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REACTIONS OF HYDROGEN AND OXYGEN WITH PHOTOSYSTEM I OF ISOLATED HETEROCYSTS FROM *ANABAENA VARIABILIS* (ATCC 29413)

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Isolated heterocysts from *Anabaena variabilis* show high rates of light- and hydrogen-dependent acetylene reduction. This heterocyst preparation also shows light-induced redox reactions of cytochromes *f*-556 and *b*-564. Both cytochromes are reversibly oxidized by light or by oxygen (in the dark). Oxygen-oxidized cytochromes assume their initially reduced state by addition of dithionite or flushing the reaction vessel with hydrogen or argon. All three agents remove oxygen from solutions more or less efficiently, thereby creating reducing conditions; hydrogen-induced reduction is slow and generally completed after about 10 min. No evidence has been obtained for a direct electron donation of hydrogen to either of the cytochromes measured. Photoreduction of nitrogen in the presence of hydrogen is explained by a combined operation of properly poised cyclic photophosphorylation providing ATP, and a light-independent hydrogenase reaction providing reductant.

Introduction

Filamentous blue-green algae (cyanobacteria) growing under aerobic nitrogen-fixing conditions produce heterocysts by transformation of 5–10% of the vegetative cells. Heterocysts provide the anaerobic environment necessary for nitrogenase to operate. The absence of an oxygen-evolving PS II and respiratory oxygen uptake are the main mechanisms to decrease oxygen concentrations inside the heterocysts (for a review see Ref. 1).

When heterocysts are isolated, high rates of nitrogen fixation (= acetylene reduction) are measured only in the presence of light and hydrogen [2,3]. Nitrogen fixation requires both ATP and a low-potential electron donor; the light reaction may provide both. Whereas cyclic photophosphorylation was demonstrated unequivocally in heterocysts [4–6], the role of hydrogen is less clearcut. Hydrogen could serve two functions: the removal of oxygen by the Knallgas reaction and as a source of electrons for nitrogen reduction [7–15]. It is open to speculation whether hydrogen is able to feed into PS I via plastoquinone [8,9,12,14–17], thereby providing reductant and ATP for nitrogenase or whether it provides electrons via a reversible hydrogenase [11,14,17–19].

Here we report for the first time on redox reactions of cytochromes *b*-564 and *f*-556 in isolated, nitrogen-fixing heterocysts. No evidence has been obtained favoring a direct interaction of hydrogen with either of the cytochromes investigated.

Materials and Methods

Anabaena variabilis Kütz. (American Type Culture Collection 29413) was grown in a light thermostat (Kniese Edwards, Marburg; light intensity

Abbreviations: Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Chl, chlorophyll; PS, photosystem.

8 W/m^2; temperature 28°C). The cultures were inoculated in an inorganic medium [39] with 1 μg Chl *a*/ml and gassed with air supplemented with 2% CO_2. After 48 h, the algae were harvested by centrifugation. Under anaerobic conditions (5% H_2/95% Ar), filaments were treated with lysozyme followed by osmotic shock and sonification in a microsonic bath [3]. Heterocysts were isolated by differential centrifugation of the algal suspension (for details of heterocyst isolation see Ref. 6).

For measurement of light-induced redox reactions, only heterocyst preparations with high activities of light-induced acetylene reduction in the presence of hydrogen were used (approx. 40 μmol C_2H_4 formed/mg Chl *a* per h, average of 68 preparations).

Heterocysts were suspended in an anaerobic reaction mixture (pH 7.0) containing (mM): sorbitol, 350; Tes, 10; sodium potassium phosphate, 10; $MgCl_2$, 10; bovine serum albumin, 0.5%. All operations were performed under the anaerobic conditions of a hydrogen atmosphere. 2.5 ml of the heterocyst suspension (20–35 μg Chl *a*/ml) were transferred into an anaerobic cuvette sealed with a rubber septum. It was possible to flush the cuvette with different gases (H_2, Ar, O_2, highest purity obtainable) through three-way Hamilton valves and butyl-rubber tubing directly connected to the gas cylinders; the gas volume of the cuvette was 1 ml. Light-induced absorbance changes were measured in an Aminco DW-2 spectrophotometer equipped with cross-illumination [20]; light intensity, defined by a 696 nm interference filter (Balzers), was 45 W/m^2. The magnetically stirred and temperature-controlled cuvette (30°C) allowed anaerobic additions of gases and solutions by means of Hamilton microliter syringes. Dithionite was freshly dissolved in the anaerobic reaction mixture.

Nitrogenase activity was determined by measuring ethylene formation in the gas phase. 0.5 ml of a heterocyst suspension (20–30 μg Chl *a*/ml) was added to 8-ml vials, previously flushed with H_2 for 15 min. The reaction was started by addition of 1 ml acetylene. Nitrogenase activity was measured during 60 min of illumination (20 W/m^2, white light) at 28°C. Ethylene was detected by gas chromatography and quantitated using a Hewlett Packard HP 3385 integrator.

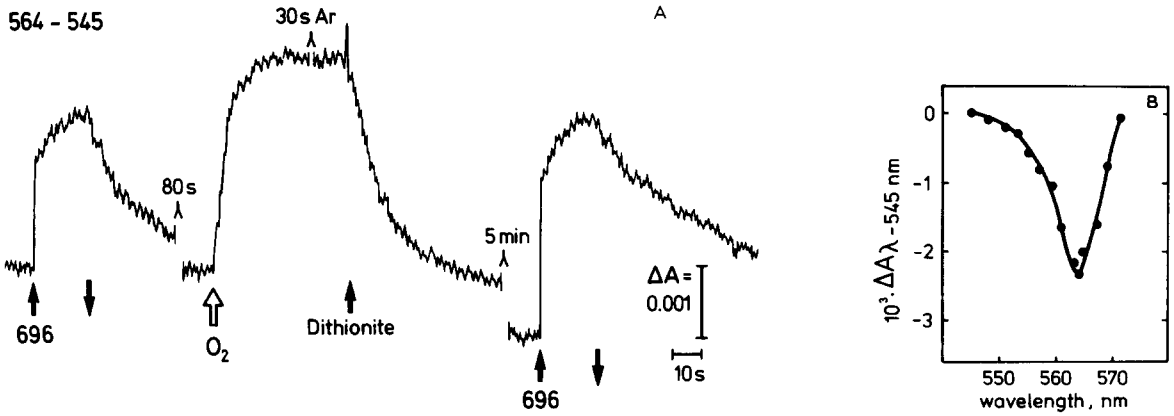

Fig. 1. (A) Redox reactions of cytochrome *b*-564 measured in isolated heterocysts of *A. variabilis* (564 − 545 nm). Upward deflection indicates an absorbance decrease. The cuvette was first flushed with hydrogen. The trace on the left shows the far-red light-induced oxidation of cytochrome *b*-564. After a 100 s dark phase, O_2 replaced H_2 for 20 s and the O_2-induced oxidation was measured (middle trace, half-time of oxidation approx. 4 s). Then, argon replaced O_2 and dithionite (0.2 mM) was added. The trace on the right shows the following light-induced absorbance change after a 5 min incubation with dithionite in the dark. Upward arrow, 696 nm light on; downward arrow, light off. Chlorophyll concentration 33 μg/ml. Activity of heterocyst preparation 41 μmol C_2H_4 formed/mg Chl *a* per h. (B) Difference spectrum for the light-induced, reversible absorbance changes measured with isolated heterocysts in the presence of 0.2 mM dithionite. Chlorophyll concentration: 26.5 μg/ml; activity: 72.4 μmol C_2H_4/mg Chl *a* per h. Ratio Chl *a*/cytochrome *b*-564: 167 ($\Delta\epsilon_{564-571nm} = 14\ mM^{-1} \cdot cm^{-1}$), calculated from this spectrum.

Results

Isolated heterocysts from *A. variabilis* reduce acetylene with high rates in the light under a hydrogen atmosphere; no further additions are necessary [3]. Light also induces a rise in the ATP content of the cell, directly demonstrating active cyclic photophosphorylation [6]. In addition, these heterocysts show light-induced cytochrome redox reactions, which will be discussed in detail in the following.

As reported previously, the thylakoid mem-

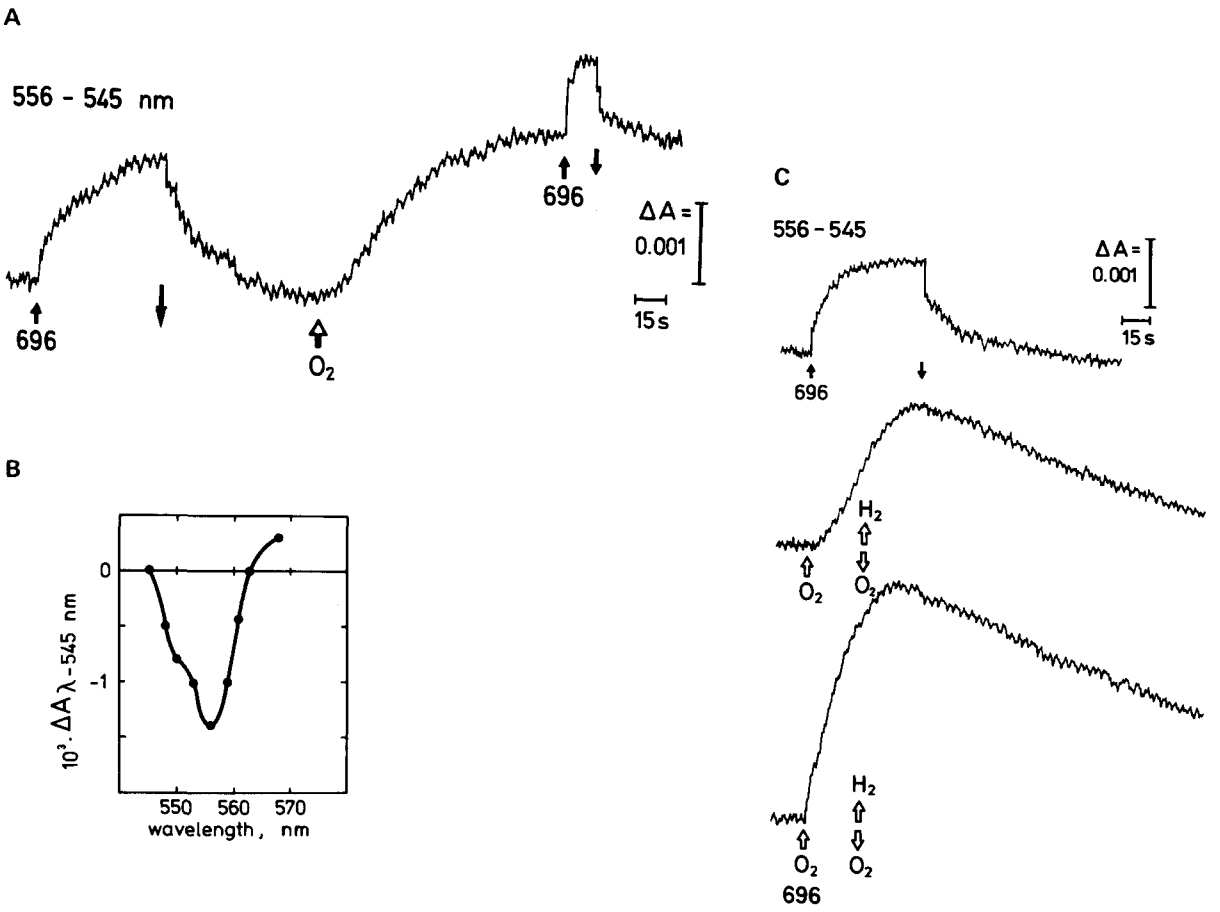

Fig. 2. (A) Redox reactions of cytochrome *f*-556 in isolated heterocysts (556–545 nm). Trace on the left: far-red light-induced oxidation under an H_2 atmosphere; 50 μl O_2 were added to the gas phase as indicated; cytochrome *f* becomes partially oxidized; in the presence of O_2, some photooxidation is still measured corresponding to about half of the amount of cytochrome *f* present. Chlorophyll concentration: 27 μg/ml; activity: 32 μmol C_2H_4/mg Chl *a* per h. (B) Difference spectrum of the light minus dark absorbance change measured with isolated heterocysts after treatment with 50 μl O_2 (cf. A). Chlorophyll concentration: 38 μg/ml; activity: 45 μmol C_2H_4/mg Chl *a* per h; reaction temperature: 15°C. (C) Oxygen-induced oxidation of cytochrome *f*-556 (556 – 545 nm) in the absence and presence of far-red illumination. After several short-time (1 min) illuminations of the heterocyst preparation, the cuvette was briefly flushed with O_2 (30 s) and immediately afterwards with H_2. Upper tracing: light-induced absorbance change (control). Middle tracing: O_2-induced oxidation and rereduction in the presence of H_2. Lower tracing: O_2-induced oxidation and H_2 reduction in the presence of far-red light. Chlorophyll concentration: 26.5 μg/ml; activity: 55 μmol C_2H_4/mg Chl *a* per h.

branes of heterocysts contain PS I and associated components only [21,22]. Some of these components have been quantitatively determined in heterocysts from *Nostoc muscorum* [22], and similar values were found with *Anabaena* heterocysts. The ratios of Chl *a* to redox component were 247 (±23) for cytochrome *f*-556, 145 (±13) for cytochrome *b*-564 ($=b_6$), and 102 (±10) for P-700 (five independent determinations).

When heterocysts are isolated under anaerobic conditions and stored under a hydrogen atmosphere, cytochrome *f* is completely reduced and cytochrome b_6 partially reduced to begin with (approx. 50% in most preparations). This is in contrast with isolated intact chloroplasts from spinach, where cytochrome b_6 is in the oxidized state intially [23]. In heterocysts we are, therefore, able to measure cytochrome b_6 photoxidation under physiological conditions.

As shown in Fig. 1A, cytochrome b_6 is rapidly oxidized by far-red light (696 nm). This light-induced absorbance change is reversible in the dark. Cytochrome *f* oxidation is hardly visible under these conditions (cf. spectrum of Fig. 1B).

Influence of oxygen

The traces in Fig. 1A show in addition that cytochrome b_6 can be completely oxidized in the dark by oxygen; this oxidation is quite rapid, the half-time in this experiment being 4 s. Thereafter, no cytochrome b_6 photoreactions are measurable. Fig. 2A shows that oxygen also causes cytochrome *f* oxidation, but this oxidation often occurs with a lag phase (10 s in this experiment) and is much slower (half-time approx. 40 s) than cytochrome b_6 oxidation. Under conditions where cytochrome b_6 is completely oxidized, cytochrome *f* is oxidized only by approx. 50%, and a rapid and reversible light-induced photooxidation is seen (Fig. 2A and spectrum in Fig. 2B).

The slow oxidation of cytochrome *f* in the dark by oxygen can be considerably accelerated in the light (Fig. 2C). This demonstrates that O_2 can serve as electron acceptor to PS I. Thylakoid isolated from heterocysts show indeed appreciable rates of light-induced oxygen uptake when supplied with an appropriate donor system such as diaminodurol [24]. Prolonged exposure to oxygen also leads to completely oxidized cytochrome *f* in the dark (cf. Fig. 3A and spectrum of Fig. 3B).

Influence of hydrogen

As shown in Fig. 3A, both cytochrome b_6 and *f*, oxidized by oxygen, resume their initially reduced state when the cuvette is flushed with hydrogen. Reduction of cytochrome *f* induced by hydrogen

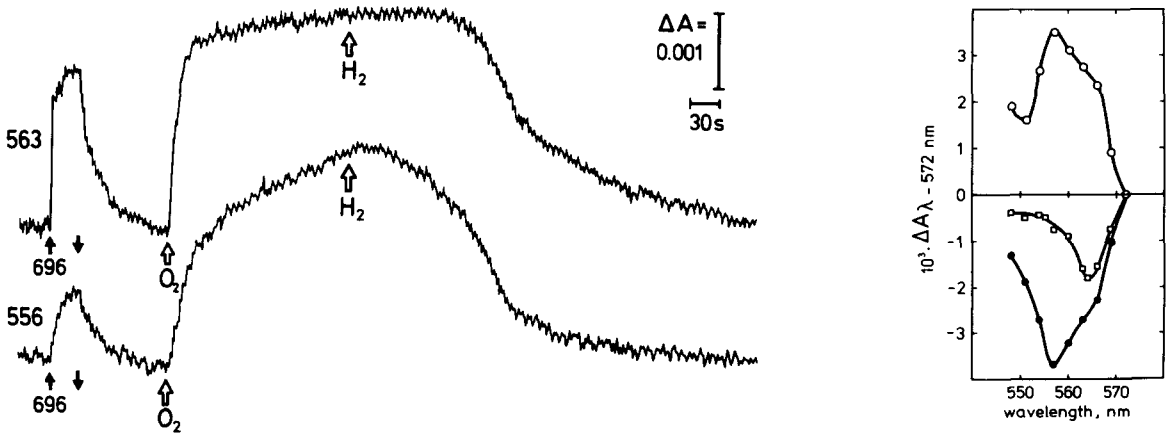

Fig. 3. (A) Kinetics of the light-, oxygen- and hydrogen-induced redox reaction of cytochrome *b*-564 (upper trace, 563−545 nm) and cytochrome *f*-556 (lower trace, 556–545 nm) measured with the same heterocyst preparation. The cuvette was flushed with O_2 for 3 min, then with H_2 for 10 min. Chlorophyll concentration: 21.5 μg/ml; activity: 32 μmol C_2H_4/mg Chl *a* per h. (B) Difference spectrum of the absorbance changes induced by light (696 nm, □——□), oxygen (●——●), and hydrogen (○——○). Chlorophyll concentration: 24 μg/ml; activity: 48 μmol C_2H_4/mg Chl *a* per h. The following ratios can be computed from the spectrum: Chl *a*/cytochrome *b*-564, 145; Chl *a*/cytochrome *f*-556, 270; light oxidizes 56% of the cytochrome *b*-564 present.

occurs slowly but immediately, whereas reduction of cytochrome b_6 is observed only after a lag phase of 1–2 min. Reduction is faster upon addition of dithionite. It should be noted that dark reduction of cytochrome b_6 after light oxidation is even slowed down by a factor of approx. 3, although dithionite is present (first and last traces in Fig. 1A). The permeability of intact isolated heterocysts to dithionite is slow, since it does not support acetylene reduction in the light or dark [2,5]. When hydrogen is present, the slow rereduction of both cytochromes is not acclerated by applying several short-time illuminations [30 s) during the 10 min reduction phase (data not shown); this experiment excludes the possibility that we are missing some light-activated steps (see also Fig. 2C).

Reduction of oxygen-oxidized cytochromes can also be achieved by flushing the cuvette with argon, which removes oxygen by slow equilibration. Here, the lag phase for cytochrome b_6 reduction was about 10 min, and the initially reduced state was obtained after 30 min; thereafter, the same light-induced, reversible redox changes of both cytochromes are measurable as in the presence of hydrogen.

Discussion

Oxygen inhibits nitrogen fixation in isolated heterocysts [3]. The nitrogenase and hydrogenase activity itself, however, is relatively insensitive to short-time exposure to aerobic conditions. A 15 min exposure of hetercysts to air resulted in an approx. 50% inhibition of light-induced acetylene-reduction activity (Ernst, A., unpublished data).

The experiments described in this report demonstrate that oxygen fairly rapidly oxidizes components of cyclic electron transport, thereby inhibiting cyclic ATP formation. It has been known for a long time that cyclic phosphorylation requires the correct poising of electron carriers involved, being most active under reducing conditions [25]. Reducing conditions are generated inside the heterocysts by respiratory removal of oxygen; in addition, oxygen-stimulated cytochrome *f* photooxidation shows that oxygen can be reduced by PS I as well (Fig. 2C). In vivo, appropriate donors to PS I, such as reduced carbon compounds, would be provided by vegetative cells. In isolated heterocysts, hydrogen is the most effective electron donor [3]. It is generally accepted that hydrogen may feed into a respiratory chain, reducing oxygen and providing ATP in the dark [10]. This reaction supports low rates of acetylene reduction [3,12,16]. Further, it has been claimed that hydrogen may feed into the photosynthetic electron-transport chain at the plastoquinone level, thereby providing ATP and a low-potential reductant upon illumination [9,12]. Our experiments, however, show that hydrogen causes only a very slow dark reduction of cytochromes b_6 and *f*, making unlikely a donor function at the high-potential site of PS I; light does not accelerate this slow rate of dark reduction. Moreover, dithionite and argon also lead to reduced cytochromes which had previously been oxidized by oxygen, the dithionite-induced reduction being, of course, much faster than reduction induced by argon.

The more or less effective removal of oxygen from solutions is a common property of all three agents, dithionite, hydrogen and argon. Dithionite reduces oxygen chemically, hydrogen via the Knallgas reaction, and argon removes oxygen by slow equilibration. The resulting reducing environment within the heterocysts leads to reduced redox components. Cytochrome *f* is reduced somewhat faster than cytochrome b_6, according to the redox potentials of both components [26–28]. From experiments done with spinach chloroplasts, a more specific pathway for redox poising of cyclic electron flow by hydrogen is suggested. Components of the electron-transport chain between both photosystems were slowly reduced when NADPH and ferredoxin had been added [25,29–33]. Particularly cytochrome b_6 was partially reduced (approx. 50%) by a hydrogen/hydrogenase system via ferredoxin [31]. Reduced ferredoxin allowed cyclic electron transport to proceed in the complete absence of PS II activity (i.e., in chloroplasts inhibited by high concentrations of DCMU) [34]. Maximum rates of cyclic phosphorylation are measured in the presence of reduced ferredoxin [25,29]. Such a situation might also exist in heterocysts. Under a hydrogen atmosphere, cytochrome *f* and probably other high-potential electron carriers are reduced completely; cytochrome b_6 is only half-reduced. These are conditions of active cyclic phosphorylation [6]. The incomplete reduction of the cyto-

chrome b_6 complement indicates two cytochrome b_6 molecules operating at different redox potentials [26], which are required for an energy-conserving Q-cycle mechanism [35].

We suggest that hydrogen has two functions in isolated heterocysts: (1) It removes oxygen through the Knallgas reaction; this not only protects the oxygen-sensitive hydrogenase/nitrogenase system, but also leads to a properly 'redox-poised' cyclic electron transport. (2) Hydrogen would additionally supply electrons for nitrogen reduction through a reversible hydrogenase, a dark reaction [11,13,17–19]. The dependence of acetylene reduction upon light and hydrogen in isolated heterocysts is explained by the availability of sufficient ATP and reductant.

The reported dibromothymoquinone sensitivity of H_2-supported acetylene reduction in the light cannot be taken as evidence for photooxidation of H_2 by PS I via plastoquinone [16]. It is known that ferredoxin-catalyzed cyclic photophosphorylation is inhibited by dibromothymoquinone [36] and the inhibition is reversed by *N*-tetramethyl-*p*-phenylenediamine [37]. Inhibition of cyclic phosphorylation would, therefore, impair the energy supply to nitrogenase.

Measurement of photoreduction of low-potential acceptors is the most direct assay of electron donation by hydrogen to PS I in heterocysts [9,12]; however, light-induced hydrogen uptake coupled to stoichiometric NADP reduction and phosphorylation has not been measured so far. A more general criticism of experiments performed with isolated thylakoids from heterocysts refers to the finding that soluble components of the electron-transport chain are likely to be lost. In membrane fractions obtained from French press-treated heterocysts, cytochrome *c*-553 is not detectable; it can be quantitatively recovered from the supernatant [22]. General agreement exists that plastocyanin (or cytochrome *c*-553) connects the cytochrome b_6-*f* complex to the reaction centers. therefore, it is difficult to understand how hydrogen could support high rates of photoreduction of NADP and other low-potential acceptors via plastoquinone, if cytochrome *c*-553 or plastocyanin is largely absent. NADP photoreduction by heterocyst thylakoids is dependent on addition of cytochrome *c* or plastocyanin [5,38].

Acknowledgements

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