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Short Communication

A Highly Active Oxygen-Evolving Photosystem II Preparation from the Cyanobacterium *Anacystis nidulans*[1]

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ABSTRACT

A highly active O_2-evolving Photosystem (PS)-II fraction has been isolated from the cyanobacterium, *Anacystis nidulans* R2, using an isolation buffer containing high concentrations of sucrose and salts and subsequent solubilization of the thylakoid membranes with the detergent Triton X-100. The isolated fraction had very high PSII activity (2500 micromoles O_2 per milligram chlorophyll per hour) and was largely depleted of PSI activity. Fluorescence emission spectra (77 K) and polypeptide analysis indicated that this preparation is highly enriched in PSII, but almost completely devoid of Cyt b_6-f and PSI complexes.

Cyanobacteria are photosynthetic prokaryotes that evolve O_2 from water. Similar to the eukaryotic algae and green plants, the membrane-bound PSII complex of cyanobacteria is involved in the process of O_2 evolution. The exact biochemical nature of the water-splitting complex is not well understood. Recently, however, O_2-evolving membrane fractions have been isolated from higher plants as well as from cyanobacteria (1, 3, 5, 12, 14). Unfortunately, the preparations from cyanobacteria differ greatly from one species to another and do not show long-term stability.

The principal light-harvesting structures for the PSII of cyanobacteria are phycobilisomes, pigment-protein complexes that are located on the surface of thylakoid membranes. Evidences from energy transfer analysis and ultrastructural studies indicate that the phycobilisomes form physical complexes with PSII particles located inside the thylakoid membrane (7). This report will detail the isolation procedure and preliminary characterization of an O_2-evolving complex from the cyanobacterium *Anacystis nidulans*. This O_2-evolving preparation has phycobilisomes attached to the PSII reaction centers and is highly active and extremely stable compared to the preparations reported earlier (5, 14).

MATERIALS AND METHODS

Cultures of *Anacystis nidulans* R2 were grown in 20-L carboys, as previously described (13). The cultures were aerated at 20 to 25°C, grown to mid- to late-log phase (3–4 d), and harvested. The procedures for the isolation of O_2-evolving membrane fragments were performed at 4°C. After harvesting, the cells were washed twice with SPC buffer[2] (6) and resuspended in the same buffer at a cell density of 0.12 g (wet weight)/ml (~350 μg Chl/ml). The cells were then passed twice through a chilled French pressure cell (Aminco) at 20,000 p.s.i. Triton X-100 was added dropwise to these broken cells with constant vortexing to a final concentration of 0.7% (w/v) giving a detergent to Chl ratio of 20:1. The mixture was immediately centrifuged at 30,000*g* for 30 min to remove unbroken cells and large fragments of membrane. Seven-ml fractions of the supernatant were then loaded on top of a sucrose step gradient (30 ml of 0.6 M, 3 ml of 1.0 M, and 15 ml of 2.0 M sucrose solutions in SPC buffer) and centrifuged for 2 h at 140,000*g* in a Ty 35 rotor (Beckman). The blue-green material that banded at the interface of 1.0 and 2.0 M sucrose layers was collected and diluted with PC buffer (0.5 M phosphate, 0.3 M citrate, pH 6.8) to a final sucrose concentration of 0.5 M. The diluted sample was centrifuged at 125,000*g* for 1 h; this yielded a blue-green pellet of PSII particles. The pellet was finally dissolved in a minimal volume of SPC buffer. All assays for optical measurements and electron transport activities were done within 4 to 6 h of the isolation, whereas part of the material was frozen at −80°C for later analysis of protein composition. Phycobilisomes were isolated according to Yamanaka *et al.* (16).

PSI and PSII activities were measured by monitoring the uptake or evolution of O_2 at 25°C, using a Clark type O_2 electrode (Yellow Spring Instruments). A Labsource QH 150 fiber optic illuminator was used to supply saturating, cool white light. H_2O→DCBQ/FeCN and DAD/Asc→MeV assays to measure PSII and PSI activities, respectively, were performed as in Guikema and Sherman (8) whereas H_2O→SiMo assays to measure PSII activity were made in the presence of 100 μM SiMo (11). The assay buffer was SPC, and each assay mixture contained 0.5 to 1.5 μg of Chl. Absorption spectra were recorded at room temperature using an Aminco-Chance DW2 dual wavelength spectrophotometer. Data from the spectra were directly used to estimate the Chl and phycocyanin contents of different samples, using the equations of Arnon *et al.* (2). Fluorescence emission spectra of undiluted samples in SPC buffer at 77 K were recorded on an SLM 8000 spectrofluorimeter (SLM Co., Urbana, IL) and corrected using correction factors supplied by the manufacturer.

For the analysis of polypeptide composition of different fractions, samples in SPC buffer were extensively dialyzed against large volumes of 10 mM Tricine-NaOH (pH 7.5) buffer. The dialyzed samples were then analyzed by electrophoresis in an LDS-polyacrylamide gel (10–20% acrylamide gradient) at 1.5 w

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[2] Abbreviations: SPC buffer, 0.5 M sucrose, 0.5 M phosphate, 0.3 M citrate, pH 6.8; DCBQ, 2,6-dichloro-*p*-benzoquinone; FeCN, ferricyanide; DAD, diaminodurene; Asc, ascorbate; MeV, methyl viologen, SiMo, silicomolybdate; LDS, lithium dodecyl sulfate; TMBZ, 3,3′,5,5′-tetramethylbenzidine.

Table I. *Photochemical Activities of Membrane Fractions*

PSI and PSII activities were measured as described in "Materials and Methods." The numbers represent the means of measurements of seven preparatons.

Fraction	Rates of O_2 Evolution or Uptake	
	PSII ($H_2O \rightarrow$DCBQ/FeCN)	PSI (DAD/Asc→MeV)
	μmol O_2 mg^{-1} Chl h^{-1}	
Thylakoid membranes	370	780
Triton X-100 supernatant	630	400
O_2-evolving fraction	2500	<50

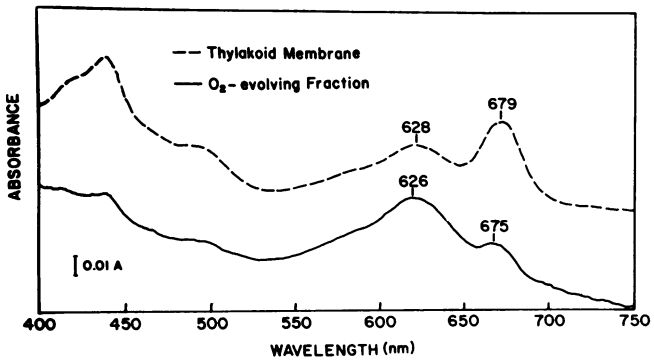

FIG. 1. Absorption spectra of *A. nidulans* thylakoid membranes with attached phycobilisomes (- - -) and the O_2-evolving fraction (——). The samples were suspended in SPC buffer, pH 6.8 (see text). Absorbance is in relative units.

at 4°C for 16 h, as described in Guikema and Sherman (10). Cyt were detected by their heme-dependent peroxidase activity in the presence of TMBZ and H_2O_2 as described previously (9). The gels were subsequently stained with silver using a protocol modified from Wray *et al.* (15).

RESULTS AND DISCUSSION

The principal light harvesting structures for PSII in cyanobacteria are the phycobilisomes, water-soluble pigment-protein complexes that are attached to the thylakoid membrane. During the isolation of an O_2-evolving PSII complex, we wanted to retain the functional attachment of phycobilisomes to the membranes for two different reasons. First, we wanted to study the PSII in its most native state, and this required the presence of its antenna structure. Moreover, studies on O_2-evolving PSII complexes from chloroplasts have demonstrated the existence of an extrinsic complex attached to the thylakoid membrane (12). Hence, conditions which maintain the functional attachment of the phycobilisomes to PSII could also stabilize other extrinsic components associated with the O_2-evolving complex. To achieve these goals, we have used the high salt conditions (SPC buffer) of Gantt *et al.* (6) to isolate *A. nidulans* thylakoid membrane fractions that had functionally attached phycobilisomes. The PSI and PSII activities of membrane fractions at different stages during the isolation procedure are given in Table I. The Triton X-100 solubilized membrane fraction had an 1.7-fold enhancement in the rate of PSII-associated O_2 evolution and a 2-fold decrease in PSI activity. The final O_2-evolving fraction had a 6.75-fold enrichment in the PSII activity, whereas its PSI activity was only 7% of that of the intact membranes. The PSII activity was completely inhibited by the presence of either 10 μM DCMU or 20 mM hydroxylamine. Monitoring PSII activity as $H_2O \rightarrow$SiMo gave rates as high as 2400 μmol O_2 mg^{-1} Chl h^{-1}. This reaction was insensitive to 10 μM DCMU but was completely inhibited by 20 mM hydroxylamine, as expected for a completely functional PSII complex (11). The half life of the preparation at 4°C

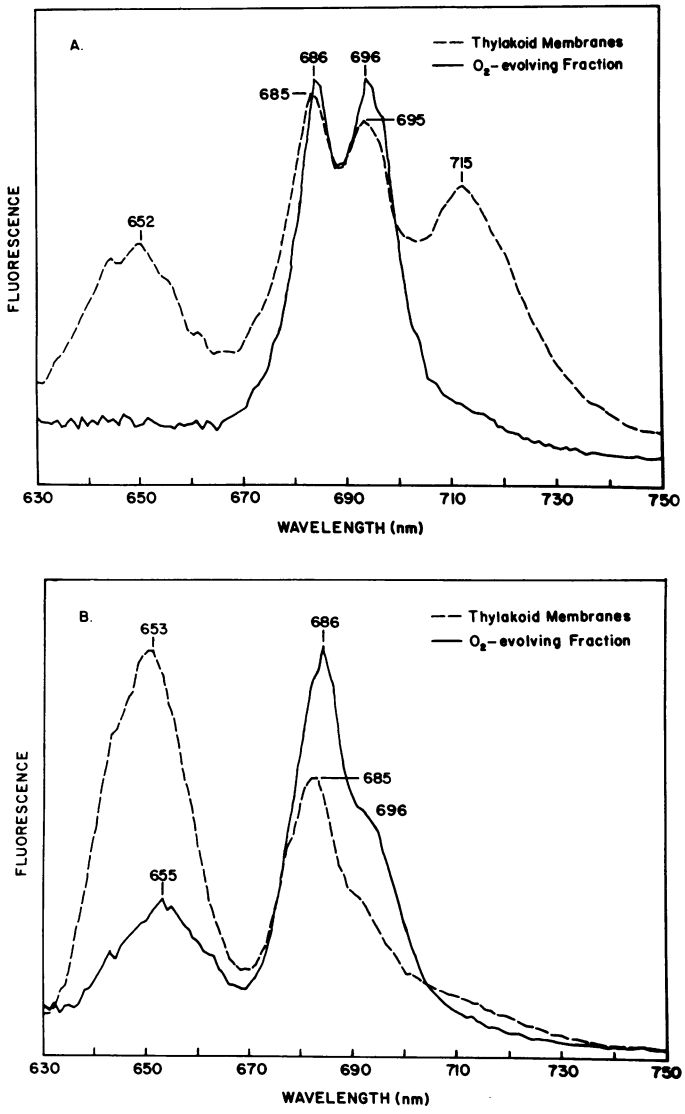

FIG. 2. Corrected 77 K fluorescence emission spectra of *A. nidulans* thylakoid membranes with attached phycobilisomes (- - -) and the O_2-evolving PSII fraction (——). The samples were suspended in SPC buffer (pH 6.8) only. Fluorescence is in relative units. A, Spectra of samples excited at 435 nm; B, spectra of samples excited at 620 nm.

was approximately 3 d (data not shown). However, it could be stored at −80°C for at least 2 months, without losing more than 30% of initial activity.

The duration of centrifugation on the sucrose step gradient ("Materials and Methods") was crucial. After 2 h, green material containing PSI material starts to come down and contaminate the PSII preparations. We have routinely used 1.5 to 2 h of

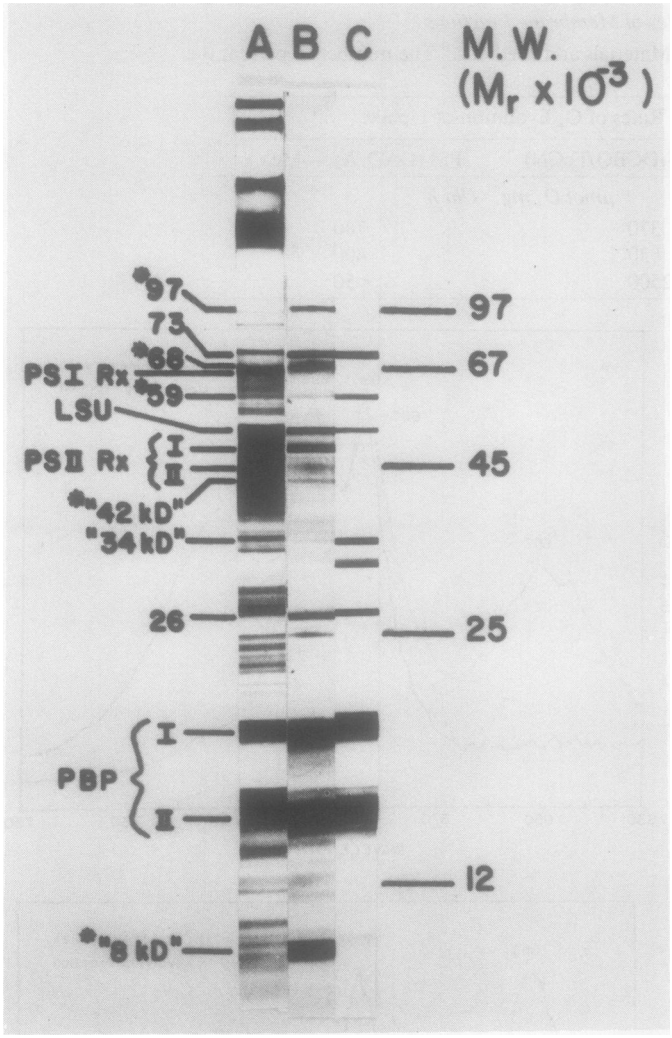

FIG. 3. Polypeptide profiles of (A) *A. nidulans* thylakoid membranes with attached phycobilisomes, (B) O_2-evolving PSII fraction, and (C) purified phycobilisomes analyzed on a 10 to 20% gradient LDS-polyacrylamide gel and stained with silver. The numbers on the left indicate apparent mol wt in kD. The numbers with * denote the polypeptides that are enriched in the O_2-evolving PSII fraction (lane B) but whose functions are not yet known. Abbreviations used: LSU, large subunit of ribulose-bisP carboxylase; PBP, phycobiliproteins; PSIRx, PSI reaction center polypeptide; PSIIRx, PSII reaction center polypeptide. Amount of proteins loaded in different lanes are 40 μg in lane A and 20 μg in each of lanes B and C.

centrifugation at this step to obtain our purest preparations.

The room temperature absorption spectra of whole membranes and the O_2-evolving fractions are shown in Figure 1. The O_2-evolving preparation had the red absorption peak of Chl shifted to 675 nm, which was similar to the spectral shift obtained with a non-O_2-evolving PSII fraction from another cyanobacterium, *Synechococcus cedrorum* (13). Also noticeable was the enrichment of the 626 nm peak arising from phycobilisomes; the ratios of phycocyanin to Chl was ~14 as compared with ~3.5 for unfractionated membranes.

The 77 K fluorescence emission spectra of membrane fractions are shown in Figure 2. With Chl excitation (435 nm), no 716 nm peak was observed (Fig. 2A). Since this peak arises from PSI-associated Chl complexes, the result implied that this O_2-evolving fraction was highly depleted of PSI. The main Chl emission peaks were at 686 and 696 nm. Phycocyanin excitation (620 nm) also gave rise to peaks at 686 and 696 nm, with a diminished peak at 655 nm as compared to whole membranes (Fig. 2B). These data showed that the phycobilisomes were functionally attached to the O_2-evolving membrane fractions.

The polypeptide compositions of the membrane fractions are shown in Figure 3. These protein-patterns were obtained by using a highly sensitive silver-staining procedure (15) that can detect many minor proteins that are not seen with conventional Coomasie-staining procedure. The O_2-evolving preparation had ~23 detectable polypeptides (lane B), including the polypeptides of the associated phycobilisomes (lane C). The few polypeptides that were unique to the O_2-evolving preparation (lane B) and not to the purified phycobilisomes (lane C) presumably belonged to the PSII and associated water-splitting complexes. The particle was enriched in a 48 kD polypeptide (one of the two Chl-binding proteins associated with the PSII reaction center) and depleted of the 67 kD apoprotein of PSI reaction center (10). A band at 8 kD was also strongly enriched in the preparation; experiments are now in progress to examine whether this protein is Cyt b_{559}. Three proteins between 32 and 36 kD as well as one at 25 kD were present in this preparation; similar polypeptides have been implicated in specific functional roles in water splitting and PSII (1). The large subunit of ribulose-bisP carboxylase (LSU) was usually present as a contaminant. TMBZ staining of gels indicated a complete lack of Cyt b_6, f, and c_{552} in this preparation (data not shown). This particle thus represents a highly enriched preparation for the analysis of numerous proteins involved with O_2 evolution and PSII function in cyanobacteria. The DCMU-binding protein in this DCMU-sensitive O_2-evolving preparation was not observed here since it stains poorly with silver (T. Bricker, personal communication). Two polypeptides migrating at 34 and 30 kD that are present in the phycobilisomes are absent or highly depleted in the O_2-evolving preparation. However, as evidenced from Figure 2B the phycobilisomes in this preparation are functionally coupled to the PSII reaction centers. Experiments are currently in progress to obtain an explanation for this apparent anomaly.

Clement-Metral and Gantt (4) have recently isolated a very similar O_2-evolving particle from the red alga *Porphyridium cruentum*. The presence of high concentrations of salts is crucial to the isolation of phycobilisome-containing membrane fractions both in their preparation and the one reported here. However, contrary to their observations, we have been able to isolate O_2-evolving membrane fractions by using a variety of nonionic detergents. The use of detergents like LDAO and Lubrol PX as well as of Triton X-100 yielded highly active O_2-evolving fractions (data not shown). Perhaps the presence of attached phycobilisomes forms a protective matrix around each PSII center and brief treatment with detergents selectively solubilizes the thylakoid membrane in such a way that the native structure of PSII remains intact. However, the Triton X-100 treated membranes yielded the cleanest PSII preparation as determined by the lack of detectable Cyt b_6-f and the presence of small amounts of PSI. Importantly, these O_2-evolving preparations have very high activity. Cyanobacteria are known to have only about 16% of their total Chl associated with PSII (7). Hence, the 6- to 7-fold enrichment in the PSII activities in the particles is physiologically meaningful, and implies that almost all of the Chl present in this fraction is functionally associated with active PSII centers.

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