Isolation and Spectroscopic Characterization of a Plantlike Photosystem II Reaction Center from the Cyanobacterium *Synechocystis* sp. 6803†

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ABSTRACT: A chlorophyll−protein complex has been isolated from the cyanobacterium *Synechocystis* sp. PCC 6803 that closely resembles higher plant photosystem II reaction centers in spectral properties. The *Synechocystis* complex has a pigment content of 5−7 chlorophyll *a* molecules:1 Cyt *b*559:2 pheophytins; an optical absorption redmost transition at ~675 nm; and a nonconservative circular dichroism red signal, with extrema at 682 (+) and 652 (−) nm. Upon illumination, the *Synechocystis* D1/D2/Cyt *b*559 complex accumulates reduced pheophytin. LDS−PAGE and/or immunoblotting showed the D1, D2, and Cyt *b*559 proteins, aggregated and degraded forms of D1 and possibly D2, and traces of ATP synthase and the CP47 photosystem II chlorophyll protein. The availability of such a *Synechocystis* preparation opens the way for employing site-directed mutagenesis in studying primary reactions of oxygenic photosynthesis.

The mechanism of photosynthetic electron transfer was elucidated following mutational analyses and structural resolution of the reaction center (RC) from nonoxygenic, photosynthetic bacteria (Parson & Warshel, 1993; DiMagno & Norris, 1993). Photosystem II (PSII) reaction centers in plants, algae, and cyanobacteria convert solar energy to electric potential in the much more prevalent process of oxygenic photosynthesis (Seibert, 1993). Application of these same techniques to study the PSII RC should have a similar impact on our understanding of oxygenic electron transfer. Unfortunately, site-directed mutagenesis of PSII reaction centers in plants is limited by the difficulty of chloroplast transformation. On the other hand, isolation of PSII RC in transformable oxygenic phototrophs such as *Synechocystis* (Williams, 1988; Vermaas et al., 1990) has been hampered by limited success in separating photosystem I (PSI) from PSII (Smart et al., 1994), although procedures for preparation of PSII particles have been suggested (Noren et al., 1991; Tang & Diner, 1994). This basic difficulty is probably responsible for there being only a single reported attempt to isolate PSII RC from cyanobacteria (Gounaris et al., 1989). In that report, despite marked similarities in activity and protein constitution between the cyanobacterial and plant PSII core complexes, the cyanobacterial PSII RC showed significantly different spec oscopic properties from the higher plant preparations (Gounaris et al., 1989). Furthermore, there is a controversy regarding the number of Cyt *b*559 complexes per PSII RC in the cyanobacteria. While some laboratories observed a ratio of approximately 2 Cyt *b*559 per RC (MacDonald et al., 1994) or 1 Cyt *b*559 per pheophytin (Phe) *a* molecule (Gounaris et al., 1989), others reported a ratio of 1 Cyt *b*559 per 2 Phe *a* molecules (Tang & Diner, 1994).

We set out to develop a new procedure for preparing cyanobacterial PSII RC. The following paper describes the purification of PSII RC particles from *Synechocystis* sp. PCC 6803 that closely resemble those of higher plants in composition and spectral properties. As such, they may be suitable for mutational analyses of PSII RC functions.

EXPERIMENTAL PROCEDURES

Separation of PSII from PSI Core Particles. Cells were grown at 30 °C and 50 μE m^{-2} s^{-1} in 12 L fermentors containing BG 11 medium (Williams, 1988) with bubbling air (at a slow flow rate). All subsequent procedures, except where indicated, were carried out at 4 °C and in dark. Cells were harvested at their late logarithmic phase. A 25 g pellet (wet weight) was washed once in solution A [50 mM Hepes (pH 6.5), 10 mM $MgCl_2$, 30 mM $CaCl_2$, 1 M sucrose, and 25% glycerol (v/v)], resuspended in 100 mL of solution A, and incubated in the dark on ice for 1−2 h. After incubation, the cells were transferred to a bead-beater homogenizing chamber (Bio-Spec Products, Bartlesville, OK) containing 350 g of 0.1 mm glass beads and broken in the dark at 4 °C by 6 cycles of 1 min homogenization followed by 10 min cooling. The cell suspension was separated from the glass beads by filtration. Cellular material was centrifuged at 6000*g* at 4 °C for 15 min, and the pellet was resuspended and centrifuged as above. The combined supernatants were centrifuged at 100000*g* at 4 °C for 45 min. The pellet was washed twice with 100−250 mL of solution B [20 mM Hepes (pH 6.35), 5 mM $MgCl_2$, 20 mM $CaCl_2$, 1 M sucrose, and 25% glycerol (v/v)] to partially remove phycobiliproteins.

To remove ATP synthase from the thylakoid preparation, the membrane fraction was treated with EDTA at 4 °C as follows: Membranes were washed with 60−80 mL of 10 mM NaCl (pH 6.5), centrifuged at 100000*g* for 45 min, resuspended in 10 mM NaCl, homogenized, and brought up

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to a chlorophyll (Chl) concentration of 0.1 mg/mL. EDTA (diluted from a stock solution of 0.25 M, pH 7.5) was added to a final concentration of 1 mM and the homogenate mixed gently while kept on ice and in the dark for 20 min. During this process, the phycobiliproteins were completely washed away. ATPase-free thylakoid membranes were centrifuged at 100000*g* for 45 min and resuspended in solution B to a Chl concentration of about 500 μg/mL. The thylakoid membrane was solubilized with dodecyl maltoside (DM) and octyl glucoside (OG) to a final detergent to Chl ratio of 5 DM:9 OG:1 Chl (w/w). DM was first added to the thylakoid preparation (from a 9% w/v stock solution), and the samples were gently mixed for 2 min in the dark at 4 °C on a magnetic stirrer. OG was then added from a 9% (w/v) stock solution while mixing for 3 min and the resulting mixture centrifuged at 160000*g* for 60 min at 4 °C.

The PSII-enriched fraction (supernatant) was carefully removed, precipitated by addition of PEG 6000 to a final concentration of 10% (from a 20% stock of PEG in solution B), and centrifuged at 170000*g* for 40 min. The pellet was suspended in solution C [20 mM Hepes (pH 6.5), 10 mM $MgCl_2$, 10 mM $CaCl_2$, and 0.5 M mannitol] with the addition of 5 mM $MgSO_4$ and 0.03% DM, to a Chl concentration of about 200 μg/mL. This suspension was loaded onto a 18 cm Q-Sepharose (Pharmacia, Sweden) column that was activated with 200 mL of solution C + 500 mM $MgSO_4$, washed with about 300 mL of solution C + 5 mM $MgSO_4$, and then equilibrated with 120 mL of solution C + 5 mM $MgSO_4$ + 0.03% DM. A temperature of 4 °C and a flow rate of 1−1.2 mL/min were maintained throughout. After sample loading, the Q-Sepharose column was washed with 20−30 mL of equilibration buffer. Washing was continued with solution C + 50 mM $MgSO_4$ + 0.03% DM. This washing initially removed some carotenoids and free Chls followed by elution of PSI core particles. When the absorption maximum of the eluting material dropped down to 674−675 nm, PSII core particles were eluted with solution C + 100 mM $MgSO_4$ + 0.03% DM. Purified PSII core particle fractions, as judged from their PAGE and 77 K fluorescence, were pooled and concentrated using 10 K centricons (Amicon). The sample was diluted 10 times with 50 mM Tris buffer (pH 7.2) and concentrated a second time.

Isolation of PSII RC. The concentrated PSII core particles were resuspended in 50 mM Tris buffer (pH 7.2) to a Chl concentration of 1 mg/mL. Triton X-100 (TX-100, Sigma) was added to the sample from a 30% stock (w/v) to a final concentration of 4% while stirring in the dark at 4 °C. After 1 h, the sample was centrifuged at 100000*g* for 60 min. The supernatant was loaded onto a preactivated (with 50 mM Tris buffer, pH 7.2, 400 mM NaCl, and 0.2% TX-100) 6 mL Fractogel TSK DEAE (Merck) column and equilibrated with 25 mL of Tris buffer (50 mM, pH 7.2, + 0.2% TX-100). After being loaded, the column was washed with 30 mM NaCl in 50 mM Tris buffer (pH 7.2) containing 0.2% TX-100 until the absorption at 430 and 411 nm indicated a constant Phe to Chl ratio. At that point, the column was washed with 20−25 mL of 50 mM Tris buffer, pH 7.2, containing 0.02% TX-100, then with 30 mL of 50 mM Tris buffer, pH 7.2, containing 0.03% DM, and, finally, with increasing salt concentrations (50−400 mM NaCl in 50 mM Tris buffer, pH 7.2, with 0.03% DM) to elute the various fractions of RC). The yield of the PSII RC preparation, in terms of Chl *a* content, is ~12% relative to the PSII core particles loaded onto the Fractogel column. This value is arrived [following Braun et al. (1990)] after calculating the Chl *a* concentration using an extinction coefficient of ~72 000 M^{-1} cm^{-1} at a maximum absorption wavelength of the Q_y transition band (674−5 nm).

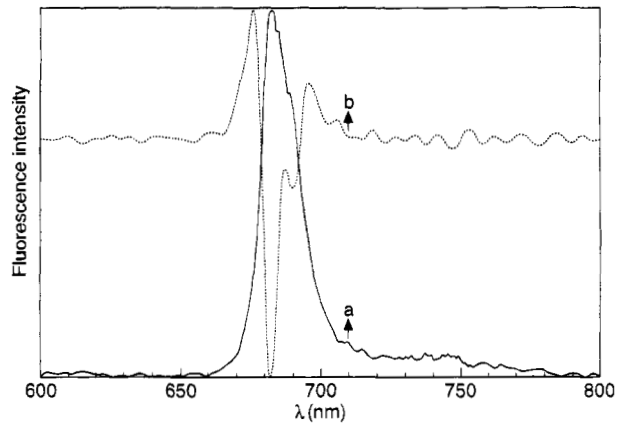

FIGURE 1: Fluorescence emission spectrum of the PSII core from *Synechocystis* 6803 complex at 77 K (a) and its second derivative (b) in solution C + 100 mM $MgSO_4$ and 0.03% DM. Excitation at 432 nm.

Spectral Analyses. Optical absorption spectra were recorded with a Milton-Roy 1201 spectrophotometer. Circular dichroism (CD) was measured with a Jasco J500 dichrograph in 1 cm cuvettes in the range of 250−750 nm. 77 K fluorescence and its second derivative were recorded using an SLM 8000 spectrofluorometer following excitation at 432 nm. The optical activity of PSII RC was measured as described by Braun et al. (1990).

Pigment Content. The content of Chls and Phes in the isolated PSII RC was calculated as previously described (Braun et al., 1990). To estimate the Cyt *b*559 content, the difference between the absorbance of oxidized and reduced RC at 556−559 nm was found according to Wasserman (1979), using an extinction coefficient of 15 mM^{-1} cm^{-1}, and compared with the Chl and Phe content as calculated from the intensity of the redmost peak.

LDS−PAGE. Samples were fractionated by LDS−PAGE (10−20% gradient gel) at 4 °C. One part was subjected to Coomassie staining, and the remaining was blotted using a semidry blotter (E&K Scientific Products). Immunoreaction was performed with primary antisera in 100 mM NaCl, 100 mM phosphate buffer, pH 7.5, containing 1% Tween-20. The signal was amplified and detected using an enhanced chemoluminescence (ECL) system (Amersham).

RESULTS AND DISCUSSION

Isolation of reaction centers critically depends on the separation of PSI from PSII core particles. Unlike the situation in higher plants, the two photosystems in cyanobacteria are not well separated in the thylakoid membrane. In addition, the ratio of PSII to PSI in cyanobacteria is ~1:5 versus ~1:1 in higher plants. PSII core particles were isolated in the present study by modification of existing methods which did not eliminate the PSI fluorescence signal (Nilsson et al., 1992; Kirilovsky et al., 1992) in combination with the procedure of Rögner et al. (1990). Our PSII core particles are practically free of PSI as inferred from their SDS−PAGE (not shown) and fluorescence spectrum (Figure 1). The spectrum shows negligible fluorescence at 720 nm

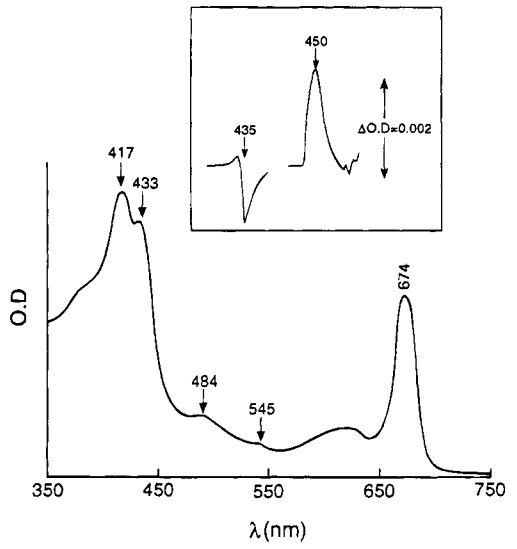

FIGURE 2: Absorption spectrum of the PSII RC from *Synechocystis* 6803 recorded at room temperature in 50 mM Tris buffer (pH 7.2) with 0.03% DM. Inset: Light-induced absorption changes at two different wavelengths, indicative of reduced Phe, formation and decay.

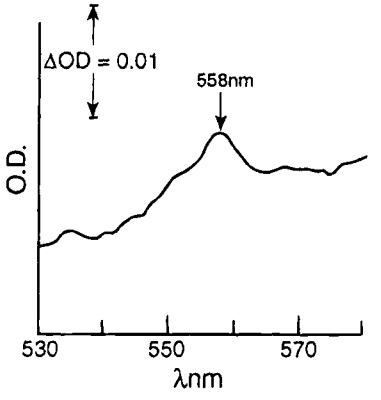

FIGURE 3: Sodium dithionite-reduced minus potassium ferricyanide-oxidized absorbance difference spectrum of cytochrome *b*559 in PSII RC from *Synechocystis* 6803 at room temperature. The difference spectrum was recorded for a sample containing 2.6 μg of Chl *a*/mL.

(PSI antenna complexes), and at 705 and 735 nm (PSI RC core antenna) (Fork & Mohanty, 1986).

RCs were isolated from the PSII core particles as described under Experimental Procedures. The optical absorption of the final PSII RC preparation is shown in Figure 2. The absorption strongly resembles that of PSII RC from the higher plant *Spirodela* (Braun et al., 1990), although the intensity at 433 nm relative to that at 417 nm is slightly higher in the *Synechocystis* PSII RC. Following Braun et al. (1990), we extracted the RC porphyrins and found 5−7 Chls per 2 Phe molecules compared to 4 (Nanba & Satoh, 1987; Braun et al., 1990; Aured et al., 1994) or 6 (Chapman et al., 1988; Kwa et al., 1994a) Chls per 2 Phes in higher plants. The isolated PSII RC accumulates Phe^- upon illumination (Figure 2, inset). However, the quantum yield (scaled to optical density at 679 nm) is about half of the activity observed in PSII RC of higher plants with a similar absorption profile (Braun et al., 1990).

The spectral difference between oxidized and reduced RC (Figure 3) is indicative of 1.1 Cyt *b*559 per 2 Phe molecules. This number agrees with the value given recently by Tang and Diner (1994) and disagrees with the reports of Gounaris

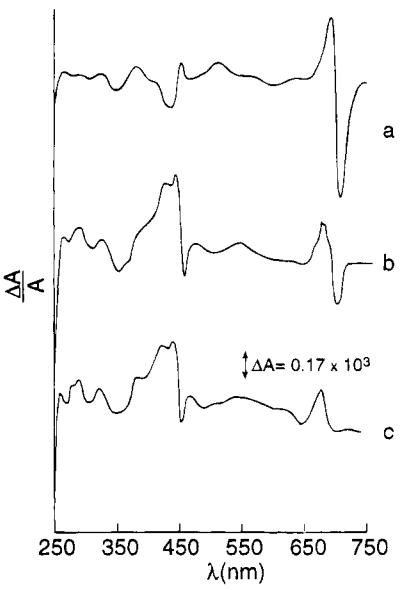

FIGURE 4: CD spectra of (a) PSII core complex in solution C + 100 mM $MgSO_4$ + 0.03% DM, (b) partially purified PSII RC fraction, and (c) purified PSII RC fraction + 2.4 μg of Chl *a*/mL. Both (b) and (c) are in 50 mM Tris buffer (pH 7.2) with 0.03% DM. All spectra were recorded at room temperature.

et al. (1989) and MacDonald et al. (1994). The former suggested that each RC contains 1 Phe and 1 Cyt *b*559. MacDonald et al. (1994) did not refer to the number of Chls and Phes, but found 1.5−2.1 Cyt *b*559 per RC.

The CD of the PSII core particles applied to the Fractogel column (Figure 4a) is compared with that of a partially-purified eluted fraction (Figure 4b), and one containing purified PSII RC (Figure 4c). On going from PSII particles to the purified PSII RC, the redmost transition loses most of the negative band on the lower energy side (this band is indicative of strongly coupled Chls in antenna complexes), shifts to the blue, and gains a negative band on the higher energy side. The new signal consists of narrow (positive) and wide (negative) Gaussians that peak at ~679 and ~669 nm, respectively. Superimposition of these two signals results in the apparent extrema at 682 and 652 nm. The broad negative signal may reflect some disintegration of the special pair as previously reported (Braun et al., 1990). The CD spectrum of the *Synechocystis* PSII RC agrees well with the CD of PSII RC from higher plants and reflects mostly the two excitonic transitions of the primary electron donor P-680 (Braun et al., 1990; Kwa et al., 1994b). Noting the similarity between the CD spectra of the RC-enriched PSII particles (Figure 4b) and Chl aggregates (Scherz et al., 1991), we suggest that the strong negative CD band at ~705 nm is due to Chl aggregates either in the CP47 complex or in antennas attached to the D1/D2/Cyt *b*559 complex.

Coomassie staining and immunoblotting of the LDS−PAGE of *Synechocystis* PSII RC (Figure 5) reveal the typical constituents of PSII RC from higher plants, including cytochrome *b*559 (~10 kDa), D1 (~32 kDa), and D2 (~36 kDa). The bands at 50 and >65 kDa are probably due to aggregated forms of the D1/D2 proteins or their fragments. Bands at similar positions, identified by immunoblotting against the D1 and D2 proteins, were previously reported by Gaunaris et al. (1989) and Tang and Diner (1994). A protein band at ~14 kDa is resolved in response to antibodies raised against the *psb*A gene product (Figure 5, lane b) and

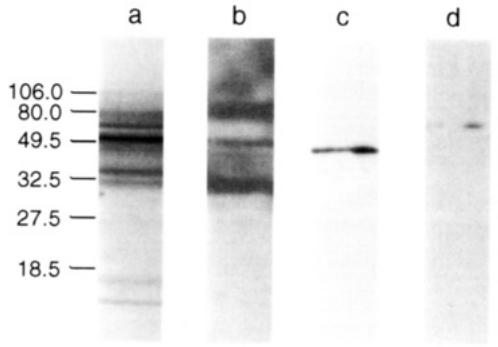

FIGURE 5: Coomassie staining (lane a) and immunoblots (lanes b–d) of the LDS–PAGE of the purified PSII RC fraction from *Synechocystis* 6803. Samples were subjected to antibodies raised against the *psb*A (D1) gene product (lane b), the CP47+CP43 proteins (lane c), and ATP synthase (lane d).

is probably a degradation product of the D1 protein (Greenberg et al., 1987). In addition, there are traces of ATP synthase (Figure 5, lanes a and d, at ~62 kDa), and CP 47 (Figure 5, lanes a and c, just below the 49.5 kDa marker). The band just below 18 kDa was not identified in the present study.

The relative amount of CP47 can be estimated from the pigment content. Assuming 4–5 Chls in each RC (Nanba & Satoh, 1987; Braun et al., 1990; Aured et al., 1994), 2–3 of the 7 Chls per 2 Phes, and 1 Cyt *b*559 found in the present report, are contributed by the CP47 complex. Provided that this complex hold 15–18 Chls (Alfonso et al., 1994), the CP47:PSII RC ratio is ~0.1 in the current preparation. On the basis of above, we suggest that the *Synechocystis* 6803 PSII RC is very similar to that of higher plants in spectra, photochemical activity, and pigment stoichiometry.

In contrast to our observations, and using a different isolation approach, Gounaris et al. (1989) have reported that PSII RC from *Synechocystis 6803* contains 8 Chls, 1 Phe, and 1 Cyt *b*559. The explanation for the large discrepancy between the two preparations may lie in differences in the content of non-RC Chls. In fact, the optical absorption spectrum of the Gounaris et al. (1989) preparation strongly resembles the spectrum of our partially purified PSII RC fraction. The CD pattern of these particles (Figure 4b) reflects the presence of aggregated Chls that are strongly coupled to each other (Scherz et al., 1991), probably as part of the CP47 complex or as part of the RC core antenna.

The similarities between the PSII RC from *Synechocystis* 6803 reported here and the PSII RC of higher plants open the way for employing site-directed mutagenesis in *Synechocystis* 6803 to study the primary reactions of oxygenic photosynthesis.

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