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AEROBIC HYDROGENASE ACTIVITY IN *ANACYSTIS NIDULANS*

THE OXYHYDROGEN REACTION

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Summary

1. The oxyhydrogen reaction of *Anacystis nidulans* was studied manometrically and polarographically in whole cells and in cell-free preparations; the activity was found to be associated with the particulate fraction.

2. Besides O_2, the isolated membranes reduced artificial electron acceptors of positive redox potential; the reactions were unaffected by O_2 levels <10—15%; aerobically the artificial acceptors were reduced simultaneously with O_2.

3. H_2-supported O_2 uptake was inhibited by CO, KCN and 2-*n*-heptyl-8-hydroxyquinoline-*N*-oxide. Inhibition by CO was partly reversed by strong light. Uncouplers stimulated the oxyhydrogen reaction.

4. The kinetic properties of O_2 uptake by isolated membranes were the same in presence of H_2 and of other respiratory substrates.

5. Low rates of H_2 evolution by the membrane preparations were found in presence of dithionite; methyl viologen stimulated the reaction.

6. The results indicate that under certain growth conditions *Anacystis* synthesizes a membrane-bound hydrogenase which appears to be involved in phosphorylative electron flow from H_2 to O_2 through the respiratory chain.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol; DSPD, disalicylidene-1,3-diaminopropane; HOQNO, 2-*n*-heptyl-8-hydroxyquinoline-*N*-oxide; SDS, sodium dodecyl sulphate; Hepes, *N*-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid.

Introduction

Hydrogenase activity of blue-green algae (cyanobacteria) has so far been studied almost exclusively in heterocystous N_2-fixing species [1–8]. For these, and also for other aerobic N_2-fixing bacteria [9,10], a so-called uptake hydrogenase has been invoked, and a close functional relationship between H_2 uptake and N_2 fixation has been proposed [1–4]. The uptake hydrogenase is believed to be particulate rather than soluble ([7]; cf. Ref. 8); in vivo, it uses O_2 as electron acceptor [1,2]. The inhibition pattern of the oxyhydrogen reaction apparently links it to respiration [1] although others [8] would not exclude the possible occurrence of a hydrogenase participating in the heterocyst's photosynthetic electron transport, too. Moveover, also the fact that in the heterocyst a hydrogenase both initiates phosphorylative electron transport from H_2 to O_2 [1,3] and anaerobic photosynthetic N_2 fixation [1,2] might suggest the occurrence of two different 'uptake' hydrogenases in blue-greens [7,8]; yet preliminary studies did not support this view [11].

An (aerobic) hydrogenase in several non-nitrogen-fixing blue-greens, among them *Anacystis*, was recently reported [11]. The respective enzyme, however, was not further characterized, and no attention was paid to anaerobic H_2-supported photoreduction (cf. Refs. 12 and 13). An examination of the latter was presented in the preceding paper ([14]; cf. also Ref. 15). The present work now describes several features of the aerobic (oxyhydrogen) hydrogenase activity in *Anacystis* thus partly confirming, and extending, the observations of Bothe and coworkers [11].

Materials and Methods

Growth of the algae. Anacystis nidulans (strain 1402-1, Göttingen, F.R.G.) was grown axenically at 25°C and 10 W/m^2 warm white-fluorescent light under H_2 supplemented with 5% CO_2; other conditions were described previously [15]. Doubling time of the algae was about 20 h. For the experiments the algae were resuspended in a buffer solution henceforth referred to as 'standard medium' (30 mM Tris/Hepes buffer; 2 mM Na_2HPO_4; 1 mM $MgCl_2$; 10 mM KCl, pH 7.4).

Measurement of gas uptake and evolution. In aerobic conditions individual rates of concomitant uptake of H_2 and O_2 were determined manometrically according to Warburg's independent two vessel method ([16]; cf. Ref. 17). The same procedure was used for the simultaneous determination of respiratory O_2 uptake and CO_2 release [17]. The individual amount of either gas exchanged was given (e.g. for gas A) by the formula

$$V_A = \frac{[h]_t \cdot k_B - [h]_t \cdot k'_B}{k_B/k_A - k'_B/k'_A}$$

where V_A, volume (μl) of gas A exchanged within given time (reduced to standard conditions, see Ref. 16); $[h]_t$, manometer reading (mm) within given time; k_A and k_B, vessel constants for gas A and B, respectively, in the smaller vessel, and k'_A and k'_B, vessel constants for gas A and B, respectively, in the larger vessel.

Values obtained by the Warburg method were usually reproducible to within about 30%. H_2 uptake was also measured by gas chromatography [2] though within the limits of error, both methods yielded identical results. The H_2-dependent uptake of O_2 by membrane preparations from *Anacystis* was most accurately measured polarographically with a Clark-type oxygen electrode: cell or membrane suspensions saturated with H_2 or other appropriate gas mixtures were filled into the chamber (2.5 ml) which was then quickly sealed by a tightly fitting glass stopper equipped with a capillary inlet through which solutions (of inhibitors, etc.) could be injected, or assay suspension withdrawn, as desired [15]. When a defined concentration of H_2 was to be established in the oxygraph suspension calibrated amounts of H_2-saturated standard medium were injected through the capillary inlet into the suspension previously flushed with $O_2/N_2 = 1/9$; this method proved best suited for determination of the stoichiometry of the oxyhydrogen reaction (Fig. 2). In case of illumination the samples contained 10 μM DCMU to prevent possible photosynthetic O_2 evolution [14,15].

Spectrophotometric hydrogenase assay. The H_2-dependent reduction of artificial redox compounds was measured spectrophotometrically as previously described [14]; also the concentrations of the acceptors used were the same except when otherwise stated. Aerobic hydrogenase activity in presence of artificial electron acceptors, i.e. the simultaneous reduction of both O_2 and an artificial redox compound, was determined in parallel with three separate algal samples; one of these was examined polarographically for O_2 uptake, in the second the reduction of the artificial acceptor was followed spectrophotometrically, and the third parallel sample was assayed for H_2 uptake by gas chromatography. The three samples were initially flushed with $O_2/H_2 = 1/9$; they were then incubated and assayed under strictly identical conditions. All hydrogenase assays were carried out at 35°C, and in darkness unless otherwise stated.

Preparation of cell-free extracts. The experiments with membrane preparations were carried out on particles isolated after brief sonication of lysozyme-treated cells as described in the preceding paper [14]; hydrogenase activity of preparations obtained by other cell fractionation procedures was substantially lower (cf. Ref. 14).

Determination of chlorophyll and protein. Measurements were done as previously described [14]. 1 mg dry weight of cells used in this study was equivalent to 3.3 μl packed cells or 0.026 mg chlorophyll or 0.61 mg protein. The protein/chlorophyll ratio in isolated membranes (free of phycocyanin) was about 10. In crude extracts the distribution of protein between the particulate and the soluble fraction was about 1 : 0.8.

Controls. For spectrophotometry samples flushed with N_2 (instead of H_2) served as controls. H_2-dependent O_2 uptake by the membrane preparations was corrected for the (very slow, 'endogenous') O_2 uptake in absence of H_2 or other substrates. H_2 uptake rates in absence of electron acceptors (i.e. either O_2 or artificial redox compounds) were always negligible. In several experiments also algae or isolated membranes heated at 90°C for 5 min served as additional controls. Unless otherwise stated 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

Gas exchange reactions by oxyhydrogen-induced intact cells

Oxygen consumption in presence of H_2 was nearly three-fold that by endogenous respiration (Table I). Thus hydrogen behaved like an efficient respiratory 'substrate'. Otherwise substrate stimulation of respiratory O_2 uptake is rare among obligately photoautotrophic blue-greens, notably in *Anacystis* (cf. Refs. 17—19). Respiratory CO_2 release is slightly reduced in presence of H_2 showing that H_2 partly suppresses endogenous respiration (Table I). The extent of anaerobic light-dependent H_2 uptake accompanied by CO_2 photoreduction apparently is very small in algae grown as described in Materials and Methods (cf. Refs. 11 and 14); in contrast, O_2-dependent H_2 consumption is appreciably high in these algae, and is hardly influenced by light (Table I). The oxyhydrogen reaction of *Anacystis*, unlike that of adapted eukaryotic algae [20—22], did not support chemosynthetic CO_2 fixation. Both the oxyhydrogen reaction and the H_2 uptake effected by artificial electron acceptors (e.g. benzoquinone, menadione or DCIP) were linear for up to at least 3 h (results not shown).

Aerobic hydrogenase activity in membrane preparations

Table II shows that more than 90% of the oxyhydrogen reaction activity present in crude extracts was recovered in the particulate fraction; the soluble fraction was virtually devoid of activity. Other test reactions used to localize the hydrogenase in cell-free extracts were the uptake of H_2 in presence of *p*-benzoquinone and of menadione, and the H_2-supported reduction of ferri-

TABLE I

GAS EXCHANGE REACTIONS OF INTACT *Anacystis* INDUCED FOR AEROBIC HYDROGENASE ACTIVITY

Uptake of H_2 and O_2, and respiratory CO_2 production were followed manometrically as described in Materials and Methods. Alternatively, H_2 uptake was determined also by gas chromatography [2]. Incorporation of CO_2 was measured radiochemically by use of labelled bicarbonate [15]. Gas exchange rates expressed as μmol/h per mg chlorophyll. Values given are the means from five independent experiments (cf. Materials and Methods). 10 μM DCMU was present in all samples.

Gas phase		H_2 uptake	O_2 uptake	CO_2 fixation	CO_2 release
$H_2/N_2/CO_2$ = 87/10/3	Light *	2.9	—	1.4	—
	Dark	<0.1	—	<0.1	—
$N_2/O_2/CO_2$ = 87/10/3	Light *	—	1.9	0.4	2.0
	Dark	—	7.4	<0.1	6.7
$H_2/O_2/CO_2$ = 87/10/3	Light *	27	15	0.4	1.8
	Dark	30	20	<0.1	5.7

* 18 W/m^2 fluorescent light.

TABLE II

LOCALIZATION OF HYDROGENASE ACTIVITY IN CELL-FREE EXTRACTS

'Particulate', activity in the 144 000 × *g* sediment. 'Soluble', activity in the 144 000 × *g* supernatant. Samples contained between 1.2 and 3.1 mg protein/ml. H_2 uptake in presence of benzoquinone or menadione was followed manometrically under 100% H_2. H_2-dependent reduction of ferricyanide or ferricytochrome *c* was measured spectrophotometrically in suspensions flushed with pure H_2. H_2-supported O_2 consumption was determined with a Clark-type oxygen electrode in samples sparged with 10% O_2 in H_2 prior to measurement. Activities expressed as nmol H_2 taken up/min per mg protein (with benzoquinone and menadione as electron acceptors) or as nmol acceptor reduced/min per mg protein (with ferricyanide, cytochrome *c* and O_2 as acceptors). Reaction rates were linear for up to at least 30 min.

Acceptor	Specific activity (nmol/min per mg protein)			Relative activity (%) (crude extract = 100%)	
	Crude extract	Particulate	Soluble	Particulate	Soluble
Oxygen	15.6	26.0	0.3	93	<1
Benzoquinone	35.8	62.1	0.2	96	<1
Menadione	25.1	42.9	0.4	95	<1
Ferricyanide	47.7	78.1	0.7	91	<1
Cytochrome *c*	33.0	51.1	0.2	86	<1

cyanide and of ferricytochrome *c*; the results, too, indicated that the hydrogenase was particulate (Table II). Besides the acceptors mentioned a variety of other redox compounds served as oxidants for the H_2-reduced hydrogenase, provided their redox potential was not too negative (Table III). Characteristically, redox compounds of strongly negative potential commonly used as electron acceptors for Photosystem I in plant photosynthesis (e.g. diquat, methyl viologen, ferredoxin or NADP) could not sustain substantial rates of the hydrogenase reaction in membranes prepared from *Anacystis* cells induced for

TABLE III

EFFECTIVENESS OF VARIOUS ELECTRON ACCEPTORS IN SUPPORTING HYDROGENASE ACTIVITY OF MEMBRANE PREPARATIONS.

(A) H_2-dependent reduction of acceptors followed spectrophotometrically or polarographically (for O_2) in samples saturated either with 10% O_2 in H_2 ('aerobic') or with 100% H_2 ('anaerobic'). No difference in rates observed between samples flushed with 100% H_2 or with 10% N_2 in H_2. (B) H_2 uptake determined manometrically under pure H_2 in presence of the acceptors. Samples contained between 0.3 and 3.6 mg protein/ml. Values in brackets give rates of O_2 reduction observed concomitantly with the reduction of DCIP, cytochrome *c* and ferricyanide, respectively.

Acceptor	E'_0 (V)	Reduction of acceptor (nmol/min per mg protein)		Acceptor	E'_0 (V)	Hydrogen uptake (nmol/min per mg protein)
		Anaerobic	Aerobic			Anaerobic
A				B		
DCIP	+0.22	21.3	10.0 (5.9)	Menadione	−0.01	23.0
Cytochrome *c*	+0.25	50.3	13.3 (9.4)	Methylene blue	+0.01	11.2
Ferricyanide	+0.36	80.6	49.1 (8.0)	Benzoquinone	+0.29	38.5
Oxygen	+0.82	—	26.8			

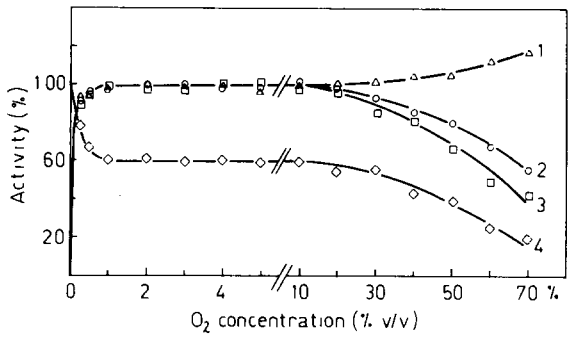

Fig. 1. Effect of increasing oxygen concentrations on the rates of (1) endogenous respiration of whole cells; (2) total gas uptake (i.e. $H_2 + O_2$) by whole cells; (3) H_2-dependent O_2 uptake by membrane preparations, and (4) H_2-dependent ferricyanide reduction by the membrane preparations. Gas phases used consisted of 30% H_2 plus 70% of appropriate mixtures of O_2 with N_2 to give final O_2 concentrations as indicated in the figure. Gas uptake was measured manometrically. Reduction of ferricyanide was determined spectrophotometrically. 100% activity was: 7.5 μmol O_2/h per mg chlorophyll for (1), 48 μmol ($H_2 + O_2$)/h per mg chlorophyll for (2), 25.9 nmol O_2/min per mg protein for (3) and 79.2 nmol ferricyanide/min per mg protein for (4).

aerobic hydrogenase activity (results not shown). The small rates found with methyl viologen and NADP in the light (4—7 nmol of acceptor reduced/min per mg protein; results not shown) probably reflected a low level of 'photoreduction hydrogenase' present also in algae induced (more or less selectively) for aerobic hydrogenase activity as described in Materials and Methods (cf. Ref. 14).

Table III also shows that in aerobic conditions the artificial oxidants were reduced by H_2 concomitantly with oxygen. The proportion between H_2-dependent reductions of redox dye and of oxygen remained constant throughout the whole range of O_2 saturation of the oxyhydrogen reaction (Fig. 1). Fig. 1 also shows that the (aerobic) hydrogenase reactions were inhibited only by oxygen levels above 10%.

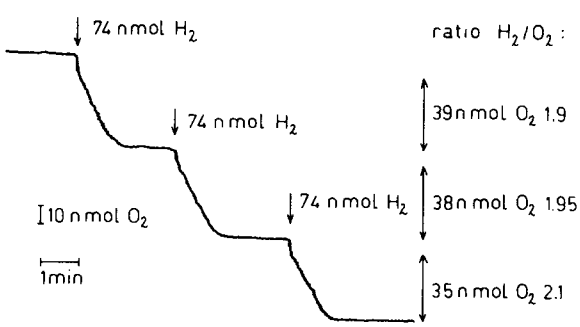

Fig. 2. Stoicheiometry of the oxyhydrogen reaction displayed by isolated membranes. The H_2-dependent reduction of O_2 was measured with a Clark-type oxygen electrode as described in Materials and Methods. At the times indicated by arrows each 0.1 ml of H_2-saturated standard medium were injected (corresponding to 74 nmol H_2). The error of roughly 4% due to dilution of the oxygraph suspension was taken into account for the calculation. Initially the sample had been flushed with 10% O_2 in N_2.

Stoichiometry of the oxyhydrogen reaction

Fig. 2 describes an experiment permitting calculation of the H_2/O_2 ratio during the oxyhydrogen reaction of isolated membranes. As the 'endogenous' (substrate-less) O_2 uptake rate of the membranes was sufficiently low the oxygen consumption effected by injection of a known amount of H_2 (as H_2-saturated buffer) could be measured accurately provided allowance was made for the dilution caused by injection of the buffer. The results indicate a stoichiometry of almost 2.0 as would be expected for the reaction $2\ H_2 + O_2 = 2\ H_2O$. Similar stoichiometries were obtained also for the oxyhydrogen reaction observed in heterocystous blue-greens [3].

Effect of inhibitors

Table IV describes the action of various compounds inhibiting selected hydrogenase-catalysed reactions in membranes prepared from oxyhydrogen-induced *Anacystis*. It is seen that typical respiratory inhibitors, e.g. HOQNO, KCN or CO, acted similarly as in the endogenous respiration of intact *Anacystis* [17,23]. Lack of an uncoupling effect by CCCP seems to reflect loss of phosphorylating capacity during preparation of the membranes since uncouplers clearly stimulated the oxyhydrogen reaction of whole cells (Table V). Dodecylic acid, a representative of lipid-soluble fatty acids and thus probably acting as a 'H^+ conductor' [24], was shown also to stimulate the endogenous respiration of intact *Anacystis* [23]. Compounds affecting H_2-supported photoreductions in appropriately induced *Anacystis* (e.g. dibromothymoquinone and disalicylidenepropanediamine; cf. Ref. 14) were without effect on reactions catalysed by the aerobic hydrogenase. On the other hand, thiol-blocking agents like *p*-hydroxymercuribenzoate or *N*-ethylmaleimide apparently inhibited both hydrogenase activities though the aerobic one seemed to be more resistant (cf. Table IV of Ref. 14).

Inhibition by CO of the oxyhydrogen reaction in membrane preparations was partly reversed by strong illumination (Fig. 3B) pointing to the same

TABLE IV

EFFECT OF INHIBITORS ON SEVERAL HYDROGENASE-CATALYSED REACTIONS IN MEMBRANE PREPARATIONS

In case of O_2 uptake the samples were sparged with 10% O_2 in H_2 before measurement. H_2 uptake was followed manometrically in presence of 2 mM benzoquinone under pure H_2. Ferricyanide reduction was followed spectrophotometrically in samples previously flushed with pure H_2. Suspensions contained 1.1—3.4 mg protein/ml. Rates expressed as nmol/min per mg protein.

Inhibitor	Oxygen uptake	Ferricyanide reduction	Hydrogen uptake
None	27.2	82.0	39.3
10 μM CCCP	27.0	82.2	39.2
10 μM HOQNO	4.5	19.2	33.0
CO	6.0 *	36.9 **	16.8 **
0.1 mM KCN	3.5	12.1	5.1
0.5 mM *N*-ethylmaleimide	3.2	9.9	9.3
0.5 mM *p*-hydroxymercuribenzoate	3.0	9.5	9.1

* Samples flushed with $H_2/CO/O_2 = 60/30/10$.

** Samples flushed with $H_2/CO = 70/30$. No difference was seen between samples flushed with $H_2/CO = 70/30$ or with $H_2/N_2/CO = 60/10/30$.

TABLE V

STIMULATION BY UNCOUPLERS OF THE ENDOGENOUS RESPIRATION (A) AND OF THE OXY-HYDROGEN REACTION (B) IN WHOLE CELLS

Gas exchange was measured manometrically. Alternatively, H_2 uptake was also assayed by gas chromatography [2]. Rates expressed as μmol/h per mg chlorophyll. Samples contained algae equivalent to 0.3—0.5 mg chlorophyll/ml.

Uncoupler	Gas phase (A) ($N_2/O_2/CO_2$ = 87/10/3)		Gas phase (B) ($H_2/O_2/CO_2$ = 87/10/3)		
	O_2 uptake	CO_2 release	H_2 uptake	O_2 uptake	CO_2 release
None	7.1	6.4	33	23	5.8
10 μM CCCP	13.8	12.3	72	45	8.9
0.5 mM dinitrophenol	12.7	11.5	64	41	8.6
20 μM dodecylic acid	10.0	8.9	48	32	7.2

(haem-type) terminal oxidase(s) being involved in the oxidation of H_2 and of endogenous substrates; illumination was shown partly to relieve the CO inhibition of endogenous respiration in intact *Anacystis* [23]. In contrast, the H_2-supported reduction of ferricyanide, when inhibited by CO, was still further inhibited in strong light (Fig. 3A). Inhibition of the hydrogenase activity with increasing light intensity (Fig. 4) was shown also for the anaerobic (photoreduction) hydrogenase (cf. Fig. 4 of the preceding paper). Fig. 5 documents the similarity of the effects of KCN and CO on O_2 uptake in presence of H_2 and of other respiratory (including endogenous) substrates; the figure also shows that the hydrogenase apparently was far less sensitive towards CO than was the terminal oxidase while both were affected in a roughly parallel way by increasing KCN concentrations.

Kinetic analysis of H_2-dependent O_2 uptake

Fig. 6 shows that the Michaelis-Menten parameters (i.e. K_m and maximal velocity) of the oxygen uptake were roughly the same for H_2, NADH or

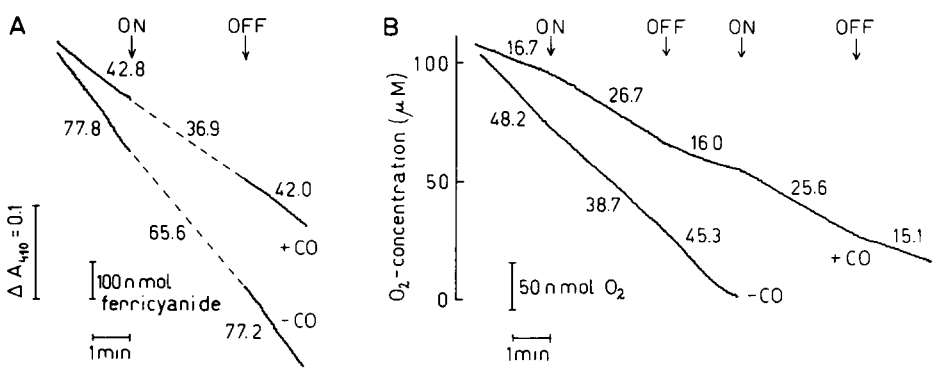

Fig. 3. Effect of illumination (400 W/m^2 incandescent light) on CO-inhibited reduction of ferricyanide (A) and of oxygen (B) by isolated membranes. O_2 reduction was followed polarographically in suspensions initially flushed with $H_2/CO/N_2$ = 60/30/10 or with H_2/N_2 = 60/40. Ferricyanide reduction was measured spectrophotometrically in anaerobic samples flushed either with 30% CO or N_2 in H_2 (cf. Ref. 14). Figures below the curves are nmol/min per mg protein.

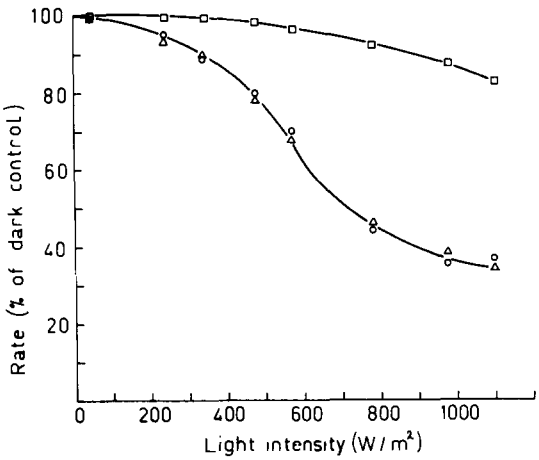

Fig. 4. Effect of light intensity on oxygen uptake reactions in membrane preparations. The following test reactions were used: ○, H_2-dependent O_2 uptake measured polarographically in samples flushed with 10% O_2 in H_2; △, H_2-supported reduction of ferricyanide measured spectrophotometrically in samples flushed with 100% H_2, and □, NADPH-dependent O_2 uptake followed polarographically in samples flushed with 10% O_2 in N_2. No difference noted between samples flushed with 100% H_2 or with 10% N_2 in H_2. Suspensions contained between 1.1 and 2.3 mg protein/ml. Illumination was by means of a slide projector's incandescent lamp. Final light intensity was varied by use of appropriate combinations of Kodak Wratten gelatin filters placed behind a KG 4 infrared filter (Schott AG, Mainz, F.R.G.), and measured with a Yellow Springs Instruments Radiometer (model 65). 100% activity corresponded to (nmol/min per mg protein) 26.8 (○), 79.8 (△) and 32.3 (□), respectively.

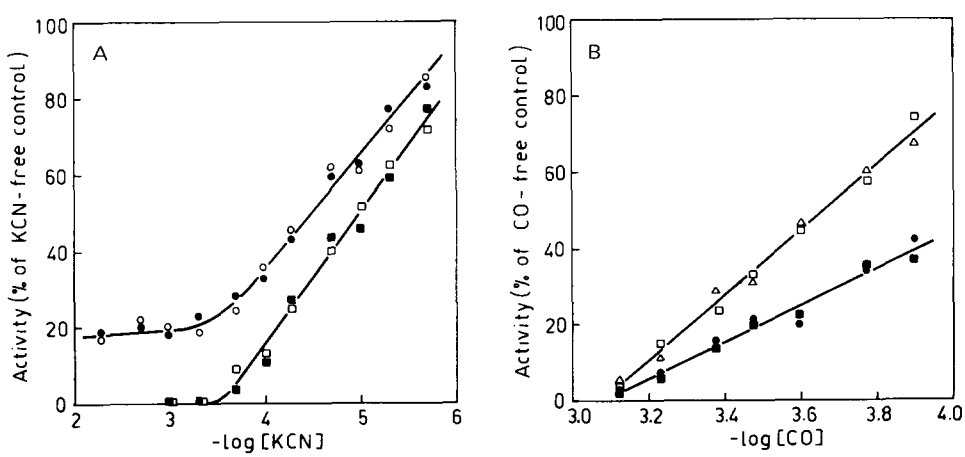

Fig. 5. Effect of cyanide (A) and of carbon monoxide (B) on various respiratory and/or hydrogenase-catalysed reactions. ●, endogenous respiration of intact cells (100% activity: 7.5 μmol O_2/h per mg chlorophyll); ○, NADPH-dependent O_2 uptake by isolated membranes (100% = 31.7 nmol O_2/min per mg protein); □, H_2-dependent reduction of ferricyanide by isolated membranes (100% = 78.8 nmol ferricyanide/min per mg protein); ■, H_2-dependent O_2 uptake by isolated membranes (100% = 26.3 nmol O_2/min per mg protein); △, H_2-dependent reduction of DCIP by isolated membranes (100% = 21.5 nmol DCIP/min per mg protein). Control experiments with appropriate N_2/H_2 mixtures assured independence of the reaction rates on H_2 content between 100% and about 10% H_2 (rest: N_2). For experimental details see Materials and Methods.

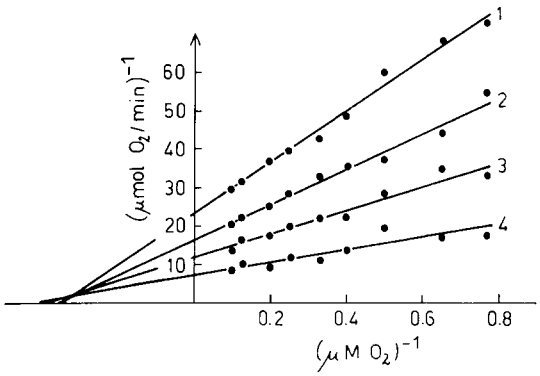

Fig. 6. Double-reciprocal plots of oxygen uptake by isolated membranes in presence of (2) 3 mM NADPH (1.6 mg protein); (3) H_2 (2.3 mg protein), and (4) 3 mM NADH (3.7 mg protein). Curve (1) gives the Lineweaver-Burk plot for the endogenous respiratory O_2 uptake by whole cells (2.2 mg cell protein).

NADPH (in membrane preparations) or for endogenous substrates (in whole cells) serving as the ultimate reductants: in case of isolated membranes V was 38.0 ± 1.2 nmol O_2/min per mg protein and K_m was 3.2 ± 0.2 μM O_2. (Considering the V value obtained with intact cells allowance must be made for the fact that only a certain fraction of the cell protein is membrane protein; cf. Materials and Methods). This result suggested that the same respiratory mechanism might have been at work with all types of substrates tested.

Hydrogen evolution

To test reversibility of the hydrogenase reaction the isolated membranes were incubated, under N_2, in presence of 10 mM dithionite. The low rates of H_2 evolution thus obtained (usually in the range of 0.8 nmol H_2/min per mg protein) were unaffected by ferredoxin but stimulated three-fold by 5 mM methyl viologen. The stimulating effect of detergents (e.g. of 1 mM sodium dodecyl sulphate or of 0.03% (v/v) Triton X-100) was much less than in the case of the photoreduction hydrogenase activity, and no indication of solubilization could be obtained (results not shown; cf. Tables V and VI of the preceding paper).

Discussion

The experiments described in the previous section present evidence for the aerobic functioning of a membrane-bound hydrogenase in appropriately induced *A. nidulans*. The enzyme catalysed H_2 uptake in presence of various external electron acceptors including molecular oxygen. At first glance the occurrence of hydrogenase activity in an aerobic organism incapable of nitrogen fixation appears puzzling. On the other hand, anaerobic H_2-supported photoreduction has been reported for several non-nitrogen-fixing blue-greens [14,15,25], and efficient reduction of O_2 by H_2 was recently found in several non-heterocystous strains, including *Anacystis*, after growth in presence of H_2 [11]. The present study attempts to characterize more closely the oxyhydrogen reaction of *Anacystis*. The results confirm the existence of an inducible,

largely O_2-resistant, membrane-bound hydrogenase initiating respiratory electron transport from H_2 to O_2 coupled to oxidative phosphorylation. In accordance with results obtained on other aerobic hydrogenase-containing organisms [3,26], oxygen could be replaced by other high potential electron acceptors, e.g. ferricyanide, ferricytochrome *c*, *p*-benzoquinone or dichlorophenolindophenol; several redox compounds of less positive potential, e.g. menadione or methylene blue, accepted electrons from H_2 possibly at a site closer to, or even immediately from, the hydrogenase itself as judged, e.g. from the far weaker inhibition by HOQNO (results not shown). Since the membrane preparations used in this study contained both plasmalemma and thylakoid fragments (cf. Ref. 14) the question of the exact localization of the aerobic hydrogenase of *Anacystis* on either type of membrane still remains open. The problem is of course intimately related to the question of the localization of the respiratory chain which is far from unraveled [27].

Comparative studies on the kinetics of O_2 reduction by the isolated membranes in presence of different respiratory substrates indicated that electron flow from H_2 to O_2 indeed proceeded via the respiratory chain. This was suggested also by the experiments with the respiratory inhibitors 2-*n*-heptyl-8-hydroxyquinoline-*N*-oxide, KCN or CO. Particularly, the terminal oxidase involved in H_2-dependent reduction of O_2 was the same as in other respiratory substrate oxidations: inhibition of the oxyhydrogen reaction by CO was reversed about 33% in strong light while the same light intensity slightly inhibited the hydrogenase-catalysed reduction of artificial electron acceptors. Similar reversal of the CO inhibition of the oxyhydrogen reaction was noted in isolated heterocysts of *Anabaena cylindrica* [3]. However, apart from the effect of CO on the terminal oxidase the hydrogenase itself seems to be affected by CO as is the case with other hydrogenases from various sources [1,20,28].

Although the aerobic hydrogenase of *Anacystis* proved remarkably stable towards O_2, concentrations in excess of 10—15% increasingly inhibited the oxyhydrogen reaction (cf. Ref. 11). Comparably weak action of O_2 was found for the uptake hydrogenase of heterocystous blue-greens [1—3] as well as for the membrane-bound hydrogenase in (aerobic) hydrogen bacteria [29—31]. The hydrogenase of adapted eukaryotic algae, however, which is likewise capable of mediating an oxyhydrogen reaction [20—22] is already inhibited by 0.5% O_2 [20]. Note that also in *Anacystis* the hydrogenase responsible for (anaerobic) photoreductions was highly susceptible towards O_2 [14].

On the side of low O_2 tensions the rate of the oxyhydrogen reaction remained essentially constant down to oxygen levels as low as about 2%. This would be expected if aerobic H_2 uptake truely mirrored respiratory O_2 consumption dependent on H_2. This result agrees with measurements on isolated heterocysts [3] but disagrees with those of Bothe and coworkers who found H_2 uptake rates to decrease below 5—10% O_2 already [11]; these lowered rates, however, might have resulted from oxygen limitation in the discontinuous experimental system used by the authors [1,2,11] rather than to a true decline of the reaction rate at low oxygen tensions. Also the high absolute rates of the oxyhydrogen reaction reported for *Anacystis* [11] could not be confirmed. Note that uptake rates around 50 μmol H_2/mg chlorophyll

per h [11], corresponding to Q_{O_2} values of 11—15 μl O_2/mg dry weight per h, would reflect unusually high rates of coupled respiration in a blue-green alga. This would be especially true of *Anacystis* whose ordinary Q_{O_2} value ranges between about 0.8 and 6.0 [17] without substantial influence of most respiratory substrates (cf. Ref. 18). In this respect, even more severe objections might be raised against the rates of the oxyhydrogen reaction in isolated heterocysts [3] reflecting exorbitantly high Q_{O_2} values of 100 or even more; but the respiratory electron transport in a heterocyst might perhaps be quite different from that in a vegetative cell, possibly capable of sustaining such fast O_2 uptake. Clearly the meaning of this unusual 'respiratory' O_2 consumption would deserve some closer consideration.

Finally, attention may be drawn to the possibility of two different hydrogenases operating, in different conditions, in *Anacystis*. Functionally different hydrogenases are well-known for certain hydrogen bacteria. In these organisms simultaneously one (soluble) hydrogenase, called hydrogen dehydrogenase, mediates the reduction of NAD by H_2 while the other (particulate) enzyme initiates the respiratory electron flow from H_2 to O_2 [31]. Similar 'division of labour' might hold for certain aerobic photoautotrophic organisms, viz. blue-green algae [7,14]; e.g. in *Anacystis* certain growth conditions apparently lead to induction of a membrane-bound hydrogenase which, in strict anaerobiosis, mediates the light-dependent electron flow from H_2 to low-potential electron acceptors (notably NADP); other conditions, in turn, seem to provoke the synthesis of another hydrogenase, which is an aerobic, membrane-bound, 'respiratory' enzyme responsible for the oxyhydrogen reaction. Details concerning the induction and further properties of the respective hydrogenases in *Anacystis* are currently being investigated in our laboratory.

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Further experimental results suggesting two functionally distinct uptake hydrogenases in *Anacystis* (cf. Ref. 14) will be discussed in Ref. 32. The function of two different uptake hydrogenases recently was emphasized also for nitrogen-fixing blue-greens [33]. Isolation and characterization of the membrane-bound uptake hydrogenases of *Alcaligenes eutrophus* [34] and *Proteus mirabilis* [35] was recently reported; the enzyme from *A. eutrophus* thus could be definitely shown to be different from the NAD^+-reducing hydrogenase present in the same organism [26].

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