Photobiotechnology: Algal Hydrogen Production and Photoconductivity of Metalized Chloroplasts

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Abstract: Sustained hydrogen photoevolution from *Chlamydomonas reinhardtii* and *C. moewusii* was measured under an anoxic, CO_2-containing atmosphere. It has been discovered that light intensity and temperature influence the partitioning of reductant between the hydrogen photoevolution pathway and the Calvin cycle. Under low incident light intensity (1-3 W m^{-2}) or low temperature (approx. 0°C), the flow of photosynthetic reductant to the Calvin cycle was reduced, and reductant was partitioned to the hydrogen pathway as evidenced by sustained H_2 photoevolution. Under saturating light (25 W m^{-2}) and moderate temperature 20 ± 5°C), the Calvin cycle became the absolute sink for reductant with the exception of a burst of H_2 occurring at light on. A novel photobiophysical phenomenon was observed in isolated spinach chloroplasts that were metalized by precipitating colloidal platinum onto the surface of the thylakoid membranes. A two-point irradiation and detection system was constructed in which a continuous beam helium-neon laser (λ = 632.8 nm) was used to irradiate the platinized chloroplasts at varying perpendicular distances from a single linear platinum electrode in pressure contact with the platinized chloroplasts. No external voltage bias was applied to the system. The key objective of the experiments reported in this report was to measure the relative photoconductivity of the chloroplast-metal composite matrix.

INTRODUCTION

Hydrogen Production

Hydrogen photoevolution by unicellular algae was first demonstrated by Gaffron and Rubin (1942). Since their pioneering discovery, the reducing power driving sustained hydrogen photoevolution under anaerobiosis has been shown to be primarily derived from the water-splitting reaction of photosystem II (Bishop *et al.*, 1977). In green algae, an oxygen labile hydrogenase couples the oxidation of reduced ferredoxin with hydrogen evolution. Hydrogenase is a critical catalyst for a number of single and multistep reactions. Experimental conditions determine which reactions proceed (Bishop *et al.*, 1977; Senger and Bishop, 1979).

Light activated:

1. Photoreduction: $CO_2 + 2H_2 + \text{light} \rightarrow CH_2O + H_2O$
2. H_2 photoproduction: $XH_2 + \text{light} \rightarrow X + H_2$

Thermally activated:

3. Oxy-hydrogen reaction: $2H_2 + O_2 \rightarrow 2H_2O$
4. Dark CO_2 fixation: $CO_2 + H_2 + \text{energy} \rightarrow CH_2O + H_2O$
5. H_2 production: $RH_2 \rightarrow R + H_2$
6. H_2 uptake: $R + H_2 \rightarrow 2H_2$.

In the absence of CO_2, the absolute light-saturated rate of electron transport is greatly reduced. Under these experimental conditions hydrogen evolution is the main pathway for the utilization of photoproduced reductant. The steady-state ratio for H_2 to O_2 evolution is usually close to two, indicating that essentially all electrons generated by the biophotolysis of water are expressed as H_2 (Greenbaum, 1984; Ward *et al.*, 1985). If CO_2 is available sustained hydrogen photoproduction does not occur (Gaffron and Rubin, 1942; Stuart and Gaffron, 1972). Gaffron and Rubin (1942) demonstrated that under anaerobic conditions dinitrophenol inhibited CO_2 reduction in *Scenedesmus* and allowed hydrogen photoproduction to occur in the presence of CO_2. Similarly, glucose and carbonyl cyanide-m-chlorophenylhydrazone stimulated H_2 photoevolution in the presence of CO_2 (Kaltwasser *et al.*, 1969). Those experiments suggested the coexistence of two reductant sinks, with the Calvin cycle being the primary sink in the presence of CO_2 and the hydrogen pathway serving as an alternate sink that functioned when CO_2 was absent or when the activity of the Calvin cycle was inhibited.

Stuart and Gaffron (1972) approached the problem of dual, competitive pathways using a closed system and chemical inhibitors. However, a closed-system approach is severely limited in the useful information that can be obtained. For example, uniform conditions are impossible to maintain for the duration of an experiment because the organisms modify their environment by liberating O_2 and H_2 and consuming CO_2. This situation leads to non-steady-state conditions that stimulate several of the aforementioned hydrogenase mediated reactions, thereby greatly complicating the interpretation of experimental results. The experiments described in this review have taken a new approach to this problem. First, continuous gas flow was used; this prevented O_2 inactivation of

hydrogenase resulting from the accumulation of O_2, which occurred in closed systems such as those used in most other studies of anaerobic photosynthesis. Second, continuous flow also removed photoproduced H_2 and O_2 so that the oxy-hydrogen and photoreduction reactions, photorespiration, and respiration did not occur. Third, the concentration of CO_2 was maintained at a constant level by bubbling a He-CO_2 gas mixture through the sample instead of supplying CO_2 from a finite bicarbonate or gas phase source (Gfeller and Gibbs, 1985; Stuart and Gaffron, 1972; Healey, 1970; Gaffron and Rubin, 1942). Fourth, the ability to perform long-term experiments in which all measured reactions were driven into steady-state provided additional information that has not been available previously. The coexistence of two pathways that directly utilize photogenerated reductant provides a unique opportunity to experimentally examine the kinetic competition between the pathways for reductant and the regulatory mechanisms that work to control and balance the activities of the Calvin cycle, the hydrogen pathway, and the photosynthesis. The key discovery of this report is control of partitioning of photogenerated reductant between the hydrogen pathway and the Calvin cycle, by variable light intensity and temperature.

Photoconductivity

Photosynthesis is vectorial photochemistry. Light quanta that are captured in photosynthetic reaction centers initiate a primary electron transfer reaction, resulting in spatial separation of electrostatic charge across the photosynthetic membrane. The vectorial nature of photosynthesis lies in the intrinsic orientation of the reaction centers embedded in the membranes. Electron flow is from the inner membrane surface of the flattened sac-like vesicles to the outer surface (Amesz, 1987). In normal photosynthesis, electrons from the reducing end of Photosystem I are used for the enzymatic reduction of NADP+ to NADPH. This reduction is mediated by ferredoxin and ferredoxin NADP-reductase. NADPH serves as the electron carrier to the Calvin cycle for the enzymatic reduction of atmospheric carbon dioxide to plant matter.

It has been shown that colloidal platinum can be precipitated onto the surface of photosynthetic membranes so that water is photobiocatalytically split into molecular hydrogen and oxygen upon illumination with light of any wavelength in the chlorophyll absorption spectrum (Greenbaum, 1985). Since no electron mediator such as ferredoxin or methyl viologen was present, the colloidal platinum must have been precipitated sufficiently close to the Photosystem I reduction site to allow interfacial electron transfer from the membrane to the platinum. The presence of a reticulated network of platinum particles embedded in the chloroplast matrix suggests that metal-like properties can, at least partially, be imparted to the chloroplasts. In this work, the relative photoconductivity of the material was measured by the flow of photocurrent in the plane of the entrapped platinized chloroplasts of uniform thickness from the point of laser irradiation to the linear platinum wire electrode.

RESULTS

Hydrogen Production

Under anaerobic conditions in the absence of CO_2, the ratio of light-saturated, steady-state H_2 and O_2 photoevolution by *C. reinhardtii* and *C. moewusii* approached two. However, when CO_2 was introduced, photosaturated O_2 evolution increased dramatically and light-driven H_2 evolution ceased, except for a transient burst that occurred when the light was turned on. These observations were consistent with those of Stuart and Gaffron (1972).

The 5—10-fold increase in light-driven O_2 production corresponded with the typical increase attributed to the bicarbonate effect on PS II activity (Vermaas and Govindjee, 1981). The lack of sustained H_2 photoevolution suggested that CO_2 also affected the activation of the Calvin cycle because CO_2 is well known to activate RuBP Carboxylase by providing substrate and positive effector (Lorimer *et al.*, 1976). The Calvin cycle provided a faster kinetic sink for reductant than the H_2 pathway thereby eliminating H_2 photoevolution under conditions where photosynthesis and the Calvin cycle were fully activated.

Hydrogenase activity in the presence of CO_2

Dark hydrogen evolution and the burst of H_2 accompanying 'light on' in the presence of CO_2 indicated that hydrogenase activity was not adversely affected by CO_2 or the high O_2 photoevolution rate in the continuous flow experimental system. The yield of H_2 represented by the burst at 'light on' was about 25 nmol for the cell suspension containing 50 μg Chl. Normalizing the data to 1 mg of Chl, the H_2 yield from the burst was 500 nmol/mg Chl. By measuring the H_2 yield associated with the burst in other, similar experiments, the yield was found to be variable, ranging from 200 to 1000 nmol/mg Chl (data not shown). This information allowed an estimation of the number of reducing equivalents contributed by each photosynthetic electron transport chain for the transient burst. The following calculation was based on the data normalized to 1 mg Chl.

500×10^{-7} mol H_2/(1.1×10^{-6} mol Chl/500 mol Chl/mol photosynthetic electron transport chains) = 227 mol H_2/mol electron transport chain.

With 2 electrons per H_2, a total of 454 electrons were spent in the burst of H_2. This number far exceeds the approx. 5-20 reducing equivalents per electron transport chain stored in a completely reduced plastoquinone pool (Joliot, 1967; Forbush and Kok, 1968; Stiehl and Witt, 1969; Greenbaum, 1979).

To determine the source of the reductant driving the burst of H_2 at light-on in the presence of CO_2, an ethanolic solution of DCMU was added to the algal cells to give a final DCMU concentration of 15 μM. This level of DCMU completely eliminated the burst of H_2 at light-on as well as O_2 photoevolution (data not shown). However, sustained light-driven H_2 evolution was observed for a few hours after the addition of DCMU demonstrating that stored reductant can be used for light-driven H_2 photoevolution.

The effect of light intensity on H_2 and O_2 photoevolution

Light intensity was varied from 0 to 25 W m^{-2}. Under low light conditions, sustained H_2 photoevolution was detected with the maximum ratio of H_2 to O_2 being

about one at 0°C, sustained, albeit low, H_2 photoproduction was observed even at the highest light intensity.

In the absence of CO_2 at 20°C and at various light intensities, H_2 and O_2 were coevolved with a hydrogen-to-oxygen ratio of about 2, as previously reported (Greenbaum, 1984). The yield of H_2 and O_2 at 0°C was similar to that seen at 20°C, and the stoichiometry of H_2-to-O_2 was approx. 2.

The effect of temperature on H_2 and O_2

Light-driven H_2 production was measured at temperatures ranging from 0 to 40°C. Pretreatment of the algae had a significant effect on O_2 and H_2 photoevolution. The cells adapted to changes in temperature by shifting their optimal photoproduction temperature. For example, cells held overnight at 0°C showed higher O_2 yields at 5°C than cells taken from 20 to 5°C within 2 h and assayed immediately. After cooling to 0°C, the algae were assayed for 1 h then the temperature was raised 5°C and the same algae were assayed for 1 h followed by another 5°C temperature adjustment and 1 h assay until 40°C was reached. The temperature changes required less than 5 min each and the steady-state rate of photoevolution was then measured after 1 h of irradiation.

At 0°C steady-state H_2 and O_2 photoevolution occurred in the presence of CO_2. The ratio of H_2 to O_2 was about 1.2, indicating that the hydrogen pathway was not the sole pathway for reductant utilization. Under these conditions, competition for reductant evidently existed between the Calvin cycle and the H_2 pathway. Moderate temperatures (>5 and <30°C) caused the cessation of H_2 photoevolution; however, at warmer temperatures (>30°C), a small portion of the photogenerated reductant was shunted to the hydrogen pathway.

In the absence of CO_2, the H_2-to-O_2 photoevolution ratio remained close to 2 until the temperature rose to >25°C. At warmer temperatures, the H_2-to-O_2 ratio significantly exceeded 2, suggesting that additional reductant was being supplied from a source other than PS II. The alternative source, which may enter the photosynthetic electron transport chain through the plastoquinone pool (Diner and Mauzerall, 1973; Gfeller and Gibbs, 1985; Peltier *et al.*, 1987), was light driven since H_2 photoevolution fell to zero when the light was turned off.

Photoconductivity

Table 1 presents data for four platinum concentrations. A 5-mL suspension of spinach chloroplasts (containing 3 mg of chlorophyll) was used. Platinization of chloroplasts is feasible because hexachloroplatinate can be converted to metallic platinum at pH 7 and room temperature (Anderson, 1975). These are experimental conditions that preserve photoactivity of the isolated chloroplasts. The platinized chloroplasts were entrapped on a thin, fiberglass filter pad (Millipore, AP40) and were moistened with Walker's assay medium (1980), in which the chloroplasts were suspended. The fiberglass filter pad was 0.3 mm thick, had an active filtration area of 10.4 cm^2, and contained no binder resins. The thickness of the chloroplast film was estimated be between 0.01 and 0.1 mm. A silver–silver chloride reference electrode was placed in pressure contact with the filter; a straight, single, platinum wire of 0.2 mm diameter was placed in pressure contact with the entrapped platinized chloroplasts. The electrodes, platinized chloroplasts, and filter paper were held together with lucite plates and compression screws. To prevent the electrochemistry of atmospheric oxygen from interfering with cathode reactions by providing an alternative electron pathway, the entire assembly was placed in a small glass chamber sealed with an O-ring. The O-ring was pierced to allow the passage of two narrow-diameter wires for establishing electrical contact with the electrodes. Premoistened helium gas flowed through the chamber to flush out atmospheric oxygen. After about 45 min, the oxygen concentration of the chamber was below 3 ppm as measured by a calibrated Hersch electrogalvanic cell. Calibration was achieved with an in-line electrolysis cell and Faraday's law of electrochemical equivalence.

TABLE 1
Vectorial Photocurrents and Electron-Transfer Distances in Platinized Chloroplasts as a Function of Platinum Loading

Line no.	I_0,[a] nA	D_{max}, mm	[Pt],[b] mg/mL
1	0	0	0.24
2	0	0	0.49
3	3.6	2.3	0.97
4	7.8	3.4	1.94

[a]I_0 is defined as the measured photocurrent when $D = 0$. This corresponded to the laser beam position when it was directly over the linear electrode and was the maximum current measured in each run.

[b]Aqueous-phase platinum concentration from which the platinum precipitation step was performed. Lines 1 and 2 are the average of two runs each. Line 3 is the average of three runs. Line 4 is the average of six runs.

The platinum precipitation step was performed in a water-jacketed reactor cell containing 8.0 mL of suspension maintained at 20°C. Molecular hydrogen was passed over the head space of the reactor while a teflon-coated magnetic stirrer was used to gently stir the chloroplast suspension in a neutral hexachloroplatinate solution. Purge times of 30 to 60 min were used. After incubation and precipitation, the reactor chamber was opened to air, the contents were filtered onto the filter pad, and the cell was assembled as described previously. This coprecipitation step was essential.

The novel observation in this research is the effect of the precipitated platinum on the photoconductivity of the chloroplast matrix. Each data point represented a steady-state flow of current. Although steady-state was achieved within a few minutes of each change in the laser beam position, sustained photocurrent could be observed for hours. Each data point represents a dwell time of 15 to 20 min.

The photocurrent had a maximum value when the laser beam was directly over the platinum wire. Although the laser beam was partially blocked by the wire electrode when the beam was positioned directly over it, this loss of

light was not sufficient to cause a drop in photocurrent. This observation is reasonable since the diameter of the laser beam is larger than the diameter of the platinum wire electrode. Also, the close physical proximity of the laser light and wire on the umbral periphery of the wire provided for efficient charge collection. The extent to which the laser beam could be moved from the wire and still generate measurable photocurrent depended on the concentration of the solution from which the platinum was precipitated. Table 1 is a summary of the data of initial currents and maximum distances that were observed for varying distances.

DISCUSSION

Hydrogen Production

These results establish the coexistence of two competitive pathways for photosynthetically generated reductant and support the findings of Gaffron and coworkers that under anaerobiosis and CO_2, inhibitors of photophosphorylation and carbon reduction stimulate H_2 photoevolution in microalgae. Light and temperature physically accomplished a result similar to that achieved by the chemical inhibitors used in prior research (Gaffron and Rubin, 1942; Stuart and Gaffron, 1972). Under low light intensities the activity of many Calvin cycle enzymes is known to be reduced (Buchanan, 1980), resulting in an imbalance between the number of reducing equivalents generated by photosynthetic water splitting and the number consumed for carbon reduction. At least part of the excess was expressed as H_2 via the hydrogen pathway. Similarly, both high and low temperature caused a disparity between production and demand, again leading to the expression of H_2. Based on these observations and those of Gaffron and colleagues (Gaffron and Rubin, 1942; Stuart and Gaffron, 1972), any event that selectively impairs the demand of the Calvin cycle for reductant should lead to the expression of the excess reducing equivalents as H_2. The experimental conditions lead to a relative imbalance between reductant demand by the Calvin cycle and reductant supply by photosynthetic water splitting. The results suggest that the activity of the photosynthetic electron transport chain and the photosystems was less sensitive to low temperatures and light regulation than the Calvin cycle. The fact that H_2 photoevolution rates under He were comparable at both 20 and 0° C indicated that hydrogenase was not limiting at the lower temperature.

Hydrogen evolution in the dark was accounted for by the dark hydrogen production reaction (Healey, 1970). The burst of H_2 at light-on represented H_2 photoevolution. The reductant expended in the burst may be from at least three different sources: (1) electrons may be released from the completely reduced plastoquinone pool. One complete release would account for about 10 hydrogen molecules per photosynthetic electron transport chain, assuming 20 PQ/pool. (2) Electrons may be released from a stored pool of reductant other than the plastoquinone pool although this reductant may enter the photosynthetic electron transport chain via the plastoquinone pool (Diner and Mauzerall, 1973; Greenbaum, 1984; Gfeller and Gibbs, 1985; Peltier *et al.*, 1987). (3) Hydrogen photoproduction may be directly driven by the biophotolysis of water. Since the amount of reductant necessary to give a hydrogen burst of the measured magnitude must equal about 450 electrons, the plastoquinone pool cannot be the sole source of reductant. An alternative pool, biophotolysis of water, or both may contribute to the transient burst. Inhibition of O_2 evolution with DCMU eliminated the burst of H_2 at light-on indicating that the burst was driven by the biophotolysis of water. However, the observation of sustained light-driven H_2 production in the absence of O_2 photoevolution indicated that stored reductant was entering the photosynthetic electron transport system under these conditions. The most likely explanation for the burst of H_2 upon illumination is a time delay between photosynthetic reductant production and activation of light regulated Calvin cycle enzymes. During the lag time, reductant is partitioned to the H_2 pathway. However, the possibility that electrons from an alternative source contribute to the burst cannot be completely excluded since H_2 evolution was observed in the presence of DCMU.

In conclusion, the coexistence of two competitive pathways for photogenerated reducing equivalents has been demonstrated in *C. reinhardtii* and *C. moewusii* adapted for H_2 evolution in the presence of CO_2. The experimental manipulation of electron partitioning between the two pathways, using the physical parameters of light and temperature, indicates that the pathways coexist under certain experimental conditions. These pathways provide the organisms with a mechanism to avoid becoming 'over reduced' under circumstances where reductant availability exceeds demand. These experiments also demonstrate that the flow of photogenerated reductant can be partially and reversibly switched from carbon fixation to hydrogen evolution for the production of a high-energy, inorganic compound by simply changing conditions in the physical environment.

Photoconductivity

The origin of the photocurrent can be understood as follows: It has previously been shown that colloidal platinum can make electrical contact with the reducing end of Photosystem I of photosynthesis. This contact was demonstrated by (1) the photocatalytic evolution of molecular hydrogen[2] and (2) the observation of photocurrent in a sandwichlike photobioelectrochemical cell (Greenbaum, 1989). Upon illumination, the platinum electrode in pressure contact with the platinized chloroplasts swung negative with respect to the silver–silver chloride electrode that was in pressure contact with the electrolyte-impregnated filter pad. No external bias was placed on the electrodes to force the direction of photocurrent flow.

A reasonable model for these results, based on the generally accepted structure of photosynthetic membranes (Marder and Barber, 1989), is that colloidal platinum precipitated onto the external surface of the thylakoid membranes forms an isopotential surface whose distance is determined by the connectivity of the reticulated colloidal particles that are the metallic component of the chloroplast-metal composite matrix. This distance is a statistical parameter whose average value is determined by the nature of the platinum precipitation process. When platinum is precipitated, it does so in a nonspecific manner on the external surface of the thylakoid

membranes. There is, however, an electrostatic interaction between the negative charge of the hexachloroplatinate ions and the local positive charge of the lysine residues constituting part of the polypeptides of the Photosystem I proteins (Colvert and Davis, 1983).

The experimental system described in this report differs qualitatively from prior research performed with photosynthesis-based bioelectrochemical cells that utilized various organelles and components to generate photocurrents. Examples of prior research include chlorophyll liquid crystals (Aizawa *et al.*, 1978; Aizawa *et al.*, 1979) pigmented bilayer membranes (Tien, 1976), chloroplasts (Haehnel and Hochheimer, 1979), chloroplast membranes (Allen *et al.* 1974; Allen and Crane, 1976; and Allen, 1977), algae (Ochiai *et al.*, 1980), and photosynthetic bacterial reaction centers (Drachev *et al.*; Janzen and Seibert, 1980). For example, a Photosystem II-enriched submembrane fraction in a photoelectrochemical cell operated in potentiostatic mode was used by Lemieux and Carpentier to generate photocurrents (1988). The cell included artificial electron acceptors acting as charge transfer mediators between the photosynthetic membrane and the working electrode. Trissl and Kunze took another approach to generating and measuring photoelectric signals; they studied primary electrogenic reactions in chloroplasts probed by picosecond flash-induced dielectric polarization (1985). Seibert and Kendall-Tobias measured photoelectrochemical properties of electrodes coated with photoactive-membrane vesicles isolated from photosynthetic bacteria (1982). In their work chromatophores, isolated from the photosynthetic bacterium *Rhodopseudomonas sphaeroides* R-26, were prepared as a film on tin oxide electrodes, and the response to red light was examined in a liquid junction photoelectrochemical cell. Alexandrowicz and Berns measured photovoltages in chloroplast extract bilayer membranes stimulated by micromolar amounts of oxidants and reductants (1980).

The distinguishing feature of the work presented in this report is that the planar composite matrix of precipitated platinum and chloroplast membranes is the conductive medium. That is to say, unlike chloroplast suspensions coupled to electrodes by redox-active mediators (there are no mediators in the preparation) or chloroplast preparations or films in close physical proximity to the electrodes, the composite photobioelectronic material itself is the photoconductive pathway. It was demonstrated that the concentration of the solution from which the platinum was precipitated directly affects the relative photoconductivity of the sample.

In conclusion, it has been demonstrated that electrical contact with the reducing end of Photosystem I was achieved by precipitating colloidal platinum in the presence of isolated chloroplasts. The presence of the platinum had a significant effect on the photoconductivity of the metal-biological composite material. This work is technologically significant because the photosynthetic reaction centers are nanometer structures with picosecond switching times. This work demonstrates that the electron transport chain of photosynthesis can be electrically contacted and that the larger structural matrix of the platinized chloroplasts demonstrates enhanced photoconductivity.

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