Hydrogen Evolution by a Chloroplast–Ferrodoxin–Hydrogenase System
(solar energy conversion/photosynthesis/electron transport)

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ABSTRACT Spinach chloroplast preparations were mixed with Clostridium kluyveri hydrogenase and ferredoxin. Hydrogen evolution could be measured in the light in the absence of any added electron donors. Inhibition of the water-splitting reaction or of photosystem II reduced the amount of H₂ evolved more than 95%, indicating that H₂O was the electron donor in this reaction. The rates of H₂ evolution observed were up to 20% of those measured in the presence of an oxygen-consuming reaction or of photosystem I electron donors. These findings indicate that hydrogen evolution from water and sunlight by photosynthetic processes could be a method for solar energy conversion.

As is well recognized, coupling the photosynthetic system of plants or algae to a hydrogenase could result in a light-driven splitting of water into hydrogen and oxygen. This proposed electron transport pathway (Fig. 1) might be useful in the conversion of solar energy into a readily utilisable nonpolluting fuel source. The possibility of such a reaction has been investigated and the results are the subject of this report.

The coupling of spinach chloroplast photosystem I to a clostridial hydrogenase with ferredoxin was noted by Arnon et al. (1). Yoch and Arnon (2) coupled heated chloroplast fragments to nitrogenase, and this system proved useful in isolating electron carriers involved in nitrogen fixation (3-5). In these experiments 2,6-dichlorophenolindophenol (DPIP)-ascorbate was the electron-donor system, but the use of water as an electron donor was not investigated. In a preliminary report (6), a 3-(3,4-dichlorophenyl)-1,1-dimethyleurea (DCMU)-sensitive nitrogenase reaction was found by mixing spinach chloroplasts with crude nitrogen-fixing Azotobacter vinelandii extracts and ferredoxin. ATP-driven nitrogenase activity by this system, measured either as acetylene reduction or hydrogen evolution, was dependent on an oxygen-consuming reaction(s) present in the crude extracts.

We have now found that hydrogen evolution by a chloroplast–ferredoxin–hydrogenase system can proceed in the absence of any added electron donors or exogenous substrate-dependent oxygen-uptake systems. Our results suggest that an efficient splitting of water into hydrogen and oxygen is a feasible process.

METHODS

Grana preparations were made by liquifying 100 g of deveined spinach leaves with 240 ml of STN buffer [0.4 M sucrose-0.05 M Tris·HCl-0.01 M NaCl (pH 7.8)] for 2 min at 4°. After it was filtered through a muslin cloth, the extract was centrifuged at 3000 × g for 1 min. The pellet was discarded and the supernatant was centrifuged at 12,000 × g for 10 min. The pellets were resuspended in 120 ml of STN buffer and the centrifugation steps were repeated. The pellets were resuspended in 8 ml of STN, and chlorophyll was estimated from the absorption coefficient determined by MacKinney (7).

Catalase (EC 1.11.1.6; 50,000 units/mg) and glucose oxidase (EC 1.1.3.4; 90 units/mg) were obtained from Calbiochem; DCMU was a gift from Prof. W. L. Butler; all other chemicals were reagent grade. Clostridium kluyveri cells were grown by Dr. G. D. Novelli. Techniques for growing large-scale cultures of these organisms will be presented elsewhere.

The hydrogenase preparation used in the experiments was obtained by chromatography of a heated and protamine sulfate-treated Clostridium kluyveri extract on a DEAE-cellulose column with a NaCl gradient according to the procedure of Nakos and Mortenson (8). Fractions containing hydrogenase were combined and concentrated by ultrafiltration to give a preparation containing 63 mg of protein per ml with a specific activity of 1.5 μmol of H₂ evolved per min per mg of protein, as measured with dithionite as reducing agent and a clostridial ferredoxin as electron carrier. Clostridium kluyveri ferredoxin was obtained by stripping the DEAE-cellulose column with 0.8 M NaCl. The ferredoxin fraction was desalted (Sephadex G-25) and concentrated (ultrafiltration). Ferredoxin concentration was estimated by assuming a millimolar extinction coefficient at 390 nm of 30.6 mM⁻¹ cm⁻¹ (9).

Hydrogen evolution was assayed in 7-ml Fernbach flasks fitted with a serum stopper and flushed with argon. All solutions were transferred under argon with syringes. The total reaction volume was 1 ml. Hydrogenase was injected to start the reactions after the flasks containing all other reagents had preincubated for 5 min in the light. The flasks were incubated on special holders at 30° in a Gilson Warburg manometer apparatus with illumination from below provided by 30-W reflector lights (light intensity measured with a Kettering Radiant Power Meter was 6.8 × 10⁴ ergs/cm² per sec). Reactions were terminated by injection of 0.3 ml of 25% Cl₂COOH.

Hydrogen was determined by injecting 1 ml of water into the assay flasks (to maintain a positive pressure in the flask), removing 1 cm³ of gas, and injecting it into a Hewlett Packard 700 gas chromatograph equipped with a 6-ft. × 1/8-in. molecular sieve 5A column. Argon was used as a carrier gas.

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyleurea; DPIP, 2,6-dichlorophenolindophenol.
the column temperature was \(30^\circ\), and hydrogen was detected by a thermal conductivity detector (filament current 130 mA) after a retention time of 30 sec. The response to hydrogen was linear over a range from 2 nmol to 20,000 nmol of hydrogen per flask (1 nmol of \(H_2\) per flask = 0.15 cm of peak height).

**RESULTS**

Mixing the grana preparation with clostridial hydrogenase and ferredoxin resulted in a significant amount of hydrogen evolution after 15 min of incubation under argon in the light (Table 1, assay 1). This hydrogen evolution was stimulated by catalase; however, catalase was not required because cyanide (1 mM) inhibition of the endogenous catalase found in chloroplasts resulted in only a slight decrease in hydrogen evolution by the basic system. The inhibitory effects of both DCMU and mild heating (45°, 10 min) of the chloroplast preparation indicated that both photosystem II and the water-splitting reaction are required for hydrogen evolution to proceed. Hydrogen evolution was also dependent on light (Table 1, assay 5) and on all three components of the basic system (see below).

Table 2 shows the effects of photosystem I electron donors (ascorbate and DPIP) and an oxygen-consuming reaction (glucose and glucose oxidase) on the basic system. The chlorophyll concentration in these experiments was half that used in Table 1. Chlorophyll concentration curves gave a linear response with the basic system and catalase up to 0.2 mg of chlorophyll (4.0 \(\mu\)mol of \(H_2\) evolved per mg of chlorophyll per hr). Higher concentrations of chlorophyll were decreasingly effective; however, the system was not completely saturated even at 1 mg of chlorophyll. Addition of the photosystem I electron donor system ascorbate–DPIP (with DCMU added to block photosystem II) resulted in a 6-fold stimulation of the rate of \(H_2\) evolution. With this electron donor system, chlorophyll concentration curves were linear up to 0.1 mg of chlorophyll (26.7 \(\mu\)mol of \(H_2\) evolved per mg of chlorophyll per hr) and completely saturated at 0.4 mg of chlorophyll (Table 2, assay 3). Other chloroplast preparations, such as the “whole” chloroplasts of Cramer and Butler (10) or the “fragmented” chloroplasts used by Yoch (11) in the chloroplast-nitrogenase assay, proved to be much less effective (by a factor of about three) in these experiments. The uncoupler NH\(_4\)Cl (2 mM) was routinely added to the assays. Although its effects in the basic system were minor, if any, it did stimulate \(H_2\) evolution from ascorbate–DPIP as much as 50%.

A stimulation of hydrogen evolution by the basic system was also observed upon addition of glucose and glucose oxidase (Table 2, assay 4). Glucose and glucose oxidase react with oxygen to give gluconate and hydrogen peroxide. Similar to the basic system, there is some stimulation by catalase of hydrogen evolution with glucose–glucose oxidase (Table 2, assay 5). The inhibitory effect of DCMU in the presence of glucose–glucose oxidase demonstrates the dependence on photosystem II of this reaction. The stimulation by glucose–glucose oxidase can thus be attributed to a consumption of the oxygen evolved by the photosystem II-dependent water-splitting reaction (see Discussion). Oxygen was a potent inhibitor (at 2% concentration) of hydrogen evolution by the basic system even in the presence of ascorbate–DPIP.

The dependence on ferredoxin by the coupled chloroplast–ferredoxin–hydrogenase system is shown in Fig. 2. The basic system (with catalase) did not saturate even with 10 nmol of ferredoxin, while with ascorbate–DPIP maximal hydrogen evolution was observed with 1 nmol of ferredoxin. The crude ferredoxin preparation used in these experiments saturated the ascorbate–DPIP-dependent hydrogen evolution at remarkably low levels, and high ferredoxin concentrations were inhibitory (Fig. 2).

The time-course of hydrogen evolution by the chloroplast–ferredoxin–hydrogenase system (+ catalase) indicates a rapid initial rate, which decreases after about 15 min. The rate of hydrogen evolution after 30 min is only one-fourth of that observed during the initial 15 min. Refluxing the assay flasks with argon after 60 min of reaction did not result in a recovery of the initial rates, and assays with dithionite indicated a severe (about 80%) inactivation of the hydrogenase. Thus, the decreased rate of the hydrogen evolution with time appeared to be a consequence of an inactivation of the system.

Inactivation of hydrogenase did not account for this observation because of the presence of this enzyme in a 10-fold excess over that required for half-saturation. In the presence of cyanide (1 mM) or with less ferredoxin, the time-course of the reaction appeared little affected during the initial period (10 min); however, hydrogen evolution decreased

![Fig. 2. Requirements of hydrogen evolution from water and DPIP for ferredoxin. Assay conditions for hydrogen evolution from water and DPIP are the same as for those of assays 1 and 3, respectively, in Table 2, except that ferredoxin concentrations were varied as indicated.](image-url)
even more than in the basic system (+ catalase) during longer assays.

**DISCUSSION**

The results presented above demonstrate that hydrogen can be evolved from water by light energy in a reaction that is dependent on both photosystems of chloroplasts, ferredoxin, and hydrogenase (Table 1). Hydrogen evolution by this basic system was stimulated 6-fold by the addition of a photosystem I electron donor or an oxygen-consuming reaction (Table 2). Sometimes 10-fold and even higher stimulations were observed; this variability apparently depended on the spinach used to prepare the chloroplasts.

We consider that oxygen produced during the reaction of the basic system accounts for the relatively low rates of hydrogen evolution compared to those observed with photosystem I electron donors. This view is supported by the inhibition of hydrogen evolution by oxygen in these experiments and by the stimulatory effect of the oxygen-consuming system, glucose-glucose oxidase. The mechanism by which oxygen might inhibit hydrogen evolution is not known. Hydrogenase inactivation during the reaction, about 50% after 15 min, could not account for the low activities since hydrogenase was always present in excess even at the end of the reaction. Reversal of the hydrogen evolution reaction—hydrogen uptake with oxygen as an electron acceptor—also was not a significant factor because with 2% H₂ and 2% O₂ no hydrogen uptake was observed in the presence of ferredoxin and hydrogenase.

The reaction of oxygen with reduced ferredoxin, resulting in superoxide formation (12, 13) which dismutates to hydrogen peroxide, is a likely mechanism for oxygen inhibition of hydrogen evolution since it results in a loss of the reductant required by hydrogenase. The need for higher concentrations of both ferredoxin and chlorophyll in the basic system than in the presence of photosystem I electron donors supports the view that the limiting factor in hydrogen evolution by the basic system is the rate at which ferredoxin reduction counterbalances ferredoxin oxidation by the oxygen produced during the reaction. Whether the hydrogenase reaction itself is inhibited by oxygen or some other oxidant such as hydrogen peroxide remains to be established.

| Table 1. Requirements for hydrogen evolution by the chloroplast-ferredoxin-hydrogenase system |
|---------------------------------------------|-------------------------------------------------|
| **Assay no.** | **μmol of H₂ evolved per 15 min** |
| 1 | Basic system | 0.25 |
| 2 | + catalase | 0.34 |
| 3 | + catalase + DCMU | 0.00 |
| 4 | heated chloroplasts (45°C; 10 ml) + catalase | 0.01 |
| 5 | light | 0.00 |

The basic system contained 0.8 mg of chlorophyll; 18 μmol of C. kuyveri ferredoxin; 470 μg of C. kuyveri hydrogenase; 15 μmol of HEPES buffer (pH 7.6); 1.5 μmol of MgCl₂; 2 μmol of NH₄Cl. The other assays also contained, as indicated, 0.4 mg of catalase and 0.1 μmol of DCMU. The assays were started with the hydrogenase (see Methods) and terminated after 15 min.

**Table 2. Effects of various additions to the chloroplast-ferredoxin-hydrogenase system**

<table>
<thead>
<tr>
<th><strong>Assay no.</strong></th>
<th><strong>μmol of H₂ evolved per 15 min</strong></th>
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<tbody>
<tr>
<td>1</td>
<td>Basic system</td>
</tr>
<tr>
<td>2</td>
<td>+ catalase</td>
</tr>
<tr>
<td>3</td>
<td>+ ascorbate + DPIP + DCMU</td>
</tr>
<tr>
<td>4</td>
<td>+ glucose + glucose oxidase</td>
</tr>
<tr>
<td>5</td>
<td>+ glucose + glucose oxidase + catalase</td>
</tr>
<tr>
<td>6</td>
<td>+ glucose + glucose oxidase + DCMU</td>
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The basic system contained 0.4 mg of chlorophyll; other additions and conditions are as in Table 1. 10 μmol of ascorbate, 0.05 μmol of DPIP, 30 μmol of glucose, 0.1 μmol of DCMU, and 0.3 mg of glucose oxidase were added as indicated.

It is possible that in the basic system no oxygen is actually evolved into the gas phase along with hydrogen. The oxygen produced could be transformed into hydrogen peroxide (giving a net reaction of 2H₂O + light → H₂ + 1/2O₂). The stimulatory effect of catalase might be due to either a Mehler reaction (14) using endogenous substrates or the decomposition of inhibiting hydrogen peroxide accumulated in the system. The stimulatory effects of glucose and glucose oxidase, explained by the oxygen-removing action of this system, give indirect evidence for oxygen production by the basic system. Therefore, even though the data presented indicate that oxygen is produced during the reaction, it remains to be established whether O₂ is actually evolved by the basic system.

The problems of ferredoxin autooxidation, hydrogenase inactivation, and photosystem II instability must be resolved before photosynthetic hydrogen evolution can be considered for solar energy conversion. The establishment of such a reaction in living algae or the development of a stable biochemical system bound to solid supports are possible means for accomplishing this goal.

Another possibility for coupling of photosynthesis with hydrogen evolution has been done in the laboratory of Prof. L. Krampitz at Case Western Reserve University. It involves the trapping of reducing equivalents generated by the photosynthesis system in a small carrier molecule (such as TPN and methylviologen) (15). Another suggestion has been made that the reducing equivalent could be used for the reduction of acetaldehyde or acetic acid to alcohol and then the alcohol could be reoxidized in a separate reactor to give hydrogen and acetaldehyde (or acetate) (16).

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