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3-(3,4-Dichlorophenyl)-1,1-dimethylurea-insensitive oxygen production in a cell-free preparation from *Phormidium luridum* that shows redox potential dependent coupling to one-electron oxidants

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SUMMARY

A cell-free preparation has been isolated from *Phormidium luridum* that evolves oxygen when coupled to one-electron oxidants, that is insensitive to 3-(3,4-dichlorophenyl)-1,1-dimethylurea, and that yields oxygen at a rate dependent on redox potential. In this preparation the Hill oxidant couples closer to the oxygen-producing apparatus than in any other cell-free system. Light saturation curve data for the cell-free preparation shows a stabilization, by the Hill oxidant, of intermediates in oxygen synthesis. In whole cells coupled to CO_2 or to $K_3Fe(CN)_6$ no such stabilization occurs and a 2nd order light intensity dependence of the oxygen-production rate is observed.

Several approaches have characterized efforts to learn about the mechanism of oxygen production in photosynthesis. The most successful has been the kinetic characterization of oxygen production in whole algal cells and in chloroplast preparations. Joliot *et al.*[1] and Kok *et al.*[2] have proposed models for oxygen evolution in which oxidizing equivalents are successively accumulated within a single reaction center. The determination of the chemical requirements for oxygen synthesis would aid in identifying these oxidizing equivalents and their relationship to the reaction center. The work of Cheniae and Martin[3] on the Mn^{2+} requirement and Izawa *et al.*[4] on the Cl^- requirement has shown that these ions act between the water-oxidation site and the photoreaction. It has been found that electron donors like NH_2OH and *p*-phenylenediamine compete with water for photo-oxidizing equivalents. By comparing the kinetics and chemical requirements of these reductants with those for water oxidation, much has been learned of the oxidizing side of the photosystem[3,5]. Finally, the direct isolation of the primary photosynthetic apparatus, while most successful in the photosynthetic bacteria[6], has met with only partial success in

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol.

green plants. Since Hill's first preparations[7], active in oxygen production, the application of physical and detergent techniques has led to appreciable separation of the photosystems[8], but the components of the oxygen-evolving system have so far escaped isolation.

We have attempted to isolate the oxygen-evolving apparatus from the blue-green alga *Phormidium luridum* var. Oliviciae (obtained through the courtesy of Dr. H.W. Siegelman) using the oxygen luminometer[9] as our assay for oxygen-evolving ability. The cells were grown at 25–28° in 20-l carboys containing Kratz and Myers[10] medium D. The cultures were continuously aerated and were illuminated with four fluorescent lamps providing a total light intensity of $3.0 \cdot 10^4$ ergs/cm^2 · sec. Cells were harvested in the late logarithmic stage of growth and were washed several times with buffer (10 mM succinate, pH 5.9, containing 23 mM NaCl and 6.7 mM $CaCl_2$). The cells were then resuspended in the same buffer, containing 2.5% Brij 35 (polyoxyethylene lauryl ether, a gift of the Atlas Chemical Co.), at a concentration such that $A_{680\,nm}$ was approximately 100. Cells were passed two times through an Aminco French pressure cell at 6000 lb/inch^2. The homogenate was spun at 2000 × *g* for 10 min to remove unbroken cells and the supernatant was made 28% of saturation in $(NH_4)_2SO_4$. After 15 min at 0°, the suspension was spun at 12 000 × *g* for 10 min. Material equally active in oxygen production, on a per chlorophyll basis, was distributed between the float and the pellet while the bulk of the phycocyanin remained in the supernatant. The float and pellet were pooled, redissolved in a minimum volume of buffer, containing 0.25% Brij 35 and placed on a Sephadex G-200 or Bio-Rad A-15m column (height/diameter = 50–100), previously equilibrated with 0.25% Brij in buffer. Three bands invariably appeared on the column upon elution with the same Brij–buffer mixture: a lead green band at the void volume with a λ_{max} at 680 nm and active in oxygen evolution coupled to $K_3Fe(CN)_6$; a second green band with a λ_{max} at 670 nm and completely inactive in oxygen production; and a third blue phycocyanin band. All of the preceding manipulations were performed at 5° unless otherwise indicated.

The lead band, hereafter called the cell-free preparation, had 10–15% of the oxygen-evolving activity of the whole cells coupled to $K_3Fe(CN)_6$, on a per chlorophyll basis. This Hill reaction showed a strong pH dependence with a sharp maximum at pH 5.9. The visible spectrum revealed the complete absence of phycocyanin but a normal complement of carotenoids. The preparation was inactive in oxygen evolution coupled to CO_2, 2,6-dichlorophenolindophenol (DCIP), Br_2, I_2 or K_2PtCl_6 but active when coupled to $K_3Fe(CN)_6$, $KMnO_4$, K_2IrCl_6 or benzoquinone. Experiments with varied ratios of $K_3Fe(CN)_6$ to $K_4Fe(CN)_6$ and the accompanying oxygen rates are given in Table I. In showing that the ferricyanide concentration was not limiting at the lower end of the redox scale we conclude that the oxygen rate was dependent on the redox potential and that a high potential was necessary for optimal oxygen production. In addition, the ease of coupling to K_2IrCl_6 and $K_3Fe(CN)_6$ (one-electron oxidants) compared to the inactivity of K_2PtCl_6 (a two-electron oxidant having similar charge and redox potential characteristics), strongly suggests that oxidant coupling occurs by a one-electron oxidation. Control experiments with mixtures of $K_3Fe(CN)_6$ and K_2PtCl_6 showed that the latter did not inhibit oxygen production.

As shown in Table II, $K_3Fe(CN)_6$-coupled, light-saturated oxygen production in the cell-free preparation was insensitive to 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), the inhibition being only 18% at $1.7 \cdot 10^{-4}$ M. Whole cells coupled to $K_3Fe(CN)_6$ or to CO_2

TABLE I

DEPENDENCE OF THE OXYGEN PRODUCTION RATE ON REDOX POTENTIAL FOR THE CELL-FREE PREPARATION IN TWO RUNS DIFFERING 10-FOLD IN FERRI- *plus* FERROCYANIDE CONCENTRATION

For each point, in both runs, the cuvette contained 7.0 μM chlorophyll in 1.5 ml. Saturating light, provided by a 750-W projector was filtered through 5 cm of 0.02% potassium chromate. Redox potential remained constant during the illumination. However, the stability of the preparation during illumination appeared to be a function of the ferricyanide concentration. Corrections, in the oxygen rates, varying from 0% at low ferricyanide concentrations to 35% at the highest concentration were made by extrapolating the declining rate to zero time. A fresh sample was used for each point.

Concentration of ferri- plus ferrocyanide: *0.50 mM*		*5.0 mM*	
Redox potential (mV)	*O_2 rate (moles O_2/min)*	*Redox potential (mV)*	*O_2 rate (moles O_2/min)*
320	$9.3 \cdot 10^{-11}$	303	$7.28 \cdot 10^{-11}$
349	$3.75 \cdot 10^{-10}$	355	$5.93 \cdot 10^{-10}$
360	$7.37 \cdot 10^{-10}$		
370	$9.18 \cdot 10^{-10}$	373	$1.21 \cdot 10^{-9}$
377	$1.12 \cdot 10^{-9}$		
378	$1.46 \cdot 10^{-9}$		
387	$1.5 \cdot 10^{-9}$	384	$1.42 \cdot 10^{-9}$
405	$2.03 \cdot 10^{-9}$		
418	$2.44 \cdot 10^{-9}$		
461	$3.28 \cdot 10^{-9}$		

TABLE II

PERCENT INHIBITION OF OXYGEN PRODUCTION RATE AT TWO CONCENTRATIONS OF DCMU

For *P. luridum* whole cells coupled to CO_2 or to $K_3Fe(CN)_6$ the chlorophyll concentration in the cuvette was 3.5 μM in 2.0 ml, while for the cell-free preparation the chlorophyll concentration was 7.0 μM in 2.0 ml. Whole cells coupled to CO_2 were assayed in 0.1 M $NaHCO_3$, pH 8.0. Whole cells coupled to $K_3Fe(CN)_6$ were suspended in the usual succinate buffer, pH 5.9, containing 4.0 mM $K_3Fe(CN)_6$ and 1.0 mM $K_4Fe(CN)_6$. The cell-free preparation was suspended in the same buffer with 0.50 mM $K_3Fe(CN)_6$. Saturating light was filtered through 5 cm of water and through a Corning red 2-64 filter.

	% Inhibition of O_2 production	
	$1.0 \cdot 10^{-6}$ M DCMU	*$1.7 \cdot 10^{-4}$ M DCMU*
Whole cells coupled to CO_2	91	>99.9
Whole cells coupled to $K_3Fe(CN)_6$	33	95
Broken prep. coupled to $K_3Fe(CN)_6$	12	18

were inhibited by this concentration of inhibitor to the extent of 95% and greater than 99.9%, respectively. These data indicate that, in the cell-free preparation, $K_3Fe(CN)_6$ is coupling either within the reaction center or between the photoreaction and the DCMU-sensitive site on the reducing side of the reaction center. As 5% activity remains in whole cells assayed under these conditions, we infer that not more than 5% of $K_3Fe(CN)_6$

coupling occurs at the site opened in the cell-free preparation. As the preparation shows some light-induced absorbance changes at 703 nm it is apparent that the photosystems are not physically separated; however, we can conclude that they are highly uncoupled with respect to electron transfer. In addition we can claim that this preparation couples to $K_3Fe(CN)_6$ closer to the oxygen-producing apparatus than in any other cell-free system.

Fig.1 compares the light saturation curves of whole *P. luridum* cells coupled to CO_2 and to $K_3Fe(CN)_6$ with that for the cell-free preparation coupled to $K_3Fe(CN)_6$. Coupling to CO_2 shows saturation, 1st order, 2nd order and possibly higher order dependence of oxygen-evolution rate on light intensity. CO_2 coupling in *Chlorella vulgaris* has been shown to have an analogous light saturation curve showing saturation, 1st order and 2nd order light dependence[11]. Whole *P. luridum* cells coupled to $K_3Fe(CN)_6$, on the other hand, show an almost direct transition from saturation to 2nd order intensity dependence.

We believe that the 2nd order light intensity dependence is indicative of a rapidly decaying intermediate in oxygen synthesis. Were each intermediate in oxygen synthesis to have an infinite lifetime, then at low light intensities each intermediate could wait indefinitely until another photooxidizing equivalent appeared to advance it to the next

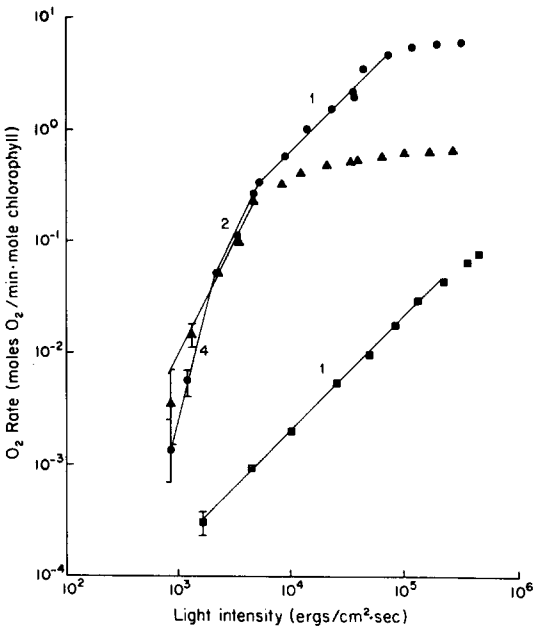

Fig.1. Light saturation curves for whole cells coupled to CO_2 (●—●) and to $K_3Fe(CN)_6$ (▲—▲) and the cell-free preparation coupled to $K_3Fe(CN)_6$ (■—■). Light provided by a 750-W projector was filtered through 5 cm of water, a Corning red 2-64 filter and a succession of neutral density Schott filters to vary the light intensity. Oxygen rates were determined, as in previous experiments, using the oxygen luminometer[9]. Light intensity was measured using a Model 65 Radiometer (Yellow Springs Instrument Co.). Orders of dependence of O_2 rate on light intensity are given next to each portion of the curves. Whole cells coupled to CO_2 were assayed in 0.1 M $NaHCO_3$, pH 8.0. Whole cells coupled to $K_3Fe(CN)_6$ were assayed in the usual succinate buffer, pH 5.9, containing 4.0 mM $K_3Fe(CN)_6$ and 1.0 mM $K_4Fe(CN)_6$. The cell-free preparation was also assayed in succinate buffer, pH 5.9, but with 0.375 mM $K_3Fe(CN)_6$ and 0.125 mM $K_4Fe(CN)_6$.

oxidation state. In such a situation, the oxygen rate would be directly proportional to the photon flux (*i.e.* the light intensity). However, should an intermediate have a finite lifetime, then at low light intensities, that intermediate could undergo reduction before photochemical advancement to the next highest oxidation state occurred. It is clear that the cooperation of a second photooxidizing equivalent, following closely enough in time to the first, is required to prevent the loss of the unstable intermediate. Where the average photon flux into a reaction center is faster than the loss rate, as at higher light intensities, a 1st order light dependence is observed. Where the average photon flux is less than the loss rate, the 2nd order dependence is obtained. At still lower light intensities, where still longer decay times of other intermediates become apparent, then still higher order intensity dependencies are observed. While the scheme of Kok *et al.*[2] is particularly supportive of this argument, we find that the lifetimes of the intermediates in both the Joliot *et al.* and Kok *et al.* models are at least 10 times longer than are required by our results. An explanation for our disagreement in lifetimes and a more quantitative discussion, relating lifetimes of intermediates to the light saturation curves, will be forthcoming[12].

In contrast with the CO_2 and $K_3Fe(CN)_6$ coupled whole cells, the cell-free preparation shows only saturation and 1st order behavior with respect to light intensity. We have seen earlier that a strong oxidant is required for oxygen activity in the preparation and that the oxidant couples either within or very close to the reaction center. We suggest that the absence of the higher order dependence on light intensity, in the cell-free preparation, is due to the stabilization of the oxygen intermediates by the Hill oxidant.

It is possible to estimate decay times given the quantum flux and the optical cross-section of the photosynthetic unit. A simpler and more accurate method is that of repetitive light flashing of variable period. Using the latter method we were able to determine that lifetimes of the oxygen intermediates for the CO_2 and $K_3Fe(CN)_6$ coupled cells were similar. Since the CO_2 coupled whole cells show a 2nd order light dependence, we thus expect and observe that $K_3Fe(CN)_6$ coupled cells also show a 2nd order intensity dependence. The two curves differ, however, in two respects. First, the light saturated rate for $K_3Fe(CN)_6$ coupled cells is 10 times lower than that for CO_2 coupling. In addition, $K_3Fe(CN)_6$ coupling shows no 1st order intensity dependence. We attribute these differences to the dark turnover time of the system. The dark turnover time is the time required to prepare the photosystem for the full utilization of a photoact following the utilization of the previous one. The turnover time is responsible for the saturated oxygen rate at high light intensity. We see that the 10-fold lower light saturated rate in $K_3Fe(CN)_6$ coupled cells as compared to CO_2 coupling results from 10-fold longer turnover times in the former case. At high light intensities, therefore, $K_3Fe(CN)_6$ coupled cells are limited by the dark turnover time until, at the breakpoint to 2nd order light dependence, the lifetime of the intermediate becomes rate determining.

Obviously, the simpler the cell-free preparation, the easier the study of the oxygen-forming chemistry becomes. Thus our efforts are now directed toward eliminating the uncoupled antenna and further reducing the size of the preparation.

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