or D2. In *Chlamydomonas* mutants, in which one of these 32- to 34-kDa proteins is absent, turnover of the remaining PSII core proteins is so rapid that they never accumulate in the membrane (22).

At this point the presence of the 32- to 34-kDa D1 and D2 proteins in the mutant has not been established; a D2 antibody was not available to us, and the cross-reactivity of an antibody raised against part of D1 (23) with *Synechocystis* D1 is not sufficiently specific to be utilized. Moreover, the D1 protein should not be detectable by [^{35}S]methionine-pulse labeling in the light in the mutant, since D1 turnover requires PSII electron transport (24). Although the lack of herbicide binding could suggest the absence of the D1 protein, it is known that herbicide binding can be modified without direct modification of the D1 protein (25). Therefore, absence of specific herbicide binding in the CP-47 mutant does not necessarily imply the absence of the D1 protein. It should be kept in mind that the fact that it is possible to isolate PSII-enriched particles from the mutant in a similar way as from wild type suggests that, except for the lack of CP-47, a relatively normal PSII complex is present in the mutant.

The Effects of the Lack of CP-47. The absence of CP-47 has severe effects on PSII function: the observations that both the light-induced chlorophyll triplets and the absorption changes attributable to P680 turnover are not found in the CP-47 mutant indicate that primary photochemistry in PSII has been inhibited completely in the mutant. Moreover, the absence of 695-nm fluorescence at 77 K in the mutant suggests that pheophytin *a* is not functionally present in the reaction center—if one accepts Breton's hypothesis (18) that pheophytin *a* is the source of 695-nm fluorescence.

These characteristics would be expected in the mutant if the hypothesis that CP-47 harbors P680 were correct. However, it is equally plausible that the lack of CP-47 prevented other proteins (such as D1 and D2) from attaining the proper conformation necessary for binding of the reaction center chlorophyll. In this context it should be noted that interruption of *psbC* (encoding CP-43) also leads to a loss of PSII activity (W.F.J.V., J.G.K.W., and C.J.A., unpublished observations). The site of electron transport inhibition has not been characterized yet in this case. This indicates that even modification or deletion of a protein that is considered to be not involved in PSII electron transport (4, 5) can lead to a loss of PSII activity.

It is obvious that from the data obtained with this mutant, a final conclusion on the localization of P680 in PSII is as yet unwarranted. Site-directed mutagenesis in certain domains of, for example, the D1, D2, and CP-47 proteins will be necessary to solve the problem of the localization of the reaction center in PSII.

We thank Dr. N.-H. Chua for sending us the antibodies against *Chlamydomonas* CP-47 and CP-43.

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