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Technical communication

Purification of highly active oxygen-evolving photosystem II from *Chlamydomonas reinhardtii*

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Abstract

A method is described for the isolation and purification of active oxygen-evolving photosystem II (PS II) membranes from the green alga *Chlamydomonas reinhardtii*. The isolation procedure is a modification of methods evolved for spinach (Berthold et al. 1981). The purity and integrity of the PS II preparations have been assesssed on the bases of the polypeptide pattern in SDS–PAGE, the rate of oxygen evolution, the EPR multiline signal of the S_2 state, the room temperature chlorophyll *a* fluorescence yield, the 77 K emission spectra, and the P700 EPR signal at 300 K. These data show that the PS II characteristics are increased by a factor of two in PS II preparations as compared to thylakoid samples, and the PS I concentration is reduced by approximately a factor ten compared to that in thylakoids.

Abbreviations: BSA – bovine serum albumin; Chl – chlorophyll; DCBQ – 2,6-dichloro-*p*-benzoquinone; DCMU – (diuron) 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DMQ – 2,5-dimethyl-*p*-benzoquinone; EDTA – ethylenediamine tetraacetic acid; EPR – electron paramagnetic resonance; Hepes – N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; MES – 2-[N-Morpholino]ethanesulfonic acid; OEE – oxygen evolving enhancer; PS II – photosystem II; SDS–PAGE – sodium dedocyl sulfate polyacrylamide gel electrophoresis

Introduction

Most studies of the oxygen-evolving complex from photosystem II (PS II) have focused on spinach (Kok et al. 1970, Cheniae and Martin 1970, Berthold et al. 1981, Dismukes et al. 1983, Casey and Sauer 1984, Ghanotakis et al. 1985, Beck et al. 1985). However, the oxygen evolving activity of PS II of spinach varies with the season (Coleman 1987, see pp 56–57). In contrast to spinach, algae can be grown in the laboratory under sterile and controlled environmental conditions (e.g., culture medium, temperature and light). The green alga *Chlamydomonas reinhardtii* is of particular interest because it is becoming amenable to molecular genetics, and several photosynthetic mutants of this organism are available (see e.g., Togasaki and Whitmarsh 1986, Erickson et al. 1989). Thus, this algal system is expected to be useful for comparative studies with higher plants and cyanobacteria.

The mechanism of O_2-evolution in *Chlamydomonas reinhardtii* has not been studied extensively due to difficulties in obtaining active and stable oxygen-evolving PS II preparations even though various degrees of success have been

obtained with thylakoid membranes (Gorman and Levine 1966, Curtis et al. 1975, Yannai et al. 1976, Spiller and Boger 1980, Mendiola-Morgenthaler et al. 1985). The PS II particles isolated from *Chlamydomonas* by Diner and Wollman (1980) do not evolve oxygen.

In this paper, we present a method for the isolation of oxygen-evolving PS II membranes from *Chlamydomonas reinhardtii* with an activity that is comparable with that of PS II from spinach, prepared under similar conditions. The purity and integrity of the PS II preparation is evident, among other things, from the polypeptide pattern in SDS–PAGE, more than ten fold reduction of PS I fluorescence emission (715 nm, 77 K), the 2-fold increase in room temperature fluorescence yield of chlorophyll *a*, and the enhanced oxygen evolution activity and low-temparature S_2 state manganese EPR signal as compared to those in thylakoid preparations. The PS II samples have 276 ± 23 Chl per 4 manganese, and thus presumably per reaction center.

Materials and methods

The cells of *C. reinhardtii* wild type were grown in liquid Tris-acetate-phosphate medium (Gorman and Levine 1965) with continuous bubbling of sterile air under bright fluorescent lamps (8×15 W) and a weak tungsten lamp (1×15 W). After 4 days' growth at 26 ± 2°C, the air pump was turned off and the cells were kept in the dark for 30 min. The cells were centrifuged for 5 min at $700 \times g$. The pelleted cells were resuspended in 20 mM Hepes (pH 7.5), 0.35 M sucrose, and 2 mM $MgCl_2$ (HSM buffer) and recentrifuged for 7 min at $750 \times g$.

All steps in the isolation procedure described below were performed in dim light and the sample was kept on ice throughout the process. Our procedure is a modification of the well-known method of Berthold et al. (1981) developed for spinach; it is adapted here for *Chlamydomonas*. An important change was the need to use higher (30 mg Triton/mg Chl) Triton concentration than used in spinach (25 mg Triton/ mg Chl) for obtaining PS I-free PS II membranes.

The resulting pellet was resuspended in the HSM buffer at a chlorophyll concentration of 1 mg/ml and passed twice through a French pressure cell at 4000 lb/in^2 (Diner and Wollman 1980) and centrifuged for 15 min at $50\,000 \times g$. The resulting pellet was resuspended, using a homogenizer, in 0.4 M sucrose, 20 mM Hepes (pH 7.5), 5 mM $MgCl_2$, 5 mM EDTA, and 1 g/l BSA, and centrifuged for 20 s at $1200 \times g$. The supernatant was centrifuged again at $11\,000 \times g$ for 12 min. The pellet was resuspended in 20 mM MES (pH 6.0), 15 mM NaCl, 5 mM $MgCl_2$, 5 mM EDTA, and 1 g/l BSA and centrifuged for 5 min at $12\,500 \times g$.

The resulting pellet was resuspended in the buffer just described at a chlorophyll concentration of 2.86 mg/ml, and 20% Triton buffer (20% w/v Triton X-100, 20 mM MES (pH 6.0), 15 mM NaCl, and 5 mM $MgCl_2$) was added dropwise (final concentration of Triton X-100 was 30 mg/mg Chl, at a chlorophyll concentration of 2 mg/ml) while the suspension was placed on ice in the cold room (4°C), stirring at low speed. After the suspension had been stirred for 30 min from the beginning of Triton buffer addition, it was centrifuged for 20 min at $40\,000 \times g$ and then washed twice with a medium containing 20 mM MES (pH 6.0), 15 mM NaCl, 5 mM $MgCl_2$, 5 mM EDTA, and 1 g/l BSA by centrifugation for 20 min at $40\,000 \times g$. The resulting pellet was resuspended in 0.4 M sucrose, 15 mM NaCl, 5 mM $MgCl_2$, and 20 mM MES (pH 6.0). The final samples were frozen quickly and stored in liquid nitrogen.

Gel electrophoresis was carried out in a 8–15% gradient or a 7% SDS-acrylamide resolving gel with 4 M urea (Laemmli 1970). The amounts of polypeptides were determined from the peak heights in the densitogram given by a densitometer.

The oxygen evolution activity was assayed on a Yellow Springs Instrument Model 53 O_2 monitor equipped with a Teflon-membrane covered Clark-type O_2 electrode in the presence of 1 mM $K_3Fe(CN)_6$ and 0.5 mM DCBQ as electron acceptors. The exciting light was provided by a Kodak 4200 projector lamp filtered through 1 inch water and a Corning yellow glass, CS2-71.

EPR spectroscopy was carried out on a Bruker ER-200D X-band EPR spectrometer. Low temperature EPR spectra were taken in a TE102 cavity by using an Oxford ESR helium flow

cryostat. The EPR spectra at room temperature were taken with 0.3 ml Wilmad Suprasil cells in a TM110 cavity.

The buffer of the EPR sample was the same as the final resuspension buffer. The 'light' samples for S_2 state EPR spectra were pre-illuminated for 2 min with a 1000 W projector lamp (filtered through 10 cm water and a Corning red glass, CS2-73) at 200 K (CO_2-ethanol bath) and kept in the dark for 30 min at 0°C and then illuminated for 5 min at 216 K with the same set-up right before taking spectra. The temperature was measured with a copper-constantan thermocouple. The 'dark' samples were pre-illuminated at 200 K and kept in the dark for 2 h at 0°C.

All samples for chlorophyll *a* fluorescence were diluted to a final chlorophyll concentration of 10 μg/ml. The 77 K-fluorescence spectra and fluorescence transient measurements were made using a laboratory-built spectrofluorometer (Blubaugh 1987). The exciting light was provided by a Kodak 4200 projector with the light filtered by two Corning blue glass filters (CS4-76 and CS5-57). Fluorescence emission was detected by a S-20 EMI 9558B photomultiplier through a Bausch and Lomb monochromator with 1 mm slit widths (bandpass: 3.3 nm) and protected from the exciting light by a red Corning filter (CS2-61). Signals were stored and analyzed by a Biomation 805 waveform recorder and a LSI-11 computer.

The amount of Mn in the PS II membranes was measured on a flame atomic absorption spectrophotometer, model IL951 of Thermo Jarrell Ash Corporation. A nitrous oxide-acetylene flame was used at the wavelength of 279.5 nm with a bandwidth of 0.5 nm. Since the sample was viscous, the technique of standard addition was used to correct for physical interference.

Results and discussion

In *C. reinhardtii*, the main polypeptides associated with the PS II are the following (Chua et al. 1975a,b, Wollman and Delepelaire 1984, De Vitry et al. 1984, Rousselet and Wollman 1986, Erickson et al. 1986, and Mayfield et al. 1987, Harris 1989): Two polypeptides of 50 and 47 kD are apoproteins of the two chlorophyll *a*-protein complexes CP III and CP IV, respectively, equivalents of CP47 and CP43 of higher plants; two polypeptides in the 30 kD region are D1 and D2; and three extrinsic polypeptides of 33, 23 and 17 kD are labeled as OEE1, OEE2, and OEE3, respectively.

Figure 1A is a 8–15% gradient gel. Lane 1 is the pattern of molecular weight standards, lane 2 is for thylakoids, and lane 3 is for isolated PS II membranes. Although exact quantitation of the polypeptide content from densitometric traces of Coomassie blue-stained gels is difficult due to overlapping bands (Miller et al. 1987), several points may be noted: CP I from PS I (70 kD) and CP 0 from peripheral antenna of PS I (22 kD) are lowered drastically ($\approx$10%); CP III and CP IV from PS II (50 kD and 47 kD) are enhanced about 2.5 fold; the 33 kD (OEE1), 23 kD (OEE2) and 17 kD (OEE3) are enhanced in PS II compared to thylakoids. Table 1 shows the ratios of the amounts of major polypeptides of PS II membranes vs. thylakoids which were determined from the peak heights of the densitometric traces of Fig. 1A. Figure 1B further demonstrates the purification of PS II mem-

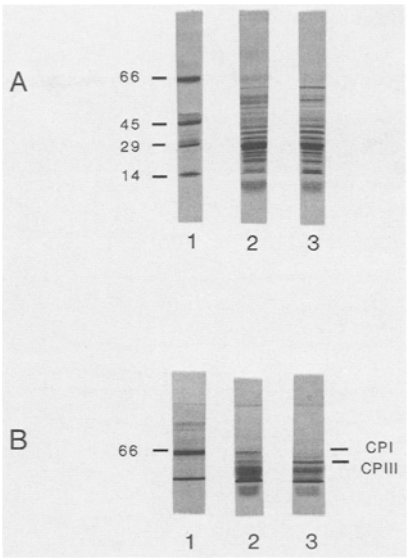

Fig. 1. SDS-polyacrylamide gel electrophoretic (PAGE) patterns of thylakoids and the purified oxygen-evolving PS II membranes from *C. reinhardtii*: (A) 8–15% gradient gel; (B) 7% gel: (1) molecular weight standards; (2) thylakoids, 18 μg (25 μl of 0.75 mg Chl/ml); (3) PS II membranes, 18 μg. CP I, a PS I component; CP III, PS II component.

Table 1. The ratios of the amounts of major polypeptides of PS II membranes vs. thylakoids which were estimated from the peak heights of the densitometric traces of Fig. 1A

	CP 0	CP I	CP III	CP IV	OEE1	OEE2	OEE3
M.W. (kD)	22	70	50	47	33	24	17
PS II/Thy (%)	10	9	262	244	182	171	175

branes. It is a 7% SDS–PAGE which resolves the higher molecular weight polypeptides (region of 50–70 kD). The 70 kD band of PS I (CP I) is absent and the 50 kD band of PS II (CP III) is enhanced in the gel of the PS II preparation.

Another test of the separation of PS II from PS I is based on room temperature EPR as shown in Fig. 2. The difference between illuminated and dark spectra indicates the $P700^+$ signal from PS I. A comparison of EPR data in thylakoids (panel A) with those of PS II membranes (panel B) shows that the latter have less than 10% of the P700 in the former.

The chlorophyll *a* fluorescence spectra and transients are indicators of the purity of the

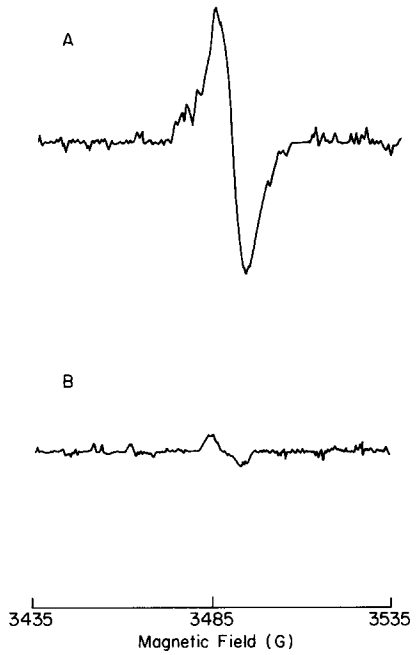

Fig. 2. Room temperature EPR spectra of 'light–dark' from *C. reinhardtii* ([Chl] = 2 mg/ml): (A) thylakoids; (B) PS II membranes. Instrumental conditions: microwave frequency = 9.70 GHz, microwave power = 20 mW, gain = 1.6×10^6 and modulation amplitude = 8 G.

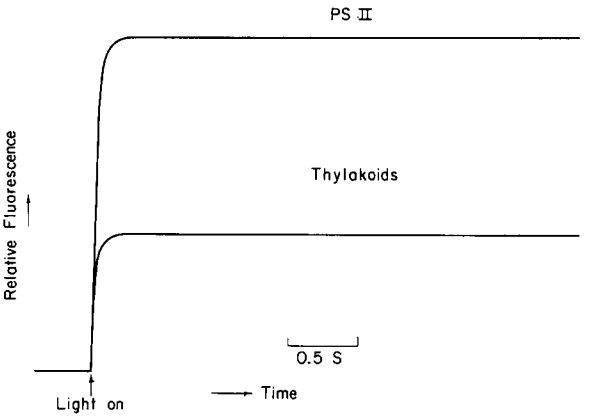

Fig. 3. Maximum Chl *a* fluorescence (F_{max}) at room temperature of *C. reinhardtii* thylakoids and PS II membranes, measured at 685 nm. Chlorophyll concentration was 10 μg/ml in 20 mM MES (pH 6.0), 15 mM NaCl, 5 mM $MgCl_2$, 0.4 M sucrose and 10 μM DCMU.

PS II. As shown in Fig. 3, the maximum fluorescence intensity, F_{max}, of PS II on chlorophyll *a* basis, measured at 685 nm, is 2.3 times that of the thylakoids. Since the ratio of PS II:PS I is approximately 1:1 in thylakoids, and PS I Chl *a* fluorescence at room temperature is extremely weak (see e.g., Briantais et al. 1986), the ratio of fluorescence yield for the same concentration of chlorophyll between thylakoids and PS II is expected to be approximately 1:2, as observed. Figures 4A and B show the uncorrected 77 K fluorescence spectra of PS II and thylakoids, respectively. In thylakoid membranes, fluorescence maxima occur at 685 nm (PS II; from the antenna complex labeled as CP IV), 695 nm (PS II; from the antenna complex labeled as CP III) and 715 nm (from a PS I complex) (Wollman and Delepelaire 1984, Briantais et al. 1986). However, in the emission spectrum of PS II membranes, the peak at 715 nm, originating in a long-wavelength antenna Chl *a* complex of PS I, is reduced to 10%. This confirms that we have succeeded in isolating a highly enriched PS II membrane preparation from *C. reinhardtii*.

The oxygen evolution activity of the thylakoid membranes in saturating yellow light from a Kodak 4200 projector right before Triton treatment was 350 ± 10 mol O_2/mg Chl per h (cf. Yamamoto 1988) and that of PS II membranes was 650 ± 15 μmol O_2/mg Chl per h in the presence of 0.5 mM DCBQ and 1 mM $K_3Fe(CN)_6$ as

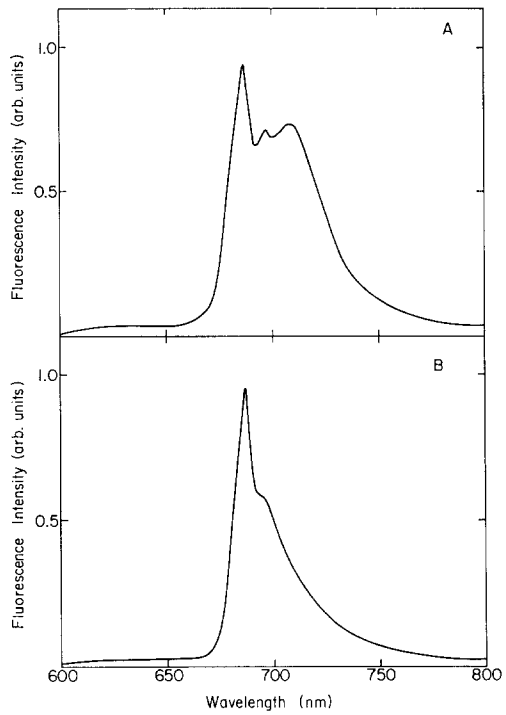

Fig. 4. 77 K fluorescence emission spectra of thylakoids and PS II membranes of *C.reinhardtii*. Chl concentration and the buffer were the same as in Fig. 3. (A) thylakoids; (B) PS II membranes.

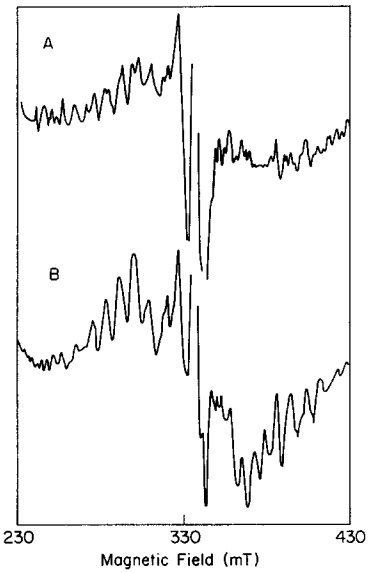

Fig. 5. EPR multiline difference spectra of the S_2 state ('light') minus the S_1 state ('dark') of (A) thylakoids and (B) PS II membranes of *C. reinhardtii* ([Chl] = 6 mg/ml) illuminated at 216 K. The method of preparation of the 'dark' and 'light' samples is given in the text. Instrumental conditions: microwave frequency = 9.46 GHz, microwave power = 0.80 mW, modulation frequency = 100 kHz, modulation amplitude = 20 G and temperature = 6.5 K. Smoothed average of 8 spectra.

electron acceptors. A spinach PS II sample, prepared by similar procedure as used for *Chlamydomonas*, produced 700 ± 20 μmol O_2/mg Chl per h.

The amount of Mn in *C. reinhardtii* PS II membranes with 2 mg Chl/ml was 1.77 ± 0.15 mg/l, equivalent to 4 Mn/276 Chl. The corresponding value for thylakoids was 1.2 ± 0.3 mg/l.

Figures 5A and B show the 'light' minus 'dark' EPR difference spectra near $g = 2$ of *Chlamydomonas* thylakoids and PS II membranes, respectively. They were obtained by subtracting the EPR spectra of the dark adapted samples from those of a sample illuminated at 216 K as described under Materials and Methods. The multiline difference signal is mainly due to the manganese in the S_2 state of the oxygen evolving complex except for the g_y component of cyt b_{559} near $g = 2.15$ (314 mT) and the Q_A^- signal near $g = 1.84$ (367 mT). The sample concentrations based on the content of chlorophyll were the same, and it is clear from Fig. 5 that the intensity of the multiline signal in PS II is doubled from that in thylakoids. This enhancement confirms further the enrichment of PS II in our preparations.

In this paper, we have documented the isolation of purified PS II membranes from *Chlamydomonas reinhardtii* and have demonstrated their usefulness for the study of the oxygen-evolving system, particularly of the S_2 state, as the preparations described here have comparable purity and activity as those from spinach.

Acknowledgements

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