

# Characterization of a Purified Photosystem II-Phycobilisome Particle Preparation from *Porphyridium cruentum*<sup>1</sup>

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## ABSTRACT

Detergent preparations isolated from thylakoids of the red alga *Porphyridium cruentum*, in a sucrose, phosphate, citrate, magnesium chloride medium consist of phycobilisomes and possess high rates of photosystem II activity. Characterization of these particles shows that the O<sub>2</sub>-evolving activity is stable for several hours and the pH optimum is about 6.5 to 7.2. Response of the system to light, electron donors and acceptors, and inhibitors verify that the observed activity, measured both as O<sub>2</sub> evolution and 2,6-dichlorophenol-indophenol reduction, is due to photosystem II. Furthermore, photosystem II is functionally coupled to the phycobilisome in this preparation since green light, absorbed by phycobilisomes of *P. cruentum*, is effective in promoting both O<sub>2</sub> evolution and 2,6-dichlorophenol-indophenol reduction. Photosystem II activity declines when light with wavelengths shorter than 665 nm is removed. Both 3-(3,4-dichlorophenyl)-1,1-dimethylurea and atrazine inhibit photosystem II activity in this preparation, indicating that the herbicide binding site is a component of the photosystem II-phycobilisome particle.

A PBS<sup>3</sup> preparation with PSII activity has been isolated from a red alga, *Porphyridium cruentum*. Phycobilisomes are the pigment protein complexes, found only in red algae and cyanobacteria, functionally analogous to Chl *a/b* complexes of higher plants. Energy absorbed by the PBS is primarily transferred to PSII. This leads to the assumption that the PBS is physically attached to PSII in the thylakoid membrane of red algae and cyanobacteria.

Early attempts to fractionate the photosynthetic electron transport chain resulted in loss or significant decline in the O<sub>2</sub> evolving activity of PSII. PSII preparations with high O<sub>2</sub> evolving activity were first isolated by Stewart and Bendall (20) from a cyanobacterium, *Phormidium laminosum*. Since then, similar preparations have been isolated from cyanobacteria and higher plants by others (2, 10, 11, 13, 19, 24). These workers did not attempt to retain the PBS light-harvesting complex. The specific interest

in our laboratory has been to ascertain if the PBS is closely associated with the PSII components as expected from energy transfer to PSII (14–16). We had already shown that in a high ionic medium (sucrose-phosphate-citrate) it was possible to retain intact PBS and PSII activity (7). Conditions normally used for PSII preparations (2, 4, 10, 11, 13, 19, 24) result in the loss of PBS by dissociation. We recently reported the successful isolation in SPCM medium, of PIIP particles with high specific rates of PSII activity (1000–3000 μmol O<sub>2</sub> · mg<sup>-1</sup> Chl · h<sup>-1</sup>) from *P. cruentum* (5). Subsequently, this same medium has been used in the isolation of PSII particles and PBS from a cyanobacterium (17).

The PIIP preparation from *P. cruentum* used in this study is deficient in PSI and has a low Chl content. By electron microscopy the preparations were shown to be discreet particles, consisting primarily of phycobilisomes (6). Thylakoid membranes were extremely rare. In this presentation we show that green light, absorbed by phycoerythrin in the PBS of *P. cruentum*, is highly effective in driving PSII in PIIP preparations. This demonstrates that PSII does not merely co-isolate with the PBS but is functionally attached. Furthermore, we show that PIIP is a true PSII particle based upon its response to light, inhibitors, and electron donors and acceptors. Inhibitor studies indicate that the herbicide binding protein is a component of the red algal photosynthetic apparatus and is also present in our PIIP preparations.

## MATERIALS AND METHODS

**Isolation of Phycobilisomes and PSII-Phycobilisome Particles.** *Porphyridium cruentum* were grown as in Dilworth and Gantt (7) and harvested after 7 d growth in the late exponential phase. The PIIP isolation was carried out in SPCM with particle solubilization using lauryl dimethylamine oxide as described in Clement-Metral and Gantt (5). PBS-(Triton)-20C and PBS-(Triton)-5C were isolated basically as in Gantt *et al.* (12) by treatment with 1% Triton X-100, followed by isolation on sucrose step gradients. The final centrifugation for 2.5 h at 254,000g was omitted. PBS-(Triton)-5C were isolated at 4°C.

**PSII Assays.** O<sub>2</sub> evolution was measured at 25°C with a Clark-type electrode. Light from a slide projector was filtered through 10 cm water and a Schott KG 1 heat absorbing filter. The assay volume was 3.0 ml and typically contained 2 mM FeCN, 1 mM DMBQ, and 0.67 to 1% ethanol.

The instrument was calibrated by the method of Robinson and Cooper (18). The solubility of O<sub>2</sub> in air-saturated SPCM was found to be about 55% of the solubility of O<sub>2</sub> in air-saturated water at 25°C. Therefore, values reported here are lower than those reported in Clement-Metral and Gantt (5) which were based on standardization of the electrode in air-saturated water.

DCPIP measurements were done in an Aminco DW2 spectrophotometer with side illumination from a Dolan Jenner model 170-D illuminator. The absorbance change of 560 minus 520 nm was measured with a 3 nm slit width and an extinction

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<sup>3</sup> Abbreviations: PBS, phycobilisome(s); PIIP, PSII-phycobilisome; SPCM, 0.5 M sucrose, 0.5 M potassium phosphate, 0.26 M potassium citrate, 10 or 15 mM MgCl<sub>2</sub>, pH 7; PBS-(Triton)-20C, phycobilisomes isolated at room temperature and solubilized with Triton X-100; PBS-(Triton)-5C, phycobilisomes isolated at 5°C and solubilized with Triton X-100; FeCN, ferricyanide; DMBQ, dimethylbenzoquinone; DCPIP, 2,6-dichlorophenol-indophenol; DPC, sym-diphenylcarbazide; I<sub>50</sub>, concentration of inhibitor required for 50% inhibition.

coefficient of  $5.75 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ . The assay was performed in 3 ml of SPCM containing  $36 \mu\text{M}$  DCPIP. Chl was determined as described in Clement-Metral and Gantt (5).

Light intensity for  $\text{O}_2$  evolution and DCPIP reduction was measured with a Gamma C9 spectral radiometer. Green light (G) was obtained with a Plexiglas filter having maximum transmittance at 528 nm and a half bandwidth of 50 nm. Red light was obtained with either a 665 or a 695 nm cut-off filter. The 665 nm cut-off filter ( $R_{665}$ ) had zero transmittance below 650 nm. The 695 nm cut-off filter ( $R_{695}$ ) had zero transmittance below 675 nm. Neutral density screens were used to attenuate light intensity.

## RESULTS

Figure 1 shows the stability of the  $\text{O}_2$  evolution activity in PIIP when the particles are kept at low temperatures ( $5^\circ\text{C}$ , or  $-80^\circ\text{C}$ ). The activity initially drops within hours to about  $770 \mu\text{mol O}_2 \cdot \text{mg}^{-1} \text{Chl} \cdot \text{h}^{-1}$  and then declines more slowly. After 25 h, 34% of the initial activity remains when the PIIP particles are kept at  $5^\circ\text{C}$  and 38% of the PIIP activity remains when the particle is kept at  $-80^\circ\text{C}$ . After 3 d at  $-80^\circ\text{C}$ , about 10% of the original activity remains. The pH optimum for the activity is about 6.5 to 7.2.

PSII activity of PIIP is further defined with various inhibitors and electron donors for PSII (Tables I and II). Table I shows that PIIP catalyzed DCPIP reduction is stimulated about 20% by

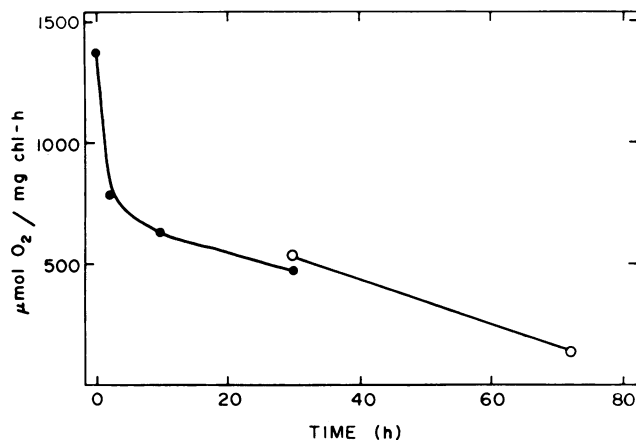


FIG. 1. Stability of  $\text{O}_2$  evolution as a function of time at  $5^\circ\text{C}$  (●) or  $-80^\circ\text{C}$  (○).  $\text{O}_2$  evolution was measured at  $25^\circ\text{C}$  as described in "Materials and Methods."

Table I. Properties of Dichlorophenol-Indophenol Reduction in PSII-Phycobilisome Particles

DCPIP measurements were made as described in "Materials and Methods." PIIP contained  $0.45 \mu\text{g}$  Chl in 3 ml SPCM; PBS-(Triton)-5C contained  $0.17 \mu\text{g}$  Chl and PBS-(Triton)-20C contained trace amounts of Chl in 3 ml of  $0.75 \text{ M}$  K-phosphate (pH 6.8). DPC was added in DMSO to a final concentration of 0.3%. DCMU was added in absolute ethanol such that the final concentration was 0.67 to 1%.

	$\mu\text{mol DCPIP} \cdot \text{mg}^{-1} \text{Chl} \cdot \text{h}^{-1}$
PIIP	215
PIIP + $90 \mu\text{M}$ DPC	261
PIIP + $3.3 \mu\text{M}$ DCMU	20
PBS-(Triton)-5C	44
PBS-(Triton)-5C, + $90 \mu\text{M}$ DPC	$438 \pm 30$
PBS-(Triton)-5C, + $90 \mu\text{M}$ DPC, + $5 \mu\text{M}$ DCMU	77
PBS-(Triton)-20C	0
PBS-(Triton)-20C, + $90 \mu\text{M}$ DPC	0

Table II. Properties of  $\text{O}_2$  Evolving Activity in PSII-Phycobilisome Particles

$\text{O}_2$  evolution was measured as described in "Materials and Methods." PBS (5C and 20C) isolated by Triton X-100 treatment contained  $0.4 \mu\text{g}$  Chl in 3 ml of  $0.75 \text{ M}$  K-phosphate (pH 6.8). PIIP particles (control) contained  $1 \mu\text{g}$  Chl in 3 ml of SPCM. SPCM contained  $15 \text{ mM}$   $\text{MgCl}_2$  in this experiment. All assays contained  $2 \text{ mM}$  FeCN and  $1 \text{ mM}$  DMBQ unless indicated differently. DCMU was added in absolute ethanol so that the final concentration was 0.3%.  $\text{O}_2$  evolution was  $765 \pm 141 \mu\text{mol O}_2 \cdot \text{mg}^{-1} \text{Chl} \cdot \text{h}^{-1}$  in the control.

	% of control
PIIP	100
PIIP, + $4 \text{ mM}$ FeCN, - DMBQ	76
PIIP, - FeCN, + $2 \text{ mM}$ DMBQ	83
PIIP, + $2 \text{ mM}$ $\text{NH}_2\text{OH}$	0
PIIP, + $1.7 \mu\text{M}$ DCMU	0
PBS-(Triton)-5C	0
PBS-(Triton)-20C	0

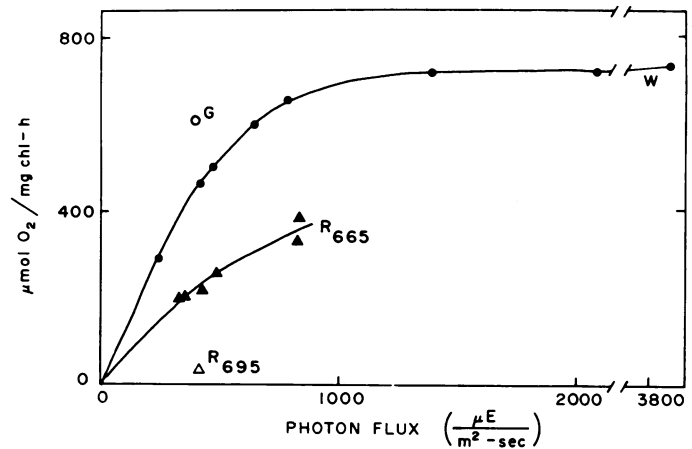


FIG. 2.  $\text{O}_2$  evolution as a function of light intensity and light quality in PSII-phycobilisome particles. G, green light (○); W, white light (●);  $R_{665}$ , red light obtained with a 665 nm cutoff filter (▲);  $R_{695}$ , red light obtained with a 695 nm cutoff filter (△).  $\text{O}_2$  evolution was measured as described in "Materials and Methods."

DPC and is inhibited about 90% by DCMU. Additionally,  $\text{O}_2$  evolution is completely inhibited in PIIP particles by DCMU and  $\text{NH}_2\text{OH}$  (Table II). If either DMBQ or FeCN are omitted from the assay, rates of  $\text{O}_2$  evolution are slightly lower. PBS isolated at  $5^\circ\text{C}$  (PBS-(Triton)-5C), by the conventional 1% Triton X-100 solubilization procedure of Gantt *et al.* (12), retain some DCPIP reduction activity (Table I). This activity is strongly stimulated by DPC, and inhibited about 80% by DCMU in the presence of DPC (Table I). PBS isolated at room temperature (PBS-(Triton)-20C) do not reduce DCPIP (Table I). Neither PBS-(Triton)-20C nor PBS-(Triton)-5C demonstrate  $\text{O}_2$  evolution activity (Table II).

Figure 2 shows the effect of photon flux density and light quality on  $\text{O}_2$  evolution activity in PIIP particles. In white light, the preparation saturates at about  $1000 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ . Half saturation occurs at about  $300 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ . Green light absorbed primarily by phycoerythrin, is highly effective in supporting  $\text{O}_2$  evolution activity.  $\text{O}_2$  evolution activity in green light appears close to full saturation at  $400 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ . At the same photon flux densities, red light, obtained either with a 665 nm filter or with a 695 nm filter, is much less effective in driving  $\text{O}_2$  evolution in PIIP particles. Although attainment of greater photon flux densities in green and red light is limited by the light source and the filter system,  $\text{O}_2$  evolution is consistently higher in green light

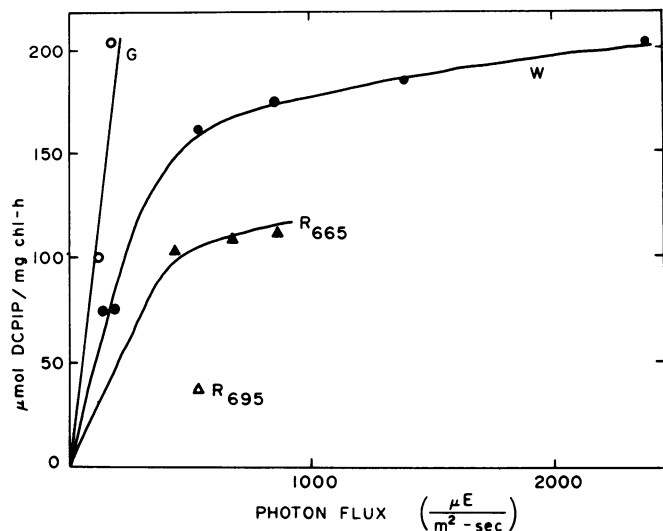


FIG. 3. Dichlorophenol-indophenol reduction in PSII-phycobisome particles as a function of light intensity and light quality. Other conditions are described in "Materials and Methods." G, green light (O); W, white light (●); R<sub>665</sub>, red light obtained with a 665 nm cutoff filter (▲); R<sub>695</sub>, red light obtained with a 695 nm cutoff filter (Δ).

compared to white light. The results clearly show that green light is more effective. The same conclusion is drawn from the results with DCPIP reduction. Figure 3 shows an analogous experiment in which DCPIP reduction by PIIP particles is measured at different light intensities in green, white, and red light. DCPIP reduction also saturates at about  $1000 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  with half saturation at about  $300 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ . Again, green light is more effective than white light in promoting DCPIP reduction, and red light is much less effective in driving DCPIP reduction by PIIP particles.

That PIIP particles contain the PSII-associated atrazine binding protein is indicated by the inhibition of the PIIP  $\text{O}_2$  evolving activity by atrazine (Fig. 4A). Fifty % inhibition occurs at  $0.05 \mu\text{M}$  concentration and the inhibition seems to level off with 80% inhibition at about  $0.4 \mu\text{M}$ , although a higher concentration of  $3.3 \mu\text{M}$  produces an inhibition of 95%. When atrazine is tested on unfractionated thylakoids (Fig. 4B), higher concentrations are required for inhibition. Fifty % inhibition occurs at about  $0.25 \mu\text{M}$ . The system saturates at about  $4 \mu\text{M}$  atrazine with 85% inhibition, and  $16 \mu\text{M}$  atrazine results in a further inhibition to 95%.

## DISCUSSION

The essential components of PSII are clearly present in PIIP particles and are directly linked to the PBS. This is shown by the light dependence of both  $\text{O}_2$  evolution and DCPIP reduction (Figs. 2 and 3) and by the response of the system to inhibitors (Tables I and II; Fig. 4) and electron donors (Table I). Rates of activity based on DCPIP reduction are lower than those based on  $\text{O}_2$  evolution. This is most likely due to the inhibition of DCPIP reduction by the high ionic strength SPCM media (22). When the PIIP preparation is diluted with  $2\times$  volume of water instead of SPCM, the rates of DCPIP reduction approximately double (data not shown).

PBS solubilized by 1% Triton X-100 retain PSII activity as measured by DCPIP reduction only when isolated at  $5^\circ\text{C}$ , not at  $20^\circ\text{C}$  (Table I). However, the absence of  $\text{O}_2$  evolution activity and the large stimulation by DPC of DCPIP reduction indicate that the PBS(Triton)-5C particles do not contain an intact electron transport chain from water to the plastoquinone pool.

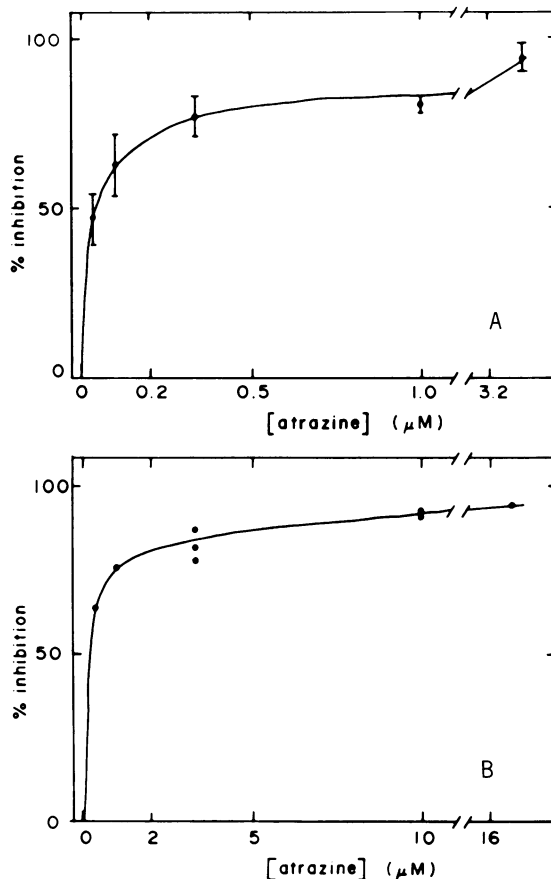


FIG. 4. Per cent inhibition of oxygen evolution activity in PSII-phycobisome particles (A) and unfractionated membranes (B) as a function of atrazine concentration. Control activity for (A) was  $805 \pm 148 \mu\text{mol O}_2 \cdot \text{mg}^{-1} \text{Chl} \cdot \text{h}^{-1}$ . Control activity for B was  $288 \pm 17 \mu\text{mol O}_2 \cdot \text{mg}^{-1} \text{Chl} \cdot \text{h}^{-1}$ .  $\text{O}_2$  evolution was measured as described in "Materials and Methods." Atrazine was added in methanol such that the final methanol concentration was 0.03 to 0.1%. Activity in the presence of atrazine was compared to appropriate controls.

Probably some component(s) on the oxidizing side of PSII have been lost. In contrast, PIIP particles evolve  $\text{O}_2$  and are stimulated only slightly by DPC. PSII components may also be present but inactive in PBS isolated at room temperature. This would explain why PBS and PIIP preparations appear similar when analyzed by SDS-PAGE (6). Alternatively, the action of Triton X-100 at room temperature may result in a particle in which PSII is not retained with the phycobisome.

The effectiveness of green light in driving PSII activity in PIIP particles (Figs. 2 and 3) is predictable if the PBS is functionally coupled to PSII since phycoerythrin, the green light absorber, comprises about 70% of the PBS in *P. cruentum* (7). Although the system appears close to saturation at  $400 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  of green light (Figs. 2 and 3), it is not verified since this is the maximum photon flux density available under our conditions. Red light is obviously less effective because there are fewer red light absorbing chromophores in the PIIP system.

The inhibition of PSII activity by atrazine (Fig. 4) and DCMU (Tables I and II) indicates that the herbicide binding protein is a component of our PIIP particles.  $I_{50}$  values observed in PIIP particles for atrazine are of the same order of magnitude as observed for cyanobacteria (10 nM) (1) and about  $10\times$  lower than the values reported for higher plants (21). The higher concentrations required for atrazine inhibition in unfractionated thylakoids are probably due to nonspecific binding.

The molar ratio of Chl/PBS is about 1200 in unfractionated thylakoids, when calculated by using the PBS mol wt of Dilworth and Gantt for *P. cruentum* (7). In PIIP the Chl/PBS ratio is about 60. If there are one to four PSII reaction centers per PBS (14, 16), this implies 15 to 60 Chl per PSII reaction center which is in agreement with results obtained using other approaches (3, 8, 9, 15, 23).

More than 98% of the original Chl is removed in the isolation of the PIIP particles. This decline in Chl content seems to reflect the preferential extraction of PSI Chl since PSI has been shown to be absent in these particles by low temperature fluorescence (5) and by absence of P<sub>700</sub> (6).

The initial decline of this activity (Fig. 1) could be due to the partial uncoupling of electron transfer components within the particles with time. It is also possible that there are two groups of O<sub>2</sub> evolving centers in our preparation—some O<sub>2</sub> evolving centers may be stabilized by small membrane vesicles, whereas another set may depend solely on the SPCM media for retention of activity. There appeared to be no further stabilization of activity if the particles were kept at -80°C rather than 5°C. The stability of our PIIP particles is comparable to that observed by Bowes *et al.* (4) for PSII particles from *P. laminosum*. However, in contrast to these workers, we are not able to increase stability of PIIP catalyzed O<sub>2</sub> evolving activity by the addition of lauryl maltoside (data not shown).

In summary, our PIIP particles demonstrate high rates of O<sub>2</sub> evolving activity in both green and white light. This shows that the PBS are functionally coupled to the PSII activity and provides evidence for the association of PBS with PSII *in vivo* in red algae and cyanobacteria. In addition, the DCMU and atrazine sensitivity of the particle indicates that the herbicide binding protein is a component of the photosynthetic apparatus in *P. cruentum* and in the PIIP particle. Experiments in progress are expected to identify specific PSII components including P680 and RC II polypeptides.

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