Biochemical and Spectroscopic Characterization of a New Oxygen-Evolving Photosystem II Core Complex from the Cyanobacterium *Synechocystis* PCC 6803†

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ABSTRACT: We describe here a new procedure permitting rapid (12–13 h) isolation of a pure oxygen-evolving photosystem II (PSII) core complex from the cyanobacterium *Synechocystis* PCC 6803. This procedure involves dodecyl maltoside extraction of thylakoid membranes followed by single-step column chromatography using a weak anion-exchanger. SDS–PAGE and immunoblotting show that the complex consists of five intrinsic membrane proteins (CP47, CP43, D1, D1, and cyt b_{559}), one extrinsic protein (MSP), and one unknown protein with a molecular mass of approximately 26 kDa. A chemical and functional analysis, normalized to 2 molecules of pheophytin *a*, indicates that this PSII core complex contains 1 photoactive plastoquinone, Q_A, 4 manganese atoms, 38 chlorophyll *a* molecules, 1 cytochrome b_{559}, 2 plastoquinone-9, and 9–10 β-carotenes. The complex exhibits high rates of oxygen evolution, typically 2400–2600 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1} in the presence of 2,5-dichlorobenzoquinone as an artificial electron acceptor with a pH optimum of 6.5. A strong light minus dark multiline EPR signal, arising from the S_2 state of the oxygen-evolving complex (OEC), is observed at 10 K following illumination at 198 K. The determination of the absolute oxygen yield per saturating microsecond flash indicates that essentially all of the PSII centers contain functional oxygen-evolving complexes. This point is further supported by the absence of photoaccumulation, upon room temperature illumination, of the immediate oxidant of the OEC, redox-active tyrosine, $Y_Z^{\bullet}$. On the basis of EPR spectra, oxidized minus reduced difference spectra, and SDS–PAGE, the preparation contains on a per mole basis with PSII only trace amounts of PSI (~0.04), cytochrome b_6/f complex (≤0.01), and ATPase (≤0.05). All of these results indicate that this PSII preparation is to date the most highly purified oxygen-evolving core complex from *Synechocystis* 6803 that retains all of the reaction centers active for oxygen evolution. As *Synechocystis* 6803 is being used extensively for site-directed mutagenesis of PSII, this preparation is particularly valuable for spectroscopic and biochemical analyses of PSII from wild-type and from site-directed mutants.

Photosystem II (PSII)[1] of oxygenic photosynthesis is the pigment–protein complex of the thylakoid membrane that drives light-induced electron transfer from water to plastoquinone. Recent studies on PSII have focused on its molecular structure and reaction mechanisms [reviewed by Babcock (1987), Diner (1991a), Debus (1992), and Rutherford et al. (1992)]. Comparison of the PSII reaction center with the three-dimensional structure of the reaction centers of the purple non-sulfur photosynthetic bacteria has allowed fairly detailed predictions to be made of the structure of the PSII reaction center, particularly with regard to the binding sites of the prosthetic groups that serve as electron acceptors (Deisenhofer et al., 1985; Trebst, 1986; Nanba & Satoh, 1987; Michel & Deisenhofer, 1988; Allen et al., 1988; Svensson et al., 1990). Less is known, however, about the structure and function of the electron donor side of PSII, that part of PSII that is least like the bacterial reaction centers. In particular, much remains to be learned about the structure of the manganese cluster that is the site of water oxidation and the nature and location of its proteinaceous ligands.

Site-directed mutagenesis and isotopic labeling have begun to play an important role in the study of PSII, including the identification of ligands for important cofactors in PSII, such as manganese, calcium, chlorophyll, pheophytin, and plastoquinone [reviewed by Diner et al. (1991a,b), Debus (1992), Nixon et al. (1992), and Pakrasi and Vermaas (1992)]. Much of this work has been carried out in the unicellular cyanobacterium *Synechocystis* PCC 6803, as this strain is readily transformable, easily incorporating DNA into its genome by homologous recombination (Williams, 1988). Through this work, several classes of amino acids in polypeptide D1 have been implicated in the assembly and coordination of the manganese cluster (Nixon & Diner, 1992; Boerner et al., 1992; Nixon et al., 1992a,b; Debus, 1992). Other amino acids in the D1 and D2 polypeptides have been implicated in the ligation of P_{680}, pheophytin, and Q_A (Svensson et al., 1990; Diner et al., 1991a,b; Nixon et al., 1992b; Ruffle et al., 1992).

Spectroscopic and biochemical analyses are required to characterize the site-directed mutants of PSII and to identify those amino acid residues involved in the ligation of important cofactors. Such analyses have been limited by the lack of adequate biochemical procedures for isolating purified and

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[1] Abbreviations: Chl, chlorophyll a; D1 and D2, polypeptides of the photosystem II reaction center; DCBQ, dichlorobenzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; ENDOR, electron nuclear double resonance; EPR, electron paramagnetic resonance; ESEEM, electron spin-echo envelope modulation; MES, 2-(*N*-morpholino)ethanesulfonic acid; MSP, manganese-stabilizing protein; OEC, oxygen-evolving complex; P_{680}, primary electron donor of PSII; P_{700}, primary electron donor of PSI; PAGE, polyacrylamide gel electrophoresis; PSI, photosystem I; PSII, photosystem II; PEG, poly(ethylene glycol) 8000; PMSF, phenylmethanesulfonyl fluoride; Q_A, primary plastoquinone electron acceptor; Q_B, secondary plastoquinone electron acceptor; SDS, sodium dodecyl sulfate; WT, wild type; Y_D, redox-active tyrosine-160 of the D2 polypeptide; Y_Z, redox-active tyrosine-161 of the D1 polypeptide.

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highly active oxygen-evolving PSII preparations from *Synechocystis* PCC 6803. A number of PSII preparations from *Synechocystis* have been described recently. Rögner et al. (1990) reported a highly purified PSII core complex that shows light-driven electron transfer from Y_Z to Q_A but which does not evolve oxygen. Burnap et al. (1989) developed the first of the oxygen-evolving core complexes from this organism, though the rates of light-saturated oxygen evolution are relatively low. Another three oxygen-evolving PSII preparations, those reported by Noren et al. (1991), Kirilovsky et al. (1992), and Nilsson et al. (1992), are reported to show good rates of oxygen evolution but contain significant contamination by other membrane protein complexes. Two promiment polypeptides near 50 000–60 000 kDa, probably the CF1 α and β subunits, as well as other polypeptides are still present in these PSII preparations. While the procedure of Noren et al. (1991) produces PSII core complexes, the latter two preparations are probably PSII-enriched membranes. Of these three, only that of Kirilovsky et al. (1992) shows strong multiline signals of the S_2 state of the oxygen-evolving complex. None of these *Synechocystis* PSII preparations have been characterized biochemically in any detail, with regard to pigment analysis and cofactors.

A major advantage of core complexes is that they can be highly concentrated, allowing the preparation of samples suitable even for physical methods with poor signal/noise ratio. We present in this paper a new procedure for rapid isolation of pure and highly active oxygen-evolving PSII core complexes from *Synechocystis* 6803. The procedure combines and optimizes certain elements of the earlier described methods of Tang and Satoh (1985), Burnap et al. (1989), Rögner et al., (1990), and Kirilovsky et al. (1992). The procedure takes only 12–13 h from cell harvesting to completion and involves a single column chromatographic step using a weak anion-exchanger. The purified complex shows high rates of oxygen evolution [2400–2600 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}] and a simplified polypeptide composition (mainly six intrinsic membrane polypeptides and one extrinsic polypeptide in addition to one unknown polypeptide). The biochemical and spectroscopic characterization of the protein complex has been carried out, and the results show that the purified PSII complex is highly purified and fully functional for electron transfer from water to the primary quinone electron acceptor, Q_A. We have recently shown, thanks to this preparation, that histidine is a ligand to the Mn cluster, responsible for photosynthetic water oxidation (Tang et al., 1994).

MATERIALS AND METHODS

Cell Growth. Cells of a glucose-tolerant strain (Williams, 1988) of *Synechocystis* PCC 6803 were grown photoheterotrophically using cool-white fluorescent lamps (7 W/m^2) at 30 °C. BG-11 medium (Rippka et al., 1979) was supplemented with 5 mM glucose and 5 mM TES (pH 8.0). The growth medium was bubbled with 5% CO_2 in air.

Preparation of Thylakoid Membranes. Cells, grown in an 18-L carboy for 4 days to an OD_{730nm} of 1.5–2.0, were concentrated to 1 L using an Amicon DC-10L concentrator coupled to a hollow fiber filter (H5MP01-43) and then pelleted by centrifugation at 4 °C for 10 min at 9000 rpm in a Sorvall GSA rotor. The pellets were washed once with buffer A [50 mM MES–NaOH, pH 6.0, 25% glycerol (w/v), 5 mM $CaCl_2$, and 5 mM $MgCl_2$] and pelleted again (9000 rpm, Sorvall GSA rotor, 15 min 4 °C). The cells were resuspended in the same buffer containing 1 mM phenylmethanesulfonyl fluoride, 1 mM benzamidine, 1 mM aminocaproic acid, and 50 μg/mL DNase I (bovine pancreas type IV, Sigma) and adjusted to a final volume of 55 mL. The cells were added to a prechilled Bead-Beater chamber (88 mL) (Bio-spec Products). The chamber was then filled with prechilled 0.1-mm-diameter glass beads (B. Braun Melsungen, AG), and the outside jacket was filled with an ice–water mixture. After a 70-min incubation in the dark, the cells were broken in darkness using 8 pulses of 15 s each, with 5-min cooling intervals. The homogenate was separated from the beads by decantation, and the beads were washed 4–5 times using a total of about 250 mL of buffer A. Unbroken cells and residual beads were removed from the membrane suspension by centrifugation for 5 min at 6000 rpm in a Sorvall GSA rotor. The thylakoid membranes were pelleted by centrifugation at 40 000 rpm for 20 min in a Beckman 45Ti rotor. The membrane pellets were then resuspended in 140 mL of buffer B (buffer A plus 15 mM $CaCl_2$) and pelleted again by centrifugation. The pelleted thylakoid membranes were resuspended in buffer B at a chlorophyll concentration of 1 mg/mL and were extracted immediately.

Extraction of Thylakoid Membranes and Purification of PSII Core Complexes by Column Chromatography. A 10% stock solution of dodecyl maltoside (Boehringer Mannheim) was added dropwise to a 1 mg of Chl/mL suspension of thylakoid membranes (typically 20–40 mg of chlorophyll) to give a final concentration of 1% in detergent. Extraction proceeded in the dark for 10 min at 0 °C with gentle stirring. The suspension was then centrifuged at 50 000 rpm in a Beckman 70Ti rotor for 30 min at 4 °C. The supernatant was loaded onto a DEAE-Toyopearl 650S column (5.5 × 17 cm, a weak anion-exchanger, Toso Haas) previously equilibrated with 500 mL of equilibration buffer (buffer B + 20 mM $MgSO_4$ + 0.03% dodecyl maltoside). The column was then washed with equilibration buffer (500–1000 mL) at a flow rate of 6–7 mL/min using a peristaltic pump (Model P-3, Pharmacia). Once the red wavelength maximum of the absorbance spectrum of the eluant dropped to ≤674.5 nm, a 500-mL linear gradient from 20 to 30 mM $MgSO_4$ in buffer B and 0.03% dodecyl maltoside was applied to the column. For mutants containing lower ratios of PSII/PSI than WT, the gradient was started earlier. In the case of wild type, fractions with chlorophyll absorbance maxima between 673.5 and 673.9 nm were pooled and immediately concentrated to 2–4 mL first with an Amicon Model 2000 and then with an Amicon 8400 ultrafiltration cell, fitted with YM-100 membranes. In the case of certain mutants, the wavelength range over which fractions were pooled needed to be shifted slightly. The sample was concentrated to 0.3–1.2 mg of Chl/mL and then frozen in liquid nitrogen and stored at –80 °C. If necessary, the sample could be further concentrated with a Centricon 100 (Amicon) or by PEG-precipitation (7.5% PEG-8000) followed by centrifugation at 47 500 rpm in a Sorvall SW50.1 rotor for 50 min at 4 °C. The material remaining on the column was eluted with 50 mM $MgSO_4$ in buffer B plus 0.03% dodecyl maltoside.

Measurement of Oxygen Evolution. Rates of oxygen evolution were measured at 25 °C in a small thermostated Clark-type oxygen electrode (Model 5356, Instech Laboratories) using continuous and flash illumination. Continuous saturating light, provided by a 150 W tungsten-light source (Model I-150, Cuda Products Inc.), was filtered through a heat-reflecting filter, a high-band-pass (>530 nm) filter, and a 10 cm × 1 cm cylindrical cuvette filled with water that acted as a light guide. Whole cells were suspended in BG11 medium plus 5 mM glucose in the presence of 2 mM 2,5-DCBQ as an artificial electron acceptor. The thylakoid membranes and

the purified PSII core complexes were suspended in 50 mM MES–NaOH (pH 6.5) buffer containing 1 mM 2,5-DCBQ, 1 M sucrose, 10 mM NaCl, and 20 mM $CaCl_2$, unless otherwise noted. Chlorophyll concentrations were typically between 5 and 10 μg/mL. The electrode was calibrated using, as standards, water equilibrated with air and water equilibrated with N_2, both at atmospheric pressure.

The concentration of active oxygen-evolving complexes was determined in thylakoid membranes and in PSII core complexes using saturating microsecond laser flash excitation [Rhodamine 590 (Exciton), Cynosure LFDL-3 dye laser]. Two of the above-mentioned oxygen electrodes were connected in a differential mode to the input amplifier (Model 5300, Yellow Springs Instrument Co.). The same samples were placed in each of the electrode chambers except that one contained 1 mM 2,5-DCBQ as electron acceptor while the other did not. The laser flashes (10–15 Hz) were passed through a 50% beam splitter (CVI Laser) such that each electrode received flashes of equal energy (15–20 mJ). In addition, tracing paper was placed over the end windows of the electrode chambers to assure homogeneous illumination of the samples. The use of the differential mode eliminated the inevitable thermal artifacts associated with illumination despite the thermostating. This method can, of course, also be used with continuous illumination.

SDS–PAGE. The polypeptide composition of the core complexes was analyzed by denaturing SDS–PAGE in the presence of 6 M urea using a 10–20% linear gradient polyacrylamide gel as described by Chua (1980). The samples were solubilized in 1% SDS sample buffer by incubation for 40 min at room temperature. The gel was run for 12–13 h at 4 °C with a constant current of 4.7 mA/cm^2. The gels were stained with Coomassie Brilliant Blue R-250, unless used for immunoblotting.

Immunoblot Analysis. Antibodies specific for the last 16 residues of the mature D1 polypeptide of *Synechocystis* 6803 were raised in rabbits as described in Nixon et al. (1992b). Antiserum specific for the D2 polypeptide was obtained by Peter Nixon through the immunization of rabbits against a dodecapeptide extending from residues 342 to 353 of the D2 polypeptide of *Synechocystis* 6803. Antisera specific for CP47, CP43, and the MSP were kindly provided by Dr. Nam-Hai Chua. For Western blot analysis, proteins separated by SDS–PAGE were transblotted onto a nitrocellulose membrane using a semi-dry system (ELECTROBLOTTER, Model IMM-1, Enprotech) and incubated with primary antibody. The blot was developed with goat anti-rabbit IgG–alkaline phosphatase conjugate (Bio-Rad) according to standard procedures (Harlow & Lane, 1988).

Optical Measurements. Absorbance spectra were recorded on a Shimadzu UV2101PC spectrophotometer. Quantification of the photoactive primary quinone electron acceptor, Q_A, was determined on a home-built flash detection spectrophotometer as described earlier (Joliot et al., 1980; Rögner et al., 1990) except that the PSII core complexes were suspended at 10 μM Chl in 25 mM MES–KOH, pH 6.5, 10 mM NaCl, and 5 mM $MgCl_2$, preincubated with 1 μM potassium ferricyanide. One minute after the addition of 2 mM in NH_2OH, Q_A was photoreduced by a train of 20 flashes given at 18 Hz. The only absorbance changes observed in the UV under these conditions arise from Q_A^-–Q_A as judged by the ratio of $\Delta A_{265nm}/\Delta A_{325nm} = -1.21$ and an isosbestic point at 290 nm (Metz et al., 1989). An extinction coefficient for Q_A^-–Q_A of 13 mM^{-1} cm^{-1} at 325 nm (van Gorkom, 1974) was used to calculate the concentration of Q_A^-. The chlorophyll *a* concentration was determined by extraction with methanol and using an extinction coefficient at 665.5 nm of 79.24 mL (mg of Chl)$^{-1}$ cm^{-1} (Lichtenthaler, 1987). The content of cytochrome b_{559} was determined from the absorbance difference spectrum, dithionite-reduced minus ferricyanide-oxidized. The P_{700}^+ content was similarly estimated, but using the ascorbate-reduced minus ferricyanide-oxidized difference. The extinction coefficient for cytochrome b_{559} was taken as 17.5 mM^{-1} cm^{-1} according to Cramer et al. (1986), and the extinction coefficient for P_{700}^+ was taken as 64 mM^{-1} cm^{-1} according to Hiyama and Ke (1972).

Pigment Analysis. Core complexes were extracted with 90% acetone at room temperature for 1 min. The solution was then centrifuged (17000*g*) at 4 °C for 5 min, and the resulting supernatant was filtered through a Millipore filter (0.5 μm, Millipore Corp.). The filtrate was immediately injected onto a reverse-phase HPLC column (Zorbax-ODS, 4.6 mm × 15 cm, Rockland Technologies). Methanol/2-propanol (3/1) at 10 °C was used for the isochratic elution of pigments according to the procedure of Eskins et al. (1977). The elution was monitored simultaneously at 255 nm (pheophytin and plastoquinone) and at 440 nm (chlorophyll and carotenoids). The absorption coefficients used were those of Eskins et al. (1977) for chlorophyll and carotenoids, of Vernon (1960) for pheophytin, and of Barr and Crane for plastoquinone (1971).

Determination of Mn Content. Purified PSII core complexes in buffer B plus 0.03% dodecyl maltoside were diluted with an equal volume of either 1 N HCl or fresh-made 40 mM hydroxylamine. The amount of manganese was then estimated by measuring by EPR the signal intensity of hexaaquomanganese (Yocum et al., 1981). Calibration curves were established using $MnCl_2$ standards in buffer B containing either 0.5 N HCl or 20 mM hydroxylamine.

EPR Measurements. The S_2 state multiline EPR signal of the oxygen-evolving complex was recorded on a Bruker ER200D spectrometer equipped with an upgraded computer software system (Model ESP-300) at 10 K using an Oxford ESR-910 helium flow cryostat. PSII core complexes were suspended in buffer B containing 0.03% dodecyl maltoside and 50 μM ferricyanide and dark-adapted for 30 min on ice. The samples were then frozen in the dark in liquid nitrogen. Illumination of the sample was performed in a dry ice/ethanol bath (198 K) for 20 min using saturating white light.

Room temperature EPR spectra were recorded at X-band on a Bruker ESP-300 spectrometer using a flat cell. The sample was suspended in buffer B plus 0.03% dodecyl maltoside and 0.3 mM ferricyanide. The $Y_Z^\bullet$ radical was measured by first treating PSII core complexes with 0.8 Tris-HCl buffer (pH 8.0) containing 1 mM EDTA and 0.03% DM for 30 min at 0 °C (Cheniae & Martin, 1970; Babcock & Sauer, 1975; Yamamoto et al., 1981) to inactivate the oxygen-evolving complex. After PEG precipitation, as described above, the protein complex was resuspended in buffer B containing 0.03% dodecyl maltoside and 0.3 mM ferricyanide. Saturating white light was used for illumination in the cavity.

RESULTS AND DISCUSSION

Purification of the Oxygen-Evolving PSII Core Complex. Two features critical to the isolation of intact PSII oxygen-evolving core complexes are the speed with which the procedure is carried out and the mildness of the extraction and purification procedures. Figure 1 schematically summarizes a simple 12–13-h procedure we have developed for isolating the oxygen-evolving PSII core complex from *Synechocystis* 6803. With

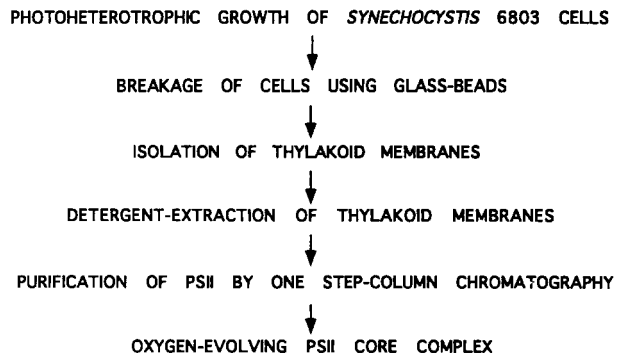

FIGURE 1: Flow diagram of the preparation of thylakoid membranes and oxygen-evolving PSII core complexes from *Synechocystis* PCC 6803.

Table 1: Purification of the Oxygen-Evolving PSII Core Complex from *Synechocystis* 6803

	O_2 evolution	PSI/PSII	total chlorophyll[c]	yield[d]
cells	510[a]		40	100
thylakoids	490,[a] 550[b]	4–5	33	83
DM extracts	480[a]		25	62
PSII complexes	2500,[a] 44[b]	0.04	1.5	3.7

[a] Micromoles of O_2 per milligram of Chl per hour. [b] Chl per 0.25 O_2 per flash (upon correction for oxygen flash yields, this number will be decreased further by 5–10%). [c] Milligrams of chlorophyll. [d] Percent chlorophyll.

the exception of some changes in the concentration of $CaCl_2$ and $MgSO_4$, the same buffer is used throughout. Activity is maintained by the choice of pH and the presence of glycerol and of the mild detergent dodecyl maltoside. The thylakoid membranes isolated by this method are highly intact, retaining near 100% Q_A to Q_B electron-transfer function (data not shown), and showing approximately the same oxygen-evolving activity as whole cells coupled to 2,5-DCBQ [400–600 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}] with a yield of cell breakage of 80–85% (Table 1). A 10-min treatment of the thylakoid membranes with dodecyl maltoside extracts about 70–80% chlorophyll. The extracts show no loss in the rate of oxygen evolution and show a PSII/PSI ratio similar to that of thylakoid membranes. Rapid freezing of the thylakoid membranes in liquid nitrogen in buffer B and storage at −80 °C result, following rapid thawing, in a loss of only 10–15% of the oxygen-evolving activity of the thylakoids. However, oxygen-evolving PSII core complexes purified from frozen and thawed thylakoid membranes show only 50–70% activity of that purified from fresh thylakoids. This observation is consistent with a previous report by Kirilovsky et al. (1992). On the basis of these observations, only fresh thylakoids are used for the isolation of fully active PSII oxygen-evolving core complexes.

Weak anion-exchange column chromatography is used for purifying the PSII core complex. DEAE-Toyopearl 650S was chosen for three reasons: (a) The weak anion-exchange resin (DEAE) permits the use of low salt concentrations to elute PSII core complexes from the column. Higher concentrations (>100 mM) of salts, such as $MgCl_2$, NaCl, or $CaCl_2$, result in inhibition of oxygen evolution in cyanobacterial PSII particles (Katoh, 1988; Pauly et al., 1992). We have checked for such inhibitory effects of $MgCl_2$, $CaCl_2$, NaCl, and $MgSO_4$ in PSII core complexes from *Synechocystis* 6803. We have found that $MgCl_2$ and $CaCl_2$ start to show inhibition of oxygen evolution when their concentration is greater than 65 mM; at 120 mM, they inhibit half of the activity (see Figure 3). NaCl also starts to show inhibition at 150 mM. $MgSO_4$ is the most gentle salt we have found for oxygen evolution, showing almost

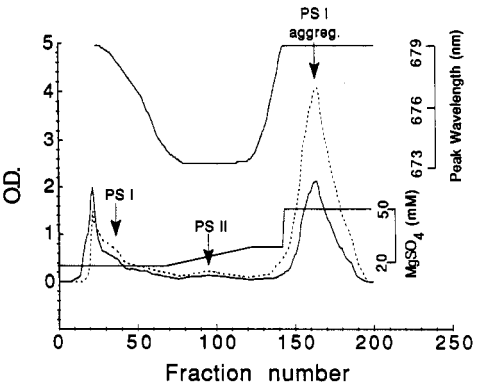

FIGURE 2: Elution profile of the dodecyl maltoside extract on a DEAE-Toyopearl 650s column. Absorbance is measured at the red absorption maximum (dashed line) and at 280 nm (solid line). Also indicated is the $MgSO_4$ concentration and the wavelength at the red absorption maximum of the eluent. See Materials and Methods and Results for further details.

no inhibition up to 90 mM. Consequently, $MgSO_4$ was ultimately chosen as the salt with which to elute PSII core complexes from the column. (b) The DEAE-Toyopearl resin is mechanically quite strong and can be run at high flow rates (up to 10 mL/min in our column, size = 5.5 × 17 cm), shortening the length of the chromatographic step. (c) DEAE-Toyopearl has been previously reported to be suitable for purifying oxygen-evolving PSII core complexes from higher plants (Tang & Satoh, 1985) and from the cyanobacteria *Synechococcus sp.* (Katoh, 1988) and *Synechocystis* 6803 (Burnap et al., 1989).

Figure 2 shows the elution profile, using DEAE-Toyopearl 650S, of a dodecyl maltoside extract of thylakoid membranes. The wash with the equilibration buffer elutes successively the free pigments, cytochrome b_6/f complex, residual phycobiliproteins, and a monomeric PSI complex. The first peak is a pink–orange color and shows very little absorbance in the red wavelength region of the visible spectrum. Further analysis showed it to contain the cytochrome b_6/f complex, free carotenoid, and a small amount of free chlorophyll. The following fractions are enriched in phycobiliproteins, with absorption peaks between 600 and 650 nm. The major peak eluted by the equilibration buffer wash is monomeric PSI complex, showing an absorbance maximum around 679 nm. Those components eluted by the 20–30 mM linear $MgSO_4$ gradient show a small peak in the elution profile. Those fractions with absorbance maxima between 673.5 and 673.9 nm are highly active in oxygen evolution, and are pooled and concentrated as described under Materials and Methods. The major component eluted at 50 mM $MgSO_4$ shows an absorption peak at 679 nm and contains aggregates of PSI core complexes, possibly a PSI trimer (Rögner et al., 1990). From the elution profile, the PSII fractions are estimated to be about 6% of the total chlorophyll loaded onto the column. The yield of this purified PSII core complex is approximately 3.7% of the total starting chlorophyll in the cells (Table 1), or 4.5% of the thylakoid membrane chlorophyll.

Activity and Stability of the Purified Oxygen-Evolving PSII Core Complex. The oxygen-evolving activities at 25 °C of the cells, thylakoids, dodecyl maltoside extracts, and purified PSII core complex are summarized in Table 1. Up until the column chromatography step, almost no change is observed in the oxygen-evolving activity, with the rate remaining at 400–600 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}. The rate of oxygen evolution in the purified PSII core complex is 2400–2600 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}, showing an approximate 5-fold

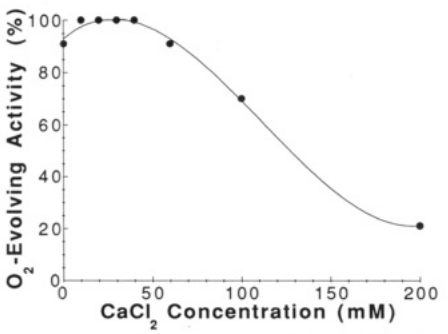

FIGURE 3: Dependence of the oxygen-evolving activity in continuous light of the purified PSII core complex on the concentration of Ca^{2+}. The core complex was washed by precipitation with PEG-8000 followed by resuspension in O_2 assay buffer containing different concentrations of Ca^{2+}. The assay medium is as described under Materials and Methods except for the variation in the concentration of $CaCl_2$.

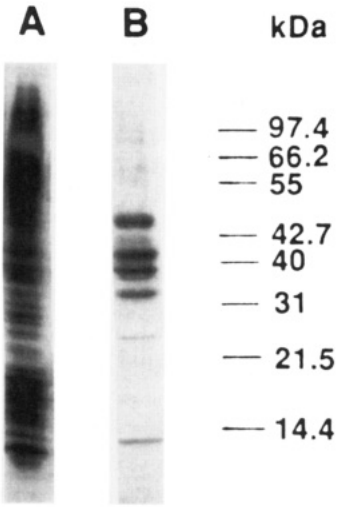

FIGURE 4: SDS–PAGE of the thylakoid membrane (A) and of the purified oxygen-evolving PSII core complex (B) from *Synechocystis* 6803. The bars on the right side indicate the positions of the molecular mass marker proteins.

increase of oxygen-evolving activity over the thylakoids. This purification factor is, however, 12.5-fold when thylakoids (550 Chl/0.25 O_2 molecules per flash) and core complexes (44 Chl/0.25 O_2 molecules per flash) are assayed using saturating flash excitation at 15 Hz, that is, under conditions where the activity is limited only by the oxygen flash yield of active oxygen-evolving core complexes and not by dark reactions involved in the turnover of core complexes. The difference between the continuous saturating light and flash measurements implies that the dark reaction allowing a center to recover following charge separation, probably the oxidation of Q_A^-, has slowed by a factor of 2.5 between membranes and core complexes in the presence of 1 mM 2,5-DCBQ. This difference may just reflect a difference in the partition coefficient of the quinone between water and the biological material. At 120 Chl/PSI in thylakoids of *Synechocystis* 6803 (Kirilovsky et al., 1992), the O_2 flash yield implies a molar ratio of 4–5 PSI/OEC in *Synechocystis* thylakoids, in agreement with previous estimates of PSI/PSII (Rögner et al., 1990; Vermaas et al., 1990).

The highest rate of oxygen evolution [2600 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}] of the purified complex in continuous light is observed at pH 6.5 using 1 mM 2,5-dichlorobenzoquinone (2,5-DCBQ) as an artificial electron acceptor in a reaction buffer containing 50 mM MES–NaOH, 1 M sucrose, 20 mM $CaCl_2$, and 10 mM NaCl. Addition of 1 mM potassium ferricyanide to the reaction buffer produces almost no change in the rate of oxygen evolution. However, only 70–80% of the maximal activity is obtained with 2,6-DCBQ as the electron acceptor. The same situation applies to the oxygen-evolving activities of the whole cells and thylakoid membranes. This observation indicates some stereospecific preference for the artificial electron acceptor on the reducing side of PSII.

One striking characteristic of the purified complex is its Ca^{2+} dependence. In general, the purified oxygen-evolving PSII core complexes lacking the 23- and 17-kDa polypeptides from higher plants and cyanobacteria require relatively high concentrations (5–50 mM) of Ca^{2+} to obtain high oxygen-evolving rates and to protect against photoinhibition (Piccioni & Mauzerall, 1976; Tang & Satoh, 1985; Ikeuchi et al., 1985; Satoh et al., 1985; Ghanotakis et al., 1984a; Pauly et al., 1992; Burnap et al., 1989; Noren et al., 1991; Kirilovsky et al., 1992). However, as shown in Figure 3, the PSII complex, isolated by the present procedure, still shows more than 90% of the maximum activity even at concentrations as low as 100 μM Ca^{2+}. Precipitation and washing the preparation with PEG-8000 followed by resuspension in assay medium lacking Ca^{2+} to lower the Ca^{2+} concentration to $\sim$1 μM still left about 50% of the oxygen activity intact at the start of illumination with some indication of rapid photoinhibition as reported by Burnap et al. (1989). These observations indicate the maintenance in the dark of tightly bound Ca^{2+}. The accelerated photoinhibition at low Ca^{2+} concentration may be explained by the release of Ca^{2+} in the higher S states of the OEC (Dekker et al., 1984; Boussac & Rutherford, 1988).

The oxygen-evolving activity of the purified PSII complex is quite stable. Almost no activity loss is observed upon overnight incubation of the concentrated complex (2–3 mg of Chl/mL) at 4 °C in the dark. Rapid freezing and thawing of the concentrated complex in liquid nitrogen also do not cause inactivation of oxygen evolution. The complex is stable enough at room temperature to do EPR measurements even with illumination in the cavity (see Figure 7). The purified oxygen-evolving PSII core complex is most stable when stored at pH 6.0, though this pH is not optimal for the measurement of oxygen evolution in continuous light. This is probably because the rate of oxygen evolution under continuous illumination is limited by the acceptor side of the PSII reaction center, which shows faster turnover at higher pH. The stability of the complex is determined by the most labile component of the complex, believed to be the manganese cluster, which begins to fall apart at higher pH.

Polypeptide Composition. The polypeptide composition of the purified oxygen-evolving PSII core complex was analyzed by SDS–PAGE containing urea and by immunoblot (Figures 4 and 5). Seven polypeptide bands could be distinguished in the presence of 6 M urea. These have molecular masses of approximately 47, 41, 38, 35, 32, 26, and 10 kDa. Antibodies raised against CP47, CP43, D1, D2, and the 33-kDa subunit (manganese-stabilizing protein) were used to identify these subunits. Cross-reactivities are observed for all five antibodies. These results allow us to assign the 47-kDa subunit to the CP47 apoprotein (*psb*B gene product), the 41-kDa subunit to the CP43 apoprotein (*psb*C gene product), the 38-kDa subunit to the manganese-stabilizing protein (*psb*O gene product), the 35-kDa subunit to D2 (*psb*D gene product), and the 32-kDa subunit to D1 (*psb*A gene product). The 10-kDa subunit presumably corresponds to the α subunit of cytochrome b_{559} (*psb*E gene product), because cytochrome b_{559} could be detected spectroscopically (see below). The β subunit (*psb*F gene product) and other low molecular mass polypeptides of PSII such as the *psb*I and *psb*K gene products

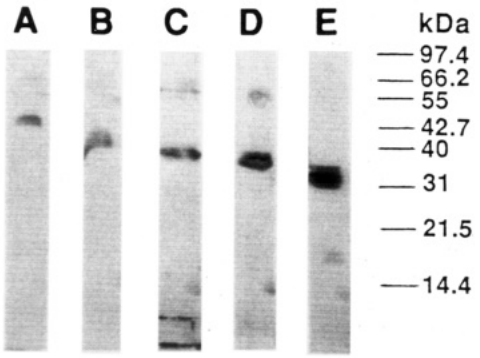

FIGURE 5: Immunoblot analysis of the purified PSII core complex. The blots were developed with antibodies against CP47 (lane A), CP43 (lane B), the 33-kDa extrinsic manganese-stabilizing protein (lane C), D2 (lane D), and D1 (lane E). The bars on the right side indicate the positions of the molecular mass maker proteins.

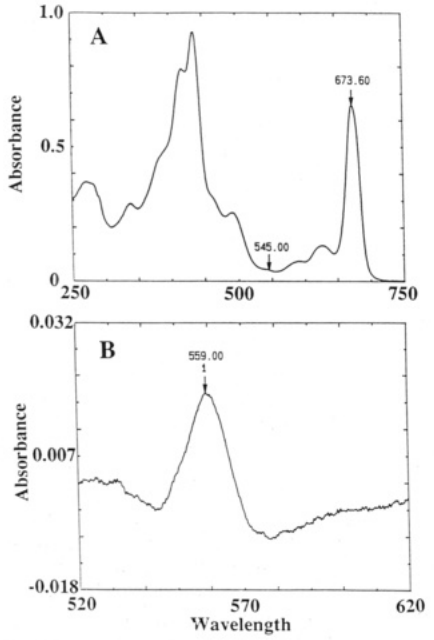

FIGURE 6: Absorbance spectrum (A) and dithionite-reduced minus ferricyanide-oxidized difference spectrum (B) of the isolated PSII core complex recorded at room temperature. The spectral bandwidth for each spectrum is 0.5 nm. The chlorophyll concentrations for (A) and (B) are 7.62 and 40.50 μg of Chl/mL, respectively.

(Koike et al., 1989) could not be resolved by this gel. We cannot at present assign a role to the 26-kDa subunit. The amount of this subunit varies between preparations, and its concentration does not appear to parallel oxygen-evolving activity. The immunoblot result also indicates that the trace amount of Coomassie Blue-stainable material at around 66 kDa is composed of aggregates of PSII subunits. The polypeptide profile clearly shows that the purified PSII oxygen-evolving core complex is essentially free of polypeptides from the PSI core complex, the cytochrome b_6/f complex, and the cyanobacterial ATPase. Subunits of the thylakoid ATPase have been found in all previous oxygen-evolving PSII particles from *Synechocystis* 6803, showing at the very least two prominent bands between 50 and 60 kDa (Burnap et al., 1989; Noren et al., 1991; Kirilovsky, 1992). Thus, the present oxygen-evolving PSII core complex appears to be the most purified, with respect to the polypeptide composition, of those isolated from *Synechocystis* 6803.

Absorbance Spectra. Figure 6A shows a room temperature absorbance spectrum of the purified oxygen-evolving PSII core complex. The red chlorophyll *a* absorption peak of this complex is observed at 673.6–673.7 nm. This is a typical absorbance spectrum of a PSII core complex, nearly identical to that of a non-oxygen-evolving PSII core complex, isolated from the same organism (Rögner, 1990), and to the oxygen-evolving PSII core complexes purified from the cyanobacterium *Synechococcus sp.* (Satoh et al., 1985) and from spinach (Satoh, 1983; Tang & Satoh, 1985). A small absorption peak near 545 nm, ascribed to pheophytin *a*, is also evident. The presence of a carotenoid species, identified as β-carotene by chemical analysis (see below), is indicated by the absorption shoulders in the Soret region (460–490 nm). This spectrum also indicates that phycobiliproteins, with absorbance maxima around 600–650 nm, have been completely removed from the complex.

Table 2: Chemical Composition of the Purified Oxygen-Evolving PSII Core Complex from *Synechocystis* 6803

component	molar ratio
pheophytin *a*	2
photoactive Q_A	1.08 ± 0.1
chlorophyll *a*	40.50 ± 2 (37.50 ± 2)[a]
OEC	1.00 ± 0.05
cytochrome b_{559}	1.16 ± 0.1
β-carotene	9.50 ± 0.5
plastoquinone-9	2.16 ± 0.2
P-700	0.04 ± 0.01
manganese	
HCl-released	3.98 ± 0.2
hydroxylamine-released	3.86 ± 0.2

[a] Intrinsic PSII Chl/PSII reaction center after correction for PSI contamination.

Chemical Composition. The chemical composition of a typical preparation is summarized in Table 2. All the components have been normalized to two pheophytin *a* as each reaction center is generally believed to contain two pheophytin molecules (Omata et al., 1984). The pigment extraction was performed rapidly and the HPLC analysis at 10 °C to avoid the artificial generation of pheophytin from chlorophyll. In a control experiment, pure chlorophyll *a* was subjected to the same extraction/HPLC procedure as in the analysis of the PSII complex. No pheophytin *a* was detected. The concentration of photoreducible Q_A is taken as a measure of the concentration of active PSII centers. That there is 1.08 ± 0.1 photoreducible Q_A per 2 pheophytins argues that there has been little chlorophyll to pheophytin conversion and reaffirms the assignment of 2 pheophytins per PSII reaction center. The small difference between these two numbers arises either from a small (≤8%) conversion and/or from experimental error. Our results therefore argue against the earlier proposal of Gournaris et al. (1989) of only one pheophytin per cyanobacterial PSII reaction center.

PSI contamination, estimated by reduced-minus-oxidized difference spectra, was found to amount to only 0.04 ± 0.01 P_{700}^+ per 2 pheophytins. This represents a substantial purification (100–125-fold) relative to the 4–5 PSI/PSII present in thylakoid membranes (Table 1). On the basis of 75 ± 5 Chl/PSI (Rögner et al., 1990) for PSI complexes isolated under similar conditions, approximately 3 of the chlorophylls per 2 pheophytins are attributable to the PSI core complex. Correcting for this number gives 37.5 ± 2 chlorophylls intrinsically associated with the PSII core complex based on 2 pheophytins and 34.7 ± 2 based on photoreduction of Q_A. This number is reduced from the 45 ± 5 Chl earlier estimated for PSII core complexes by Rögner et al. (1990), based on Q_A photoreduction, and implies that in the earlier preparations some 16–22% of the centers were inactive for photoreduction of Q_A. A similar conclusion was independently

arrived at recently by Matthias Rögner (personal communication), who has found 39 ± 2.4 Chl per 2 pheophytins in *Synechocystis* core complexes isolated according to his procedure (Rögner et al., 1990). The number of chlorophylls per center (35–38) is similar to that reported for *Chlamydomonas* core complexes (40 Chl per 2 pheophytins; de Vitry et al., 1987) but contrasts with that observed in higher plant PSII core complexes (~48 Chl per 2 pheophytins; Yamada et al., 1987), implying either the possible presence in higher plant core complexes of a chlorophyll protein complex(es) outside of CP47, CP43, D1, and D2 or a greater packing of chlorophylls into these subunits.

Measurements of the yield of oxygen per microsecond flash upon steady-state flash illumination (at 10 and 15 Hz) indicate 44 chlorophylls per 0.25 O_2 molecule per flash (Table 1). Allowing for 90–95% flash yield associated with the normal functioning of the OEC (Kok et al., 1970), this would give ~41 Chl/OEC, in agreement with the number of chlorophylls present per 2 pheophytins. A further correction for the small PSI contamination gives 38 PSII Chl/OEC, as found above per 2 pheophytins. This measurement indicates that all of the PSII reaction centers contain functional oxygen-evolving complexes. This finding is further supported (see below) by EPR measurements at room temperature showing the absence of photoaccumulation of redox-active tyrosine $Y_Z^{\bullet}$. Flash excitation at 30 Hz decreases the O_2 yield per flash by 15–20%, indicating that complete recovery of the centers from charge separation requires ≥67 ms under the assay conditions used (see Materials and Methods).

Four manganese per two pheophytin molecules are detected by measuring the EPR signal intensity of the hexaaquomanganese released by 0.5 M HCl. No hexaaquomanganese six-line signals are observed in the EPR spectra of these samples before the addition of HCl, indicating that, prior to acid treatment, all the manganese are tightly bound to the protein complex. Furthermore, a nearly identical number is obtained using 20 mM hydroxylamine to release manganese from the PSII protein complex. Hydroxylamine is used as a reductant in an attempt to release only the high oxidation state manganese ions that are specifically associated with the water-oxidizing systems in PSII (Yocum et al., 1981; Ghanotakis et al., 1984b; Tamura & Cheniae, 1985; Mei & Yocum, 1991). The fact that both procedures release nearly identical quantities of manganese indicates that all manganese in the purified oxygen-evolving PSII core complex is associated with the water-oxidizing system to form the manganese cluster. As the oxygen flash measurements indicate that all of the OEC are active for oxygen evolution, we confirm that there are four manganese per OEC, the most widely accepted number (Debus, 1992). We disagree with a recent estimation of Pauly and Witt (1992) using flash excitation of six Mn per OEC in PSII core complexes from *Synechococcus sp.* The simplest explanation for this discrepancy is, as suggested by Debus (1992), that because no more than 50% of the PSII centers are active for oxygen evolution in the *Synechococcus* preparations, these authors cannot exclude that inactive centers still contain bound Mn. The present measurements avoid this pitfall as all of the centers are active for O_2 evolution.

We observe one cytochrome b_{559} molecule per reaction center (Table 2), as determined by the reduced minus oxidized difference spectrum (Figure 6B), consistent with previous observations (Sandusky et al., 1983; de Vitry et al., 1987; Miyazaki et al., 1989; Buser et al., 1992; Fotinou et al., 1993); however, there are a number of reports of two cytochromes b_{559} (Murata et al., 1984; Dekker et al., 1989; Haag et al., 1990), including one case of an oxygen-evolving preparation from *Synechocystis* 6803 (Boerner et al., 1992). We note, however, that the 63–65 Chl per 2 cytochromes b_{559}, reported by Boerner et al. (1992), is identical to the quantification reported here. We attribute the two cytochromes per center estimate of these authors to the presence of inactive centers containing cytochrome b_{559} and the consequent overestimation of the number of Chl and cytochromes per active center. The earlier estimate of Rögner et al. (1990), based on Q_A photoreduction, of 1.4 cytochromes b_{559} per center is somewhat overestimated as indicated above by the presence of 16–22% of the PSII centers inactive for Q_A photoreduction, assuming that inactive centers still contain this cytochrome. The number we obtain here in the *Synechocystis* core complexes is consistently, very slightly higher (1.2) than 1 cytochrome b_{559} per center and may reflect a small systematic error, coming, for example, from an underestimation of the extinction coefficient. We note that the difference spectrum of Figure 6B also indicates the complete absence of the cytochrome b_6/f complex from the preparation of purified PSII core complex.

The only carotenoid species we find in the purified PSII complex is β-carotene. The same carotenoid composition and stoichiometry have been reported in PSII particles from spinach (Yamada et al., 1987).

Two plastoquinones per center are found in the purified complex. One of these must correspond to Q_A. However, the other plastoquinone appears to lie outside the Q_B binding site as no more than 10% of the centers show oxidation of Q_A^- in the millisecond time range. The light-saturated rate of oxygen evolution, measured in the presence of 2,5-DCBQ, is inhibited only 70–80% by 10 μM DCMU. Either the Q_B site in a fraction of the centers no longer binds DCMU or the electron on Q_A^- or the pheophytin anion is accessible to 2,5-DCBQ through a pathway independent of the Q_B binding site. The latter situation has been observed in reaction centers of *Rhodobacter sphaeroides* (Warncke & Dutton, 1991).

In conclusion, the chemical composition of this PSII core complex isolated from *Synechocystis* contains, per 2 pheophytins *a*, a mole ratio of 1 photoactive Q_A, 1 photoactive OEC, 41 chlorophylls *a* (3 of which arise from residual PSI contamination), 2 plastoquinone-9, 9–10 β-carotenes, 1 cytochrome b_{559} heme, and 4 manganese. This complex closely resembles that isolated from higher plants (Yamada et al., 1987), further indicating the similarity of PSII between higher plants and cyanobacteria.

Redox-Active Tyrosines, Y_D and Y_Z. Figure 7 shows the room temperature EPR spectra of the redox-active tyrosines, Tyr160 (Y_D) of the D2 polypeptide (Barry & Babcock, 1987; Debus et al., 1988a; Vermaas et al., 1988) and Tyr161 (Y_Z) of the D1 polypeptide (Debus et al., 1988b; Metz et al., 1989), recorded, respectively, in oxygen-evolving PSII core complexes and in the same complex inactivated by Tris wash. In the presence of 0.3 mM potassium ferricyanide, the PSII complex exhibits in the dark a typical $Y_D^{\bullet}$ EPR spectrum (Babcock & Sauer, 1973) with the characteristic partially resolved hyperfine structure (Figure 7A, solid line). No PSI P_{700}^+ signal (7–8-G line width at $g = 2.0025$) (Beinert et al., 1962), normally formed in the presence of ferricyanide, is found in this spectrum, indicating that the core complex is free of PSI contamination at the EPR-detectable level. The spectrum recorded under illumination (Figure 7A, dashed line) is very similar to the dark spectrum except for minor changes at the center. Such changes may arise from chlorophyll radicals of PSII, possibly accessory chlorophylls in the D1/D2 polypep-

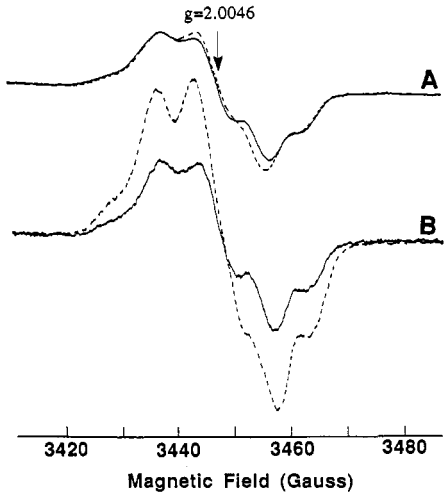

FIGURE 7: Room temperature EPR spectra of the redox-active tyrosines in the purified oxygen-evolving PSII core complex (A) and of the same following Tris-wash inactivation of the oxygen-evolving complex (B). Spectra were recorded either in the dark (solid line) or under illumination (dashed line). See Materials and Methods for illumination conditions. The chlorophyll concentrations for (A) and (B) are 1.0 and 0.93 mg of Chl/mL, respectively. The scale factors for (A) and (B) are 1 and 1.5, respectively. Instrument conditions: microwave power, 4 mW; field modulation, 4 G; time constant, 1 s; scan time, 3 min; single scan.

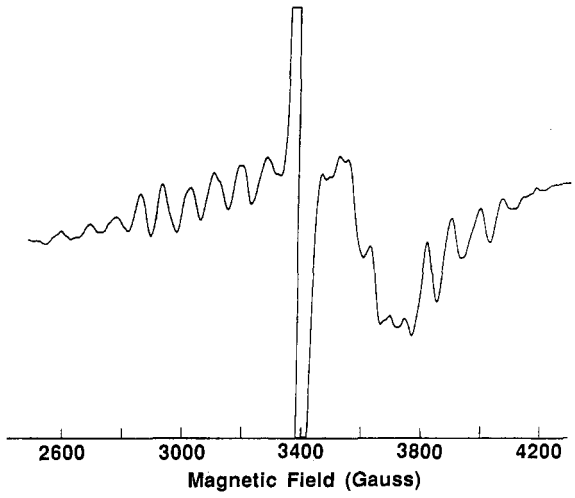

FIGURE 8: Light minus dark difference EPR spectrum of the S_2 state multiline signal of the oxygen-evolving complex in the purified PSII core complex from *Synechocystis* 6803. See Materials and Methods for illumination conditions. The chlorophyll concentration is 1.3 mg/mL. Instrument conditions: microwave power, 32 mW; time constant, 300 ms; number of scans, 4; field modulation, 20 G; microwave frequency 9.483 GHz.

tides, which could be oxidized under strong illumination. Such PSII radicals have been widely observed (Visser et al., 1977; Malkin & Bearden, 1983; Miller & Brudvig, 1990; Thompson & Brudvig, 1988; Nugent et al., 1990). The light and dark spectra show nearly identical amplitudes of the redox-active tyrosine signal at lower and higher fields. This is the expected result if the rereduction of $Y_Z^\bullet$ by the water-oxidizing system is rapid relative to the rate of Y_Z photooxidation. This result is consistent with the flash determination of 38 PSII Chl/OEC, arguing that practically 100% of the purified oxygen-evolving PSII core complexes contain an intact and active manganese cluster. This experiment also indicates that the present preparation is quite stable to room temperature illumination. Figure 7B shows the EPR spectrum of the PSII core complexes obtained following Tris wash inactivation of the oxygen-evolving complex. While the illumination conditions are the same as in Figure 7A, there is now an approximately 95% increase in the signal upon illumination. This light-induced increase is caused by the accumulation of the $Y_Z^\bullet$ radical, which now has a much longer lifetime upon inactivation of the oxygen-evolving complex. As in Figure 7A, the appearance of a small contribution from a narrow signal, possibly arising from a PSII chlorophyll radical, is evident in the spectrum of the Tris-washed sample, recorded under illumination (Figure 7B). The near one-to-one spin concentration ratio of $Y_D^\bullet$ to $Y_Z^\bullet$ observed in Figure 7B also indicates that the light intensity used in those experiments is saturating.

State S_2 Multiline Signal. The functional oxygen-evolving complex normally exists in five different oxidation states (S_0–S_4), where the subscript corresponds to the number of stored oxidizing equivalents (Joliot et al., 1969; Kok et al., 1970). One of these, the S_2 state, shows a characteristic broad (~1700 G wide) multiline signal centered at $g = 2$ (Dismukes & Siderer, 1981; Hansson & Andreasson, 1982; Brudvig et al., 1983; Zimmermann & Rutherford, 1984). A light minus dark S_2-state multiline EPR spectrum recorded after 198 K illumination of a dark-adapted PSII core complex is shown in Figure 8. Superimposed on this signal is $Y_D^\bullet$ (truncated, 20 G wide at 3380 G) and Q_A^- (broad feature at 3600 G). Potassium ferricyanide (50 μM) was added to the sample to assure oxidation of Q_A, found to be partially reduced in purified PSII particles in the dark (Rögner et al., 1990). The amplitude of the multiline signal is >75% of that observed in PSII-enriched spinach BBY preparations (Berthold et al., 1981; Ford & Evans, 1983) on a per center basis, assuming a 5-fold higher Chl/PSII reaction center in BBYs compared to the present preparation [e.g., see Buser et al. (1992)]. The signal amplitudes were compared both by measuring individual lines of the S_2 EPR multiline signal and by measuring the S_2 multiline signal field swept spin-echo spectrum (R. David Britt, personal communication). The hyperfine structural features of the S_2 multiline spectrum are nearly identical to those previously observed in higher plants (Dismukes & Siderer, 1981; Brudvig et al., 1983) and the cyanobacteria *Synechococcus sp.* (McDermott et al., 1988; Tang et al., 1993) and *Synechocystis* 6803 (Noren et al., 1991; Kirilovsky et al., 1992). The strong multiline EPR signal from this PSII complex is enabling us to use the magnetic resonance techniques of ESEEM and ENDOR to directly investigate the protein coordination of the manganese cluster. A good ESEEM spectrum of the multiline signal, showing nitrogen and proton hyperfine components, has been observed from this core complex (Tang et al., 1994). We have recently used this purification procedure to isolate PSII preparations containing specifically labeled histidine from a histidine-tolerant strain of *Synechocystis* 6803. Using the EPR/ESEEM technique, we have succeeded in showing that histidine is a ligand to manganese of the water-oxidizing complex of PSII (Tang et al., 1994). We have already begun using this preparation to characterize site-directed mutants that show altered behavior of the oxygen-evolving complex and expect that it will play an important role in the biochemical and spectroscopic analyses of PSII in the wild-type strain and in site-directed mutants of the cyanobacterium *Synechocystis* 6803.

CONCLUSION

A new procedure has been developed to purify the oxygen-evolving PSII core complex from the cyanobacterium *Syn-*

echocystis PCC 6803. This procedure requires only a single purification step beyond detergent extraction of the thylakoid membrane and takes 12–13 h to complete. Biochemical and spectroscopic characterization of the preparation is described here, and the results show that the purified complex is highly purified and fully intact with regard to PSII electron transfer, including oxygen evolution. Considering both its purity and its activity, this is an excellent PSII preparation from *Synechocystis* PCC 6803, and suitable for biochemical and spectroscopic analyses of the large number of site-directed mutants already constructed in this strain.

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REFERENCES

Allen, J. P., Feher, G., Yeates, T. O., Komiya, H., & Rees, D. C. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8487–8491.

Babcock, G. T. (1987) in *New Comprehensive Biochemistry: Photosynthesis* (Amesz, J., Ed.) pp 125–158, Elsevier, Amsterdam.

Babcock, G. T., & Sauer, K. (1973) *Biochim. Biophys. Acta 325*, 483–503.

Babcock, G. T., & Sauer, K. (1975) *Biochim. Biophys. Acta 376*, 315–328.

Barr, R., & Crane, F. L. (1971) *Methods Enzymol. 23*, 372–408.

Barry, B. A., & Babcock, G. T. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7099–7103.

Beinert, H., Kok, B., & Hoch, G. (1962) *Biochem. Biophys. Res. Commun. 7*, 209–212.

Berthold, D. A., Babcock, G. T., & Yocum, C. F. (1981) *FEBS Lett. 165*, 156–162.

Boerner, R. J., Nguyen, A. P., Barry, B. A., & Debus, R. J. (1992) *Biochemistry 31*, 6660–6672.

Boussac, A., & Rutherford, A. W. (1988) *FEBS Lett. 236*, 432–436.

Brudvig, G. W., Casey, J. L., & Sauer, K. (1983) *Biochim. Biophys. Acta 723*, 366–371.

Burnap, R., Koike, H., Sotiropoulou, G., Sherman, L. A., & Inoue, Y. (1989) *Photosynth. Res. 22*, 123–130.

Buser, C. A., Diner, B. A., & Brudvig, G. W. (1992) *Biochemistry 31*, 11441–11448.

Cheniae, G. M., & Martin, I. F. (1970) *Biochim. Biophys. Acta 197*, 219–239.

Chua, N.-H. (1980) *Methods Enzymol. 69*, 434–446.

Cramer, W. A., Theg, S. M., & Widger, W. R. (1986) *Photosynth. Res. 10*, 393–403.

Debus, R. J. (1992) *Biochim. Biophys. Acta 1102*, 269–352.

Debus, R. J., Barry, B. A., Sithole, I., Babcock, G. T., & McIntosh, L. (1988a) *Biochemistry 27*, 9071–9074.

Debus, R. J., Barry, B. A., Babcock, G. T., & McIntosh, L. (1988b) *Proc. Natl. Acad. Sci. U.S.A. 85*, 427–430.

Deisenhofer, J., Epp, O., Miki, K., Huber, R., & Michel, H. (1985) *Nature 318*, 618–624.

Dekker, J. P., Ghanotakis, D. F., Plijter, J. J., Gorkom, H. J. V., & Babcock, G. T. (1984) *Biochim. Biophys. Acta 767*, 515–523.

Dekker, J. P., Bowlby, R. N., & Yocum, C. F. (1989) *FEBS Lett. 254*, 150–154.

de Vitry, C., Diner, B. A., & Lemoine, Y. (1987) in *Progress in Photosynthesis Research* (Biggins, J., Ed.) Vol. II, pp 105–108, Martinus Nijhoff Publishers, Hague, The Netherlands.

Diner, B. A., Petrouleas, V., & Wendoloski, J. J. (1991a) *Physiol. Plant. 81*, 423–436.

Diner, B. A., Nixon, P. J., & Farchaus, J. W. (1991b) *Curr. Optin. Struct. Biol. 1*, 546–554.

Dismukes, G. C., & Siderer, Y. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 274–278.

Eskins, K., Scholfield, C. R., & Dutton, H. J. (1977) *J. Chromatogr. 135*, 217–220.

Ford, R. C., & Evans, M. C. W. (1983) *FEBS Lett. 160*, 159–164.

Fotinou, C., Kokkinidis, M., Fritzsch, G., Haase, W., Michel, H., & Ghanotakis, D. F. (1993) *Photosynth. Res. 37*, 41–48.

Ghanotakis, D. F., Babcock, G. T., & Yocum, C. F. (1984a) *FEBS Lett. 167*, 127–130.

Ghanotakis, D. F., Topper, J. N., & Yocum, C. F. (1984b) *Biochim. Biophys. Acta 767*, 524–531.

Gounaris, K., Chapman, D. J., & Barber, J. (1989) *Biochim. Biophys. Acta 973*, 296–301.

Haag, E., Irrgang, K. D., Boekema, J. E., & Renger, G. (1990) *Eur. J. Biochem. 189*, 47–53.

Hansson, O., & Andreasson, L.-E. (1982) *Biochim. Biophys. Acta 679*, 261–268.

Harlow, E., & Lane, D. (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory: Cold Spring Harbor, NY.

Hiyama, T., & Ke, B. (1972) *Biochim. Biophys. Acta 267*, 160–171.

Ikeuchi, M., Yuasa, M., & Inoue, Y. (1985) *FEBS Lett. 185*, 316–322.

Joliot, P., Barbieri, G., & Chabaud, R. (1969) *Photochem. Photobiol. 10*, 309–329.

Joliot, P., Béal, D., & Frilley, B. (1980) *J. Chem. Phys. 77*, 209–216.

Katoh, S. (1988) *Methods Enzymol. 167*, 263–269.

Kirilovsky, D. L., Boussac, G. P., van Mieghem, F. J. E., Ducruet, J.-M. R. C., Sétif, P. R., Yu, J., Vermaas, W. F. J., & Rutherford, A. W. (1992) *Biochemistry 31*, 2099–2107.

Koike, H., Mamada, K., Ikeuchi, M., & Inoue, Y. (1989) *FEBS Lett. 244*, 391–396.

Kok, B., Forbush, B., & McGloin, M. (1970) *Photochem. Photobiol. 11*, 457–475.

Lichtenthaler, H. K. (1987) *Methods Enzymol. 148*, 350–382.

Malkin, R., & Bearden, A. J. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 294–297.

McDermott, A. E., Yachandra, V. K., Guiles, R. D., Cole, J. L., Dexheimer, S. L., Britt, R. D., Sauer, K., & Klein, M. P. (1988) *Biochemistry 27*, 4021–4031.

Mei, R., & Yocum, C. F. (1991) *Biochemistry 30*, 7836–7842.

Metz, J. G., Nixon, P. J., Roger, M., Brudvig, G. W., & Diner, B. A. (1989) *Biochemistry 28*, 6960–6969.

Michel, H., & Deisenhofer, J. (1988) *Biochemistry 27*, 1–7.

Miller, A.-F., & Brudvig, G. W. (1990) *Biochemistry 29*, 1385–1392.

Miyazaki, A., Shina, T., Toyoshima, Y., Gounaris, K., & Barber, J. (1989) *Biochim. Biophys. Acta 975*, 142–147.

Murata, N., Miyao, M., Omata, T., Matsunami, H., & Kuwabara, T. (1984) *Biochim. Biophys. Acta 765*, 363–369.

Nanba, O., & Satoh, K. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 109–112.

Nilsson, F., Gounaris, K., Styring, S., & Andersson, B. (1992) *Biochim. Biophys. Acta 1100*, 251–258.

Nixon, P. J., & Diner, B. A. (1992) *Biochemistry 31*, 942–948.

Nixon, P. J., Chisholm, D. A., & Diner, B. A. (1992a) in *Plant Protein Engineering* (Shewry, P. R., Gutteridge, S., Eds.) pp 93–163, Cambridge University Press, Cambridge, U.K.

Nixon, P. J., Trost, J. T., & Diner, B. A. (1992b) *Biochemistry 31*, 10859–10871.

Noren, G. H., Boerner, R. J., & Barry, B. A. (1991) *Biochemistry 30*, 3943–3950.

Nugent, J. H. A., Telfer, A., Demetriou, C., & Barber, J. (1990) *FEBS Lett. 255*, 53–58.

Omata, T., Murata, N., & Satoh, K. (1984) *Biochim. Biophys. Acta 765*, 405–405.
Pauly, S., & Witt, H. T. (1992) *Biochim. Biophys. Acta 1099*, 211–218.
Pauly, S., Schlodder, E., & Witt, H. T. (1992) *Biochim. Biophys. Acta 1099*, 203–210.
Piccioni, R. G., & Mauzerall, D. C. (1976) *Biochim. Biophys. Acta 423*, 605–609.
Rippke, R., Deruelles, J., Waterbury, J. B., Herdman, M., & Stanier, R. Y. (1979) *J. Gen. Microbiol. 111*, 1–61.
Rögner, M., Nixon, P. J., & Diner, B. A. (1990) *J. Biol. Chem. 265*, 6189–6196.
Ruffle, S. V., Donnelly, D., Blundell, T. L., & Nugent, J. H. A. (1992) *Photosynth. Res. 34*, 287–300.
Rutherford, A. W., Zimmermann, J.-L., & Boussac, A. (1992) in *The Photosystems: Structure, Function and Molecular Biology* (Barber, J., Ed.) pp 179–229, Elsevier Science Publishers, B.V., Amsterdam.
Sandusky, P. O., De Selvious, R. C. L., Hicks, D. B., Yocum, C. F., Ghanotakis, D. F., & Babcock, G. T. (1983) in *The Oxygen Evolving Complex of Photosynthesis* (Inoue, Y., Crofts, A. R., Govindjee, Murata, N., Renger, G., & Satoh, K., Eds.) pp 189–199, Academic Press, Tokyo.
Satoh, K. (1983) in *The Oxygen Evolving System of Photosynthesis* (Inoue, Y., Crofts, A. R., Govindjee, Murata, N., Renger, G., & Satoh, K., Eds.) pp 27–38, Academic Press, Tokyo.
Satoh, K., Ohno, T., & Katoh, S. (1985) *FEBS Lett. 180*, 326–332.
Svensson, B., Vass, I., Cedergren, E., & Styring, S. (1990) *EMBO J. 7*, 2051–2059.
Tamura, N., & Cheniae, G. M. (1985) *Biochim. Biophys. Acta 809*, 245–259.
Tang, X.-S., & Satoh, K. (1985) *FEBS Lett. 179*, 60–64.
Tang, X.-S., Sivaraja, M., & Dismukes, G. C. (1993) *J. Am. Chem. Soc. 115*, 2382–2389.
Tang, X.-S., Diner, B. A., Larsen, B. S., Gilchrist, M. L., Jr., Lorigan, G. A., & Britt, R. D. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 704–708.
Thompson, L. K., & Brudvig, G. W. (1988) *Biochemistry 27*, 6653–6658.
Trebst, A. (1986) *Z. Naturforsch. 41C*, 240–245.
van Gorkom, H. J. (1974) *Biochim. Biophys. Acta 347*, 439–442.
Vermaas, W. F. J., Rutherford, A. W., & Hansson, O. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8477–8481.
Vermaas, W. F. J., Charité, J., & Shen, G. (1990) *Z. Naturforsch. 45C*, 359–365.
Vernon, L. P. (1960) *Anal. Chem. 32*, 1144–1150.
Visser, J. W. M., Rijersberg, C. P., & Gast, P. (1977) *Biochim. Biophys. Acta 460*, 36.
Warncke, K., & Dutton, P. L. (1991) *Biophys. J. 59*, 146a.
Williams, J. G. K. (1988) *Methods Enzymol. 167*, 766–778.
Yamada, Y., Tang, X.-S., Itoh, S., & Satoh, K. (1987) *Biochim. Biophys. Acta 891*, 129–137.
Yamamoto, Y., Doi, M., Tamura, N., & Nishimura, M. (1981) *FEBS Lett. 133*, 265–268.
Yocum, C. F., Yerkes, C. T., Blankenship, R. E., Sharp, R. R., & Babcock, G. T. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7507–7511.
Zimmermann, J.-L., & Rutherford, A. W. (1984) *Biochim. Biophys. Acta 767*, 160–167.