J. Biochem. 123, 644-649 (1998)

Hydrogen Evolution by Direct Electron Transfer from Photosystem I to Hydrogenases[1]

Hugh McTavish

Department of Biochemistry, University of Minnesota, 1479 Gortner Ave., St. Paul, Minnesota 55108, USA

Received for publication, October 20, 1997

H_2 evolution by direct electron transfer from the dithionite-reduced photosystem I (PSI) complex to both hydrogenase I and hydrogenase II from *Clostridium pasteurianum* was observed. Evidence indicates that the electron carriers on PSI that transfer electrons to hydrogenase in this system are the F_A/F_B iron-sulfur clusters on the PsaC polypeptide, the terminal bound electron acceptors in PSI. Light-dependent H_2 evolution was also observed, using high potential electron donors to PSI, from a combination of hydrogenase I and either solubilized purified PSI or thylakoids. Mediators capable of transferring electrons from the PSI complex to hydrogenase were not necessary for H_2 evolution, indicating again that the mechanism of H_2 evolution is direct electron transfer from PSI to hydrogenase, and that this can occur with light-reduced as well as chemically reduced PSI, and with PSI in thylakoids as well as the solubilized complex. Light-dependent H_2 evolution was also observed from a mixture of thylakoids and the oxygen-resistant hydrogenase of *Rhodococcus* sp. MR11. These results suggest that direct electron transfer from PSI to hydrogenase could be engineered to occur *in vivo* in a photosynthetic organism to create an organism that would efficiently produce H_2 from H_2O.

Key words: *Clostridium pasteurianum*, *Rhodococcus* sp. MR11.

Solar generation of H_2 has long been recognized to be an ideal means of generating energy. Sunlight and water are both free and almost limitless. And hydrogen is almost pollutionless, yielding only water vapor when burned.

The most efficient system for converting solar energy to chemical form yet developed is the system developed by green plants: photosynthesis. Thus, perhaps the most efficient way to generate hydrogen by photolysis of water would be to adapt the photosynthetic system, modifying it so that the reducing equivalents generated, rather than reducing $NADP^+$ to NADPH and then generating carbohydrates, are used to reduce protons to H_2. This has been accomplished *in vitro* by Benemann *et al.* (*1*), who showed light-induced hydrogen evolution with water as an electron donor from a system of thylakoids, ferredoxin, and the hydrogenase from *Clostridium kluyveri*. Photosystem I (PSI) in the thylakoids reduced ferredoxin, which donated electrons through hydrogenase to H^+. Methyl viologen (*2*) and cytochrome c_3 (*3*) could replace ferredoxin as electron carriers in this system.

The results indicated that the rate and quantum yield of H_2 evolution were limited by the rate of electron transfer between PSI and hydrogenase (*2*). Hydrogenase was not the rate-limiting factor, because if dithionite was added in the dark to chemically reduce all the methyl viologen or ferredoxin, the rate of hydrogen evolution increased by a factor of four (*2*). Rather, reduced ferredoxin and methyl viologen did not accumulate to high levels (*2, 4*). The electron carriers under aerobic conditions were oxidized by oxygen. Even under anaerobic conditions, oxidation of these carriers, most likely their oxidation by the donor side of PSI to give cyclic electron transfer, competed effectively with oxidation by hydrogenase (*2, 4*).

Hydrogen evolution in this system would be more efficient if the electron carrier could be eliminated, if hydrogenase could accept electrons directly from PSI. Presumably that would mean accepting electrons from the F_A/F_B iron-sulfur clusters, held on the PsaC protein, which are the terminal bound electron acceptors in PSI (*5*). The primary advantage of direct electron transfer from PSI to hydrogenase would be to reduce or eliminate electron flow to destinations other than hydrogen production. A second advantage is that direct electron transfer would give a greater thermodynamic driving force. F_A and F_B, the terminal bound electron carriers on PSI, have redox potentials of -530 and -550 mV (*6, 7*), *i.e.* significantly lower than the H^+/H_2 E_o' of -420 mV, whereas spinach ferredoxin's E_o' is -400 mV.

PsaC, the polypeptide that holds the terminal bound electron acceptors of PSI, is an acidic extrinsic membrane protein of 8.8 kDa in spinach that holds two 4Fe-4S clusters, the F_A and F_B clusters (*5, 8, 9*). To be stably held onto the PSI core, PsaC requires the PsaD protein, a basic extrinsic membrane protein (*5*). PsaC is approximately the same size as and exhibits sequence homology with bacterial ferredoxins, including the one from *C. pasteurianum*, which also hold two 4Fe-4S clusters (*9*). Since ferredoxin is the natural electron donor to the Fe-only hydrogenases I and II

[1] This work was supported by the United States Department of Agriculture, grant number 94-37500-1073.

Abbreviations: PSI, photosystem I; DCMU, *N'*-(3,4-dichlorophenyl)-*N,N*-dimethylurea.

of *C. pasteurianum* (*10–12*), the latter would seem to be good candidates for accepting electrons from the PsaC protein in the PSI core.

A drawback to the *C. pasteurianum* hydrogenases is that, like almost all hydrogenases, they are inhibited by O_2. This would make them unsuitable for a practical H_2 production system without mutation to an oxygen-resistant form. Since O_2 resistance is so important, the hydrogenase from *Rhodococcus* sp. MR11 (formerly *Nocardia opaca* 1b) (*13*), which is able to be turned over in the presence of oxygen, was also tested for H_2 evolution by direct electron transfer from PSI.

MATERIALS AND METHODS

C. pasteurianum—Winogradsky 5 ATCC 6013 was grown under N_2, at 35°C in YEM medium (*14*). *Rhodococcus* sp. MR11 was grown at 30°C in minimal medium (*13*), pH 7.0, supplemented with 0.2% glycerol and 0.4% fructose until the H_2 oxidizing activity of the culture stopped increasing, which was approximately 36 h after the culture reached the stationary phase.

All steps for *C. pasteurianum* hydrogenase purification were performed anaerobically with 2 mM sodium dithionite added to all buffers. Hydrogenase I was purified as in Ref. *10*. Partially purified hydrogenase II was collected as the peak of hydrogenase activity eluted with approximately 100 mM NaCl from the first DEAE column during the purification of hydrogenase I (*11*). It was distinguishable from hydrogenase I by its higher ratio of H_2 oxidation to H_2 evolution activity. Spinach ferredoxin was purified as in Ref. *15*. *Rhodococcus* sp. MR11 hydrogenase was purified as in Ref. *13*.

To prepare the photosystem I complex, thylakoids were prepared from spinach (*16*), and then resuspended in 20 mM sodium phosphate, pH 6.3, 5 mM $MgCl_2$ at 2 mg chl/ml (300 mg chl total). Triton X-100 (25 mg/mg chl) was added and the thylakoids were solubilized for 30 min at 23°C. The mixture was centrifuged at 18,000 rpm for 30 min in a JA-20 rotor. The supernatant was collected and its pH adjusted to 7.5 with 100 mM Tris-HCl, pH 8.3. 100 mM Tris-HCl, pH 7.5, and water were added to give final concentrations of 20 mM Tris and 10 mM phosphate. The mixture was loaded onto a 2.5×13 cm column of DEAE-macroprep (BioRad) and eluted with a 0-500 mM NaCl gradient in 20 mM Tris-HCl, pH 7.5, 0.2% (w/v) Triton X-100. The green P700-containing fractions were pooled, Triton X-100 was added to a final concentration of 1.2% (w/v), and the mixture was dialyzed against 20 mM Tris- HCl, pH 7.5, at 4°C. It was then loaded onto another 2.5×13 cm DEAE-macroprep column and eluted with the same salt gradient. P700-containing fractions were concentrated to 6 ml by ultrafiltration and then passed through a 1.5×40 cm Toyopearl HW55TSK (Tosohaas, Montogeryville, PA) column equilibrated with 20 mM Tris-HCl, pH 7.5, 300 mM NaCl, 0.2% Triton X-100. The PSI fractions were then dialyzed against 50 mM Tris-HCl, pH 8.0, and concentrated by filtration with a Centriprep 3 unit (Amicon, Beverly, MA).

To prepare thylakoids from chloroplasts that had been completely broken and washed free of ferredoxin, 200 g deribbed spinach was ground in a Waring blender in 500 ml 50 mM Tris-HCl, pH 8.1, and the slurry was filtered through 4 layers of cheesecloth. Debris and unbroken chloroplasts were pelleted by centrifugation at 2,000 rpm for 2 min in a Beckman JA-10 rotor. The supernatant was removed and centrifuged at 8,000 rpm for 20 min to pellet the thylakoids. The pellet was resuspended in 25 mM Tris-HCl, pH 8.1, to osmotically burst any unbroken chloroplasts, followed by centrifugation at 2,000 rpm for 2 min. The supernatant was removed, 4 M NaCl was added to it to a final 0.5 M, and then it was centrifuged at 8,000 rpm for 20 min. The pellet was washed once in 25 mM Tris-HCl, pH 8.1, and the resuspended in the same buffer.

PsaC from *Synechococcus* sp. PCC 7002 was overexpressed from plasmid pET36C in *Escherichia coli* BL21 (DE3), purified and reconstituted as in Ref. *17*. PsaD from *Nostoc* sp. PCC 8009 was overexpressed from plasmid pET-3a/D as in Ref. *17*. pET36C and pET-3a/D were gifts from Prof. Donald A. Bryant.

To remove PsaC, PsaD, and other extrinsic polypeptides from PSI, PSI was treated with 6 M urea in 50 mM Tris-HCl, pH 8.0, at 0.25 mg chl/ml (*18*), concentrated to 5 ml, and then passed through a 1.5×40 cm Toyopearl HW55-TSK column equilibrated with 50 mM Tris-HCl, pH 8.0, 5 M urea. The green fractions were then dialyzed against 50 mM Tris-HCl, pH 8.0, 0.05% Triton X-100. For reconstitution, 1.7 ml of 0.94 mg chl/ml urea-stripped PSI was mixed with 0.9 mg reconstituted purified PsaC and 3.1 mg purified PsaD in 50 mM Tris-HCl, pH 8.0, 0.05% Triton X-100, incubated 10 min at 23°C, and then filtered and diluted three times with a Centricon 100 unit (Amicon) to remove unbound PsaC and PsaD.

Protein was measured as in Ref. *19*. *C. pasteurianum* hydrogenase I H_2 evolution activity was measured at 23°C in 40 mM Tris-HCl, pH 8.0, with 5 mM dithionite and 1.33 mM methyl viologen as electron donors or with other electron donors, as specified. H_2 evolution or uptake was measured amperometrically (*20*). The concentration of reconstituted PsaC was calculated using an ε_{410} of 15 mM^{-1} (*8*). A P700 difference ε of 64 mM^{-1} (*21*) was used to determine the concentration of PSI. In the light-driven H_2 evolution assays, *C. pasteurianum* hydrogenase I, which was stored in 2 mM dithionite, was added to the reaction mixture and then the reaction mixture was stirred and exposed to air for 20 s to oxidize all dithionite prior to adding glucose oxidase, sealing the chamber anaerobically and starting the assay. Saturating illumination was provided by a film projector lamp. PSI activity was measured as light-dependent O_2 consumption in 50 mM Tris-HCl, pH 8.1, 2 mM ascorbate, 3 μM DCMU, 100 μM 2,6-dichlorophenol-indophenol, 0.6 mM methyl viologen.

RESULTS

The evolution of H_2 gas was observed with the dithionite-reduced spinach photosystem I complex and *Clostridium pasteurianum* broken cell extracts in the dark (Table I). The fact that more H_2 evolution was observed with PSI than with dithionite alone added to the *C. pasteurianum* extract indicates that PSI is a direct electron donor to the *C. pasteurianum* hydrogenase or hydrogenases, and does not transfer electrons through *C. pasteurianum* ferredoxin, which would be fully reduced by dithionite alone.

H_2 evolution was also observed with purified *C. pasteurianum* hydrogenase I mixed with 5 mM dithionite and PSI

core complex at 0.96 mg chl/ml or 22.5 μM P700 (610 nmol H_2 evolved·min^{-1}·mg hydrogenase^{-1} at 23°C). Only 2 nmol H_2·min^{-1}·mg^{-1} was observed when PSI was absent and dithionite alone served as the electron donor to hydrogenase I.

It was important to insure that the true electron donor to hydrogenase in these assays was not some contaminating spinach ferredoxin, rather than PSI. The PSI purification procedure included a size-exclusion chromatography step, which should easily separate the 11.5 kDa spinach ferredoxin from the approximately 200 kDa PSI complex. Thus, if any ferrodoxin happened to contaminate the PSI preparation after the two ion-exchange chromatography steps, it should not have after the size-exclusion chromatography step.

One approach for ruling out that the hydrogen evolution observed with PSI as the electron donor was due to contaminating spinach ferredoxin was to precipitate the PSI complex with 40% saturated ammonium sulfate. This concentration completely precipitated the PSI but was found to not precipitate any ferredoxin when tested with purified ferredoxin. After precipitation with 40% saturated ammonium sulfate, the pellet and supernatant were separately dialyzed (1,100-molecular-weight cut-off membrane) against 50 mM Tris-HCl, pH 8.0, and then tested for the ability to support H_2 evolution with hydrogenase I (Table II). The supernatant had only 12% of the ability to support H_2 evolution of the starting PSI prior to precipitation. If the ability of the PSI preparation to support H_2 evolution had been entirely due to contaminating ferredoxin, this value should have been close to 100%. The precipitated PSI also exhibited much decreased ability to support H_2 evolution compared to the unprecipitated PSI. This might be due to irreversible aggregation of PSI, that renders the F_A and F_B iron-sulfur clusters sterically inaccessible to hydrogenase. Ammonium sulfate precipitation is based on a hydrophobic interaction, and it is easy to imagine that the extremely hydrophobic PSI complex might be irreversibly aggregated.

I hypothesize that the electron carriers on PSI that serve as the immediate electron donors to hydrogenase are the F_A and F_B iron-sulfur clusters on the PsaC polypeptide. To test this, free recombinant PsaC was expressed in *E. coli*, reconstituted, purified, and then tested for its ability to support H_2 evolution with hydrogenase I when reduced with dithionite. PsaC at 18 μM supported an H_2 evolution rate of 540 nmol H_2·min^{-1}·mg hydrogenase^{-1}. This can be compared to the rate with dithionite-reduced PSI as the electron donor (22.5 μM P700 supporting 610 nmol H_2/min/mg hydrogenase), which was assayed with the same hydrogenase preparation. PsaC and PSI at approximately the same molar concentration supported approximately the same rate of hydrogen evolution, which supports the idea that PsaC is the part of PSI serving as the electron donor to hydrogenase I.

TABLE I. **Rate of H_2 evolution catalyzed by *C. pasteurianum* extracts.** 0.2 mg protein in 50 mM Tris-HCl, pH 8.3, 100 mM NaCl, 5 mM sodium dithionite in the dark, 1.5 ml reaction volume, 23°C.

Electron donor	V (nmol H_2/min/mg protein)
Dithionite alone	33
14.5 μM PSI (0.59 mg chl/ml)	160
2 mM methyl viologen	1,520

The rate of H_2 evolution with PsaC as the electron donor linearly increased with increasing PsaC concentration up to a velocity of 2.9 μmol H_2·min^{-1}·mg hydrogenase^{-1} at 89 μM PsaC (data not shown), indicating that these concentrations of PsaC as a substrate for hydrogenase I are not saturating. It was also found that with dithionite-reduced PSI as the electron donor for hydrogen evolution catalyzed by crude *C. pasteurianum* extracts, the rate of hydrogen evolution increased with PSI concentration up to at least 39 μM, indicating that these concentrations of PSI as an electron donor are not saturating (data not shown). For comparison, the concentration, 2 mM, of methyl viologen used as the electron donor in Table I is close to saturating. Thus, with PsaC or PSI as the electron donor to hydrogenase I, the rate limiting step for hydrogen production under the conditions tested is the binding of PSI or PsaC to hydrogenase I.

F_A and F_B have rather low redox potentials (−530 and −580 mV) when bound to PSI (*6, 7*). Thus, there was some doubt as to what extent they would be reduced by dithionite in the dark. To determine whether it was possible that F_A and F_B were the electron donors to hydrogenase in this system, it was necessary to determine whether they were reduced. EPR spectra of the PSI core complex at pH 8.0 in 5 mM dithionite in the dark indicate that F_A and F_B are mostly reduced (Fig. 1). The spectrum shown in Fig. 1

TABLE II. **Rate of H_2 evolution catalyzed by *C. pasteurianum* hydrogenase I.** In 50 mM Tris-HCl, pH 8.0, 5 mM sodium dithionite, 23°C, 1.5 ml assay volume.

Electron donor	V (nmol H_2/min/mg hydrogenase)
Dithionite alone	23
PSI (525 μg chl)	442
Supernatant of 40% saturated ammonium sulfate precipitation of PSI (525 μg chl)	74
Precipitate of 40% saturated ammonium sulfate precipitation of PSI (525 μg chl)	44

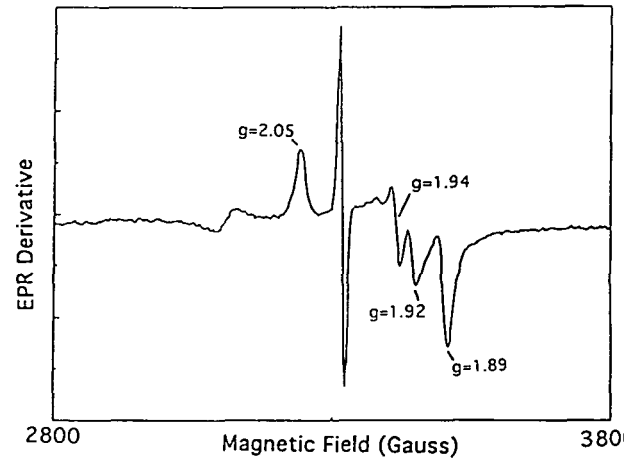

Fig. 1. **Electron paramagnetic resonance spectrum of the photosystem I core complex in 50 mM Tris-HCl, pH 8.0, 5 mM dithionite, in the dark prior to freezing.** Conditions: temperature, 18 K; microwave frequency, 9.230 GHz; microwave power, 0.6 mW; gain, 8,000; scan time, 4 min; time constant, 0.128 s; modulation amplitude, 10 G; modulation frequency, 100 kHz.

matches the reported spectrum for the F_A and F_B clusters, with the $g=1.89$ peak arising in part from interaction between the two reduced clusters (*5, 7*). Double integration of the spectrum in Fig. 1, excluding the $g=2.0$ radical signal, and comparison to a Cu-EDTA standard, gave a spin concentration of 21 μM, which would be 1.85/P700. One would predict 2 unpaired e^-/P700 if F_A and F_B were fully reduced. The amplitude of the spectrum shown in Fig. 1 increased by a factor of 1.42 if the sample was illuminated with freezing in liquid N_2. That treatment was reported to fully reduce the F_A and F_B clusters (*6, 7*), which would mean that in the dark they were 70% reduced by dithionite.

The redox potentials of the F_A and F_B iron-sulfur clusters on PsaC (−530 and −580 mV) (*6, 7*) are lower than the redox potentials of *C. pasteurianum* hydrogenase I's F iron-sulfur clusters (−420 mV) or the active site H cluster (−400 mV) (*22*), and lower than the H_2/H^+ redox couple ($E_o' = -420$ mV). Thus, the redox potentials of the iron-sulfur clusters on PsaC are low enough to support hydrogen evolution.

One approach for demonstrating that the PSI complex, and specifically the PsaC polypeptide on the PSI complex, was capable of donating electrons directly to hydrogenase was a reconstitution experiment. Purified PSI was stripped of its small extrinsic polypeptides, including PsaC and PsaD, by treatment with 6 M urea, followed by size-exclusion chromatography. Then a portion of the urea-stripped PSI was reconstituted with recombinant PsaC and PsaD. The urea-stripped PSI was found to support no more H_2 evolution than dithionite alone with *C. pasteurianum* cell extracts. But the PSI reconstituted with PsaC and PsaD did support some H_2 evolution, almost as much as would be predicted on comparison with the rate with a somewhat greater concentration of free PsaC (Table III). In Table III, as well as in Table I, the rate of hydrogen evolution with dithionite alone added to the extracts is a significant fraction of the rate with dithionite plus PSI or PsaC added. This background hydrogen evolution could arise because dithionite alone can act as an electron donor to hydrogenase or because of electron donation by the ferredoxin contained in crude *C. pasteurianum* extracts. Nonetheless, an increase in hydrogen evolution is clear when reconstituted PSI is added.

In Table III, the concentration of reconstituted PSI was 12.4 μM or 59% of the 21 μM free PsaC concentration shown in Table III. After subtracting the rate with dithionite alone, the reconstituted PSI was found to support 32% of the rate of H_2 evolution supported by free PsaC in Table III (30 nmol/min/mg for reconstituted PSI compared to 94 nmol/min/mg for free PsaC), fairly close to the velocity that was predicted. Because the reconsituted PSI was filtered three times through a 100 kDa cut-off filter, all free PsaC would have been removed (*17*). Only PsaC bound to PSI should be available to support H_2 evolution.

Like hydrogenase I, partially purified hydrogenase II of *C. pasteurianum* was also able to produce H_2 with dithionite-reduced PSI (3.2 nmol $H_2 \cdot min^{-1} \cdot mg$ hydrogenase II^{-1} at 23°C). Here, interestingly, the velocity was increased when assayed in 2 M urea, whereas there was a decrease in velocity in 2 M urea when ferredoxin was the electron donor (data not shown). This again indicates that the PSI complex, and not any contaminating ferredoxin, was the electron donor.

In addition to showing that solubilized chemically reduced PSI could be an electron donor to hydrogenase, it was important also to determine whether light-reduced PSI and PSI in the thylakoid membrane could be electron donors to hydrogenase. Figure 2 shows that the light-reduced solubilized PSI complex can donate electrons directly to *C. pasteurianum* hydrogenase for hydrogen evolution. In that system, ascorbic acid and dithiothreitol served as electron donors to PSI. Again, there was no electron mediator added to transfer electrons from PSI to hydrogenase. The quantity of hydrogenase used in Fig. 2 was 46 μmol/min total H_2 evolution activity, as assayed with dithionite-reduced methyl viologen.

Light-dependent H_2 evolution was also seen with thylakoids replacing solubilized PSI in the assay system used in Fig. 2. With thylakoids (145 μg chl, 0.44 nmol P700), 2 mM ascorbate, 50 mM dithiothreitol (DTT), 2 μM DCMU, to inhibit photosystem II, and 5.1 mg hydrogenase in 1.5 ml, the rate of H_2 evolution in the light peaked at 62 nmol/min, after 16 min illumination, during which time the rate gradually increased (data not shown). This rate represents 6% of the rate of maximal light-driven electron transfer through PSI in the thylakoids, as measured with artificial electron acceptors.

TABLE III. **Ability of urea-stripped and reconstituted photosystem I to support H_2 evolution by *C. pasteurianum* extracts.** The reaction mixtures comprised *C. pasteurianum* extracts (30 μg protein per ml) in 50 mM Tris-HCl, pH 8.0, and 5 mM sodium dithionite.

Electron donor	*V* (nmol H_2/min/mg protein)
Dithionite alone	53
12.4 μM urea-stripped PSI	56
12.4 μM urea-stripped PSI reconstituted with PsaC and PsaD	83
21 μM free PsaC	147

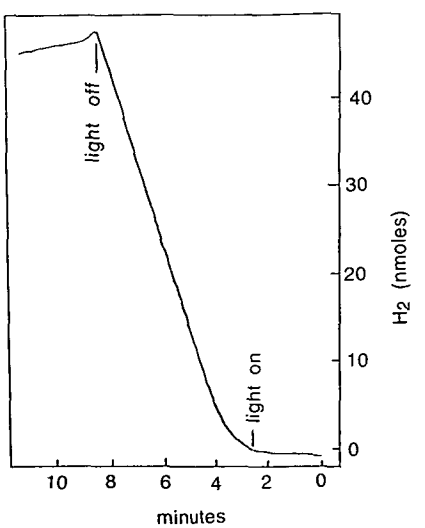

Fig. 2. **Light-dependent H_2 evolution *via* direct electron transfer from photosystem I to *C. pasteurianum* hydrogenase I.** The assay mixture of 1.5 ml comprised purified PSI (190 μg chl, 3.6 nmol P700), 5.1 mg hydrogenase I, 25 mM Tris-HCl, pH 8.1, 60 mM NaCl, 2 mM ascorbate, 50 mM dithiothreitol, 5 mM glucose, and 4.5 μg glucose oxidase. Temperature, 23°C.

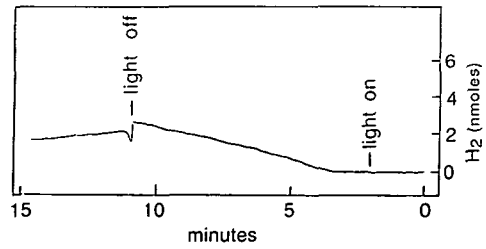

Fig. 3. **Light-dependent H_2 evolution with thylakoids and *Rhodococcus* sp. MR11 hydrogenase.** The assay mixture of 1.5 ml comprised thylakoids (145 μg chl), *Rhodococcus* sp. MR11 hydrogenase (0.28 mg), 50 mM Tris-HCl, pH 7.5, 5 mM $MgSO_4$, 2 mM ascorbate, and 50 mM dithiothreitol. Temperature, 23°C.

The amount of light-driven hydrogen evolution shown in Fig. 2 with purified solubilized PSI is 2.1 nmol H_2/min/mg hydrogenase at 2.4 μM PSI. At 0.63 μM P700 and 0.21 mg hydrogenase in 1.5 ml, under the conditions in Fig. 2, 1.24 nmol H_2/min was produced in the light. This corresponds to a rate of 9.4 nmol·min^{-1}·mg hydrogenase I^{-1}·μM PSI^{-1}. With 0.63 μM PSI, at hydrogenase I concentrations above 0.55 mg/ml, the rate of light-driven hydrogen production actually decreased with increasing hydrogenase concentration (data not shown). This would be explicable if the one-electron reduced hydrogenase can be an electron donor to the oxidized photosystem I. Hydrogenase would thus catalyze cyclic electron transfer before it could accept a second electron and produce H_2.

Consistent with this explanation, when thylakoids were used instead of solubilized PSI, the light-driven hydrogen production rate always increased with increasing hydrogenase concentration. This would be consistent with the above explanation because with thylakoids hydrogenase would not have access to the oxidizing side of PSI. It would only have that access, and therefore only be able to catalyze cyclic electron transfer, with solubilized PSI. This would explain the relatively faster rates of light-driven hydrogen production with thylakoids than with purified PSI. The light-driven H_2 production rate with thylakoids and hydrogenase I under the conditions described above, 62 nmol/min, can also be expressed as 12.1 nmol·min^{-1}·mg hydrogenase I^{-1} or 41.7 nmol·min^{-1}·mg hydrogenase I^{-1}·μM PSI^{-1}. This can be compared with the rate of hydrogen production with dithionite-reduced PSI and hydrogenase I of 610 nmol H_2·min^{-1}·mg hydrogenase I^{-1} at 22.5 μM P700, or 27 nmol H_2·min^{-1}·mg hydrogenase I^{-1}·μM PSI^{-1}. Thus, expressed as nmol H_2·min^{-1}·mg hydrogenase I^{-1}·μM PSI^{-1}, light-reduced PSI in thylakoids, light-reduced solubilized PSI, and dithionite-reduced solubilized PSI support similar rates of hydrogen evolution by direct electron transfer to hydrogenase I.

A lower level of light-driven H_2 evolution was also observed with thylakoids and the purified *Rhodococcus* sp. MR11 hydrogenase, an enzyme that is not affected by oxygen (Fig. 3). Since no electron carriers were added to this assay, the H_2 evolution observed here is again most likely due to direct electron transfer from PSI in the thylakoids to hydrogenase. Although the thylakoids were prepared in such a way as to insure the removal of ferredoxin, ferredoxin contamination was less of a concern with *Rhodococcus* sp. MR11 hydrogenase than with *C. pasteurianum* hydrogenase because no hydrogen evolution could be detected with dithionite-reduced spinach ferredoxin as the electron donor to *Rhodococcus* sp. MR11 hydrogenase.

DISCUSSION

Light-driven hydrogen evolution can occur by direct electron transfer from the PSI complex to hydrogenases I and II of *C. pasteurianum*. PSI can be in either a solubilized form or in the thylakoid membrane.

Evidence indicates that the redox centers on PSI that are the immediate electron donors to hydrogenase are the F_A and F_B iron-sulfur clusters on the PsaC polypeptide. First, EPR showed that these clusters are 70% reduced by dithionite at pH 8.0, and are thus available as electron donors to hydrogenase. Second, PSI stripped of the extrinsic polypeptides, PsaC and PsaD, loses its ability to support H_2 evolution, but it regains that ability when it is reconstituted with PsaC and PsaD. Third, approximately equimolar concentrations of dithionite-reduced PSI and PsaC supported approximately the same rate of hydrogen evolution with hydrogenase I.

Two previous EPR studies on PSI-containing membrane fragments showed only a small level of reduction of F_A and F_B by dithionite in the dark at pH 8.0 or 9.0 (*6, 7*). But the preparation described in this paper involved a second solublization step after the first DEAE chromatography step, and the more extensive solubilization could easily cause a small shift in the redox potential that would explain the discrepancy. Also, lower concentrations of dithionite were used in this study, which is known paradoxically to give a solution with a more negative redox potential (*23*).

Light-driven hydrogen evolution was observed in this study with thylakoids and *Rhodococcus* sp. MR11 hydrogenase in the absence of any diffusible electron carriers. Most likely this also occurs by direct electron transfer from PSI to hydrogenase.

Previously, *in vitro* photosynthetic hydrogen evolution was reported to require an electron carrier to transfer electrons from PSI to Clostridial hydrogenase (*1, 2, 24*). The probable reason why direct electron transfer from PSI to Clostridial hydrogenase was not detected in those studies is that the concentrations of hydrogenase and/or PSI were too low. Benemann *et al.* (*1*) used 0.7 unit of hydrogenase per ml in their assays, whereas 30 units/ml was used in the light-dependent hydrogen evolution assays with thylakoids or purified PSI described here.

The V_{max} could not be determined with either PSI or PsaC as the electron donor to hydrogenase I, as the substrate concentrations were not saturating. With PsaC as the electron donor, the rate of H_2 evolution increased linearly with PsaC concentration up to at least 89 μM. This indicates that for the most efficient electron transfer, hydrogenase and PSI would have to be at fairly high concentrations around each other. High local concentrations of hydrogenase around PSI might be achieved *in vivo* by expressing a recombinant hydrogenase at high levels in a cyanobacterial cell. In that way a cyanobacterium might be engineered that would use direct electron transfer from PSI to hydrogenase to efficiently produce hydrogen from water.

The native hydrogenase I of *C. pasteurianum* would be unsuitable from a practical standpoint in any photosynthetic hydrogen production scheme such as this, because it is inhibited and irreversibly inactivated by O_2. But it might

be possible to engineer this hydrogenase for oxygen resistance, as the *Azotobacter vinelandii* hydrogenase was (*25*), or a naturally oxygen-resistant hydrogenase such as *Rhodococcus* sp. MR11 hydrogenase might be used.

I with to thank Professor Larry Wackett for the use of his laboratory space and for the helpful discussions. I also thank Professor John Lipscomb for the use of his EPR spectrometer. I also thank two anonymous reviewers for their helpful suggestions.

REFERENCES

1. Benemann, J.R., Berenson, J.A., Kaplan, N.O., and Kamen, M.D. (1973) Hydrogen evolution by a chloroplast-ferredoxin-hydrogenase system. *Proc. Natl. Acad. Sci. USA* **70**, 2317-2320
2. Rosen, M.M. and Krasna, A.I. (1980) Limiting reactions in hydrogen photoproduction by chloroplasts and hydrogenase. *Photochem. Photobiol.* **31**, 259-265
3. Hall, D.O., Adams, M.W.W., Morris, P., and Rao, K.K. (1980) Photolysis of water for H_2 production with the use of biological and artificial catalysts. *Phil. Trans. R. Soc. London A* **295**, 473-476
4. Nikandrov, V.V., Van Nhi, C., Brin, G.P., and Krasnovskii, A.A. (1979) Investigation of the conditions of photoreduction of methylviologen by chloroplasts. *Mol. Biol. (Moscow)* **12**, 1278-1287
5. Golbeck, J.H. and Bryant, D.A. (1991) Photosystem I. *Curr. Top. Bioenerget.* **16**, 83-177
6. Ke, B., Hansen, R.E., and Beinert, H. (1973) Oxidation-reduction potentials of bound iron-sulfur proteins of photosystem I. *Proc. Natl. Acad. Sci. USA* **70**, 2941-2945
7. Heathcote, P., Williams-Smith, D.L., Sihra, C.K., and Evans, M.C.W. (1978) The role of the membrane-bound iron-sulphur centres A and B in the photosystem I reaction centre of spinach chloroplasts. *Biochim. Biophys. Acta* **503**, 333-342
8. Wynn, R.M. and Malkin, R. (1988) Characterization of an isolated chloroplast membrane Fe-S protein and its identification as the photosystem I Fe-S_A/Fe-S_B binding protein. *FEBS Lett.* **229**, 293-297
9. Oh-Oka, H., Takahashi, Y., Kuriyama, K., Saeki, K., and Matsubara, H. (1988) The protein responsible for center A/B in spinach photosystem I: isolation with iron-sulfur clusters and complete sequence analysis. *J. Biochem.* **103**, 962-968
10. Chen, J.-S. and Mortenson, L.E. (1974) Purification and properties of hydrogenase from *Clostridium pasteurianum* W5. *Biochim. Biophys. Acta* **371**, 283-298
11. Adams, M.W.W. and Mortenson, L.E. (1984) The purification of hydrogenase II (uptake hydrogenase) from the anaerobic N_2-fixing bacterium *Clostridium pasteurianum*. *Biochim. Biophys. Acta* **766**, 51-61
12. Adams, M.W.W. (1990) The structure and mechanism of iron hydrogenases. *Biochim. Biophys. Acta* **1020**, 115-145
13. Schneider, K., Cammack, R., and Schlegel, H.G. (1984) Content and localization of FMN, Fe-S clusters and nickel in the NAD-linked hydrogenase of *Nocardia opaca* 1b. *Eur. J. Biochem.* **142**, 75-84
14. Palosaari, N.R. and Rogers, P. (1988) Purification and properties of inducible coenzyme A-linked butyraldehyde dehydrogenase from *Clostridium acetobutylicum*. *J. Bacteriol.* **170**, 2971-2976
15. Buchanan, B.B. and Arnon, D.I. (1971) Ferredoxins from photosynthetic bacteria, algae, and higher plants in *Methods in Enzymology* (San Pietro, A., ed.) Vol. 23, pp. 413-440, Academic Press, New York
16. Kuwabara, T. and Murata, N. (1982) Inactivation of photosynthetic oxygen evolution and concomitant release of three polypeptides in the photosystem II particles of spinach chloroplasts. *Plant Cell Physiol.* **23**, 533-539
17. Li, N., Zhao, J., Warren, P.V., Warden, J.T., Bryant, D.A., and Golbeck, J.H. (1991) PsaD is required for the stable binding of PsaC to the photosystem I core protein of *Synechococcus* sp. PCC 6301. *Biochemistry* **30**, 7863-7872
18. Parrett, K.G., Mehari, T., Warren, P.G., and Golbeck, J.H. (1989) Purification and properties of the intact P-700 and F_x-containing photosystem I core protein. *Biochim. Biophys. Acta* **973**, 324-332
19. Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254
20. Sweet, W.J., Houchins, J.P., Rosen, P.R., and Arp, D.J. (1980) Polarographic measurement of H_2 in aqueous solutions. *Anal. Biochem.* **107**, 337-340
21. Marsho, T.V. and Kok, B. (1980) P700 detection in *Methods in Enzymology* (San Pietro, A., ed.) Vol. 69, pp. 280-289, Academic Press, New York
22. Adams, M.W.W. (1987) The mechanism of H_2 activation and CO binding by hydrogenase I and hydrogenase II of *Clostridium pasteurianum*. *J. Biol. Chem.* **262**, 15054-15061
23. Mayhew, S.G. (1978) The redox potential of dithionite and SO_2^- from equilibrium reactions with flavodoxins, methyl viologen, and hydrogen plus hydrogenase. *Eur. J. Biochem.* **85**, 535-547
24. Rao, K.K., Gogotov, I.N., and Hall, D.O. (1978) Hydrogen evolution by hydrogenase-chloroplast systems: improvements and additional observations. *Biochimie* **60**, 291-296
25. McTavish, H., Sayavedra-Soto, L.A., and Arp, D.J. (1995) Substitution of *Azotobacter vinelandii* hydrogenase small-subunit cysteines to serines can create insensitivity to inhibition by O_2 and preferentially damages H_2 oxidation over H_2 evolution. *J. Bacteriol.* **177**, 3960-3964