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Inhibition of respiration and nitrate assimilation enhances photohydrogen evolution under low oxygen concentrations in *Synechocystis* sp. PCC 6803

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Abstract

In cyanobacterial membranes photosynthetic light reaction and respiration are intertwined. It was shown that the single hydrogenase of *Synechocystis* sp. PCC 6803 is connected to the light reaction. We conducted measurements of hydrogenase activity, fermentative hydrogen evolution and photohydrogen production of deletion mutants of respiratory electron transport complexes. All single, double and triple mutants of the three terminal respiratory oxidases and the *ndhB*-mutant without a functional complex I were studied. After activating the hydrogenase by applying anaerobic conditions in the dark hydrogen production was measured at the onset of light. Under these conditions respiratory capacity and amount of photohydrogen produced were found to be inversely correlated. Especially the absence of the quinol oxidase induced an increased hydrogenase activity and an increased production of hydrogen in the light compared to wild type cells. Our results support that the hydrogenase as well as the quinol oxidase function as electron valves under low oxygen concentrations. When the activities of photosystem II and I (PSII and PSI) are not in equilibrium or in case that the light reaction is working at a higher pace than the dark reaction, the hydrogenase is necessary to prevent an acceptor side limitation of PSI, and the quinol oxidase to prevent an overreduction of the plastoquinone pool (acceptor side of PSII). Besides oxygen, nitrate assimilation was found to be an important electron sink. Inhibition of nitrate reductase resulted in an increased fermentative hydrogen production as well as higher amounts of photohydrogen.

Keywords: Hydrogenase; Cyanobacteria; Fermentation; Nitrate reductase; Cytochrome c oxidases; Quinol oxidase

1. Introduction

The unicellular, non-nitrogen fixing cyanobacterium *Synechocystis* sp. PCC 6803 is a photosynthetic model organism. It was found to grow heterotrophically and to be naturally transformable. Initially, these properties made this strain an ideal system for mutational analysis of the photosystems [1,2]. But since the publication of its complete genome sequence [3], the investigations broadened to study cyanobacterial metabolism in general as well as gene homologs of higher plants [4]. At present, there is probably no other cyanobacterium, which has been investigated in such detail, making it interesting for biotechnological applications [5].

Hydrogenases split hydrogen into protons and low potential electrons or combine protons and electrons to hydrogen. *Synechocystis* contains only one hydrogenase [6] that is coupled to the photosynthetic light reaction. It is working as an electron valve in transition states such as those during shifts from dark anaerobiosis to light [7]. It was found that this type of cyanobacterial hydrogenase is inactive in the presence of oxygen and activated in less than a minute when cells are depleted of oxygen [7,8]. In vitro the enzyme can be activated within minutes by NADH and NADPH [9,10]. Except the soluble NAD-reducing hydrogenase of *Cupriavidus necator* (the former *Alcaligens eutrophus* and *Ralstonia eutropha*) [11,12], all other NiFe-hydrogenases are inactivated by oxygen and their reactivation in the presence of hydrogen and reducing equivalents takes hours (see for example [13,14]), thus making the cyanobacterial hydrogenase an exceptional enzyme. During a dark-to-light transition, hydrogen was found

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to be produced for 10 to 20 s, followed by a period of hydrogen uptake until the hydrogenase is inactivated by oxygen evolved in the light reaction. In the *ndhB*-mutant, which lacks the NDH-1 complex (M55 mutant) [15], the uptake was nearly absent and the production period was prolonged to minutes [8,10]. This suggests that cyclic electron transport and respiration via the NDH-1 complex and the hydrogenase are competing for reducing equivalents. Thus, this enzyme is working truly bidirectional under physiological conditions, the direction of the catalysed reaction depending on the redox conditions in the cell.

Although these results already allowed a promising glimpse on the potentials of *Synechocystis* in the sense of biohydrogen production, there is still a lack of systematic analysis of modifications of the electron transport and their effects on hydrogen production.

Due to a number of studies carried out on all known respiratory electron transport complexes in this cyanobacterium, the knowledge of the different pathways has considerably increased. A tight interconnection of respiratory and photosynthetic electron transport became apparent. For a schematic representation of the different complexes and their redox partners see Fig. 1.

Analysis of the membranes revealed the presence of at least three different types of NDH-1 complexes involved in respiration, cyclic electron transport and the CO_2-concentrating mechanism [16–18]. These findings reflect the presence of different genes homologous to *ndhD* and *ndhF* in the genome [3]. There are three homologs of type II NADH-dehydrogenase also called NDH-2 (slr0851, slr1743, sll1484), which seem to be involved mainly in redox regulation rather than respiration [19], and two different succinate dehydrogenases [20,21]. Deletion mutagenesis revealed the presence of three respiratory terminal oxidases [22–24]. The first is a classical cytochrome *c* oxidase, CtaI, the second, an alternative cytochrome *c* oxidase, CtaII, [24] and the third a quinol oxidase, Cyd [23,25]. The quinol oxidase is localized in the thylakoid membrane [25] and a subunit of the alternative cytochrome oxidase CtaCII was found in the plasma membrane by proteomic analysis [26]. Measurements of the uptake of radioactively labeled 3-*O*-methyl-glucose in double and triple mutants of the oxidases suggest that they are all localized in the plasma membrane [24]. However, the relative distribution and amount of the different oxidases in the different membranes is still unclear.

In this study we examined the influence of the inhibition of respiratory electron transport and nitrate assimilation on the

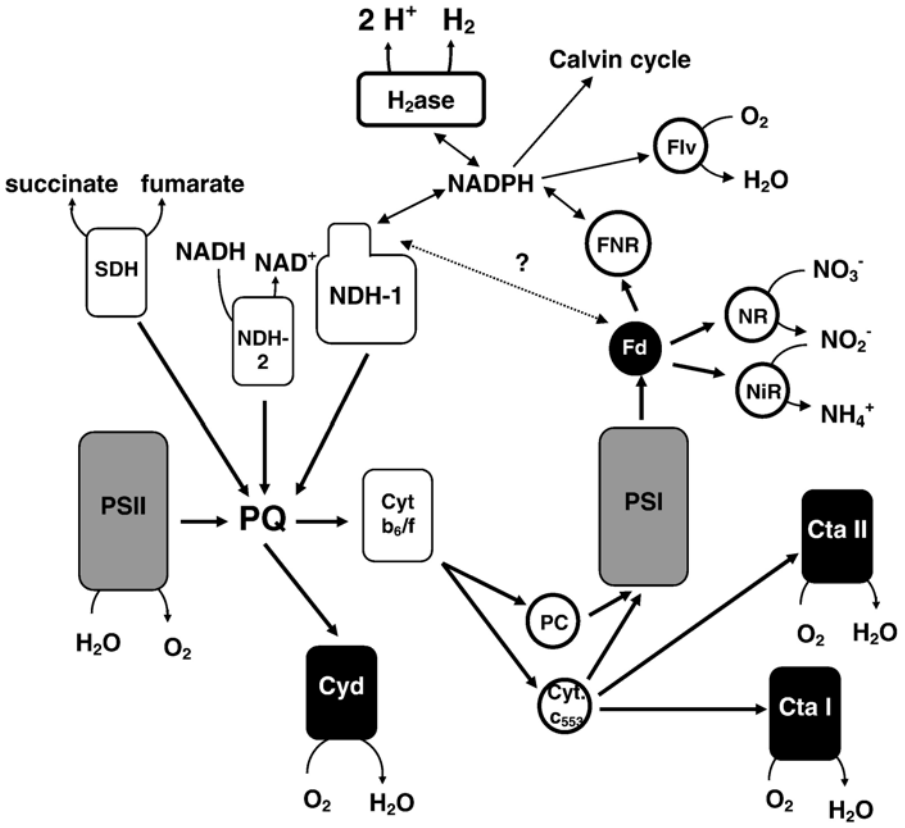

Fig. 1. Schematic model of the different electron transport routes in *Synechocystis*. Arrows represent electron transport between complexes or redox reactions. If the bidirectional hydrogenase is coupled to complex I is still a matter of debate. The redox reaction between ferredoxin and hydrogenase is shown by a dashed arrow and a question mark to indicate that this pathway is not proven yet. The abbreviations are H2ase: bidirectional hydrogenase, NDH-1: NADPH-dehydrogenase (complex I), SDH: succinate dehydrogenase, NDH-2: type 2 NADH-dehydrogenase, PSII: photosystem II, PSI: photosystem I, Cyt b6/f: cytochrome b6/f complex, PQ: plastoquinone, Fd: ferredoxin, FNR: ferredoxin:NADP reductase, PC: plastocyanin, Cyt. c_{553}: cytochrome c_{553}, Flv: flavoprotein, NR: nitrate reductase, NiR: nitrite reductase, Cyd: quinol oxidase, CtaI: cytochrome *c* oxidase and CtaII: alternative cytochrome *c* oxidase.

hydrogen metabolism of *Synechocystis*. Specifically, we investigated the effects of deletion mutagenesis of one, two or all three respiratory oxidases on hydrogen production in *Synechocystis*. To compare their activities with a very good photohydrogen producer [10], the *ndhB*-mutant M55 that does not contain any NDH-1 complex [18] was also included. Apart from oxygen, the influence of nitrate as an electron sink was examined as well.

2. Materials and methods

2.1. Strains and growth conditions

Throughout this study *Synechocystis* sp. PCC 6803 was grown in BG-11 [27] supplemented with 5 mM TES pH 8. Cultures of 50 ml volume were grown in 100 ml Erlenmeyer flasks on a rotary shaker at 100 rpm, 28° C and 50 μE m^{-2} s^{-1}. The media of the mutant strains of the respiratory terminal oxidases (for construction of mutants see 22,31) and the *ndhB* mutant M55 [15] were supplemented with 50 μg ml^{-1} kanamycin, 20 μg ml^{-1} chloramphenicol, 20 μg ml^{-1} spectinomycin and 25 μg ml^{-1} erythromycin when necessary. For physiological measurements an aliquot was taken from these cultures, inoculated in 250 ml BG-11 without antibiotics and bubbled with air. At an OD_{730} of 3 to 4 (corresponding to 2.0 to 2.6×10^9 cells ml^{-1}) cells were harvested and resuspended in fresh BG-11 medium for measurement. To grow cells with an inhibited nitrate reductase molybdate was omitted from the trace metal mix of $BG\text{-}11_0$ and tungstate added at a concentration of 4,8 μM [28,29]. This corresponds to 3 times the concentration of molybdate compared to the normal BG-11 medium. In this case NH_4Cl was added as a nitrogen source at a concentration of 5 mM. To grow cells in the absence of nitrate, $BG\text{-}11_0$ with 5 mM NH_4Cl was used.

2.2. Hydrogen measurements

Hydrogenase activity was measured as previously described [7], but with a Clark-type-electrode from Hansatech (DW 1 Liquid Clark electrode, Hansatech Inst., Norfolk, UK). The electrode was connected to a lab-made control box set to a voltage of –600 mV. To measure the activity of the bidirectional hydrogenase aliquots were taken directly from the air bubbled cultures and measured immediately in the presence of 5 mM methylviologen and 10 mM sodium dithionite. To determine photohydrogen production or hydrogen uptake a total volume of 1 ml containing 100 μg ml^{-1} chlorophyll was made anaerobic by either adding 10 μl of a freshly prepared 100 mM solution of sodium dithionite or by adding 40 U of glucose oxidase, 50 U of catalase and 1 mM glucose. After the completion of fermentative hydrogen production the suspension was illuminated at 800 μE m^{-2} s^{-1} to induce photohydrogen production. For hydrogen uptake measurements, 100 μl of H_2-saturated water was added first. When the signal had reached its plateau, the sample was depleted of oxygen by adding 10 mM glucose. The rate of photohydrogen production or of the hydrogen uptake given in the figures is the maximal slope measured. It is important to note that the Clark electrode has a certain response time. Therefore, the measured kinetics might not accurately represent the rapid processes at the onset of light. But a comparison of our data with the data collected at the mass spectrometer [10] showed that the measured hydrogen amount is in the same range.

Measurements of the ratio of OD_{730} to the concentration of chlorophyll of a given culture were found to be the same for all the strains used in this investigation. Since the standard deviation of the chlorophyll determinations is lower all the data is given as per chlorophyll.

2.3. Photosynthesis

The rate of photosynthesis was measured as net oxygen evolution using an oxygen electrode (DW1 liquid Clark electrode, Hansatech Inst., Norfolk, UK). The cell suspension was diluted to 5 μg ml^{-1} chlorophyll, and $NaHCO_3$ was added to a final concentration of 1 mM. To ensure that saturated photosynthesis occurred, measurements were carried out at three different light intensities in the range of 1000 to 1500 μE m^{-2} s^{-1}, applied with a projector (Prado Universal, Leitz, Wetzlar, Germany).

2.4. 77 K fluorescence measurements

Deep temperature fluorescence spectra were recorded at a Hitachi F-4500 fluorescence spectrophotometer with the low temperature measuring unit (Hitachi, Tokyo, Japan). To determine the stoichiometry of the photosystems the samples were diluted to 2 μg chlorophyll/ml and excited at 440 nm. The spectrum was recorded between 650 and 750 nm. The ratio of the fluorescence measured at 725 nm to the fluorescence at 695 nm was shown to be related to the ratio of PSI to PSII [30]. To determine the state change capacity of the strains the samples were incubated either in far red light for 5 min to induce state 1 or for 30 min in the dark to induce state 2 and immediately frozen in liquid nitrogen. In this case the samples were excited at 580 nm and the spectra were recorded between 600 and 750 nm.

2.5. Quinol oxidase measurements

The presence of the quinol oxidase was detected by measuring the variable fluorescence in the presence of 5 mM ascorbate and 20 μM DBMIB as described by Berry et al. [25]. In this case a cell suspension of 5 μg chlorophyll/ml was used and the chlorophyll fluorescence was measured with the PAM fluorimeter (Walz, Germany) equipped with the ED-101US/M unit for liquid samples. The measuring light was pulsed at 1.6 kHz at an intensity set to 6. After adding ascorbate the variable fluorescence was measured in the dark, in 50 μmol m^{-2} s^{-1} light of a red diode connected to the PAM-102 unit and after adding DBMIB in 50 μmol m^{-2} s^{-1} red light by giving saturating light flashes of 8,000 μmol m^{-2} s^{-1} of 800 ms of a KL-1500 (Schott, Germany). After adding DBMIB every 30 s a saturating light flash was given.

3. Results

3.1. Hydrogenase activity of mutant strains

The hydrogenase activity of all possible single, double and triple mutants of the three terminal oxidases and a mutant lacking the complex I (M55) was investigated. The measurements were

Table 1
Hydrogenase activity of wild type cells and different oxidase mutants

WT	1149±76
Δ*ctaI*	1181±24
Δ*cyd*	1580±93
Δ*ctaII*	1116±18
Δ*ctaI*Δ*cyd*	2104±111
Δ*ctaI*Δ*ctaII*	1273±87
Δ*cyd*Δ*ctaII*	2218±110
Δ*ctaI*Δ*cyd*Δ*ctaII*	1789±13
M55	165±35
Δ*rpaC*	1118±23
Δ*hoxH*	5.3±4
Δ*hoxEFUYH*	4.7±4

Besides the oxidase mutants Δ*ctaI* (cytochrome *c* oxidase less), Δ*cyd* (quinol oxidase less), Δ*ctaII* (alternative cytochrome *c* oxidase less), the respective double and triple mutants, the *ndhB*-mutant M55, the Δ*rpaC*, that does not perform a state change, and the deletion mutants of *hoxH* (hydrogenase large subunit) and the whole *hox*-operon *hoxEFUYH* of *Synechocystis* sp. PCC 6803 were measured. Cell suspensions were directly taken from the cultures and their hydrogen evolution was immediately measured in the presence of methylviologen and dithionite. Two measurements of two independent cultures were made. Values are given in nmol H_2 mg Chl^{-1} min^{-1}.

conducted in the presence of dithionite and methylviologen at the hydrogen electrode (Table 1). Dithionite immediately causes anaerobic conditions and reduces methylviologen. Under these conditions hydrogen evolution starts after a short lag phase [7]. The evolution of hydrogen is independent of the electron transport activities in the cell since the reduced methylviologen directly delivers electrons to the hydrogenase [31]. We found the maximal rate to be proportional to the amount of hydrogenase protein in the cells.

The measurements showed an increased hydrogenase activity when the quinol oxidase was deleted (Δ*cyd*, Δ*ctaI*Δ*cyd*, Δ*cyd*Δ*ctaII* and Δ*ctaI*Δ*cyd*Δ*ctaII*). In the respective double mutants, the increase was more pronounced than in the single mutant or in the mutant of all three terminal oxidases. The activity of the hydrogenase in the *ndhB* mutant M55 was consistently found to be reduced to 15% of the wild type level.

3.2. Photosynthesis and hydrogen production

Net photosynthesis, rate of hydrogen production in the light (photohydrogen) and the amount of hydrogen produced were recorded for all mutants. We found the bidirectional hydrogenase to be rapidly activated in the dark in anaerobiosis [7], which is in agreement with the results of Cournac et al. [8,10]. To measure hydrogen evolution cells were first depleted of oxygen by addition of glucose oxidase, catalase and glucose. Under these conditions the cells started to produce hydrogen in darkness within almost a minute. When the curve leveled off, light was applied at an intensity of 800 μE m^{-2} s^{-1} to induce photohydrogen production, and the measurement continued until hydrogen uptake finished. For a complete curve including the fermentative hydrogen production phase refer to Fig. 8, trace A, for wild type cells. The amount of hydrogen produced in darkness was not reproducible and varied between experiments. Therefore, only hydrogen evolution in the light is depicted. Data traces are shown in Fig. 2.

All single mutants as well as the wild type cells showed a similar behavior with respect to the rate of net photosynthesis, rate of hydrogen production and amount of hydrogen produced in the light. Therefore, only the traces of the double mutants, the triple mutant and the *ndhB*-mutant M55 are shown in Fig. 2. With the exception of M55, all strains show a short production phase between 20 and 40 s, followed by a phase of hydrogen uptake in the same time range. Since the rate of photohydrogen production of M55 is considerably slower compared to that of the oxidase mutants, it is shown on a different time scale. It produced 36 times more hydrogen than wild type cells at three times the rate of the wild type cells.

It should be taken into account that because of the preceding fermentative hydrogen production, not all of the produced hydrogen was consumed before hydrogenase inactivation by oxygen from photosynthesis occurred.

The net photosynthesis of the three double mutants (Δ*ctaI*Δ*cyd*, Δ*ctaI*Δ*ctaII*, and Δ*cyd*Δ*ctaII*) and the M55 was impaired (Fig. 3). In spite of this the rate of hydrogen production and the amount of hydrogen produced of the strains without the quinol oxidase are considerably increased. Especially the Δ*ctaI*Δ*cyd* mutant had a 12 times higher production rate and produced 12 times as much hydrogen as wild type cells. Complementary to this result, the double mutant of both cytochrome *c* oxidases (Δ*ctaI*Δ*ctaII*), which still contains the

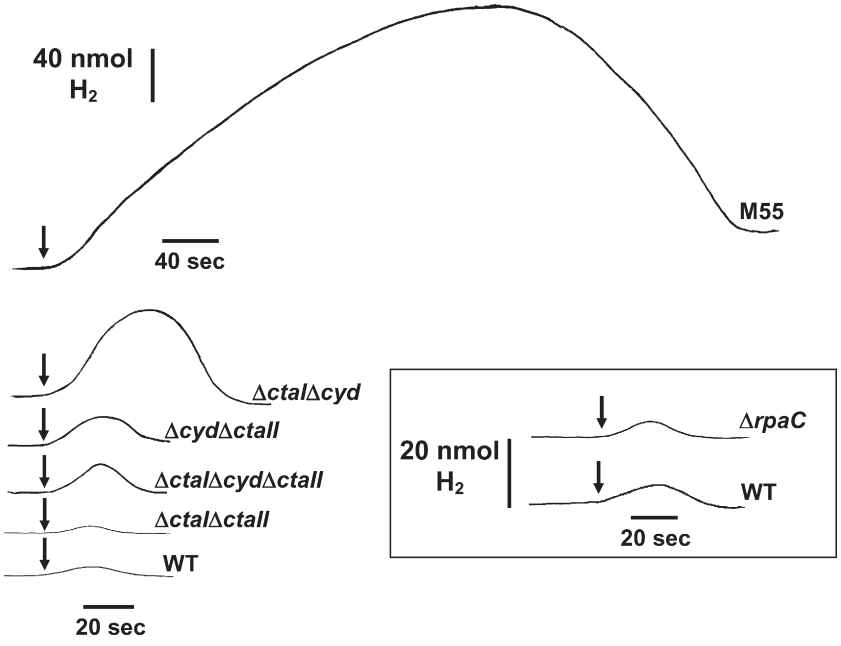

Fig. 2. Original recorder traces of photohydrogen evolution of WT, the double and triple oxidase mutants and M55. Cell suspensions of 100 μg Chl ml^{-1} were made anaerobic by the addition of glucose oxidase, catalase and 1 mM glucose. After cessation of the dark fermentative hydrogen production light was switched on as indicated by the arrows at an intensity of 800 μE m^{-2} s^{-1}. Only this part of the curve of photohydrogen production is shown. A complete curve of wild type cells is shown in Fig. 8. Note the different time scale used for the M55. The boxed inset shows the traces of the wild type cells and the *rpaC*-mutant that is not able to perform a state change from an additional set of experiments.

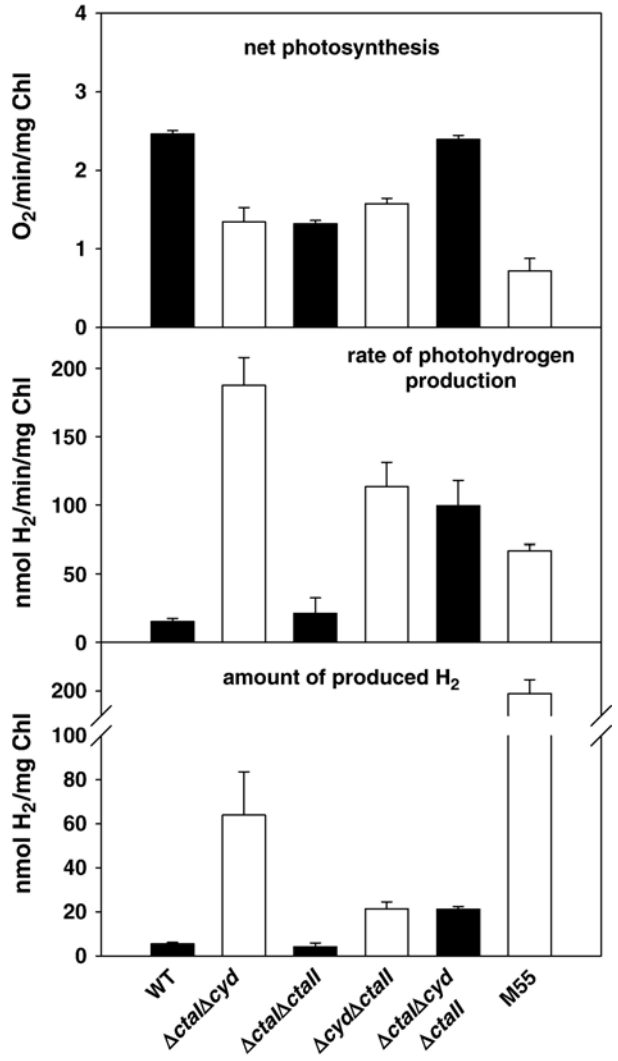

Fig. 3. Net photosynthesis, rate of photohydrogen production and the amount of hydrogen produced in the light for wild type cells, all double and the triple oxidase mutant and M55. Mean values of at least two measurements of two independent cultures are given.

quinol oxidase, is similar to the wild type with respect to its hydrogen production capacity.

3.3. Photosystem stoichiometry and state change

The deletion of main complexes of the electron transport could induce a changed stoichiometry of the photosystems or an impaired state change. This was especially shown for the M55 mutant, which is locked in state 1 [32]. Since these effects could result in changed redox conditions they might influence hydrogen production of the mutant strains. We tested the ratio of the photosystems in the different strains by measuring 77 K fluorescence spectra with an excitation wavelength of 440 nm. The ratio of F725 to F695 is a measure of the ratio of PSI to PSII [30]. It was found to be lower in the Δ*cyd*Δ*ctaII* mutant but it was higher in the Δ*ctaI*Δ*cyd* whereas both are good photohydrogen producers (Fig. 4). In the Δ*rpaC*, which is unable to perform a state change and which is probably locked in state 1 [33], the ratio was reduced compared to wild type cells.

The spectra recorded at an excitation of 580 nm of samples incubated either in far red light for 5 min to induce state 1 or in the dark for 30 min to induce state 2 revealed that all the mutants except the M55 and the Δ*rpaC* were able to perform a state change. Moreover photohydrogen evolution and hydrogenase activity of the Δ*rpaC* mutant was not different to wild type cells (Fig. 2). Therefore, there is no correlation of photohydrogen production and the stoichiometry of the photosystems or their capacity to perform a state change.

3.4. Activity of quinol oxidase

The quinol oxidase activity was found to be changed according to the culturing conditions and the light regime [25]. It was hypothesized that it is differentially localized to the cytoplasmic and thylakoid membrane due to a small regulatory protein encoded by sll1717 [34]. To ensure that the quinol oxidase is active in the thylakoids fluorescence measurements were performed (Fig. 5). In the presence of 5 mM ascorbate and 20 μM DBMIB (2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone) plastoquinone can no longer deliver electrons to the cytochrome b6/f complex [25]. The only way to oxidise the PQ-pool under these conditions is the quinol oxidase activity. As visible in Fig. 5 the wild type cells still have a variable fluorescence when saturating light flashes are applied after the addition of DBMIB whereas the quinol oxidase mutant (Δ*cyd*) has not. This proves the presence of a quinol oxidase activity in the thylakoids under the culturing conditions used in this study.

3.5. Effect of nitrate reduction on hydrogen production

Under normal conditions two fifths of the low potential electrons generated in the light reaction are delivered to the

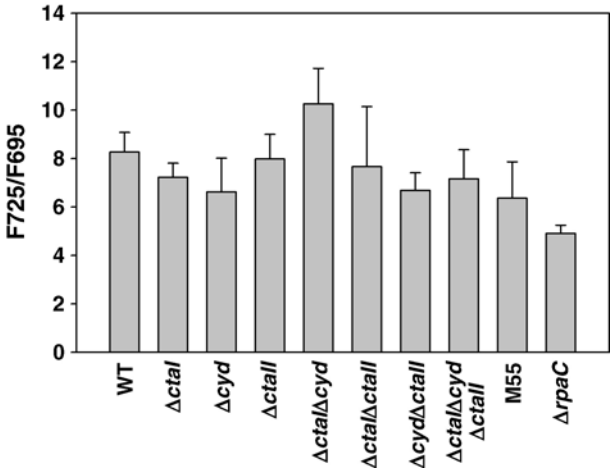

Fig. 4. Ratio of F735/F695 of 77 K fluorescence spectra of wild type cells, the different oxidase mutants, M55 and the rpaC. This ratio is proportional to the stoichiometry of the photosystems [30]. Mean values of at least three measurements of two independent cultures are given.

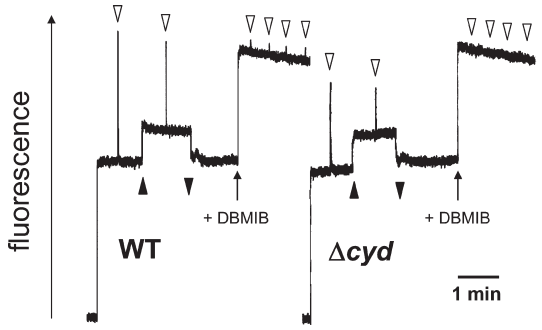

Fig. 5. Fluorescence measurements at the PAM fluorimeter of wild type cells and the quinol oxidase mutant Δ*cyd*. The curves were recorded in the presence of 5 mM ascorbate. At the upward pointing black triangle light of 50 μmol/m2/s was switched on and at the downward pointing black triangle it was switched off again. At the arrow light of 50 μmol m^{-2} s^{-1} was switched on and DBMIB was added to a final concentration of 20 μM. The open triangles indicate saturating flashes of 800 ms.

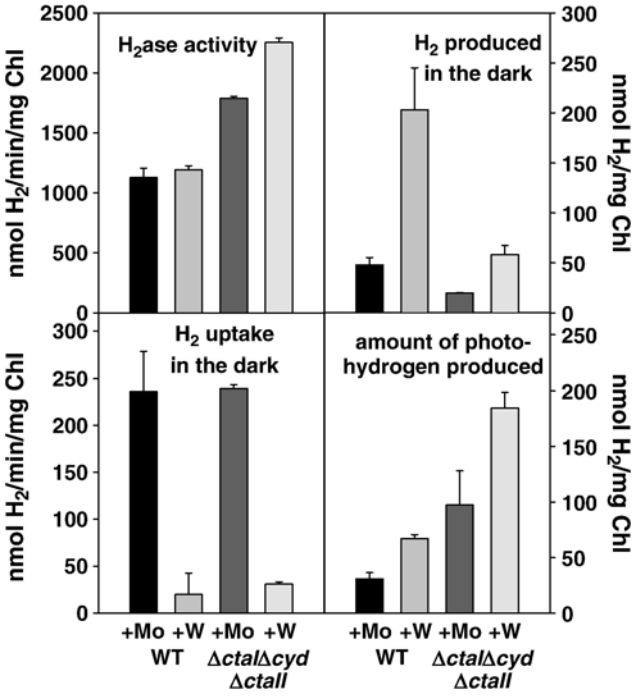

Fig. 6. Hydrogenase activity, H_2 uptake in the dark, H_2 production in the dark by fermentation and the amount of photohydrogen produced for wild type cultures and the triple oxidase mutant grown either in normal BG-11 (+Mo) or in BG-11_0 with 5 mM NH_4Cl in the presence of tungstate (+W). The latter inhibits the nitrate reductase. Mean values of two measurements of two independent cultures are given.

reduction of nitrate to ammonia [35]. Therefore, nitrate is an important electron sink that might compete with the hydrogenase under the conditions used in this study. In order to investigate the effect of an impaired nitrate assimilation we inhibited the first enzyme in this pathway, i.e. the nitrate reductase, which is a molybdoenzyme [36,37] by adding tungstate to the medium instead of molybdate [28,29]. The genome of *Synechocystis* is completely sequenced. The only known Mo-enzyme in *Synechocystis* to date is NarB (ferredoxin:nitrate oxidoreductase) [3]. There is a number of Mo-enzymes known that are mainly oxotransferases [36,37]. Under oxygen depleted conditions these enzymes should not work. Therefore, it is hard to imagine that any of these enzymes could be an as effective electron sink as the nitrate reductase. The conditions used in our assays should monitor specifically the effect of inhibiting nitrate reduction.

Fig. 6 shows the hydrogenase activity, hydrogen uptake in the dark, fermentative hydrogen production in the dark and photohydrogen production of wild type cultures and the triple oxidase mutant grown either with molybdate or tungstate. After harvesting cultures the cells were resuspended in fresh BG-11, containing 17 mM nitrate. The triple oxidase mutant was used to exclude any hydrogen uptake due to the oxyhydrogen reaction [31] and to investigate the effect of depleting the cells of two main electron sinks.

The hydrogenase activity of the wild type cells remained unchange irrespective of the media, whereas its activity was increased in the oxidase mutant in the presence of tungstate. In both strains, the hydrogen uptake was only one tenth when tungstate was added compared to the uptake in the normal medium (Figs. 6 and 7, trace A and B). Interestingly, fermentative hydrogen production could be consistently increased (4 times) in the absence of active nitrate reductase in wild type cells (Figs. 6 and 8, trace A and B). Thus, a considerable proportion of reducing equivalents produced in fermentation should be delivered to nitrate reduction under normal conditions. A less pronounced increase of hydrogen production was also found for the oxidase mutant. This might be attributed to a decreased dissimilatory metabolism in this strain due to the lack of respiration. Addition of tungstate also caused an increased photohydrogen production in wild type and mutant cells.

To confirm the role of nitrate reductase in hydrogen uptake, wild type cells were grown in BG-11_0 supplemented with 5 mM ammonia. Their uptake activity in an assay mixture containing ammonia was low and could be rapidly increased by adding nitrate (Fig. 7, trace C).

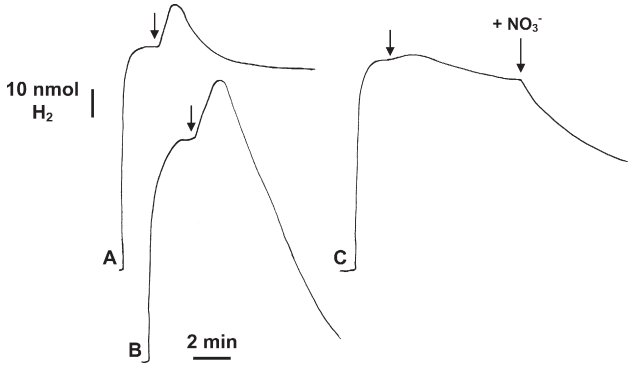

Fig. 7. Hydrogen uptake measurements of wild type cultures either grown in BG-11_0 with 5 mM NH_4Cl in the presence of tungstate (A), in normal BG-11 (B) or in BG-11_0 with 5 mM NH_4Cl (C). The steep rise of the curve represents the addition of 100 μl H_2 saturated water. The arrows indicate addition of 10 mM glucose to deplete the oxygen. At the arrow $+NO_3^-$ nitrate was added to a final concentration of 17 mM which is the concentration used in BG-11 medium. In this case cell suspensions of 60 μg Chl ml^{-1} were used.

4. Discussion

A set of mutational investigations has revealed the complexity of the electron transport chain in *Synechocystis*. A schematic representation of the involved complexes and their interconnectedness is given in Fig. 1. The most important electron sink under normal conditions besides the Calvin cycle is oxygen. It is either reduced by the respiratory terminal oxidases or a Mehler type reaction catalysed by a special flavoprotein at the acceptor side of photosystem I [38]. Isotopic fractionation studies of the three oxygen isotopes allowed to determine the relative contributions of the dark reaction, the photoreduction of oxygen and respiration in the absence of photorespiration to the overall electron flow. It was estimated that 40% of the electrons leaving photosystem II are delivered to the photoreduction of oxygen whereas respiration contributes only 6% [39]. In anaerobiosis this main electron sink is not available to regulate photosynthetic electron flow. Under these conditions the bidirectional hydrogenase gets activated and delivers electrons to protons to form hydrogen. This is true in the dark under fermentative conditions (Fig. 8) and in the light when the cells start photosynthesis (Figs. 2 and 8).

The 77 K fluorescence spectra did not reveal a correlation of PS-stoichiometry (Fig. 4) and the evolution of photohydrogen (Fig. 2). An impaired capacity to perform a state change can also be ruled out to elicit an increased hydrogen production as seen in the comparison of wild type cells and the Δ*rpaC* (Fig. 2, boxed inset). Our measurements show that the deletion of the quinol oxidase in combination with one of the other two oxidases causes a prolonged H_2 production phase in the light and a higher maximal amount (Figs. 3 and 4). Even when the cells are depleted of oxygen as in our experiments, trace amounts of O_2 evolved by PSII seem to be sufficient to turn photohydrogen production in short time into hydrogen uptake due to the possibility to transfer electrons to O_2. Our experiments show that the quinol oxidase alone can fulfil this role since the Δ*ctaI*Δ*ctaII* mutant shows the same behavior as wild type cells (Table 1 and Fig. 2). This is reminiscent of the cytochrome bd oxidase in *E. coli*, which was shown to be preferentially expressed at an oxygen tension of 2% [40] and found to have the highest affinity for oxygen when the reduction state of the quinone pool was close to saturation [41]. Therefore, our data suggest that the quinol oxidase is important as an electron valve under low oxygen concentrations.

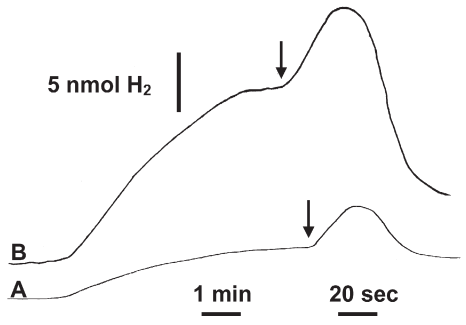

Fig. 8. Fermentative hydrogen production and photohydrogen production in wild type cells either grown in normal BG-11 (A) or BG-11$_0$ with 5 mM NH_4Cl in the presence of tungstate (B). At the arrows light was switched on of 800 μE m^{-2} s^{-1}. For a better resolution the time scale was changed when the light was turned on. The cell suspensions were set to 100 μg Chl ml^{-1} in normal BG-11.

The exceptionally high photohydrogen production capacity of the *ndhB*-mutant M55 also described by Cournac et al. [10] can be attributed to the absence of NADPH oxidation by NDH-1 on the one hand. On the other hand its net photosynthesis is severely reduced (Fig. 3) since its CO_2-concentrating mechanism is inhibited [15] which in turn results in a reduced rate of the Calvin cycle. These effects keep the NADP-pool completely reduced as measured by Cooley and Vermaas [21] and support a long photohydrogen production phase (Fig. 2).

In cells with normal photosynthesis, the turnover of electrons at PSI is severely limited due to acceptor side limitation as long as the dark reaction is not activated [42]. Parallel measurements of PSII and PSI activity showed that after the onset of the light reaction there is a period of rereduction of PSI of several 100 ms. In this phase, PSII activity is higher than PSI activity due to acceptor side limitation, since this effect could be relieved by a previous activation of the dark reaction or by addition of methylviologen [43,44]. The same phase was found to be considerably prolonged in a hydrogenase lacking mutant Δ*hoxH* [7] indicating that the hydrogenase drains off the acceptor side of PSI under these conditions.

The results in this work strongly support the role of the hydrogenase as an electron valve in transition states from anaerobic or microaerobic conditions in the light. They also suggest that the quinol oxidase is fulfilling a similar function. Whereas the hydrogenase prevents an acceptor side limitation of PSI especially under anaerobic conditions the quinol oxidase might be necessary to avoid an acceptor side limitation of PSII as soon as low concentrations of oxygen have built up.

Our measurements revealed a significant increase of hydrogenase activity in all deletion strains of the quinol oxidase and wild type levels of enzyme activity if the quinol oxidase was still present (Table 1). The expression of a functional hydrogenase needs at least 7 accessory proteins that insert the active site bimetallic center [45]. The respective genes have recently been identified in *Synechocystis* [46]. The time needed from the translation of the mRNA to the functional enzyme is therefore expected to be in the order of minutes and not appropriate to cope with rapidly changing conditions. Our results suggest that the cells might already take precautions by expressing a higher amount of hydrogenase in the absence of the quinol oxidase. At this stage it is difficult to pinpoint the signal that ultimately elicits the increased activity but these findings will be important for all future studies on the regulation of the hydrogenase that was found to be transcriptionally activated by LexA [47,48].

The low hydrogenase activity of the M55 mutant is remarkable (Table 1). It was shown that this mutant does not express any of the Ndh-subunits known to be part of the NDH-1L or NDH-1M complex [18]. Therefore, the absence of the NdhB subunit seems to induce their degradation. The same might also be applicable to the bidirectional hydrogenase, which was suggested to be the NADPH oxidizing module of the NDH-1 on the basis of sequence comparisons [6]. But since

more direct evidence is still lacking we cannot rule out that other signals cause a reduced hydrogenase activity in the M55.

Hydrogenase activity, fermentative hydrogen production, and photohydrogen production were significantly increased and hydrogen uptake was reduced when the nitrate reductase was inhibited or nitrate absent from the medium (Figs. 6, 7 and 8). This shows that nitrate reduction is withdrawing electrons from hydrogen production. Since the cyanobacterial nitrate and nitrite reductase rely on reduced ferredoxin as electron donor [35,49], the hydrogenase might be using ferredoxin as its redox partner. This is also indicated by its role in preventing acceptor side limitation of PSI as discussed above. Nevertheless, this interpretation needs further experiments to be proven, since in both cases electrons could also commute via NADPH and FNR, although this would be thermodynamically less feasible.

After the addition of glucose in the course of hydrogen uptake measurements we repeatedly observed a brief period of hydrogen production. The amount produced was higher in the cells grown in normal BG-11 (Fig 8, trace A and B). In both cases, the same amount was taken up again. It will be important to unravel alternative electron acceptors other than nitrate, that are also used in the presence of ammonium (Fig. 8, trace C before addition of nitrate).

Our experiments show that there are four different electron sinks (protons, oxygen, nitrate and the Calvin-cycle) that compete with each other and that the depletion of oxygen, nitrate and inorganic carbon supply increases hydrogen production. These are important findings for further attempts to increase biohydrogen production in cyanobacteria. By inventing the cyanobacterial bidirectional hydrogenase nature has created a device able to use hydrogen as a temporary buffer for low potential electrons that is also able to use it up again when conditions are getting more oxidizing, thus preventing a waste of energy. Major challenges in the future will be to control the flow of electrons into the dark reaction, getting a sustained flow of low potential electrons to the hydrogenase and to prevent its inactivation by oxygen.

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