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Photosynthetic oxygen production and the size of the photosynthetic unit in a cell-free preparation from *Cyanidium caldarium*

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SUMMARY

A cell-free preparation has been isolated from a mutant of *Cyanidium caldarium*, grown under conditions such that there is 15 times less chlorophyll per photosynthetic unit than in normal green algae. The preparation is sensitive to 3-(3,4-dichlorophenyl)-1,1-dimethylurea and shows the well-characterized oscillation of O_2 yield, from saturating flashes, following a period of dark adaptation. Greening experiments with dark-grown, wild-type *Cyanidium* show that the synthesis of photosynthetic units precedes that of bulk chlorophyll and that the O_2-producing system is assembled before the total system coupled to CO_2. No large-scale cooperation of chlorophyll molecules is required for O_2 production.

It has long been the goal of investigators of photosynthesis to isolate highly purified, antenna pigment-free preparations that are capable of the primary photochemistry. Cell-free preparations have been isolated by many workers from green plants[1–5] which show varying degrees of separation or uncoupling of the two photosystems. In all of these preparations, however, the chlorophyll antenna have remained intact, though possibly uncoupled, from the reaction center[5]. Isolation of an antenna-free particle would facilitate the study of the absorption spectra and kinetics of the primary photoprocesses, no longer masked by the 250–300 chlorophylls per center. Such preparations have been achieved in the photosynthetic bacteria[6,7] and have led to a clear identification of bacteriochlorophyll as the primary photoactive species. They have also permitted determination of the number and kind of pigment molecules associated with the reaction center[8]. Our particular interest is in the isolation of an antenna chlorophyll-free preparation which would permit study of

Abbreviation: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

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the primary photochemistry related to the evolution of O_2. Our criterion for what constitutes the best preparation is that which shows the highest ratio of O_2 activity to chlorophyll content.

Our initial attempts to isolate a low chlorophyll preparation involved a number of fractionating treatments of a particle we isolated from *Phormidium*[5]. These procedures included organic solvent extraction, enzyme digestion with chlorophyllase, lipases and proteases, growth in the presence of chlorophyll synthesis inhibitors and growth in high light intensity. All of these treatments led to, at best, a 2-fold improvement in the O_2 activity on a per chlorophyll basis in the *Phormidium* preparation.

We know that some algae, when grown in the dark, fail to synthesize chlorophyll. Burr and Mauzerall[9] in experiments with the 610 mutant of *Chlorella*, showed that greened cells, in heterotrophic medium, when placed in the dark dilute out their chlorophyll and photosynthetic apparatus as the cells divide. Herron and Mauzerall[10] found that once placed in the light, these cells resynthesize chlorophyll and photosynthetic apparatus, but at different rates. Photosynthetic activity increases rapidly and with no apparent lag period, while chlorophyll synthesis is slower and begins after a definite lag. By greening dark-grown *Chlorella* in dim light, Herron and Mauzerall[11] obtained cells containing one-tenth of normal chlorophyll, but capable of the usual light-saturated O_2 production rate per cell, coupled to CO_2 uptake. These experiments suggest that the synthesis of photosynthetically active reaction centers precedes the synthesis of the antenna. A high ratio of O_2 formation to chlorophyll content was also found by Schmid and Gaffron[12] in yellow mutants of tobacco.

We decided to use a different alga, a mutant of *Cyanidium caldarium*, prepared by Nichols and Bogorad[13]. The III-C mutant (generously provided by Dr L. Bogorad) does not synthesize phycocyanin and when grown in the light contains only 40% of the chlorophyll content of wild-type cells. Neither the mutant nor the wild-type cells synthesize chlorophyll in the dark.

Wild-type *Cyanidium* cells, grown in the dark, then transferred to the light in mineral and glucose media and assayed with CO_2 or benzoquinone, all show an increase in O_2 activity on a per chlorophyll basis (Fig. 1). After 48 h, cells greened in mineral medium show an O_2-producing activity that is higher for coupling to CO_2 uptake and lower for coupling to benzoquinone reduction than those greened on glucose medium. At this time point, the O_2 production rate, per chlorophyll, coupled to CO_2 uptake, is 3 times greater for cells grown in mineral medium than in glucose medium, while O_2 production coupled to benzoquinone reduction is 7 times greater for cells grown in glucose medium than in mineral medium. After 72 h, O_2 production rate, coupled to benzoquinone reduction, is 20 times greater in glucose medium than in mineral medium. It is clear from the increase in specific activity (O_2 evolution rate per chlorophyll) in all cases, that the synthesis of reaction centers precedes the synthesis of chlorophyll and that this precedence is most obvious where cells are least reliant on photosynthesis for cell growth in the light (glucose-grown cells). It is also likely, considering the much greater O_2 evolution activity coupled to benzoquinone reduction than to CO_2 uptake, at 33 and 48 h in glucose-grown cells, that

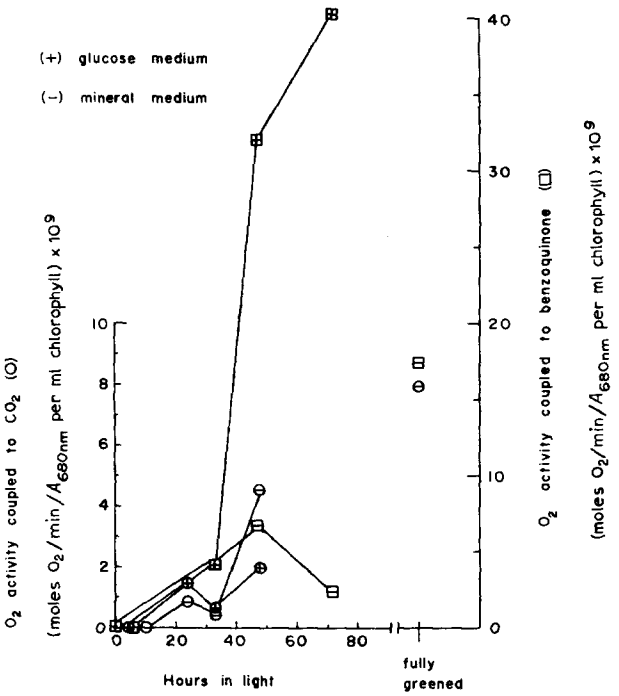

Fig. 1. Greening experiment with wild-type *Cyanidium* in the presence or absence of glucose. Cells were grown on 1% glucose medium[14], pH 2.4 at 35 °C in the dark for 5 days with continuous shaking. Half the cells were then transferred to mineral medium (−) while the other half remained in glucose medium (+). Cells were greened under a Lucalox lamp (General Electric Co.), at an incident light intensity of 10^5 ergs·cm^{-2}·s^{-1}. At various times following the start of illumination cells were assayed for O_2 activity in the O_2 luminometer[9] under saturating light and their spectra taken. O_2 assays were performed in either 0.05 M $NaHCO_3$, pH 8.0 (○) or in 10 mM succinate (pH 5.9), 23 mM NaCl, 6.7 mM $CaCl_2$ with 0.5 mM benzoquinone (□). Because of the difficulty in extracting chlorophyll from these cells, O_2 activity, in moles O_2/min is expressed per A_{680} nm per ml instead of per mg chlorophyll.

Photosystem II reaction centers are completed before the remainder of the photosynthetic apparatus. This is because benzoquinone can act as electron acceptor at a point between the photoreactions (Diner, B. and Mauzerall, D., unpublished) and bypass much of the electron transport chain. The use of CO_2 as the ultimate electron acceptor requires that the entire photosynthetic system be intact. At 72 h enhancement of O_2-evolving activity on a per chlorophyll basis was approximately 3-fold for coupling to benzoquinone reduction as compared to fully greened cells. Similar results were obtained with the III-C mutant. Because photocyanin replaces about half the antenna chlorophyll as accessory pigment in *Cyanidium* and because the chlorophyll, in the mutant, is about 2.5 times depleted as compared to wild-type cells, the overall chlorophyll antenna size is decreased about 15 times from fully greened plant cells. The partially greened mutant thus provides an ideal starting material for a chlorophyll-depleted cell-free preparation.

The procedures developed in the greening experiments were scaled up to provide sufficient cells for making a cell-free preparation. Mutant III-C cells were inoculated into

15 l of Allen medium[14], pH 2.4, containing 1% glucose. Cells were grown in the dark for about 6 days at 39 °C and with continuous aeration (approx. 700 ml/min). The dark-grown cells (approx. 0.005 ml packed cell volume per ml of medium), which were light yellow in appearance, were then exposed to a Lucalox lamp ($1 \cdot 10^5$ ergs·cm^{-2}·s^{-1}, incident intensity, General Electric Co.) for 48 h with continued aeration. Cells were harvested and washed 2 times with buffer (10 mM succinate (pH 5.9), 23 mM NaCl, 6.7 mM $CaCl_2$) and once with 2.5% Brij 35 (polyoxyethylene lauryl ether, a gift of the Atlas Chemical Co.) in the same buffer. The cells were resuspended in 2.5% Brij 35 containing 1 mg DNAase and 1 mg RNAase per 200 ml at a concentration such that $A_{680\ nm} \approx 2$ (approx. 100 ml packed cell volume in 180 ml). Cells were passed twice through an Aminco French pressure cell at 6000 lb/inch^2. The homogenate was incubated for 1 h at 0 °C and then given two 15-s bursts at maximum power (20 kcycles/s on a sonicator (Measuring and Scientific Equipment Ltd, 100-W Ultrasonic Disintegrator). The homogenate was spun at 4000 x *g* for 20 min to remove unbroken cells and cell walls. The supernatant was poured off and spun at 12 000 X *g* for 30 min. The supernatant was again poured off and spun at 200 000 X *g* for 40 min. All the O_2 activity was in the high speed pellet, which was resuspended in buffer with the aid of a teflon homogenizer and stored in liquid N_2. The isolation procedure was performed at 5 °C unless otherwise indicated.

The light-saturated specific activity of the resulting preparation with $K_3Fe(CN)_6$ was $1 \cdot 10^{-7}$ moles O_2 evolved per min/$A_{680\ nm}$ per ml chlorophyll, or approximately $1 \cdot 10^{-5}$ moles O_2 per min/mg chlorophyll. This specific activity is about 100 times that of the *Phormidium* cell-free preparation[5], $1.2 \cdot 10^{-7}$ moles O_2 per min/mg chlorophyll and 6 times that of wild-type *Cyanidium* cells coupled to benzoquinone.

Taking the result of the early Emerson and Arnold experiments[15] which indicated 2500 chlorophylls cooperating in the synthesis of an O_2 molecule, photosynthesis workers have conveniently divided by 8–10 (the quantum requirement of O_2 production) to give 250–300 chlorophylls per reaction center. The rapid transient increase in effective fluorescence yield of chlorophyll also has a cross-section of about 350 chlorophylls[16]. We determined the optical cross-section of the reaction centers in the *Cyanidium* preparation by means of pulsed light saturation experiments. A one-hit Poisson analysis should give, in the linear region of the pulsed light saturation curve, the following dependence of the O_2 yield per flash on light intensity:

$$O_2/\text{flash} = (\text{max. } O_2/\text{flash}) \cdot \sigma I$$

where I is the average light intensity of the light flash and σ is the optical cross-section of the reaction center and its associated antenna. This experiment was performed in the steady state using an O_2 polarograph (Diner, B. and Mauzerall, D., unpublished), in a 3 ppm O_2 atmosphere and with 8-μs flashes, given at a rate of 20 per s. The preparation *plus* $K_3Fe(CN)_6$ were separated from the platinum surface by a teflon membrane and covered with a cellophane membrane. Integrated light intensities were measured with a Light Mike (Model 560B EG and G, Inc.). The slope of the O_2 yield per flash *versus* intensity plot yielded an optical cross-section σ at 680 mm of 47 ± 1 Å^2. We estimate the optical cross-section of an individual chlorophyll molecule to be 3 Å^2 at this wavelength. Thus the

number of chlorophylls associated with each reaction center is about 15. This antenna size is about one-twentieth of the 300 chlorophylls per reaction center existent in most fully greened algae and plants.

Further support for this claim comes from fluorescence induction and O_2 activation experiments. 10^{-5} M 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was added to dark-adapted *Cyanidium* cell-free preparation. The time course of fluorescence induction was measured in blue light (Schott BG 38) and compared to that shown by DCMU-poisoned *Chlorella* under identical light conditions. *Chlorella* cells showed a half-rise time to F_{max} that was 15 times more rapid than that shown by the preparation.

Also compared was the time course of O_2 activation in modulated light (interference filter, 582 nm, half-width 13 nm) for dark-adapted preparation and spinach chloroplasts, placed directly on the platinum electrode of the Joliot polarograph[19]. The *Cyanidium* preparation showed the same time course for O_2 production in unattenuated light as spinach chloroplasts showed for light attenuated 16 times.

Both of the above experiments clearly show that the photosynthetic unit size in the preparation is decreased at least 15-fold relative to that observed in *Chlorella* cells or spinach chloroplasts, assuming similar quantum efficiencies of the different preparations.

While the association of the reaction center with its antenna has been greatly simplified, the components of the photosynthetic electron transport chain appear to have remained with the preparation, in view of its sensitivity to DCMU. O_2 evolution activity coupled to benzoquinone reduction is inhibited 99.9% by $1.7 \cdot 10^{-4}$ M DCMU while O_2 evolution activity coupled to $K_3Fe(CN)_6$ reduction is inhibited somewhat less, 93% with the same inhibitor concentration. A further difference between these two oxidants is that the light-saturated, $K_3Fe(CN)_6$ reduction coupled, O_2 evolution rate is 2.7 times that with benzoquinone. Whether this difference is a matter of turnover or quantum yield will have to be established by repetitive single flash experiments.

The real advantage of the *Cyanidium* preparation is the great reduction in the photosynthetic unit size, a feature from which we conclude that no large-scale cooperation of chlorophyll molecules is needed for the production of O_2. The loss of most of the antenna chlorophyll will facilitate the study of the reaction center chlorophyll spectrum and kinetics.

After 15 min of dark adaption, this preparation shows a sequence of O_2 yields from saturating flashes, closely resembling that described by Joliot *et al.*[17] and Kok *et al.*[18]. This sequence is essentially the same in the presence or absence of 1 mM $K_3Fe(CN)_6$. Thus this preparation is ideal for determining whether the successive oxidations on the oxidizing side of Photosystem II show concomitant spectral changes.

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