

# Sustained Photoevolution of Molecular Hydrogen in a Mutant of *Synechocystis* sp. Strain PCC 6803 Deficient in the Type I NADPH-Dehydrogenase Complex

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The interaction between hydrogen metabolism, respiration, and photosynthesis was studied *in vivo* in whole cells of *Synechocystis* sp. strain PCC 6803 by continuously monitoring the changes in gas concentrations (H<sub>2</sub>, CO<sub>2</sub>, and O<sub>2</sub>) with an online mass spectrometer. The *in vivo* activity of the bidirectional [NiFe]hydrogenase [H<sub>2</sub>:NAD(P) oxidoreductase], encoded by the *hoxEFUYH* genes, was also measured independently by the proton-deuterium (H-D) exchange reaction in the presence of D<sub>2</sub>. This technique allowed us to demonstrate that the hydrogenase was insensitive to light, was reversibly inactivated by O<sub>2</sub>, and could be quickly reactivated by NADH or NADPH (+H<sub>2</sub>). H<sub>2</sub> was evolved by cells incubated anaerobically in the dark, after an adaptation period. This dark H<sub>2</sub> evolution was enhanced by exogenously added glucose and resulted from the oxidation of NAD(P)H produced by fermentation reactions. Upon illumination, a short (less than 30-s) burst of H<sub>2</sub> output was observed, followed by rapid H<sub>2</sub> uptake and a concomitant decrease in CO<sub>2</sub> concentration in the cyanobacterial cell suspension. Uptake of both H<sub>2</sub> and CO<sub>2</sub> was linked to photosynthetic electron transport in the thylakoids. In the *ndhB* mutant M55, which is defective in the type I NADPH-dehydrogenase complex (NDH-1) and produces only low amounts of O<sub>2</sub> in the light, H<sub>2</sub> uptake was negligible during dark-to-light transitions, allowing several minutes of continuous H<sub>2</sub> production. A sustained rate of photoevolution of H<sub>2</sub> corresponding to 6 μmol of H<sub>2</sub> mg of chlorophyll<sup>-1</sup> h<sup>-1</sup> or 2 ml of H<sub>2</sub> liter<sup>-1</sup> h<sup>-1</sup> was observed over a longer time period in the presence of glucose and was slightly enhanced by the addition of the O<sub>2</sub> scavenger glucose oxidase. By the use of the inhibitors DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea] and DBMIB (2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone), it was shown that two pathways of electron supply for H<sub>2</sub> production operate in M55, namely photolysis of water at the level of photosystem II and carbohydrate-mediated reduction of the plastoquinone pool.

Cyanobacteria are oxygenic photosynthetic prokaryotes containing two photosystems, PS II and PS I, linked by a linear electron transport chain similar to that of higher plant or algal chloroplasts. Much attention has been focussed on H<sub>2</sub>-producing cyanobacteria in the hope that the photosynthetic reactions producing reductant from water can be coupled to the reduction of protons to generate molecular hydrogen at the expense of solar energy (3, 11, 24, 31, 32, 39). In cyanobacteria, two different enzymes capable of H<sub>2</sub> production *in vivo* have been described, nitrogenase and reversible hydrogenase. The first enzyme, which occurs only in N<sub>2</sub>-fixing strains, reduces protons to H<sub>2</sub> concomitantly with the reduction of N<sub>2</sub> to NH<sub>4</sub><sup>+</sup>. In these organisms, however, H<sub>2</sub> production is limited due to an efficient recycling of H<sub>2</sub> by an uptake [NiFe]hydrogenase, which is present in all known N<sub>2</sub>-fixing strains (31, 39). Accordingly, it has been demonstrated that mutations impairing the activity of uptake hydrogenase (26, 41) or inactivating the structural *hupSL* genes (13) can stimulate H<sub>2</sub> photoproduction in N<sub>2</sub> fixers. The other type of hydrogenase found in cyanobacteria is the reversible [NiFe]hydrogenase (42). The first molecular characterization of this enzyme in *Anabaena* and *Anacystis* species showed that it belonged to the class of NAD(P)-reducing hydrogenases homologous to the previously described

HoxFUYH complex of *Ralstonia eutropha* or *Nocardia opaca* (33). The reversible hydrogenase is not ubiquitous enzyme in cyanobacteria, but it has been found both in N<sub>2</sub> fixers and non-N<sub>2</sub> fixers.

The complete genome sequence of *Synechocystis* sp. strain PCC 6803 (18, 27) and a survey based on a molecular approach (40) showed the presence of genes encoding the reversible hydrogenase and the absence of both nitrogenase and uptake hydrogenase. The *Synechocystis* reversible hydrogenase is a pentameric [NiFe] enzyme utilizing NAD(P) as a substrate (4, 34). The HoxY and HoxH subunits form the [NiFe]hydrogenase moiety, while three other subunits (HoxU, HoxF, and HoxE), homologous to subunits of complex I of respiratory chains, contain NAD(P), flavin mononucleotide, and FeS binding sites (3, 6, 18, 33, 34; also reviewed in reference 44). Since no hydrogenase activity has been found in a hydrogenase deletion mutant ( $\Delta$ *hoxH*), it has been concluded that in *Synechocystis* sp. strain PCC 6803 only the bidirectional HoxEFUYH hydrogenase functions for H<sub>2</sub> uptake or H<sub>2</sub> production (2).

In *Synechocystis*, following anoxic incubation in the dark, H<sub>2</sub> photoproduction has been described as limited to a low-amplitude transient outburst (1, 9). The authors hypothesized that it was driven by counteracting evolution and uptake reactions. As only the reversible hydrogenase is present in *Synechocystis* strain PCC 6803, variations in amplitude and direction of the H<sub>2</sub> flux may rely on two main factors: the hydrogenase activity (related to enzyme amounts and to its activation status) and

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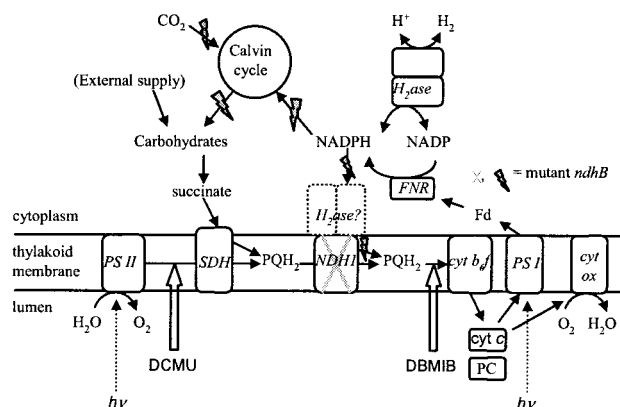


FIG. 1. Proposed electron transfer pathways in the light involved in  $H_2$  production and uptake in *Synechocystis* sp. strain PCC 6803. Abbreviations:  $H_2ase$ , hydrogenase; PS I, photosystem I; PS II, photosystem II; cyt, cytochrome; *cyt b<sub>6</sub>f*, cytochrome *b<sub>6</sub>f* complex; cyt ox, cytochrome *aa<sub>3</sub>* oxidase; SDH, succinate dehydrogenase; NDH-1, type I NAD(P)H-dehydrogenase; PQ, plastoquinone; PC, plastocyanin; FNR, ferredoxin-NADP<sup>+</sup> reductase; Fd, ferredoxin. Electron transfers are represented by solid arrows; DCMU and DBMIB inhibition sites are indicated by thick white arrows. Enzyme names are in italics. Grey flash arrows indicate pathways which are impaired in the *ndhB* mutant M55.

the redox status of the donor/acceptor NAD(P)H/NAD(P) pool.

The activity of hydrogenases is generally inhibited by  $O_2$ . The reversible hydrogenases of cyanobacteria are considered to be more  $O_2$  sensitive than the uptake hydrogenases (14, 39). In the case of *Synechocystis* strain PCC 6803,  $H_2$  production has been observed only in anoxia (1, 9), but interestingly, the hydrogenase is constitutively expressed in the presence of  $O_2$  and its suppression was reported to induce a perturbation in photosynthetic activity (2), indicating that it might be active under such conditions.

In cyanobacterial cells, the redox status of the NAD(P)H/NAD(P) pools is essentially controlled by the activities of photosynthesis and respiration. Photosynthetic electron transport takes place in the thylakoid membrane, while respiratory electron transport occurs in both the thylakoid and the cytoplasmic membranes. In the thylakoids, the respiratory and photosynthetic electron transport chains have electron carriers in common (cytochrome *b<sub>6</sub>f* complex and the plastoquinone [PQ] pool; see Fig. 1). The photosynthetic electron transport chain reduces NADP to NADPH, which is the electron donor used for photosynthetic  $CO_2$  fixation and is also the preferential substrate of the respiratory complex I of cyanobacteria, also called the type I NADPH-dehydrogenase complex NDH-1 (8, 25), which is largely confined to the thylakoid membrane (30).

NDH-1 is encoded by the *ndh* genes, which number only 11 in the *Synechocystis* genome (18, 27), instead of the 14 found in other, related bacterial complexes. The three missing genes are those coding for the peripheral diaphorase subunits. It has been suggested that these subunits might be used simultaneously by the reversible hydrogenase and the respiratory complex I (3, 4, 6, 35), as is shown in Fig. 1. On the other hand, the bidirectional hydrogenase is absent in a significant number of cyanobacterial strains (40), and there is evidence that there is not a common use of the diaphorase subunits by the hydroge-

nase and NDH-1 (5). Different cyanobacterial mutants affected in the NDH-1 complex have been obtained. The large hydrophobic subunit, NdhB, is a core membrane component of the complex, and the *ndhB* gene is present in a single copy in the genome. Therefore, the *ndhB*-deficient mutant M55 is totally devoid of the NDH-1 complex (28), in contrast to other *ndh* mutants affected in multicopy genes, such as *ndhD* and *ndhF* (22, 29). M55 has a high  $CO_2$ -requiring phenotype and is characterized by a low respiration rate and a reduced activity of cyclic electron flow around PS I (28). As a result, M55 shows a highly impaired photosynthetic activity under normal  $CO_2$  concentrations (Fig. 1), with the NADP pool remaining almost entirely in the reduced state (8).

The goal of this study was to understand the nature of the interactions between hydrogenase function, respiratory activity, and photosynthesis, a prerequisite to looking for appropriate strains allowing economical and efficient  $H_2$  production. To this end, we have used time-resolved mass spectrometry to measure  $H_2$ ,  $O_2$ , and  $CO_2$  fluxes in wild-type (WT) *Synechocystis* and in the NDH-1-deficient mutant M55 and to monitor hydrogenase activity by the proton-deuterium (H-D) exchange reaction in the  $D_2$ - $H_2O$  system. While a low and transitory rate of  $H_2$  production was observed in the WT during dark-to-light transition, the NDH-1-deficient mutant was able to sustain significant  $H_2$  production in the light. We discuss the mechanisms underlying the competition between NDH-1,  $CO_2$  fixation, and hydrogenase for reducing equivalents in *Synechocystis* (Fig. 1) and their potential use for improving the  $H_2$ -producing capacities of this organism.

## MATERIALS AND METHODS

**Strains and growth conditions.** WT *Synechocystis* strain PCC 6803 and mutant strains were grown autotrophically in liquid aerated modified Allen's medium (16) at 34°C under continuous illumination, using two fluorescent tubular lamps, which provided an average light intensity of 70  $\mu\text{mol}$  of photons  $\text{m}^{-2} \text{s}^{-1}$ . The high  $CO_2$ -requiring mutant M55 (*ndhB::Km<sup>r</sup>* cartridge) (kindly provided by T. Ogawa) was grown in medium supplemented with 100  $\mu\text{g}$  of kanamycin  $\text{ml}^{-1}$  and 10 mM  $\text{NaHCO}_3$  (28).

**Cell fractionation.** Cells grown autotrophically were pelleted, resuspended in 50 mM morpholineethanesulfonic acid (MES)-KOH buffer, pH 6, supplemented with 2% (wt/vol) bovine serum albumin before two passages through a French press (16,000 psi). Cell debris was removed from the cell extract by centrifugation at 3,000  $\times g$  for 10 min.

**Mass spectrometric measurements of gas exchange.** Cultures were harvested in the exponential or plateau phase of growth, centrifuged, washed in distilled water, and resuspended in 35 mM HEPES-NaOH buffer, pH 7.2, or in 50 mM MES-KOH buffer, pH 6.0. The cell suspension was placed in the measuring chamber (1.5 ml) of a mass spectrometer (model MM 8-80; VG Instruments, Cheshire, United Kingdom). The bottom of the chamber (Hansatech electrode type) was sealed by a Teflon membrane, allowing dissolved gases to be directly introduced through a vacuum line into the ion source of the mass spectrometer. The chamber was thermostated at 30°C, and the cell suspension was stirred continuously by a magnetic stirrer. Light was supplied to the suspension by a fiber optic illuminator (Schott, Mainz, Germany). Experiments were performed at 300  $\mu\text{mol}$  of photons  $\text{m}^{-2} \text{s}^{-1}$  of incident light. Inhibitors (DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea] and DBMIB [2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone]) when used were introduced directly into the chamber with a Hamilton syringe about 10 min before experiments. Before hydrogenase activity was studied, cells were subjected to anaerobic dark adaptation in HEPES-NaOH buffer (pH 7.2); after transfer to the measuring chamber of the mass spectrometer, the cells were sparged with argon, the  $\text{Ar}$  was removed, the vessel was closed, and the cells were left to consume the residual  $O_2$  (or, when indicated,  $O_2$  was removed by  $O_2$  scavengers).

The principle of the kinetic measurements of  $H_2$  production,  $H_2$  uptake, H-D exchange,  $O_2$  uptake or production, and  $CO_2$  uptake has been described previously (17). The spectrometer sequentially scans the abundance of the different

TABLE 1. Simple model for H-D exchange combined with H<sub>2</sub> production or uptake

Equation	Exchange	Production ( $d\Sigma/dt > 0$ )	Uptake ( $d\Sigma/dt < 0$ )
1	$\frac{d[D_2]}{dt} = -V_{\text{exch}} \frac{[D_2]}{\Sigma}$	+0	$+\frac{d\Sigma}{dt} \cdot \frac{[D_2]}{\Sigma}$
2	$\frac{d[HD]}{dt} = V_{\text{exch}} \frac{[D_2]}{\Sigma} - \frac{1}{2} V_{\text{exch}} \frac{[HD]}{\Sigma}$	+0	$+\frac{d\Sigma}{dt} \cdot \frac{[HD]}{\Sigma}$
3	$\frac{d[H_2]}{dt} = \frac{1}{2} V_{\text{exch}} \frac{[HD]}{\Sigma}$	$+\frac{d\Sigma}{dt}$	$+\frac{d\Sigma}{dt} \cdot \frac{[H_2]}{\Sigma}$

gases (H<sub>2</sub>, D<sub>2</sub>, HD, O<sub>2</sub>, and CO<sub>2</sub>) by automatically adjusting the magnet current to the corresponding mass peaks ( $m/e = 2, 4, 3, 32,$  and  $44,$  respectively). Measuring one mass peak typically takes 0.5 s. Mass peaks are continuously recorded during the experiment, every 2 s for fast-kinetic measurements, e.g., for light-to-dark transitions, or every 10 s for measuring slower kinetics, e.g., during hydrogenase induction. The amperometric signal collected by the spectrometer is directly proportional to the gas concentration in the chamber, the proportionality coefficient varying from one mass to the other according to the ionization properties of the corresponding gas. In order to calculate the rates of gas exchange, the time derivation of gas concentrations has to be corrected for the

slow but significant rate of gas consumption by the mass spectrometer, which is superimposed on production or uptake rates. The consumption of gases by the mass spectrometer was assayed in cell-free buffer. It showed first-order kinetics, with time constants around 0.09 min<sup>-1</sup> for H<sub>2</sub>, 0.08 min<sup>-1</sup> for D<sub>2</sub>, and 0.024 min<sup>-1</sup> for O<sub>2</sub>; CO<sub>2</sub> consumption by the apparatus was negligible under the test conditions. The setup also allowed us to determine the in vivo activity of hydrogenase by the use of hydrogen isotopes for the H-D exchange reaction, in which D<sub>2</sub> disappears and is quantitatively replaced by HD and H<sub>2</sub>. Calculation of hydrogen gas exchange has been elaborated as described below.

**Modeling of the H-D exchange reaction catalyzed by hydrogenase enzymes in the presence of various relative concentrations of D<sub>2</sub> and of H<sub>2</sub>-consuming or H<sub>2</sub>-producing processes.** As a first approximation for modeling H-D exchange, we will assume that the cleavage is heterolytic and that the D<sup>+</sup> concentration in water is negligible, so that each D<sub>2</sub> exchange event leads to 1 HD molecule and each exchange of an HD molecule leads with a 0.5 probability to either 1 H<sub>2</sub> or 1 HD molecule, the H<sub>2</sub> interaction with the hydrogenase being neutral in this respect. When reducing or oxidizing equivalents are provided to the hydrogenase, H<sub>2</sub> production or uptake can be superimposed on the H-D exchange activity. We will consider that production and uptake are mutually exclusive at a given time and are therefore equal to the net rate of change in hydrogen concentration (all isotopic species combined). We will also assume that the probability that one hydrogen species interacts with the hydrogenase (either for H-D exchange or for uptake) is equal to its proportion in the mix and that H<sub>2</sub> production, apart from the H-D exchange, yields only H<sub>2</sub> coming from water protons. In the following equations, the total concentration of hydrogen species ( $\Sigma = [D_2] + [HD] + [H_2]$ );  $d\Sigma/dt$  is then the net production or uptake of

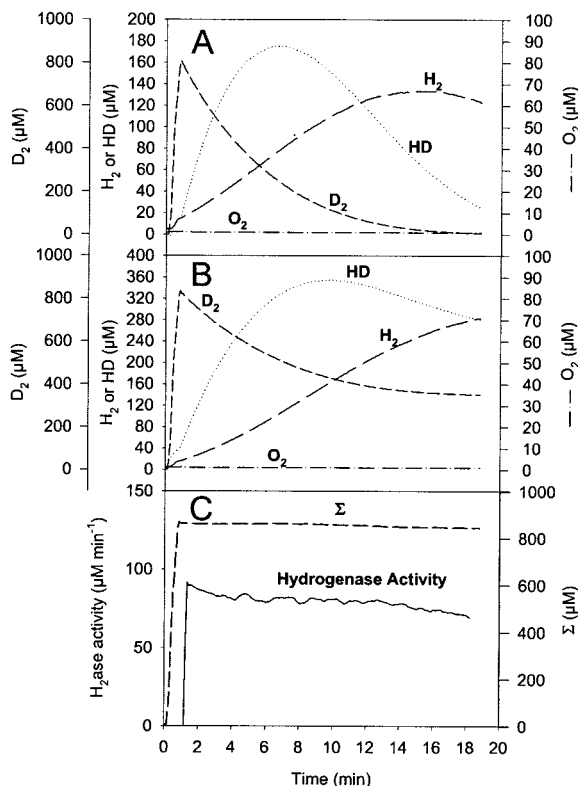


FIG. 2. H<sub>2</sub> and HD production in exchange with D<sub>2</sub> uptake catalyzed by *Desulfovibrio fructosovorans* [NiFe]hydrogenase. (A) Real concentrations of the hydrogen species present in the vessel (D<sub>2</sub> concentration reaches zero). (B) Gas concentration changes corrected for consumption by the apparatus (shown is the equivalence between D<sub>2</sub> uptake and H<sub>2</sub> plus HD production). (C) Hydrogenase activity, calculated as  $\frac{1}{\tau} \left( 2 \frac{d[H_2]}{dt} + \frac{d[HD]}{dt} \right)$ , is expressed in nanomoles per milliliter per minute (or micromolar per minute). The sum ( $\Sigma$ ) of D<sub>2</sub> plus H<sub>2</sub> plus HD concentrations is calculated as described in Materials and Methods.  $\Sigma$  remains constant since only the exchange reaction between hydrogen isotopes and protons of the medium is taking place here.  $V_{\text{exch}}$  and hydrogenase activity are then confounded.

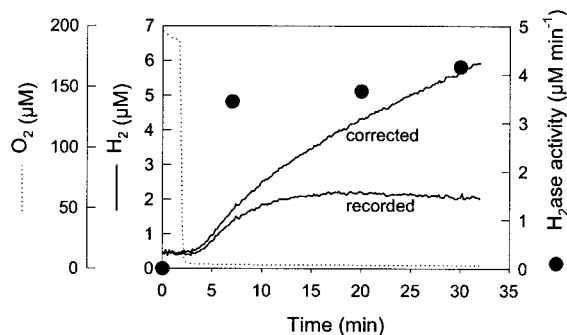


FIG. 3. Anaerobic H<sub>2</sub> production in darkness. A cell suspension of *Synechocystis* sp. strain PCC 6803 (10 μg of Chl ml<sup>-1</sup>) in 35 mM HEPES buffer, pH 7.2, was placed in the measuring chamber of the online mass spectrometer, and catalase (500 U), glucose (5 mM), and glucose oxidase (30 U) were added to make the medium totally anaerobic. The concentrations of H<sub>2</sub> (straight line, “recorded”) and O<sub>2</sub> (dotted line) were recorded every 10 s at mass peaks 2 and 32, respectively. The curve labeled “corrected” shows H<sub>2</sub> evolution in the medium corrected for the consumption by the apparatus and thus directly reflects H<sub>2</sub> production by the cells. For the determination of hydrogenase activity by the H-D exchange reaction (●), the cells were preincubated in darkness under the same conditions as those described above for the indicated periods of time and then sparged with D<sub>2</sub> and hydrogenase activity, based on the rates of H<sub>2</sub> and HD formation, was calculated as described in Materials and Methods and in the legend to Fig. 2C.

hydrogen (<0 in case of uptake, >0 in case of production);  $V_{\text{exch}}$  is the turnover rate of hydrogen species at the hydrogenase active site leading to the H-D exchange reaction (i.e., the H-D exchange activity of the enzyme); and  $\tau$  is the isotopic ratio of D in hydrogen:  $\tau = ([D_2] + 1/2[HD])/\Sigma$ . A simple model for H-D exchange combined with hydrogen production or uptake can then be formulated (Table 1) (in order to simplify the equations, the rates described are those corrected for consumption by the mass spectrometer).

The hydrogenase activity can then be deduced from  $D_2$ , HD, and  $H_2$  concentration changes, by simple rearrangement of the equations (Table 1). In the case of a simple H-D exchange ( $d\Sigma/dt = 0$ ), adding equations 2 and 3 ( $\times 2$ ) (Table 1, Exchange column) gives

$$2 \frac{d[H_2]}{dt} + \frac{d[HD]}{dt} = V_{\text{exch}} \frac{[D_2]}{\Sigma} + \frac{1}{2} V_{\text{exch}} \frac{[HD]}{\Sigma} = V_{\text{exch}} \cdot \tau$$

which gives

$$H_2\text{ase activity} = V_{\text{exch}} = \frac{1}{\tau} \left( 2 \frac{d[H_2]}{dt} + \frac{d[HD]}{dt} \right)$$

An example of calculations in the case of the H-D exchange reaction only (in the absence of electron donors or acceptors) is shown in Fig. 2. These results, obtained with a pure hydrogenase sample, validate the calculations and show that the method is suitable for assaying the H-D exchange rate for a wide range of  $D_2$  concentrations.

When  $H_2$  production occurs simultaneously with H-D exchange ( $d\Sigma/dt > 0$ ), addition of equations 2 and 3 ( $\times 2$ ) (Table 1, Exchange and Production columns) yields

$$2 \frac{d[H_2]}{dt} + \frac{d[HD]}{dt} = V_{\text{exch}} \frac{[D_2]}{\Sigma} + \frac{1}{2} V_{\text{exch}} \frac{[HD]}{\Sigma} + 2 \frac{d\Sigma}{dt} = V_{\text{exch}} \cdot \tau + 2 \frac{d\Sigma}{dt}$$

which gives

$$V_{\text{exch}} = \frac{1}{\tau} \left( 2 \frac{d[H_2]}{dt} + \frac{d[HD]}{dt} - 2 \frac{d\Sigma}{dt} \right)$$

Then

$$H_2\text{ase activity} = V_{\text{exch}} + \frac{d\Sigma}{dt}$$

When uptake occurs simultaneously with H-D exchange ( $d\Sigma/dt < 0$ ), addition of the terms of equations 2 and 3 ( $\times 2$ ) (Table 1, Exchange and Uptake columns) finally yields

$$V_{\text{exch}} = \frac{1}{\tau} \left( 2 \frac{d[H_2]}{dt} + \frac{d[HD]}{dt} - 2(1 - \tau) \frac{d\Sigma}{dt} \right)$$

Then

$$H_2\text{ase activity} = V_{\text{exch}} - \frac{d\Sigma}{dt}$$

The mathematical treatment developed in this report extends the H-D exchange interpretation method, previously essentially applied to the initial rates of  $H_2$  and HD production, to the whole period during which isotopes are present (i.e., about 20 to 30 min in our setup). The treatment allows monitoring of changes in hydrogenase activity over time and enables us to evaluate whether the measured concentration changes of the hydrogen species are simply related to the H-D exchange reaction or are accompanied by  $H_2$  production or uptake.

**Other measurements.** Pigments were extracted by methanol from pelleted cells, and the chlorophyll concentration in the centrifuged cell extract was measured at 665 nm (23).

**Chemicals.** Chemicals were all supplied by Sigma-Aldrich (Saint Louis, Mo.).

## RESULTS

**Anaerobic hydrogenase activation and  $H_2$  production in darkness.** *Synechocystis* cells were placed in the dark in the online measuring chamber of the mass spectrometer. No  $H_2$  was detected in the medium when  $O_2$  was present. The cells were then made anaerobic by the addition of glucose and glucose oxidase in the presence of catalase.  $H_2$  gas production began around 1 to 2 min after  $O_2$  was completely depleted from the medium (Fig. 3). The  $H_2$  concentration in the medium increased progressively, reaching a stable value when  $H_2$

production by the cells was compensated for by  $H_2$  consumption by the mass spectrometer. The maximal  $H_2$  production rate (around 0.35 nmol of  $H_2$  ml<sup>-1</sup> min<sup>-1</sup>) was sustained during 2 to 3 min at the beginning of the production period and progressively decreased to a lower steady-state rate (around 0.15 nmol of  $H_2$  ml<sup>-1</sup> min<sup>-1</sup>). The activity of hydrogenase was measured by the H-D exchange reaction assay in separate experiments repeated under identical conditions. In the presence of  $O_2$ , H-D exchange was not detectable (Fig. 3). Five minutes after anaerobiosis had been reached, the hydrogenase activity assayed by the H-D exchange reaction was 3.5 nmol ml<sup>-1</sup> min<sup>-1</sup> (or 350 nmol min<sup>-1</sup> mg of chlorophyll (Chl)<sup>-1</sup>) and increased only slightly during the following 30 min of incubation. This fast activation indicated that the hydrogenase was present in cells taken from oxic conditions but required anaerobiosis to become active. Moreover, the rate of  $H_2$  production in the dark represented only a fraction of the potential hydrogenase activity, indicating that the limiting factor for  $H_2$  production was not hydrogenase but rather the NAD(P)H supply, which under these conditions is produced from fermentation (38).

**$O_2$  and  $H_2$  exchange during dark-light transitions in WT *Synechocystis*.**  $O_2$  and  $H_2$  exchange were then measured during dark-light transitions in WT *Synechocystis* cells adapted to anaerobic conditions. Anaerobiosis was obtained by flushing the sample with argon, closing the vessel, and letting the cells consume the residual  $O_2$  in the dark. When  $O_2$  was fully consumed,  $H_2$  production started. After stabilization of the  $H_2$  level (about 10 min), corresponding to a production rate of 0.1  $\mu$ mol of  $H_2$  mg of Chl<sup>-1</sup> h<sup>-1</sup>, the light was switched on. A sudden, short (less than 30-s) burst of  $H_2$  (initial rate, 1.2  $\mu$ mol of  $H_2$  mg of Chl<sup>-1</sup> h<sup>-1</sup>) was observed, followed by a rapid  $H_2$  uptake consuming all  $H_2$  present in the medium (Fig. 4A). This burst preceded photosynthetic  $O_2$  production, which began after 1 min of illumination. When the light was switched off,  $O_2$  was consumed by respiration and  $H_2$  production resumed once anaerobiosis was achieved. Figure 4B shows results of the same type of experiment carried out in the presence of DCMU, an inhibitor of PS II. In the light, the addition of DCMU prevented photosynthetic  $O_2$  production (Fig. 4B) while the  $H_2$  exchange pattern was not modified.  $H_2$  production started as soon as the light was switched off, probably due to the absence of  $O_2$ .

**Oxygen inhibition of hydrogenase activity, measured by the H-D exchange reaction.** Hydrogenase activity was assessed during dark-light-dark transitions by injecting  $D_2$  and monitoring the H-D exchange (Fig. 5). Under these conditions, the  $O_2$  exchange pattern (Fig. 5 A and B) was similar to that observed in the previous experiment. In the light, hydrogenase activity (Fig. 5C) was inhibited as soon as  $O_2$  appeared in the medium and was restored in the dark once  $O_2$  had been consumed (Fig. 5A). In this experiment, the global hydrogen flux can be followed on the curve representing the sum  $[D_2] + [H_2] + [HD]$ , corrected for instrument consumption ( $\Sigma$ ). When hydrogenase was active, significant hydrogen consumption was observed in the light. When the hydrogenase activity was inhibited, the hydrogen consumption stopped, with the total hydrogen concentration ( $\Sigma$ ) remaining constant (Fig. 5C). In the presence of DCMU, no  $O_2$  was produced (Fig. 5B) and the hydrogenase activity remained practically constant during the light period,

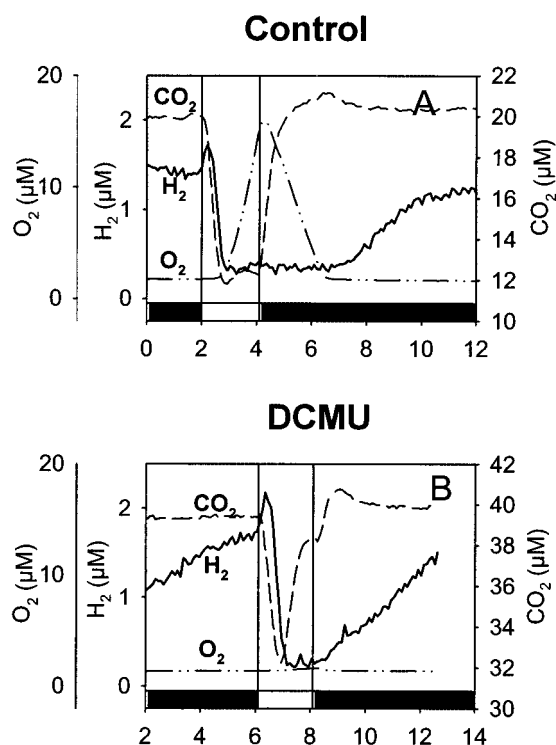


FIG. 4. Mass spectrometric measurements of H<sub>2</sub>, O<sub>2</sub>, and CO<sub>2</sub> exchange during dark-light-dark transitions in WT *Synechocystis* cells adapted to dark anaerobic conditions. Shown are effects of the PS II inhibitor DCMU. (A) Transitory H<sub>2</sub> output and H<sub>2</sub> uptake at the onset of light. Cells of *Synechocystis* sp. strain PCC 6803 (10 μg of Chl ml<sup>-1</sup>) were preincubated anaerobically in darkness in the measuring chamber of the mass spectrometer, and H<sub>2</sub> production was allowed to proceed in the dark. The cells were then illuminated briefly (2 min), and the concomitant changes in concentrations of dissolved gases H<sub>2</sub>, O<sub>2</sub>, and CO<sub>2</sub> were measured every 4 s. (B) Same conditions as for panel A but in the presence of 75 μM DCMU. Dark periods are represented on the x axis by black bars, and the light period is represented by a white bar.

resulting in a continuous hydrogen (Σ) uptake in the light (Fig. 5D).

**Involvement of hydrogenase in CO<sub>2</sub> fixation.** CO<sub>2</sub> exchange was also recorded in the gas exchange experiments (Fig. 4 and 5). In control experiments, the light-induced CO<sub>2</sub> exchange pattern observed in the presence of D<sub>2</sub> (Fig. 5A) qualitatively resembled that in the absence of D<sub>2</sub> (Fig. 4A): a rapid drop in CO<sub>2</sub> concentration at light onset due to the activation of CO<sub>2</sub>-concentrating mechanisms, followed by a secondary CO<sub>2</sub> uptake process corresponding to CO<sub>2</sub> fixation by the Calvin cycle (19, 28). In the presence of DCMU, only a transient CO<sub>2</sub> uptake, corresponding to the concentration phase, was observed in the absence of D<sub>2</sub> (Fig. 4B) while a sustained CO<sub>2</sub> uptake, resulting in a net CO<sub>2</sub> consumption (compare differences in steady-state CO<sub>2</sub> levels in the dark before and after illumination), was recorded in the presence of D<sub>2</sub> (Fig. 5B). These differences in CO<sub>2</sub> uptake can be compared to the differences in hydrogen uptake patterns. In Fig. 4B, the small H<sub>2</sub> amount produced in the dark was rapidly consumed. On the other hand, in Fig. 5D, the high D<sub>2</sub> concentration added to measure H-D exchange allowed sustenance of continuous hy-

drogen (Σ) consumption during the light period. The amount of CO<sub>2</sub> fixed in the light in the presence of DCMU (Fig. 5B) was about 3 μM, which corresponds to ca. 30 μM of dissolved carbon (CO<sub>2</sub> + HCO<sub>3</sub><sup>-</sup>) at pH 7.2, while the consumption of hydrogen (decrease of Σ during the light period) was about 70 μM. This indicates that under these conditions hydrogen is the main source of electrons for CO<sub>2</sub> fixation (2 H<sub>2</sub> molecules provide 4 reducing equivalents, which are needed for reduction of 1 CO<sub>2</sub> molecule). Interestingly, this amount is of the same order of magnitude as the photosynthetic O<sub>2</sub> production measured in the control experiment (Fig. 5A). Light-driven H<sub>2</sub>, O<sub>2</sub>, and CO<sub>2</sub> exchange were inhibited by the cytochrome *b<sub>6</sub>f* inhibitor DBMIB (20 μM) (data not shown), indicating that electron transfer from the PQ pool to PS I is essential for all the light-dependent activities discussed here. (It was checked that neither DCMU nor DBMIB affected hydrogenase activity, as monitored by H-D exchange in the dark in intact cells and extracts.)

**H<sub>2</sub> production in the light in the *Synechocystis* mutant M55, deficient in the NDH-1 complex.** Since NADPH produced by photosynthetic electron transfer or by oxidation of H<sub>2</sub> may enter the respiratory chain via NDH-1, we studied photobiological gas exchange in the NDH-1-deficient mutant M55 (Fig. 6). When the light was switched on after anaerobic adaptation, the M55 mutant showed H<sub>2</sub> production in the light, with an initial rate (5 μmol of H<sub>2</sub> mg Chl<sup>-1</sup> h<sup>-1</sup>) higher than that observed in the WT and with a much longer duration (Fig. 6A). This H<sub>2</sub> production was accompanied by a simultaneous O<sub>2</sub> production, while CO<sub>2</sub> uptake was negligible. The O<sub>2</sub> production rate was 25-fold lower than in the WT. The absence of apparent H<sub>2</sub> uptake in the NDH-1-deficient mutant during illumination indicates that the uptake observed in the WT was related to the activity of the NDH-1 complex. H<sub>2</sub> production in M55 stopped however after 4 to 5 min of illumination. The light-dependent H<sub>2</sub> production was completely inhibited by DCMU (Fig. 6B), indicating that essentially electrons originating from water photolysis at PS II were used for H<sub>2</sub> production. Addition of glucose to DCMU-treated cells increased the light-dependent H<sub>2</sub> production (Fig. 6C), showing that electrons originating from the fermentative metabolism of glucose can reach PS I by a PS II-independent electron transfer pathway. DBMIB inhibited light-induced H<sub>2</sub> production occurring in the presence of glucose (Fig. 6D), showing that both PS II-dependent and PS II-independent pathways rely on PQ reoxidation by the cytochrome *b<sub>6</sub>f* complex (Fig. 1).

Glucose addition also had significant effects on H<sub>2</sub> exchange in the dark. In M55, hydrogenase induction under anaerobiosis in the dark (assayed by H-D exchange) was comparable to that observed in the WT (data not shown), but H<sub>2</sub> production in darkness was barely detectable (Fig. 6A and B) unless exogenous substrate (succinate or glucose) was added (Fig. 6C and D). This is probably due to the poor CO<sub>2</sub> fixation capacity of M55 cells, which do not accumulate high levels of endogenous substrates during the growth phase and therefore cannot support significant H<sub>2</sub> production in the dark. However, dark H<sub>2</sub> production in the absence of an exogenous supply was observed with M55 cells taken from the stationary phase, probably because, having stopped growing, they had accumulated endogenous substrates (data not shown).

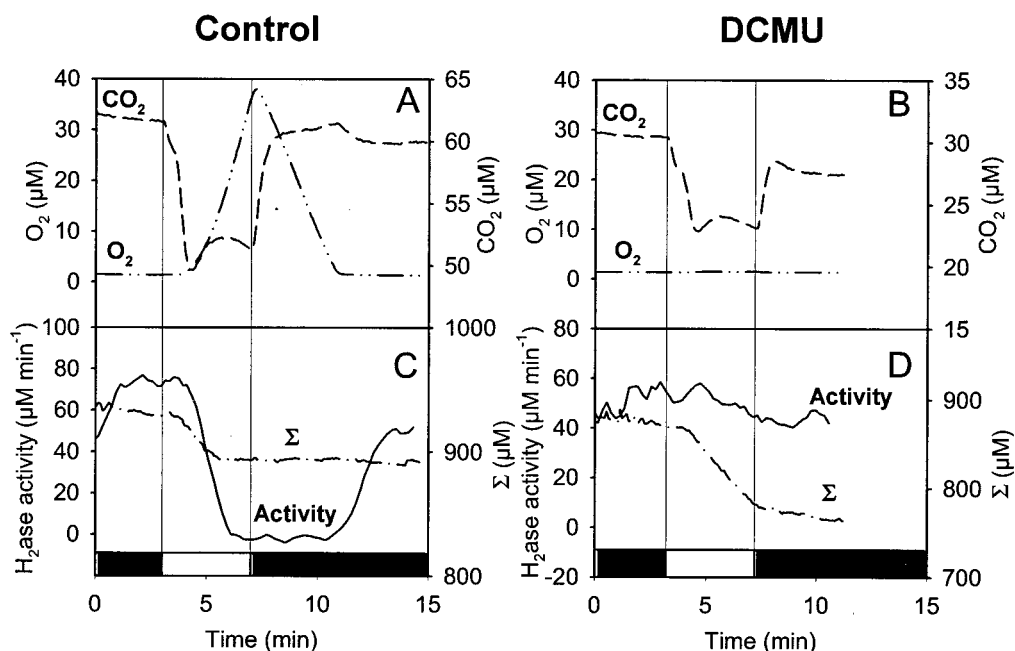


FIG. 5. Mass spectrometric measurements of  $O_2$  and  $CO_2$  exchange and H-D exchange during dark-light-dark transitions in WT *Synechocystis* cells adapted to dark anaerobic conditions in the presence of  $D_2$ . Shown are effects of the PS II inhibitor DCMU. (A and B) Time course of  $O_2$  and  $CO_2$  concentration changes. (C and D) Time course of hydrogenase activity measured by the H-D exchange reaction and of the sum ( $\Sigma$ ) of  $D_2$  plus  $H_2$  plus HD concentrations, corrected for the apparatus consumption, determined in the presence of  $400 \mu M D_2$  without further addition (A and C) or in the presence of  $75 \mu M$  DCMU (B and D). Dark periods are represented on the x axis by black bars, and the light period is represented by a white bar.

**Sustained  $H_2$  photoproduction in the light in the *ndhB*-defective *Synechocystis* M55 mutant.** As shown in Fig. 7A, light-induced  $H_2$  production in M55 stopped after a few minutes. This might have been due to the observed increase in  $O_2$  concentration, which could have inhibited hydrogenase. In the presence of glucose, the concentration of  $O_2$  remained low and  $H_2$  production was sustained in the light for at least 25 min (Fig. 7B). The addition of glucose oxidase, by further decreasing the  $O_2$  level, slightly stimulated the rate of  $H_2$  production (Fig. 7C). In this case a production rate of ca.  $6 \mu mol$  of  $H_2$   $mg$   $Chl^{-1} h^{-1}$  (or  $2 ml$  of  $H_2$   $liter^{-1} h^{-1}$ ) was observed during the whole light period, a value in the upper range of hydrogenase-mediated  $H_2$  production by cyanobacteria (31). In the presence of glucose, DCMU had a limited effect (ca. 20% inhibition) on  $H_2$  photoproduction by M55 during the whole light period (data not shown), indicating that both PS II-dependent and PS II-independent pathways of PQ reduction may durably contribute to the supply of electrons for  $H_2$  production.

**Both NADH and NADPH act as electron donors and activators for the bidirectional hydrogenase.** The ability of NADH or NADPH to provide electrons for  $H_2$  production was tested in cell extracts. As observed in intact cells, no  $H_2$  was evolved before  $O_2$  was totally removed from the medium. Both nucleotides could serve as electron donors for  $H_2$  production with comparable efficiencies. Final rates of  $H_2$  production were around  $0.09 \mu mol$  of  $H_2$   $mg$   $Chl^{-1} h^{-1}$  with  $1 mM$  of either NADPH or NADH (Fig. 8). The low rate found in cell extracts may be explained by a partial inactivation of hydrogenase by  $O_2$  during the preparation of the extract. A lag time of a few

minutes was observed between NADPH addition and  $H_2$  production (Fig. 8), which might correspond to the time necessary to activate the enzyme.

The ability of reduced nucleotides to activate the enzyme was tested by measuring the hydrogenase activity with the H-D exchange assay. In aerobic extracts saturated with  $D_2$ , no activity was detected until  $O_2$  was removed. When the extract was placed under anaerobic conditions, hydrogenase activity was barely detectable. Upon NADH addition, the hydrogenase activity rose rapidly (Fig. 9, left panel), reaching its steady-state level within minutes. When NADH was added before anaerobiosis was reached, no activation of the hydrogenase was observed in the presence of  $O_2$  but hydrogenase activity started as soon as  $O_2$  was removed (data not shown). NADPH was also able to activate the hydrogenase to comparable levels, but after a lag phase of a few minutes (Fig. 9 right panel), accounting for the delay in  $H_2$  production observed in the experiment for which results are shown Fig. 8. On the other hand,  $NAD^+$  or  $NADP^+$  added at a high concentration ( $5 mM$ ) did not interfere with the NADH or NADPH activation process (data not shown). At pH 6, the  $K_m$  value for hydrogenase activation was  $12 \mu M$  for NADH and  $100 \mu M$  for NADPH. Since these experiments were conducted with cell extracts, it cannot be excluded that NADH can be formed from NADPH by a transhydrogenase reaction and that NADH is the true activator of the hydrogenase. The cellular localization of hydrogenase was assessed by the H-D exchange assay performed with cell extracts in membrane and in cytosolic fractions (after NADH activation). The membrane fraction was found to retain 10 to

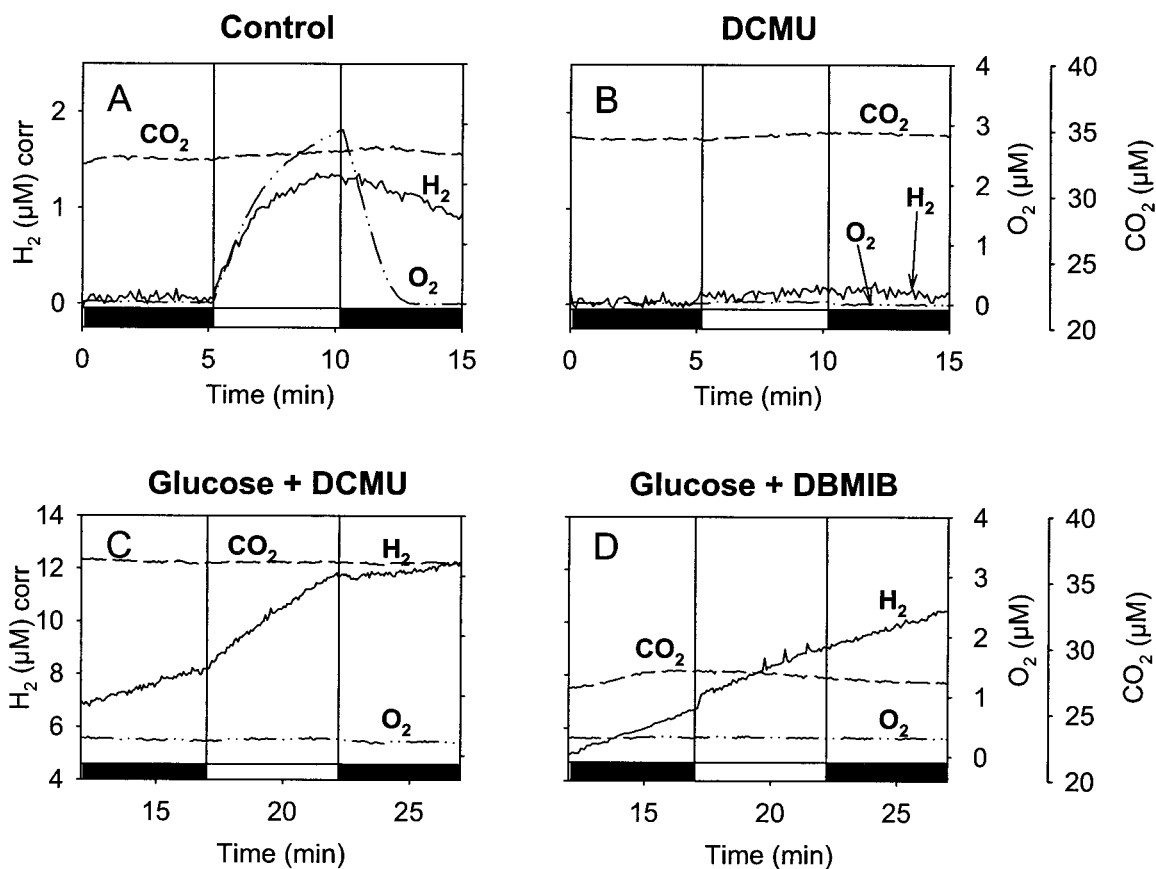


FIG. 6. H<sub>2</sub> and O<sub>2</sub> concentration changes in darkness and during a 5-min illumination period in the *Synechocystis ndhB* mutant M55. A culture of M55 (optical density at 730 nm = 1.5) was adapted to dark anaerobic conditions (A), in the presence of DCMU (75 μM) (B), in the presence of glucose (10 mM) and DCMU (75 μM) (C), or in the presence of glucose and DBMIB (20 μM) (D) in a closed reaction vessel connected to a mass spectrometer. The concentrations of H<sub>2</sub>, O<sub>2</sub>, and CO<sub>2</sub> in the medium were continuously recorded. Dark periods are represented on the x axis by black bars, and the light period is represented by a white bar.

25% of hydrogenase activity, and the supernatant fraction retained around 70% (in WT as well as in mutant cells).

## DISCUSSION

**How does the NDH-1-deficient mutant of *Synechocystis* sustain photobiological hydrogen gas production?** During a dark-to-light transition in anoxia, a low and transitory H<sub>2</sub> production was observed in WT *Synechocystis* cells. In contrast, the NDH-1-deficient mutant M55 was able to sustain significant H<sub>2</sub> production in the light. Suppression of the NADPH-consuming enzyme NDH-1 therefore provoked a long-term accumulation of reducing equivalents in the light which was sufficient to maintain activity of the NADPH-dependent, reversible hydrogenase. Due to the difference in electrochemical potentials (ca. 320 mV for the NAD(P)/NAD(P)H couple and 420 mV for H<sup>+</sup>/H<sub>2</sub>), the reversible hydrogenase can evolve H<sub>2</sub> only in the case of a very high (ca. 99.9%) reduction of the NAD(P) pool. It is likely therefore that H<sub>2</sub> production depends on the ability of the cells to maintain such a high reduction state. In *Synechocystis* strain PCC 6803, the size and redox state of the pyridine nucleotide pools have been determined in aerobically grown WT and NDH-1 mutant cells (8). While the NAD reduction level was 70% in both strains, the reduction level of

NADP shifted from 50% in the WT to 100% in the NDH-1 mutant (8), indicating that the mutant is specifically impaired in NADPH oxidation. In anoxia, it is likely that reduction of the NAD(P) pools is even higher, making H<sub>2</sub> evolution possible. The total NADP content was found to be more than 10 times that of NAD, in both WT and NDH-1 mutant cells (8). As the enzyme catalyzes electron transfer at comparable rates using NADH or NADPH (34; this study), the predominance of the NADP pool suggests that H<sub>2</sub> uptake or production observed in our study is essentially due to reactions of the hydrogenase with NADP or NADPH.

Apart from its interaction with hydrogenase, NADP reduction in *Synechocystis* is driven in the dark essentially by carbohydrate metabolism and in the light by photosynthesis. On the other hand, NADPH oxidation is primarily driven in the dark by respiration (through NDH-1) and in the light by the Calvin cycle. Recently, two functionally distinct classes of the NDH-1 complex have been identified. The first class, containing the NdhD1 or NdhD2 subunit, catalyzes NADPH-dependent PQ reduction and is involved in PS I cyclic electron transport and respiration, while the second class, containing the NdhD3 or NdhD4 subunit, does not directly oxidize NADPH but is involved in CO<sub>2</sub> uptake (22, 29). In NdhB<sup>-</sup> mutants such as M55,

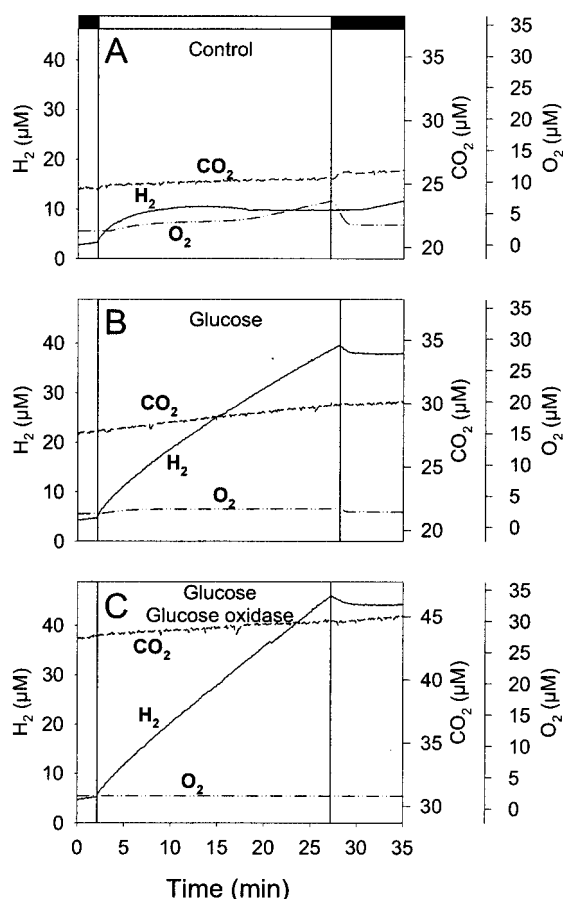


FIG. 7. Mass spectrometric measurements of  $H_2$ ,  $O_2$ , and  $CO_2$  exchange in the *Synechocystis ndhB* mutant M55 in darkness and during a 25-min illumination period. Cells were adapted for 1 h under anaerobiosis in the dark in a closed vessel connected to a mass spectrometer, and the concentrations of  $H_2$ ,  $O_2$ , and  $CO_2$  in the medium were continuously monitored. (A) Control, (B) with 10 mM glucose, and (C) with 10 mM glucose plus glucose oxidase plus catalase. Conditions were the same as for Fig. 6 except for the duration of the illumination period. Dark periods are represented by the x axis by black bars, and the light period is represented by a white bar.

both types of NDH-1 complex are absent. However, it would be of interest to determine how independent alteration of the NdhD1/NdhD2 or NdhD3/NdhD4 subunits, might interfere with  $H_2$  production.

**Sources of reducing power for  $H_2$  production.** Hydrogenase-dependent  $H_2$  evolution in cyanobacteria can be supported in the dark by endogenous substrates or by exogenous electron donors such as glucose (14). This was also the case with *Synechocystis* in our experiments. In the light, the existence of simultaneous  $O_2$  and  $H_2$  outputs in the NDH-1-deficient mutant and their inhibition by DCMU (Fig. 6) show that the water-oxidizing photosynthetic electron transport chain can also serve as an electron donor for  $H_2$  production. In vivo,  $H_2$  evolution associated with water photolysis via a reversible hydrogenase was first observed by Laczkó (20) with *Anabaena cylindrica* cells grown at high light intensities.

In the M55 mutant strain, electron flow from PS II to hydrogenase stopped after a few minutes. Glucose addition al-

lowed sustained  $H_2$  production, probably through two main mechanisms. First, the presence of glucose considerably lowers net  $O_2$  production in the light. This can be explained either by a stimulation of respiratory  $O_2$  consumption or by an inhibition of PS II due to PQ overreduction. We showed that addition of glucose to PS II-inhibited M55 cells restored  $H_2$  photoproduction by a pathway involving PQ. This indicates that glucose-stimulated PS II-independent reduction of PQ can also provide electrons to PS I for  $H_2$  production. The identity of this pathway remains to be elucidated. In any case, it must lead to an increase in the level of reduction of the NAD(P) pool, suggesting that the mechanism for PQ reduction is different from those classically described for cyclic electron transfer around PS I, which consume NADPH or reduced ferredoxin. A probable explanation for our observations is that nonphotochemical reduction of PQ by products of glucose metabolism such as succinate (7, 8) could sustain NADPH production by PS I and therefore play a significant role in  $H_2$  production.

**Sinks of reducing power for  $H_2$  uptake.** In the WT,  $H_2$  is efficiently taken up in the light before inactivation of the hydrogenase by photosynthetic  $O_2$  (Fig. 4).  $H_2$  uptake did not occur at significant rates in the NDH-1-deficient mutant (Fig. 6 and 7), which is essentially affected in NADPH-mediated PQ reduction and in  $CO_2$  uptake. Therefore, the  $H_2$  oxidation observed in the WT proceeded essentially via oxidation of NADPH by the electron transport chains and by  $CO_2$  fixation (Fig. 1). In the presence of high amounts of hydrogen ( $D_2$ ), under microaerobic conditions, hydrogen oxidation could be sustained in the light and efficiently serve as an electron donor for  $CO_2$  fixation, hydrogen being almost as efficient as PS II in this case (Fig. 5). Therefore, although it has been stated that in cyanobacteria,  $CO_2$  fixation by the Calvin cycle proceeds at a much higher rate than  $H_2$  uptake or production by the bidirectional hydrogenase (35), it appears that under our experimental conditions, in anoxia (or microaerobiosis) and in the presence of hydrogen, hydrogen consumption is of the same magnitude as photosynthetic activity and could therefore significantly contribute to  $CO_2$  fixation.

**Characteristics of *Synechocystis* hydrogenase and physiological implications.** In cell extracts, *Synechocystis* hydrogenase was inactivated by  $O_2$  in a reversible manner. It could be reactivated in anoxia in the presence of hydrogen, this activation being considerably stimulated (within minutes) by NADH

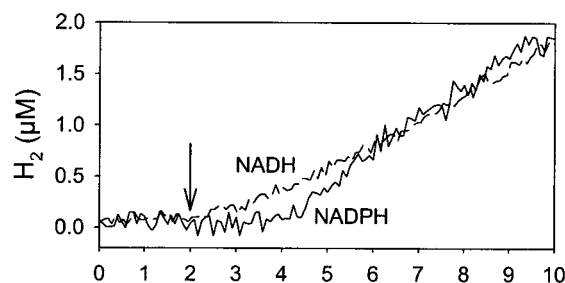


FIG. 8.  $H_2$  evolution catalyzed by a *Synechocystis* cell extract with NADH or NADPH as the electron donor. The extract (200  $\mu$ g of Chl) was gassed with argon, the chamber was closed, and  $O_2$  (monitored at mass peak 32) was removed by the addition of catalase, glucose, and glucose oxidase. NADH (1 mM) (dashed line) or NADPH (1 mM) (solid line) was added at 2 min (arrow).



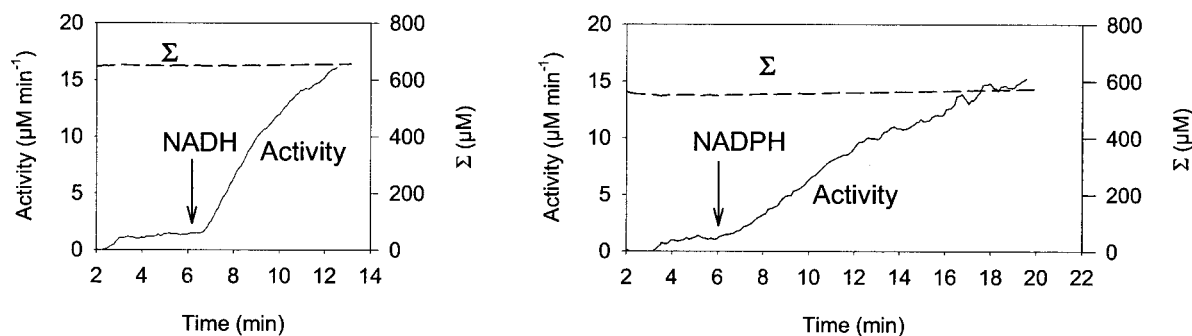


FIG. 9. Activation by NADH (left) or NADPH (right) of the hydrogenase activity in cell extracts of WT *Synechocystis* sp. strain PCC 6803 at pH 6.0. NADH or NADPH (0.2 mM) was added after the extract (73 µg of Chl) saturated with D<sub>2</sub> was made anaerobic as described in the legend to Fig. 3. Two minutes after the O<sub>2</sub> concentration had reached zero, the concentration changes in D<sub>2</sub>, HD, H<sub>2</sub>, and O<sub>2</sub> were measured at mass peaks 4, 3, 2, and 32, respectively. Hydrogenase activity and the corrected total concentration of hydrogen species ( $\Sigma$ ) were calculated as described in Materials and Methods and depicted in Fig. 2.

or NADPH at catalytic concentrations. Most [NiFe]hydrogenases are inactive in the presence of O<sub>2</sub>. They need to be reductively activated, for example by a long incubation (several hours) with H<sub>2</sub> in the absence of O<sub>2</sub> and in the presence of a reductant such as dithionite (10, 21) or NADH (14, 15, 37). But the activation that we observed in *Synechocystis*, occurring at low concentrations of NAD(P)H and being unaltered by NAD(P)<sup>+</sup> addition, appears to be a specific phenomenon.

The activation observed *in vivo* in anoxia in the dark is then probably due to a combination of O<sub>2</sub> scavenging and of NAD(P) reduction within the cells. Inactivation of hydrogenase in the light *in vivo* can be attributed to a direct effect of PS II-produced O<sub>2</sub>. Other interpretations, such as an independent consequence of illumination or a deactivation through the decrease of the NAD(P)H/NAD(P)<sup>+</sup> ratio, can be discarded. Indeed, in the light and in the presence of DCMU, the hydrogenase was not inactivated, although the Calvin cycle was functional and was able to reoxidize NADPH quickly. Therefore, although the bidirectional hydrogenase in *Synechocystis* is constitutively expressed in the presence of O<sub>2</sub> (2), it probably plays a role mainly under anaerobic or microaerobic conditions and, as suggested by Appel et al. (2), at the onset of light before the enzyme is inactivated by photosynthetic O<sub>2</sub>.

We can compare the features of the *Synechocystis* hydrogenase to those of the homologous NAD<sup>+</sup>-dependent hydrogenase of *R. eutropha* (formerly *Alcaligenes eutrophus*), which has been extensively studied and is often referred to as a prototype for this kind of hydrogenase. The *R. eutropha* enzyme is highly insensitive to O<sub>2</sub> (36), is clearly NAD preferring, and is activated by NADH, but activation is prevented by NAD<sup>+</sup> (37). Thus, despite sequence homologies between the *Synechocystis* and *R. eutropha* enzymes, their characteristics are not identical. Another example of a [NiFe]hydrogenase insensitive to O<sub>2</sub> is the H<sub>2</sub> sensor HupUV of *Rhodobacter capsulatus*, which is able to catalyze H-D exchange in the presence of high levels of O<sub>2</sub> (43). In both cases, O<sub>2</sub> insensitivity has been assumed to result from steric hindrance preventing O<sub>2</sub> access to Ni. In the case of the *R. eutropha* enzyme, additional CN<sup>-</sup> ligands bound to the NiFe metalcenter may protect the enzyme against O<sub>2</sub> inactivation (12). In the case of the H<sub>2</sub> sensor HupUV, a partial blocking of the gas channel close to the active site, due to the presence of bulkier amino acid residues, might limit access to

Ni to very small molecules (e.g., H<sub>2</sub>) (45). These recent advances in unraveling the O<sub>2</sub> resistance mechanism of [NiFe]hydrogenases could open a way to increase the O<sub>2</sub> resistance of the *Synechocystis* hydrogenase.

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