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A HIGHLY ACTIVE OXYGEN-EVOLVING PHOTOSYSTEM II PREPARATION FROM SYNECHOCOCCUS LIVIDUS

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A PS II preparation highly active in oxygen generation was prepared from the cyanophyte, *Synechococcus lividus*. This preparation was enriched in Hill reaction activity, manganese, cytochrome *b*-559, and possessed only trace amounts of cytochrome *b*-563. This non-phosphorylating, visually clear preparation appears to be a promising system for the detailed study of Photosystem II.

Introduction

During the past several years, the process of oxygen generation has been under intense scrutiny. The structure and function of membrane components involved in the oxidation of water is still not clearly understood. A role for manganese in this process has been clearly established [1,2], yet efforts to isolate and reconstitute the proposed manganese-protein into the photosynthetic membrane have met with limited success, probably due to the lability of the water-splitting complex. In this report, we characterize an oxygen-evolving PS II preparation from the thermophilic cyanophyte, *Synechococcus lividus*. This soluble preparation is highly enriched in oxygen evolution capacity and appears to be a promising system for the eventual isolation of the oxygen-evolving complex.

Materials and Methods

Synechococcus lividus (Type II) was cultured at 52.5 °C in Castenholz media at pH 7.5 for 11–13 days at a constant light intensity of 19 $J \cdot m^{-2} \cdot s^{-1}$. Cells were harvested by centrifugation, washed in 0.5 M sorbitol, 10 mM Hepes-NaOH buffer at pH 7.50, 10 mM $MgCl_2$, 3.5 mM K_2HPO_4, and 1.5 mM $NaHPO_4$ (sorbitol-Hepes buffer), and resuspended in the above buffer containing 12.5 mM EDTA at a $\delta A_{440-540}$ of 0.20. After the cells were incubated for 1 h at 40 °C in the presence of 1 mg/ml lysozyme with gentle shaking, they were centrifuged for 10 min at 6000 *g* and resuspended in sorbitol-Hepes buffer.

Membranes were then prepared by hypotonic shock according to the procedure of Stewart and Bendall [3] except that cells were exposed to hypotonic medium for only 1 h. The PS II preparation was isolated as previously described [4], except that the membranes were treated with LDAO (critical micelle concentration = $2.2 \cdot 10^{-3}$ M) at chlorophyll a to detergent ratios of 2.5 to 3.1 : 1 (wt./wt.) at 0 °C in the dark for 40 min. The last step in the isolation consisted of addition of LDAO to the PS II preparation at a detergent to Chl a

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Abbreviations: PS, Photosystem; Chl, chlorophyll; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3,4-dichloro-phenyl)-1,1-dimethylurea; DMBQ, 2,5-dimethyl-*p*-benzoquinone; $K_3Fe(CN)_6$, potassium ferricyanide; LDAO, lauryldimethylamine oxide.

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ratio of 8 : 1, followed by incubation in the dark for 1 h on ice.

Oxygen generation was monitored polarographically under saturating light intensity with a Clark-type electrode at 32.5 °C with 2 mM potassium ferricyanide and 1 mM 2,5-dimethyl *p*-benzoquinone at pH 5.00. PS I electron transport was measured according to Izawa [5]. Photophosphorylation was measured in a medium containing 250 mM sucrose, 50 mM NaCl, 20 mM Tricine at pH 7.50 and 5 mM sodium phosphate/potassium phosphate at 32.5 °C by ascending chromatography [6].

Manganese content of charred samples was measured with a Perkin-Elmer Model 303 atomic absorption spectrophotometer equipped with a graphite furnace. Membrane-bound cytochromes were assayed by difference spectroscopy in 5 mM NaCl, 2 mM $MgCl_2$, 5 mM K_2HPO_4 and 50 mM Tricine at pH 7.00. Reductants and oxidants were the same as those used by Stewart and Bendall [4] except that a freshly prepared anaerobic solution of sodium hydrosulfite was used to reduce cytochrome *c*-549. P-700 content was measured by difference spectroscopy at pH 7.00 [7].

Chlorophyll *a* content was determined by absorbance at 663 nm in 80% acetone [8]. For protein assays, chlorophyll *a* was removed by the addition of 0.5 ml aliquots of cold 90% acetone. The solution was mixed and centrifuged for 15 min at 15 500 *g* at −5 °C. The blue-gray pellet was dissolved in deionized water and protein content was determined according to Lowry et al. [9] at 750 nm.

Phosphate was also determined by the method of Lowry et al. [10] in which samples were washed three times with deionized water, dried at 90 °C, charred, and treated with sulfuric acid to convert the organic phosphates to orthophosphoric acid. After drying, the samples were redissolved in deionized water and assayed for phosphate.

SDS-gel electrophoresis was carried out according to the procedure of Laemmli [11]. The separating gel contained 12.5% acrylamide and 6 M urea. Samples were pretreated for electrophoresis by freezing for 48 h at −30 °C followed by heating for 1 h at 50 °C to fully denature all chlorophyll-protein complexes. Heme-dependent peroxidase-like activity was assayed according to Guikema and Shermann [12] in the presence of 2-mercaptoethanol.

For estimation of molecular weight, the PS II preparation was rechromatographed at 4 °C on Sepharose 6B which had been equilibrated with 25% glycerol, 7.5 mM Hepes at pH 7.50, 10 mM $MgCl_2$, and 5 mM sodium phosphate/potassium phosphate (Hepes-glycerol buffer).

Results and Discussion

The main objective of this study was to characterize an oxygen-evolving PS II preparation from *S. lividus*, a thermophilic cyanophyte that lives at the apparent upper temperature limit for oxygenic photosynthesis [13]. Table Ia shows the yield of total oxygen-generating activity in thylakoid membranes, in the LDAO-released supernatant and corresponding pellet, and in the PS II preparation. Table Ia also shows that total activity for oxygen generation was increased in the PS II preparation and that LDAO released most of the oxygen-evolving enzyme into the supernatant after ultracentrifugation. Table Ib lists the corresponding Hill reaction activity rates observed during the isolation of the PS II preparation on both a Chl *a* and total protein basis. These Hill Reaction rates are similar to the values reported by Stewart and Bendall for a PS II preparation from *Phormidium laminosum* [3] (1342 μmol O_2 evolved/mg Chl *a* per h) and several-fold greater than the values reported for O_2-evolving preparations from spinach [14] (320 μmol O_2 evolved/mg Chl *a* per h).

Rates of Hill activity were approx. 4–5-fold greater in PS II preparations than thylakoid membranes (Table II). PS I electron transport rates were very low, approx. one-seventh the rate observed in membranes. DCMU inhibited approx. 90% of total electron transport through PS II, while 2 μM DBMIB inhibited approx. 9% of the electron transport. Addition of 5 mM ammonium chloride caused a reproducible inhibition rather than a stimulation of electron transport through PS II, suggesting that the preparation was uncoupled. This lack of stimulation with NH_4Cl was consistent with the observation that the PS II preparation yielded insignificant rates of photophosphorylation (3.3 nmol of ATP formed per μg Chl *a* per hour with $K_3Fe(CN)_6$ and 2,5-di-

TABLE IA

PURIFICATION

OXYGEN GENERATION ASSAYS CARRIED OUT AT pH 5.00 AND AT 32.5 °C WITH FeCy AND DMBQ

Chl *a* concentration was 5 μg/ml for the thylakoid membranes and 1 μg/ml for PS II preparation and active superntant. The column chromatography step consisted of passage of the active supernatant through a Sepharose 6B column at 4°C equilibrated with Hepes-glycerol buffer. Ultracentrifugation of the eluted green fractions was carried out at 181000 × *g* for 3 h.

Sample	nmol O_2 evolved $\cdot h^{-1}$	mg total protein	mg protein/ total Chl *a* ratio
Thylakoid membranes	207	26.9	2.50 10.7
LDAO treatment and centrif.			
Active supernatant	515	8.7	0.292 30.0
Less active pellet	98	18.1	2.21 8.2
Column chromatography, ultracentrifugation	249	4.33	0.225 19.2

TABLE IB

SPECIFIC ACTIVITIES

Sample	nmol O_2 evolved (μg Chl *a*)$^{-1}$ $\cdot h^{-1}$	nmol O_2 evolved (μg protein)$^{-1}$ $\cdot h^{-1}$
Thylakoid membranes	82.7	7.7
LDAO treatment and centrif.		
Active supernatant	1763	59
Less active pellet	44.4	5.4
Column chromatography and ultracentrifugation	1107	58

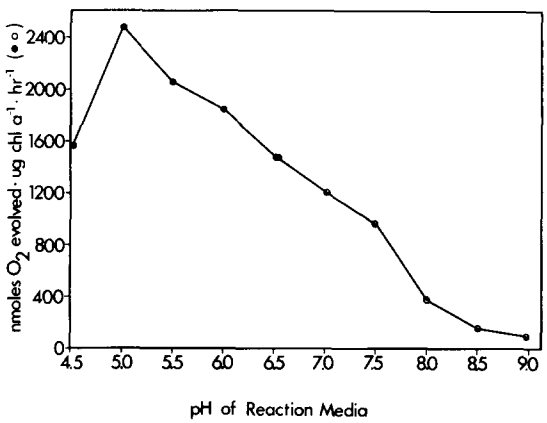

Fig. 1. Hill reaction activity in PS II preparation as a function of pH. ○, 10 mM Hepes; ●, 10 mM Mes.

methyl-*p*-benzoquinone and 0.8 nmol of ATP formed per μg Chl *a* per hour with pyocyanine/ascorbate as the electron acceptors). As expected, we have been unable to detect any light-induced proton pumping in the preparation [15].

Fig. 1 shows that the PS II preparation possessed a sharp and acidic pH optimum. If the pH optimum in uncoupled (leaky to protons) preparations for Hill activity reflects the pH of the water oxidation site as has been proposed [16,17], then this low pH optimum may be indicative of the pH at which oxygen generation occurs in vivo. The rate of oxygen generation rapidly decreased below pH 5, possibly due to a disturbance at the water oxidation site and P-680 [18] or to loss of manganese [19].

TABLE II

ELECTRON TRANSPORT IN THYLAKOID MEMBRANES AND PS II PREPARATIONS

DBMIB = 2 μM, DCMU = 10 μM. Chl *a* concentrations = 1 μg/ml for PS II prep. Thylakoid membranes measured at 45°C at pH 7.50.

System	Reaction	nm O_2 evolved or consumed: (μg Chl *a*)$^{-1}\cdot$(h)$^{-1}$
Thylakoids	H_2O to $K_3Fe(CN)_6$/DMBQ + NH_4Cl	312
PS II Prep	H_2O to $K_3Fe(CN)_6$/DMBQ	1830
	H_2O to $K_3Fe(CN)_6$/DMBQ + NH_4Cl [a]	1722
	H_2O to $K_3Fe(CN)_6$/DMBQ + DBMIB	1668
	H_2O to $K_3Fe(CN)_6$/DMBQ + DCMU	136
	DCMU + asc/DPIP to MeV	41

[a] Control value normalized to 1830 nmol O_2/μg Chl *a*/h.

Table III shows the results of manganese analysis in the PS II preparation. Based on the average results of four samples, two manganese atoms were found per 33 Chl *a* molecules. For comparison, thylakoid membranes yielded 0.750 atoms of manganese per 50 Chl *a* molecules. Analysis of membrane-bound cytochromes (Table III) indicated a high concentration of both high and low potential cytochrome *b*-559 while cytochrome *b*-563 in the PS II preparation was present in only trace amounts. Cytochrome *c*-549 was found in only two of four PS II preparations and this uncertainty may be due to either the specific conditions necessary to induce the difference spectrum or to the absence of cytochrome *c*-549 in some PS II preparations. A decrease in P-700 content was also observed in the PS II preparation, since thylakoid membranes yielded 5.06 molecules of P-700 per 1000 Chl *a* molecules. These results indicate that one is not just removing Chl *a* from the membrane but is actually enriching for PS II through detergent fractionation.

Fig. 2 shows an absorption spectrum of the PS II preparation. A side peak at 659 nm was observed which probably represented chlorophyll- or protein-bound core phycobilisome subunits of allophycocyanin, since this side peak is near the wavelength maximum of free allophycocyanin B from *Synechococcus* sp. [20]. The presence of allophycocyanin is consistent with an assumed role for a form of this pigment in oxygen generation in red algae [21].

TABLE III

MANGANESE, CYTOCHROME AND P-700 ANALYSIS IN PS II PREPARATIONS

Manganese values are atoms of Mn per 1000 molecules of Chl *a*. Cytochrome and P-700 values are molecules per 1000 molecules of Chl *a*.

Component	Concentration	Number of samples
Manganese	60.40	4
b-559 HP	13.19	4
b-559 LP	5.25	4
b-559 Total	18.44	4
b-563	0.06	3
c-549	4.94	2
f	3.22	4
P-700	1.90	1

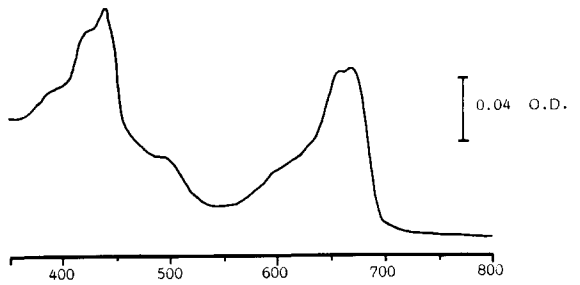

Fig. 2. Absorption spectrum of PS II preparation.

Total phosphate relative to Chl *a* and protein was also measured. The results indicated the presence of 0.044–0.048 μg of phosphate per μg Chl *a* in the PS II preparation. If the mono-phosphate phosphatidylglycerol is the only phospholipid in *S. lividus* thylakoid membranes [22], then there are approx. 0.41 mol of phosphate per mol Chl *a* in our PS II preparation. We also found 19–23 μg of protein per μg Chl *a* in our preparation. Thus the PS II preparation does not seem to contain enough organic phosphate to be composed of phospholipid vesicles.

Gel-electrophoresis in the presence of SDS indicated the presence of 27 separate bands when stained with Coomassie brilliant blue (Fig. 3). The two major bands at 13.5 and 15.5 kDa (lanes a and b) may be due to the alpha and beta subunits of allophycocyanin and reflect the high protein-chlorophyll *a* ratios found in our preparation. These two major bands were blue in color in unstained gels. In our gel system, the oxygen-evolving PS II preparation also contained major bands at 30, 43.9, 53.1, 59.9, 64.3, and 81.9 kDa. Heme-dependent peroxidase-like activity was detected in one band at 27 kDa (lane c). Since under denaturing conditions only covalently-bound heme groups will stain positively for cytochromes (see lane d), this band probably corresponds to cytochrome *f*.

When the PS II preparation was rechromatographed on Sepharose 6B which had been preequilibrated with Hepes-glycerol buffer, the active preparation eluted as a single peak at a void volume identical to that for blue dextran of molecular weight $2 \cdot 10^6$.

These results indicate that a PS II preparation which is highly active in oxygen generation can be

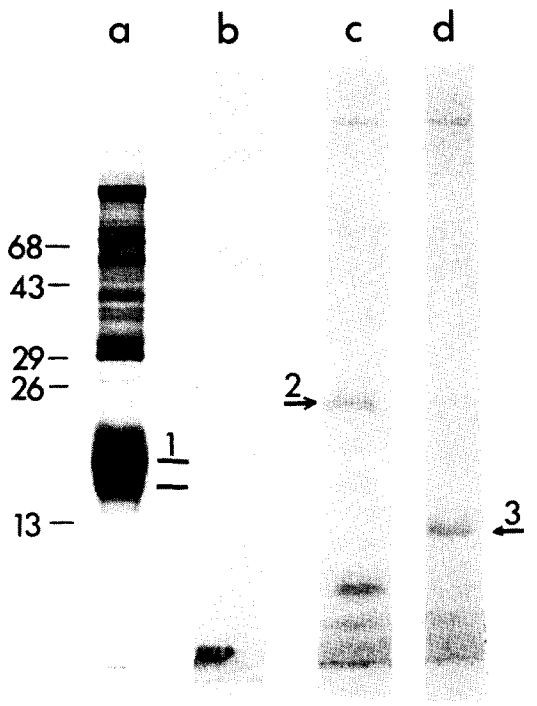

Fig. 3. SDS-gel electrophoresis in PS II preparation. (a) Stained with Coomassie brilliant blue. (b) Unstained gel. (c) Stained with tetramethylbenzidine. Arrow 2 indicates positively stained band. (d) Tetramethylbenzidine-stained gel of catalase, hemoglobin, and cytochrome *c* (arrow 3). Numbers to left of lane a (×1000) correspond to molecular weight.

isolated from *S. lividus*. This preparation is enriched in PS II components and is partially depleted in PS I components and activities. This visually clear, long wavelength bile-containing preparation appears to be a promising system for the detailed study of PS II and for the eventual isolation of the oxygen evolving complex.

References

1 Cheniae, G.M. (1980) Methods in Enzymology 69, 349–363
2 Yamamoto, Y. and Nishimura, M. (1983) Biochim. Biophys. Acta 724, 294–297
3 Stewart, A.C. and Bendall, D.S. (1979) FEBS Lett. 107, 308–312
4 Stewart, A.C. and Bendall, D.S. (1981) Biochem. J. 194, 877–887
5 Izawa, S. (1980) Methods in Enzymol. 69, 413–434
6 Wang, J.H. (1979) Methods in Enzymol. 55, 536–540
7 Markwell, J.P., Thornber, J.P. and Skrdla, M.P. (1980) Biochim. Biophys. Acta 591, 391–399
8 Mackinney, G. (1941) J. Biol. Chem. 140, 315–322
9 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
10 Lowry, O.H., Roberts, N.R., Leiner, K.Y., Wu, M.L. and Farr, A.L. (1954) J. Biol. Chem. 207, 1–17
11 Laemmli, U.K. (1970) Nature 227, 680–685
12 Guikema, J. and Shermann, L. (1981) Biochim. Biophys. Acta 637, 189–201
13 Brock, T. (1967) Science 158, 1012–1019
14 Berthold, D.A., Babcock, G.T. and Yocum, C.F. (1981) FEBS Lett. 134, 231–234
15 Smutzer, G. (1983) Ph.D. Thesis, State University of New York, Buffalo, NY
16 Harth, E., Reimer, S. and Trebst, A. (1974) FEBS Lett. 42, 165–168
17 Papageorgiou, G.C. and Isaakidou, J. (1982) FEBS Lett. 138, 19–24
18 Renger, G., Glaser, M. and Buchwald, H.E. (1976) Biochim. Biophys. Acta 461, 392–402
19 Pullar, M.P.J., Van Gorkom, H.J. and Verschoor, G.A.M. (1976) Biochim. Biophys. Acta 440, 98–106
20 Lundell, D.J. and Glazer, A.N. (1981) J. Biol. Chem. 256, 12600–12606
21 Stewart, A.C. and Larkum, A.W. (1983) Biochem. J. 210, 583–589
22 Fork, D., Murata, N. and Satoh, N. (1979) Plant Physiol. 63, 524–530