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ANAEROBIC HYDROGENASE ACTIVITY IN *ANACYSTIS NIDULANS*

H_2-DEPENDENT PHOTOREDUCTION AND RELATED REACTIONS

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Summary

1. Anaerobic hydrogenase activity in whole cells and cell-free preparations of H_2-induced *Anacystis* was studied both manometrically and spectrophotometrically in presence of physiological and artificial electron acceptors.

2. Up to 90% of the activity measured in crude extracts were recovered in the chlorophyll-containing membrane fraction after centrifugation (144 000 × *g*, 3 h).

3. Reduction of methyl viologen, diquat, ferredoxin, nitrite and NADP by the membranes was light dependent while oxidants of more positive redox potential were reduced also in the dark.

4. Evolution of H_2 by the membranes was obtained with dithionite and with reduced methyl viologen; the reaction was stimulated by detergents.

5. Both uptake and evolution of H_2 were sensitive to O_2, CO, and thiol-blocking agents. The H_2-dependent reductions were inhibited also by the plastoquinone antagonist dibromothymoquinone, while the ferredoxin inhibitor disalicylidenepropanediamine affected the photoreduction of nitrite and NADP only. 3-(3,4-Dichlorophenyl)-1,1-dimethylurea did not inhibit any one of the H_2-dependent reactions.

6. The results present evidence for a membrane-bound 'photoreduction' hydrogenase in H_2-induced *Anacystis*. The enzyme apparently initiates a light-

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Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; Chl *a*, chlorophyll *a*; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone: DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; DSPD, *N*,*N*′-disalicylidene-1,3-diaminopropane; HOQNO, 2-*n*-heptyl-8-hydroxyquinoline-*N*-oxide; PMS, *N*-methylphenazonium sulphate; SDS, sodium dodecyl sulphate.

driven electron flow from H_2 to various low-potential acceptors including endogenous ferredoxin.

Introduction

The term hydrogenase has been somewhat loosely applied to enzymes catalysing the metabolism of molecular hydrogen [1-3]. However, also nitrogenase, the enzyme responsible for dinitrogen fixation, may evolve H_2 under certain conditions [4—6]. H_2 uptake and evolution are widespread among anaerobic bacteria [7], and hydrogenases similar to the clostridial type (H_2:ferredoxin oxidoreductase, EC 1.12.7.1) may also occur in H_2-adapted eukaryotic algae [8].

In the aerobic 'hydrogen bacteria' hydrogenases are essential for chemolithotrophic energy conservation and for chemoautotrophic CO_2 fixation [9]. Two types of hydrogenases have been encountered in hydrogen bacteria: a soluble H_2:NAD^+ oxidoreductase (EC 1.12.1.2), and a particulate enzyme initiating respiratory electron flow from H_2 to O_2, similar to the 'uptake hydrogenase' responsible for H_2 uptake in the N_2-fixing bacteria *Rhizobium* and *Azotobacter* [10,11], and in the likewise aerobic N_2-fixing blue-green algae (cyanobacteria), e.g. *Anabaena cylindrica* [12—14]. H_2-supported reduction of NAD^+ in photosynthetic bacteria [15,16] strikingly differs from the functionally corresponding activity in hydrogen bacteria as most photosynthetic bacteria require additional energy from light to reduce NAD with H_2 [17].

Among blue-green algae hydrogenase activity has so far received attention almost exclusively in the context of N_2 fixation, and a functional relationship between hydrogenase and nitrogenase has been proposed [12,14]. Usually, in aerobic conditions both enzymes are most active in heterocysts only [18—20]; studies with isolated heterocysts [14,19,20] pointed to participation of hydrogenase in the heterocyst's photosynthetic electron transport [19]. The enzyme is believed to be particulate [19,21]. Whether H_2 can support N_2 fixation has not yet been firmly established [12—14,20,22]. Also a possible role of hydrogenase for CO_2 photoreduction was questioned [13,22,23].

However, among non-heterocystous non-nitrogen-fixing species anaerobic CO_2 photoassimilation with H_2 could be demonstrated in *Oscillatoria limnetica* and *Aphanothece halophytica* [24], and also in *Anacystis nidulans* [25,26]. Notes on H_2-dependent CO_2 photoreduction in blue-greens had already appeared earlier [27]. Bothe and coworkers recently confirmed hydrogenase to be inducible in *Anacystis* but emphasized its role in an oxyhydrogen reaction rather than in photoreduction [20,21].

The present study describes the hydrogenase-catalysed reduction of physiological and artificial electron acceptors by whole cells and cell-free preparations of *Anacystis* whose aerobic hydrogenase activity was only about 10—30% of the total hydrogenase activity. The actual contributions of anaerobic (photoreduction) and of aerobic (oxyhydrogen) hydrogenase activities were strongly influenced by the growth conditions of the algae (Peschek, G.A., unpublished results). Some features of H_2-supported CO_2 photoreduction by intact *Anacystis* were reported previously [26]. A companion paper will describe investigations on the oxyhydrogen reaction of *Anacystis*.

Materials and Methods

Growth of the alga. Axenic *A. nidulans* (strain 1402-1, Göttingen, F.R.G.) was grown photoautotrophically at 41°C and 10 W/cm^2 warm white fluorescent light in presence of H_2 as previously described [26] except that nitrate was the nitrogen source; doubling time of the (light-limited) cultures was 5.3 h. Photosynthetic O_2 evolution by the algae remained unimpaired during growth in presence of H_2 [26]. At the end of logarithmic growth the algae were harvested [26], washed twice with a buffer solution henceforth referred to as 'standard medium' (30 mM Tris/Tricine buffer; 2 mM Na_2HPO_4; 1 mM $MgCl_2$; 10 mM KCl, pH 7.8), and finally resuspended herein. The suspensions were always immediately used for the experiments. All handling was done under H_2 as far as possible.

Measurement of H_2 uptake and evolution. Gas exchange was followed manometrically at 35°C in a Photo-Warburg apparatus (Braun Apparatebau, Melsungen, F.R.G.), model F 166. In case of illumination light intensity was 18 W/cm^2 at the bottom of the vessels, measured with a Yellow Springs Instruments Radiometer (model 65). Conical Warburg flasks, approx. 14 ml volume, with one side arm closed by a serum cap equipped with a hypodermic needle, were shaken at 100 strokes/min and 3 cm amplitude. The vessels contained 2.0 ml standard medium together with all other compounds required; they were gassed through the manometers for 15 min with O_2-free H_2, or with the desired mixtures of H_2, N_2, O_2 and CO. While gassing, 0.5 ml of the algal suspension or cell-free preparation was filled into the side arm through the hypodermic needle. After gassing had been stopped the contents of the side arm were dipped in, the side arm was rinsed twice, and the manometer readings were started. In some experiments the sensitivity of the manometer readings was improved by a factor of 1.5 using 2,2,4-trimethylpentane (instead of Brodie's solution) in the manometers (see Ref. 28). Most of the experiments were done in strict anaerobiosis under H_2 with 0.15 ml alkaline pyrogallol in the center well. O_2 evolution by illuminated whole cells was prevented by 10 μM DCMU.

Spectrophotometric hydrogenase assay. Apart from manometry, hydrogenase activity was also assayed by measuring the H_2-dependent reduction of several redox compounds spectrophotometrically at 35°C in the dark with a Perkin-Elmer recording spectrophotometer, model 200. Light-dependent reductions were studied discontinuously by separately illuminating the cuvettes containing the assay samples (2.5 ml) outside the spectrophotometer at 35°C with 20 W/cm^2 tungsten light from a slide projector for the desired time intervals; for each measurement the cuvettes were put back into the cuvette chamber and absorbance was read at the proper wavelength. All experiments were performed in cuvettes (1 cm light path) closed with serum stoppers permitting the use of hypodermic needles to flush for 10 min with O_2-free H_2 or other gas mixtures. Control experiments with N_2/H_2 mixtures of different composition showed reaction rates to be independent of H_2 tension between 100% and at least 25% H_2 (rest: N_2). 2.5 ml standard medium, together with other compounds required, were placed in the cuvettes before flushing. Reactions were started by injecting 0.1—0.25 ml of anaerobic algal suspension, extract or membrane preparation. The reference cuvette contained the same

assay mixture as the measuring cuvette except that flushing was by O_2-free N_2 insteady of H_2. In several experiments acceptor-free assay suspensions served as additional references. The difference in absorbances (ΔA) between reference and sample cuvettes was recorded as a function of time with a Perkin-Elmer recorder (model 56). Only absolute values of ΔA will be given in the figures.

The following wavelengths (nm) and millimolar absorption coefficients ($mM^{-1} \cdot cm^{-1}$) were used: methyl viologen, 578, 9.7; NADPH, 366, 3.3; DCIP, 522, 8.6 (isosbestic point); ferricyanide, 410, 1; methylene blue, 600, — ; cytochrome *c*, 550, 19.7 ($\epsilon_{red} - \epsilon_{ox}$); PMS_{red}, 387, — . The H_2-dependent reduction of O_2 by isolated membranes was measured with a Clark-type oxygen electrode after flushing the samples for 10 min with 5% O_2 in H_2.

In the experiments with cell-free systems the acceptor compounds were used at the following concentrations (mM) throughout: methyl viologen, 5; diquat, 5; neutral red, 5; NAD, 3; NADP, 3; menadione, 0.05; methylene blue, 5; PMS, 5; DCIP, 0.1; cytochrome *c*, 0.5; benzoquinone, 2; ferricyanide, 0.8. Separate experiments had shown that the reactions were independent, within wide limits, of acceptor concentrations which in each case were sufficient to ensure maximal rates.

Colorimetric determination of nitrite. Nitrite was determined with sulphanilic acid and 1-aminonaphthalene according to Lunge and Lwoff [29]; nitrate was first converted to nitrite with Zn/HCl.

Preparation of cell-free extracts. Harvested algae were suspended in standard medium at a chlorophyll concentration of 0.1—0.2 mg/ml. The following methods were used for cell breakage: (a) Sonication. Algal suspensions were sonicated after addition of 0.3 M mannitol and 1 mM dithiothreitol, at 4°C under H_2 with a Branson Sonifier at the maximal power output of the mitrotip for ten times 1 min, with 1 min intervals to allow for cooling. The overall yield of broken cells was about 85% as judged from the phycocyanin content of intact cells compared to that of 'crude extracts' (see below). (b) Lysozyme digestion. After adding 3 mM Na_2EDTA, 0.3 M mannitol and lysozyme (5 mg/mg Chl *a*) the algal suspension was incubated at 37°C under H_2 for 2 h with occasional shaking. About 90% of the algae had formed microscopically discernible sphaeroplasts after this treatment. Sphaeroplasts were centrifuged at 500 × *g* and 4°C for 15 min, washed once with standard medium containing 0.3 M mannitol and 1 mM dithiothreitol, and the resulting pellet was resuspended and stirred in a 1:20 dilution of this medium at 4°C for 15 min. The overall yield of broken cells after lysozyme digestion and osmotic shock was approx. 70%. (c) Cells were pretreated with lysozyme as described above (b), but Na_2EDTA was omitted and incubation was for 30 min only. Then the suspension was sonicated for five times 30 s as described under (a). When the H_2-supported photoreduction of NADP was used as test reaction the rates obtained with membranes prepared according to (a), (b) or (c) were in the ratio of about 0.3 : 0.1 : 1, respectively; in case of ferricyanide reduction the ratio was 0.4 : 0.7 : 1. Consequently, all experiments on isolated membranes were done with preparations obtained by short sonication of lysozyme-pretreated *Anacystis* cells in a medium of moderate osmolarity.

The cell-free extracts were immediately centrifuged at 8000 × *g* and 4°C for 10 min to remove whole cells and cell debris. The resulting supernatant ('crude

extract') was then centrifuged at 144 000 × *g* and 4°C for 3 h; the pellet was resuspended in standard medium containing 0.3 M mannitol, and immediately used for the experiments. Photosynthetic O_2 evolution by the isolated membranes always was less than 0.2 pmol/min per mg protein which corresponded to the sensitivity limit of polarographic O_2 determination when performing measurements at 35°C and 20 W/cm^2 white light in presence of any one of the electron acceptors used in this study. Obviously, the procedure of cell breakage employed was just harsh enough to eliminate the water-splitting system while still preserving sufficient activities of the membrane-bound hydrogenase.

Determination of chlorophyll and protein. Chlorophyll was determined according to Arnon [30]. Protein content was determined by a modified biuret method [31]. 1 mg dry weight was equivalent to 3.1 μl packed cells [26] or 0.024 mg Chl *a* or 0.56 mg protein. The protein/chlorophyll ratio in isolated membranes (free of phycocyanin) was 12. In crude extracts the distribution of protein between the particulate and the soluble fraction was approx. 1 : 0.86.

Controls. In the experiments on H_2-supported acceptor-reduction samples flushed with O_2-free N_2 served as controls. Anaerobic H_2 uptake followed manometrically in presence of acceptors was compared with endogenous H_2 uptake which was zero with membrane preparations and small even with whole cells (see Fig. 1). In several runs also algae or isolated membranes heated at 90°C for 5 min were used as additional controls. Isolated membranes were incapable of photosynthetic O_2 evolution (see above); however, to ensure that Photosystem II actually was not involved in H_2-dependent photoreduction in experiments with isolated membranes, too, 10 μM DCMU was included in the reaction mixtures; no difference was seen between samples with and without DCMU (cf. Table IV).

All values in the figures and tables are the means from at least three independent representative experiments; maximal deviations of single determinations from the corresponding mean were 10–15% of the mean for each given set of experiments.

Chemicals. All chemicals used were of the highest purity available. Substances poorly soluble in water were dissolved in freshly distilled dimethylsulphoxide. Samples containing dimethylsulphoxide alone (at most 1%, v/v) gave the same results as dimethylsulphoxide-free controls.

Results

Hydrogen uptake by whole cells

Whole cells of *Anacystis* grown as described in Materials and Methods took up H_2 in the light in presence of bicarbonate, nitrite and nitrate (Fig. 1); endogenous H_2 uptake in the light was very small and ceased after about 3 h. In the dark no measurable H_2 uptake occurred either in presence or absence of the oxidants. The reactions with bicarbonate and nitrate were linear over at least 3 h. Also artificial electron acceptors permitted H_2 uptake by whole cells (Fig. 1). While H_2 uptake with methyl viologen and diquat was observed exclusively in the light, *p*-benzoquinone permitted almost equal rates in the light and in the dark. H_2 uptake in presence of *N*-methylphenazonium sulphate was stimulated by light. Table I shows the stoichiometries between H_2 consumption

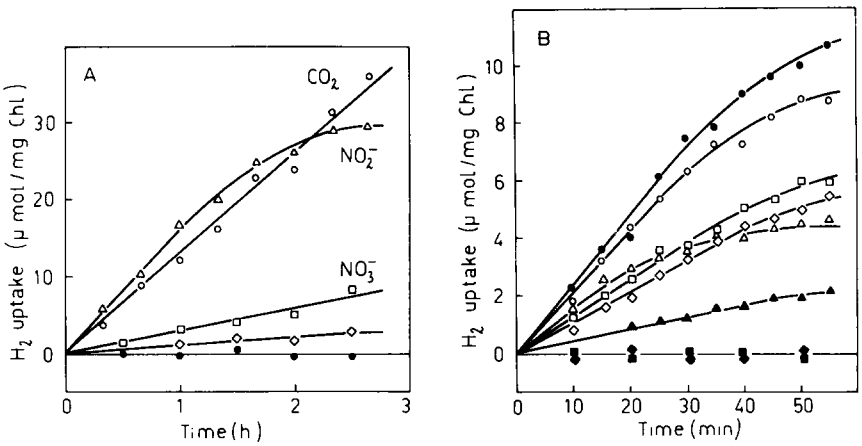

Fig. 1. Anaerobic H_2 uptake by whole cells in presence of physiological and artificial electron acceptors. (A) 20 mM $NaHCO_3$ (○), 3 mM $NaNO_2$ (△), 10 mM $NaNO_3$ (□), endogenous (◇). (B) 2 mM *p*-benzoquinone (○), 15 mM methyl viologen (□), 15 mM diquat (◇), 15 mM *N*-methylphenazonium sulphate (△). Open symbols: light (18 W/m^2). Full symbols: dark. H_2 uptake was measured manometrically under 100% H_2. No H_2 uptake was observed in the dark with $NaHCO_3$, $NaNO_2$, $NaNO_3$, methyl viologen, diquat or without added acceptors.

and the corresponding amount of acceptor photoreduced by intact cells. It is seen that the H_2 consumed is almost stoichiometrically used for the reduction of the oxidant.

Hydrogen uptake by membrane preparations

The localization of hydrogenase activity in cell-free systems from *Anacystis* was studied by use of the H_2-supported reduction of methyl viologen, NADP, nitrite and ferricyanide as hydrogenase test reactions (Table II). In addition, H_2-dependent uptake of O_2 (i.e. oxyhydrogen activity) was also tested. About 70—90% of the hydrogenase activity present in crude extracts were recovered in the membrane fraction. Activity in the supernatant was always negligible.

TABLE I

STOICHIOMETRIES OF H_2-SUPPORTED PHOTOREDUCTION OF CO_2, NITRITE AND METHYL VIOLOGEN BY WHOLE CELLS

Algae from one and the same harvest were assayed in parallel for H_2 uptake and for reduction of acceptor compounds (see Materials and Methods). Reaction rates in N_2-flushed controls and in dark controls were always negligible.

Acceptor added	(μmol/h per mg Chl)		μmol H_2/μmol acceptor
	H_2 uptake	Acceptor reduction	
None	1.6	—	—
20 mM $NaHCO_3$	14.2 *	6.7	2.12 (2) **
3 mM $NaNO_2$	13.9 *	5.0	2.78 (3) **
15 mM methyl viologen	5.6 *	13.1	0.43 (0.5) **

* Corrected for endogenous H_2 uptake.

** Values in brackets: theoretical stoichiometries.

TABLE II

LOCALIZATION OF HYDROGENASE ACTIVITY IN CELL-FREE EXTRACTS

'Particulate', activity of the 144 000 × *g* sediment. 'Soluble', activity of the 144 000 × *g* supernatant. Cell-free preparations contained between 0.9 and 2.3 mg protein/ml. Spectrophotometric hydrogenase assay as described in Materials and Methods. O_2 uptake measured with a Clark-type O_2 electrode. n.d., not determined.

Acceptor		Specific activity (nmol/min per mg protein)			Relative activity (%) (crude extract = 100%)	
		Crude extract	Particulate	Soluble	Particulate	Soluble
Methyl viologen	Light	26.5	41.2	0.9	83.5	1.6
	Dark	0.5	0	0.8	0	70
Nitrite	Light	1.8	2.3	0.8	68	20
	Dark	0.4	0	0.7	0	80
NADP	Light	12.3	19.1	0.3	84	1.1
	Dark	0.3	0	0.5	0	78
Ferricyanide	Light	34.1	57.1	0.2	91	0.2
	Dark	34.0	58.9	0.3	90	0.3
Oxygen (22 μM)	Light	— *	6.3 **	n.d.	—	—
	Dark	3.2 **	4.9 **	n.d.	85	—

* High rates of O_2 uptake in crude extracts were observed on illumination regardless whether flushing was by H_2 or N_2. The reaction therefore probably reflects some kind of photooxidation rather than an oxyhydrogen reaction.

** Values corrected for the 'endogenous' O_2 uptake (measured under N_2/O_2 = 95/5) which was between 8 and 13% of the total O_2 consumption in presence of H_2.

Washing of the membranes with standard medium led to complete loss of nitrite and NADP photoreduction without significantly affecting the reduction of methyl viologen or ferricyanide (not shown). Exogenous ferredoxin (cf. Table III) not only stimulated the photoreduction of NADP and nitrite by freshly prepared membranes but also nearly completely restored these reactions with washed membranes, and the same influence of ferredoxin was seen in the manometric hydrogenase assay but there was no effect when methyl viologen or ferricyanide served as acceptors (results not shown).

Table III summarizes the effects of physiological and artificial redox compounds as electron acceptors for the hydrogenase reaction observed with isolated membranes. The results again document the obligatory participation of light in the H_2-dependent reduction of compounds with strongly negative redox potential (cf. Fig. 1). Photosystem II apparently does not contribute to the photoreductions as seen from the lack of inhibition by DCMU. Moreover, no Emerson enhancement has been found for H_2-supported photoreduction by intact *Anacystis* [26].

Inhibition by CO and O_2. Reversibility of the O_2 effect

Dependence of the inhibition of ferricyanide and $NADP^+$ reduction mediated by the photoreduction hydrogenase on the concentration of CO and O_2 is given in Fig. 2. Both reactions were inhibited by some further 20–25% upon illumination with 400 W/m^2 white light (results not shown; cf. Fig. 4). Therefore the effect of CO on reactions catalysed by the photoreduction hydrogenase

TABLE III

HYDROGENASE ACTIVITY OF ISOLATED MEMBRANES

(A) H_2-supported reduction of electron acceptors followed spectrophotometrically. (B) H_2 uptake measured manometrically in presence of acceptors. Samples contained between 0.5 and 3.4 mg protein/ml. The reactions were followed for up to 30 min and remained linear during this time.

Acceptor	Reduction of acceptors (nmol/min per mg protein)			
	E'_0 (V)	H_2/light	H_2/dark	N_2/dark
A				
Nitrite	—	7.5	0.3	0.4
Methyl viologen	−0.44	35.4	0.4	0.4
NAD	−0.32	0.6	0.3	0.3
NADP	−0.32	18.2	0.4	0.3
DCIP	+0.22	25.2	24.8	0.6
Cytochrome *c*	+0.25	5.2	4.8	0.1
Ferricyanide	+0.36	45.7	45.3	0.5

Acceptor	Hydrogen uptake (nmol/min per mg protein)		
	E'_0 (V)	Light	Dark
B			
Endogenous	—	0	0
Ferredoxin *	−0.43	18.6	0.1
Diquat	−0.35	14.4	0.1
Neutral red	−0.34	0.1	0
Menadione	−0.01	26.2	17.2
Methylene blue	+0.01	7.5	6.9
PMS	+0.08	21.4	11.2
Benzoquinone	+0.29	28.7	28.0

* Ferredoxin was extracted and purified from *A. nidulans* following procedures published for *Anacystis* [39] and for *Nostoc* [40]. Ferredoxin isolated by either method behaved identically in the experiments described here; final concentration was 1.0 mg/ml. H_2-dependent reduction of ferredoxin was tested spectrophotometrically at 423 nm [39].

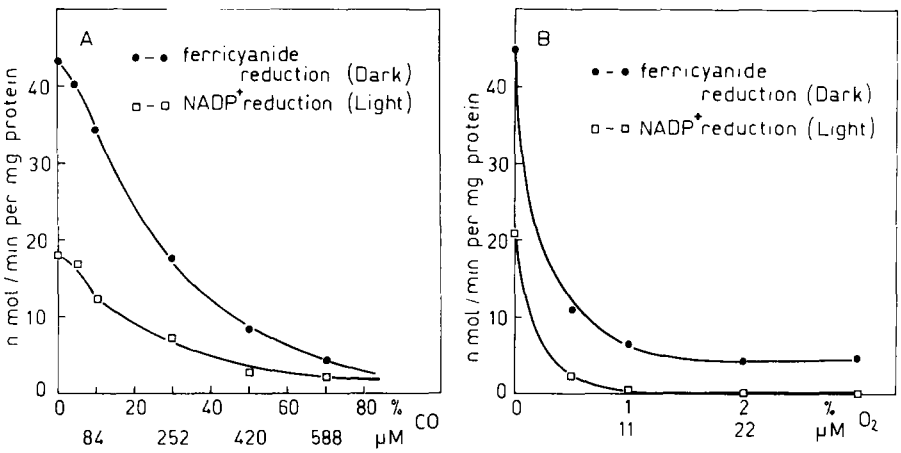

Fig. 2. Inhibition of H_2-dependent reduction of ferricyanide and of $NADP^+$ by CO (A) and O_2 (B). Spectrophotometric hydrogenase assay as described in Materials and Methods. Before injecting 0.25 ml of anaerobic membrane suspensions (equivalent to 0.55–0.75 mg protein) through the serum stoppers the cuvettes containing 2.5 ml of standard medium (plus acceptors) were flushed for 10 min with gas mixtures composed of 30% H_2, 0—70% CO and 70—0% N_2, respectively, or with 0—3% O_2 in H_2. From this, the molar concentrations of CO and O_2 were calculated using the respective Bunsen absorption coefficients [28]. Control experiments with H_2 containing 0–70% N_2 revealed no dependence of the reaction rate on H_2 concentration. 400 W/m^2 white incandescent light further inhibited the H_2-supported, CO-inhibited reduction of ferricyanide and of $NADP^+$ by about 20—25% (results not shown; cf. Fig. 4) showing that the effect of CO was not reversed in strong light and, hence, probably was not mediated by some CO-sensitive respiratory cytochrome(s) (cf. Fig. 3 of Ref. 41).

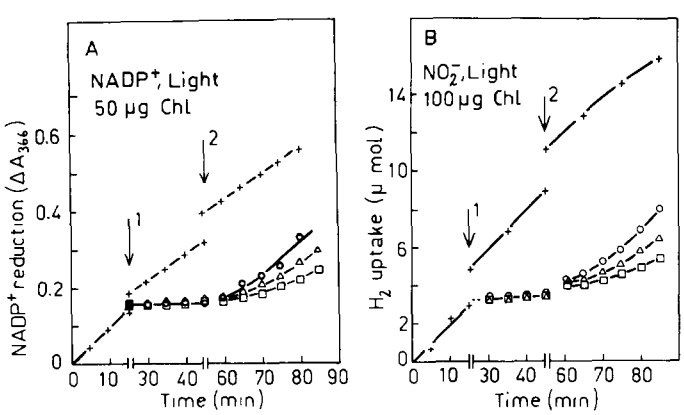

Fig. 3. Reversibility of O_2 inhibition and protective effect of dithiothreitol. H_2-dependent reduction of NADP (A) and H_2 uptake in presence of nitrite (B) by isolated membranes were measured as described in Materials and Methods. At the time indicated by arrow 1, samples were sparged with 1% (○———○), 5% (△———△) or 10% (□———□) O_2 for 10 min in the dark without removing them from the thermostatted (35°C) water bath. After allowing 5 min for equilibration the readings were continued for another 15—20 min. At the time indicated by arrow 2, pure H_2 (free of O_2) was bubbled through the samples for 10 min. Thereafter, readings were continued with the completely anaerobic suspensions. +———+, suspensions contained 1 mM dithiothreitol from the beginning; other procedures as given above; no difference was seen between samples with or without dithiothreitol during strict anaerobiosis (0—15 min). Appropriate control experiments revealed lack of donor function with dithiothreitol in the absence of H_2 (cf. Table V).

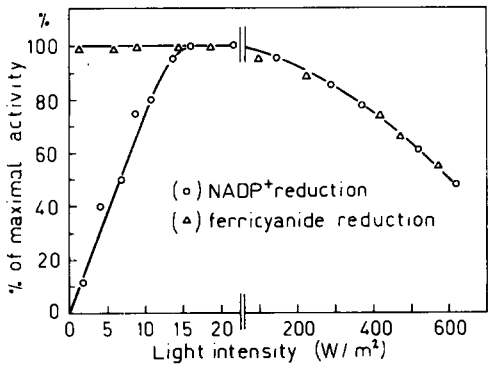

Fig. 4. Influence of light intensity on the H_2-dependent reduction of NADP and ferricyanide by isolated membranes. Spectrophotometric hydrogenase assay as described in Materials and Methods. Illumination was by a slide projector lamp. Final light intensity was varied by means of suitable combinations of Kodak Wratten gelatin filters placed behind a KG 4 infrared filter (Schott AG, Mainz, F.R.G.). In each case the absorbance was measured in the dark immediately before and after illuminating the samples at 35°C for 15 min. Fresh samples (0.25 mg protein/ml) were used for each determination. Maximal activities (100%) corresponded to 17.7--20.2 nmol NADP and to 50.3- 59.7 nmol ferricyanide reduced/min per mg protein.

present in the *Anacystis* membrane preparations used in this study appeared to be due to inhibition by CO of the hydrogenase enzyme, proper, and not to some CO-sensitive respiratory cytochrome(s).

Fig. 3 shows that the light-dependent reduction of $NADP^+$ and of nitrite was completely inhibited by less than 2% O_2, and that this inhibition was reversible: almost the same reaction rate as before was re-established when, after a short period of oxygenation with 1% O_2, pure H_2 was introduced again. Reversibility decreased, however, with increasing O_2 concentrations and prolonged incubation periods: after 30 min exposure to air recovery under pure H_2 was less than 1% (results not shown). Conflicting results of reactivation experiments with O_2-inactivated hydrogenases from various sources have appeared in the literature [3,32,33]. The inactivating effect of low O_2 concentrations was partly counteracted by dithiothreitol (Fig. 3). Control experiments established that 1 mM dithiothreitol neither affected H_2-supported photoreductions nor acted as electron donor in the absence of H_2 (cf. Table V). Effects similar to those shown in Figs. 2 and 3 were found with ferricytochrome *c* or DCIP serving as terminal electron acceptors for the hydrogenase reaction either in the light or in the dark (results not shown; cf. Table III).

Evidence for the involvement of photosynthetic electron flow

Participation of photosynthetic electron transport in H_2-dependent (photo)-reductions as deducible from the results presented so far was also suggested by the action of specific inhibitors (Table IV). Effects of the inhibitors were the same in the spectrophotometric and in the manometric hydrogenase assays (results not shown). Inhibitor studies recently conducted on H_2-supported photoreduction of CO_2 by intact *Anacystis* [26] did not allow clear-cut conclusions.

The plastoquinone antagonist DBMIB (1 μM) inhibited the light-dependent reduction of methyl viologen, NADP and nitrite by about 70%; also the dark

TABLE IV

ACTION OF INHIBITORS ON HYDROGENASE-CATALYSED REDUCTIONS BY MEMBRANE PREPARATIONS

H_2-dependent reduction of methyl viologen (MV), nitrite and NADP in the light, and of ferricyanide (FeCy) and DCIP in the dark, was measured spectrophotometrically as described in Materials and Methods. Samples contained between 1.3 and 2.2 mg protein/ml.

Inhibitor	Reduction of acceptors (nmol/min per mg protein)				
	MV	NO_2^-	NADP	FeCy	DCIP
None	39.3	7.0	17.9	51.8	27.2
10 μM DCMU	38.8	7.1	17.8	52.1	27.0
1 μM DBMIB	12.6	2.2	5.6	17.5	10.0
10 μM DBMIB	8.7	1.5	3.8	24.7	12.8
0.5 mM DSPD	35.0	1.4	3.6	47.0	24.8
10 μM HOQNO	26.4	4.8	11.5	34.2	19.0
10 μM CCCP	38.8	7.4	17.2	50.2	25.9
0.5 mM *p*-hydroxymercuribenzoate	2.0	0.4	0.9	2.6	1.3
0.5 mM *N*-ethylmaleimide	2.8	0.5	1.2	3.5	1.9

reduction of ferricyanide and DCIP by H_2 was inhibited 66 and 63%, respectively. Thus both in the light and in the dark electrons from H_2 apparently have to pass a, or the, DBMIB-sensitive site. H_2-dependent photoreductions were still further inhibited by $\geqslant$10 μM DBMIB while the rates of ferricyanide or DCIP reduction were raised above their levels at 1 μM DBMIB (Table IV); H_2 uptake was stimulated by 10 μM DBMIB when compared with 1 μM DBMIB, either in presence or absence of any one of the acceptors used (results not shown). Therefore it was concluded that $\geqslant$10 μM DBMIB per se can function as a terminal electron acceptor in the hydrogenase reaction of *Anacystis* membranes. Reduced DBMIB in turn may then reduce suitable high-potential acceptors, e.g. DCIP or ferricyanide (Table IV). Similar conclusions had been drawn in case of DBMIB photoreduction by isolated chloroplasts [34].

The ferredoxin inhibitor DSPD depressed photoreduction of NADP and nitrite more than 80% reflecting the involvement of ferredoxin. Methyl viologen appeared to be photoreduced by H_2 without participation of ferredoxin; also the dark reduction of ferricyanide and DCIP by H_2 was only slightly inhibited (Table IV). The uncoupler CCCP was without major influence on the hydrogenase-catalysed reactions of isolated membranes. Also in whole cells there was neither stimulation nor inhibition by 10 μM CCCP or 0.5 mM dinitrophenol (results not shown); therefore, phosphorylation either is not involved in the electron flow from H_2 to the acceptors tested or, more likely, is not rate limiting for the overall reaction.

The inhibitory effect of thiol-blocking agents, e.g. *p*-hydroxymercuribenzoate or *N*-ethylmaleimide (Table IV) is reminiscent of an involvement of 'active' thiol groups in catalysis by hydrogenases from both bacteria [32] and (eukaryotic) algae [35].

Hydrogen evolution

Evolution of H_2 by crude extracts and isolated membranes was tested under

TABLE V

EVOLUTION OF H_2 BY MEMBRANE PREPARATIONS IN PRESENCE OF VARIOUS ELECTRON DONORS IN THE LIGHT AND IN THE DARK. EFFECT OF SODIUM DODECYL SULPHATE

Light intensity was 18 W/m^2. H_2 evolution was measured manometrically under O_2-free N_2. The gas formed was identified as H_2 by gas chromatography and by adsorption onto activated palladium. Samples contained 1.2—4.2 mg protein/ml. H_2 evolution expressed as nmol/min per mg protein. No activity was detected with controls containing heat-denatured membranes. H_2 evolution rates were depressed by some 30% under an atmosphere of 100% H_2.

Donor	Light	Dark	Light + 1 mM SDS	Dark + 1 mM SDS
10 mM dithionite	0.4	0.4	2.3	2.3
10 mM dithionite + ferredoxin (0.5 mg/ml)	0.4	0.3	2.1	2.2
10 mM dithionite + 5 mM methyl viologen	1.2	1.3	6.7	6.6
10 mM cysteine	0	0	0	0
5 mM dithiothreitol	0	0	0	0
10 mM ascorbate + 0.5 mM DCIP	0	0	0	0
10 mM ascorbate + 0.5 mM DCIP + ferredoxin (0.5 mg/ml)	0	0	0.2	0
10 mM ascorbate + 0.5 mM DCIP + 5 mM methyl viologen	1.0	0	6.0	0

N_2 with several reductants in the light and in the dark (Table V; results given for isolated membranes). Only dithionite and reduced methyl viologen proved effective. In particular, unlike other hydrogenase-containing photosynthetic systems (see Ref. 36) the *Anacystis* membranes did not show photoproduction of H_2 with cysteine, dithiothreitol or ascorbate-reduced DCIP. Also ferredoxin reduced either by dithionite or by membranes illuminated in presence of ascorbate plus DCIP, could not serve as electron donor to the hydrogenase

TABLE VI

ACTIVATION AND SOLUBILIZATION OF THE MEMBRANE-BOUND HYDROGENASE BY TREATMENT WITH DETERGENTS

Spectrophotometric hydrogenase assay: 0.5 ml anaerobic membrane suspensions equivalent to 4.9—6.5 mg protein were injected through a serum cap into a cuvette containing 2.0 ml 5 mM methyl viologen in standard medium previously flushed with purified N_2 through hypodermic needles and brought to A_{578} = 0.5—0.6 by injecting a few drops of dithionite solution. For solubilization experiments aliquots of the original membrane preparation (about 0.2 mg protein/ml) were incubated with 1 mM SDS or deoxycholate, or with 0.03% (v/v) Triton X-100 at 4°C under N_2 for 20 min, then centrifuged at 144 000 × *g* for 3 h. Controls were incubated without detergents. No change in absorbance was seen with samples containing no membranes or heated membranes or membranes prepared from algae not previously induced for hydrogenase.

Membranes incubated:	Specific hydrogenase activity (nmoles of reduced methyl viologen oxidized/min per mg protein)		
	Before centrifugation	After centrifugation	
		Sediment	Supernatant
Without detergent	2.6	2.4	0
+ dodecyl sulphate	12.8	4.2	86.0
+ Triton X-100	5.1	5.0	0.6
+ deoxycholate	13.3	1.9	94.7

while photoreduced methyl viologen was nearly as effective as dithionite-reduced methyl viologen (Table V). H_2 evolution showed a similar response to the inhibitors CO and mercuribenzoate as did H_2 uptake; also high light intensities inhibited both reactions (results not shown). Evolution of H_2 by isolated membranes or by crude extracts was not influenced by 3 mM ATP, but was inhibited about 30% by 100% H_2 (results not shown). Therefore it is concluded that the evolution of H_2 demonstrated here for *Anacystis* preparations was due to the reversible action of the hydrogenase, and not to a nitrogenase: nitrogenase-catalysed H_2 evolution is inhibited by N_2 but not by H_2, is ATP dependent, and is not inhibited by CO. Anyway nitrogenase is thought to be absent from *Anacystis* [20] as well as from most of the other *Synechococcus*-type blue-greens recently tested [18].

The evolution of H_2 by the membrane preparations was markedly stimulated by detergents, and part of the H_2-evolving activity was recovered in the supernatant after treatment with 1 mM dodecyl sulphate or deoxycholate (Table VI) indicating partial solubilization of the hydrogenase normally tightly bound to the photosynthetic lamellae.

Discussion

The results of the present study provide evidence for a membrane-bound hydrogenase associated with the photosynthetic electron transport in *A. nidulans*. The enzyme catalysed the H_2-dependent photoreduction of methyl viologen, diquat, ferredoxin, NADP, nitrite, *N*-methylphenazonium sulphate and menadione by chlorophyll-containing membrane ('thylakoid') preparations under strictly anaerobic conditions. This implies that all the enzymes and cofactors necessary for hydrogenase-mediated (photo)reduction of the compounds remained bound to the membranes during isolation. On the other hand the preparations were completely devoid of photosynthetic O_2 evolution (see Materials and Methods). Requirements for retaining optimal hydrogenase activity were sufficiently mild cell breakage, e.g. by short sonication of lysozyme-pretreated cells, and appropriately high osmolarity of the medium throughout the whole isolation procedure (see Materials and Methods). In particular, also (endogenous) ferredoxin, NADP : ferredoxin oxidoreductase [37] and nitrite reductase [38] had apparently been retained by the membranes during isolation. Washing of the membranes predominantly removed ferredoxin as in washed membranes the ferredoxin-dependent reactions, i.e. NADP and nitrite photoreduction, were lacking but could be restored by exogenous ferredoxin. Involvement of ferredoxin in H_2-dependent NADP and nitrite photoreduction by the isolated membranes was also derived from the action of the ferredoxin inhibitor disalicylidenepropanediamine (Table IV).

The light dependence of the reduction of, e.g. methyl viologen (E'_0 = —0.44 V), diquat (E'_0 = —0.35 V), ferredoxin (E'_0 = —0.43 V) or NADP (E'_0 = —0.32 V) by H_2 (E'_0 = —0.42 V) might not be completely explained by mere thermodyanamics; usually the reduction of such compounds by hydrogenases from other sources indeed is a dark reaction [8,32]. Therefore it may simply be the spatial organization within the cell that prevents a hydrogenase from acting on a redox level it should display thermodynamically: provided, e.g. the hydro-

genase is membrane bound and integrated into an electron flow chain at a site of comparatively high redox potential then the electrons which it extracts from H_2 will immediately be diverted to the reduction of an adjacent electron carrier of still more positive potential. Only further promotion of the electrons by additional energy input, e.g. via a Photosystem I light reaction, might eventually 'restore' the original reducing power of H_2. Incidentally, similar arguments might apply in the case of photosynthetic bacteria whose photoautotrophic growth on H_2 is light dependent [17].

For *Anacystis* the results suggest that in vivo H_2 photoreduced primarily endogenous ferredoxin. If indeed reduced ferredoxin is the immediate electron donor to the nitrogenase of heterocystous blue-greens then reduction of ferredoxin by H_2 should be a prerequisite for the H_2-supported N_2 fixation found by several investigators [13,22]. However, as outlined above this does not presuppose direct reactivity between ferredoxin and the hydrogenase. Rather, no evidence for such coupling was found in the present experiments (cf. Table V), nor seems direct coupling between ferredoxins and hydrogenases (both from various sources) to be the rule except perhaps in case of clostridial hydrogenases (cf. Ref. 36).

Inhibition of H_2-dependent (photo)reductions by the plastoquinone antagonist DBMIB suggested a site of action for the hydrogenase before plastoquinone (cf. Ref. 2). Note, however, that this cannot be on the oxidizing side of Photosystem II as the latter was not involved in the reaction; DCMU was without effect on the photoreductions, and no dichromatic enhancement was observed for H_2-supported photoreduction of CO_2 in intact *Anacystis* [26]. The fact that DBMIB not only inhibited the photoreductions, but also the dark reductions tested may indicate that, in the algal material used in the present study, electrons from H_2 initially follow the same flow chain in the light and in the dark. Eventually they may be taken over by some suitable acceptor compound from an electron transport component of appropriate redox potential. Such partially identical pathways are also suggested by the similar degree of inhibition by O_2 and by other inhibitors listed in Table IV, when comparing photoreductions with dark reductions.

The inhibitory effect of CO on the H_2-supported reduction of ferricyanide and of $NADP^+$ was not reversed in strong light which appears to rule out an involvement of respiratory cytochromes in the hydrogenase-catalysed reactions described here for the *Anacystis* membranes used in this study. On the other hand, both uptake and evolution of H_2 by the membranes were inhibited to corresponding degrees by CO and in strong light, and H_2 evolution was independent of ATP but inhibited under an atmosphere of pure H_2; from this, also the participation of a nitrogenase in the hydrogenase activities described in this paper appears to be excluded.

A last point to which attention may be drawn in the present study is the reversible inactivation by O_2 of the photoreduction hydrogenase of *Anacystis*. Hydrogenases from different sources may show pronounced differences in O_2 sensitivity: e.g. the enzymes involved in the oxyhydrogen reaction in Knallgas bacteria [9,32] and in N_2-fixing blue-greens [12—14] appear to be remarkably stable towards O_2. In contrast, with adapted green algae O_2 concentrations below 1% already lead to inactivation of the hydrogenase (see Ref. 8); also the

enzyme involved in photoreductions by whole cells of *Anacystis* [26], and by the membrane preparations used in the present study, was completely inhibited by <2% O_2.

With adapted eukaryotic algae the O_2 effect probably reflects a 'reversible de-adaptation' rather than an irreversible destruction [8], but there may be exceptions [3]. A highly purified soluble hydrogenase from *Alcaligenes eutrophus* H16 was inactivated by O_2 (presumably by oxygenation of disulphide bridges), and subsequently reactivated by reducing agents [32]. When inactivated by <2% O_2 the enzyme from *Anacystis* could be perfectly reactivated simply by treatment with pure H_2, and the reducing agent dithiothreitol prevented the deleterious effect of O_2. Recalling the effect of thiol-blocking agents, an obligatory involvement of SH groups in the function of the photoreduction hydrogenase of *Anacystis* thus seems likely. Involvement of 'active' SH groups in hydrogenases of both bacteria [32] and eukaryotic algae [35] has been envisaged.

The results of the work presented here apparently are the first to describe a membrane-bound hydrogenase to be involved in anaerobic photoreductions (and related reactions) in a blue-green alga. However, predominance of either photoreduction or oxyhydrogen activity in *Anacystis* strongly depended on the conditions under which the organisms had been induced for hydrogenase (Peschek, G.A., unpublished). The companion paper deals with the oxyhydrogen reaction of *Anacystis*, and the data presented may altogether point to the possible occurrence in *Anacystis* of two different hydrogenases, an O_2-sensitive 'photosynthetic' hydrogenase responsible for photoreduction, and an O_2-insensitive 'respiratory' hydrogenase responsible for the oxyhydrogen reaction. The results obtained so far at least do not necessarily support the view that one and the same hydrogenase couples, according to conditions, sometimes with the photosynthetic, sometimes with the respiratory electron transport.

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