# Facultative $H_2$-dependent anoxygenic photosynthesis in the unicellular cyanobacterium *Gloeocapsa alpicola* CALU 743

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ABSTRACT. When cells of the unicellular cyanobacterium *Gloeocapsa alpicola* CALU 743 are deprived of nitrate, the phycobilisomes are actively degraded by a proteolytic process termed chlorosis, which accompanied by decrease of rates of oxygen evolution and carbon dioxide fixation, increase of amount of stored glycogen and increase of hydrogenase activity. Suspensions of such cells exhibited a capacity for light-dependent inorganic carbon photoassimilation under anaerobic conditions in the presence of hydrogen and DCMU. The rate of $^{14}C$ incorporation was commensurable with that for nitrate-sufficient cells at oxygenic photosynthesis and reached 35–38 $\mu$mol $^{14}C$ $h^{-1}$ $mg^{-1}$ Chl *a*. Incubation of *G. alpicola* grown aerobically in the presence of limiting concentrations of nitrate under anaerobic conditions (Ar, $CO_2$, DCMU) in the light with addition of nitrate and $H_2$, resulted in the increase of cellular protein, evidencing that *G. alpicola* cells with high level of hydrogenase activity are able to perform $H_2$-dependent anoxygenic photosynthesis at levels supporting the growth of this cyanobacterium.

## 1. INTRODUCTION

Cyanobacteria (blue-green algae) are plant-type phototrophs which carry out water-splitting oxygenic photosynthesis based on light-mediated electron flow through photosystem II (PSII) and photosystem I (PSI) thereby generating ATP and reducing power for photoassimilation of inorganic carbon. In addition to oxygenic photosynthesis a facultative $CO_2$ photoassimilation using sulfide as electron donor has been demonstrated in many different cyanobacteria [1–3]. This type of photosynthesis is called anoxygenic photosynthesis, since it involves PSI only and no oxygen is evolved. Detailed studies of this process in *Oscillatoria limnetica* show that induction of sulfide-dependent $CO_2$ photoassimilation requires *de novo* protein synthesis, specifically an inducible sulfide-quinone oxidoreductase [4–7]. Molecular hydrogen, being as sulfide highly reducing electron donor, can be utilized to photoreduction of $CO_2$ by not only cyanobacteria but by eucaryotic algae as well [8, 9]. This reaction requires hydrogenase participation and is driven by PSI, although possible involvement of PSII has been indicated [10].

In spite of many species of cyanobacteria have the ability to photooxidize sulfide and $H_2$ for $CO_2$ reduction, actual growth exclusively at the expense of sulfide oxidation has been demonstrated only for two species of *Oscillatoria* [4, 7]. There is no information about $H_2$-dependent anaerobic phototrophic growth.

The unicellular non-nitrogen-fixing cyanobacterium *Gloeocapsa alpicola* possesses the reversible-type hydrogenase, which activity increases significantly during light- or nitrate-limited growth [11]. The cells grown under nitrate-limitation are able to produce $H_2$ under dark anaerobic conditions due to fermentation of stored glycogen and to take it up under illumination [11, 12]. The light-dependent $H_2$ uptake was observed in the presence of exogenous hydrogen also and was not sensitive to the inhibitor of PSII, DCMU [12].

The present communication reports experiments showing the use of $H_2$ as electron donor for $CO_2$ photoassimilation in *G. alpicola* and the possibility of $H_2$-dependent growth of this cyanobacterium in anaerobic conditions.

## 2. MATERIALS AND METHODS

The unicellular non-nitrogen-fixing cyanobacterium *G. alpicola* CALU 743 (=Synechocystis 6308) was obtained from the Alga Collection of St. Petersburg University and was grown in BG11$_0$ [13] supplemented with 1.5 $\mu$M $NiCl_2$ and 2 or 15 mM $KNO_3$ as nitrogen source. Cultivation was performed at 30 °C in 0.55-l cylindrical glass flasks two-thirds full of medium and exposed to the light of luminescent lamps with an intensity of 165 $\mu$E $m^{-2}$ $s^{-1}$. Growing cultures were bubbled with gas mixture (300 ml/min): Air + 2% $CO_2$ or Ar + 2% $CO_2$ + 30% $H_2$. Cells from late-log-phase cultures were corrected for Chl *a content* and used in comparative experiments.

Photosynthetic $O_2$ evolution was monitored with a Clarke-type $O_2$ electrode at 25 °C and illumination (120 $\mu$E $m^{-2}$ $s^{-1}$). Culture aliquots (5 ml) of 7–10 $\mu$g Chl *a* $ml^{-1}$ were used for measurements.

For determination of photosynthetic $CO_2$ assimilation the vessels (15 ml) with cell suspensions (5 ml, 10 $\mu$g Chl *a* $ml^{-1}$) were flushed in the dark with Ar for 15 min and then injected, if noted, with DCMU

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(10 $\mu$M), $KNO_3$ (10 mM), flushed with $H_2$ and incubated on the light (120 $\mu$E $m^{-2}$ $s^{-1}$) for 5 min before injection with $Na_2{}^{14}CO_3$ to a final concentration of 20 mM (0.8 $\mu$Ci/ml). Each 30 min aliquots (0.5 ml) of cell suspensions were withdrawn for estimation of $^{14}C$ incorporation.

*In vivo* $H_2$ uptake was detected amperometrically with a Hansatech DWI hydrogen electrode at 30 °C and 120 $\mu$E $m^{-2}$ $s^{-1}$ [12]. Cell suspensions of 10–15 $\mu$g Chl *a* $ml^{-1}$ were used.

Hydrogenase activity was assayed in cell-free extracts by determining the rate of reduced methyl viologen (MV)-dependent $H_2$ evolution by gas chromatography [11]. NAD(P)H-dependent $H_2$ evolution was assayed by the same method, using NADH or NADPH instead of MV.

Cell-free extracts were prepared by ultrasonic treatment of cell suspensions in 50 mM Tris-HCl buffer, pH 8.0, at 70 W for 10 min with intermittent cooling on ice. The resulting homogenate was centrifuged (4000 × g, 40 min) and supernatant was used in hydrogenase reactions.

## 3. RESULTS AND DISCUSSION

The cyanobacterium *G. alpicola* strain CALU 743 is a unicellular, non-nitrogen-fixing, obligate photoautotroph requiring only inorganic nutrients and light for growth. Like some other cyanobacteria, in cells of *G. alpicola* deprived of essential nutrient, such as nitrogen (nitrate), the light-harvesting antennae (the phycobilisomes) are actively degraded by a proteolytic process termed chlorosis [14–17]. The absorbance spectra (Figure 1(A) and (B)) demonstrate a significant reduction in phycocyanin absorbance (peak at 620 nm) in cells of *G. alpicola*, grown under nitrate-limitation in comparison with nitrogen-sufficient cells. Some physiological properties of such cultures are summarized in Table 1. Nitrate-starvation resulted in inactivation of photosystem II (PSII), which the decrease of photosynthetic oxygen evolution rate (it did not compensate the respiration) and photosynthetic $CO_2$ fixation rate evidence about. Inactivation of PSII was reversible and was recovered when nitrate was replenished (not shown) [18]. The low redox potential intracellular medium in nitrate-starved *G. alpicola* developed due to inactive PSII and unaltered respiration induced the increase of hydrogenase activity (Table 1) [12]. Suspensions of such cells exhibited a capacity for *in vivo* light-dependent hydrogen consumption in the presence of $CO_2$. The process was insensitive to DCMU, an inhibitor of PSII, although DBMIB, inhibitor of plastoquinone oxidation, prevented this reaction (Table 1).

The direct measurements of carbon photoassimilation by nitrogen-starved cells under anoxic conditions clearly demonstrate the dependence of the process on the presence of $H_2$ (Figure 2). The rate of $H_2$-dependent $^{14}C$ incorporation ($H_2$, DCMU) was

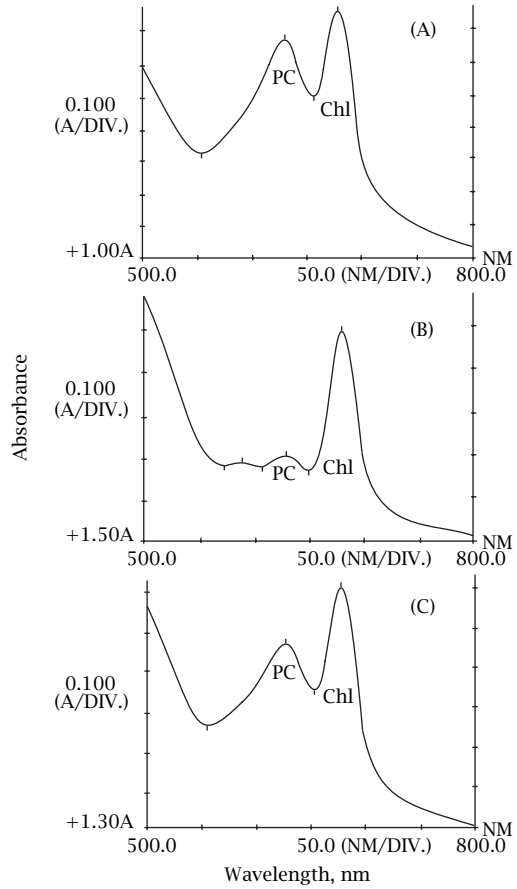


Figure 1. *Whole-cell absorbance spectra of G. alpicola. (A) - nitrogen-sufficient culture; (B) - nitrogen-limited culture; (C) - nitrogen-limited culture after incubation under anoxic conditions in the presence of* $H_2$, $CO_2$ *and* $KNO_3$.

similar to control (oxygenic photoreduction of $CO_2$). The addition of $NO_3^-$ resulted in the decrease of $H_2$-dependent carbon photoassimilation. It might be due to the competition between $CO_2$ fixation and nitrate reduction for assimilatory power, when electrons from $H_2$ are spent for both physiological reactions. The $H_2$-dependent photoreduction of $CO_2$ was not observed in the dark, evidencing that hydrogen is taken up by light-dependent reaction of PSI.

The ability to use $H_2$ for $CO_2$ photoassimilation is not in itself sufficient for anaerobic growth. For example, the well known photoreduction with $H_2$ in eucaryotic alga [8, 9] has never been proved to support their growth. On the other hand anaerobic photoautotrophic growth with highly reducing electron donor, such as $H_2S$, has been demonstrated and studied in details in cyanobacterium *Oscillatotia limnetica* [4–7]. In order to check the growth of *G. alpicola* under $H_2$-dependent anoxygenic photosynthesis the cyanobacterium was grown aerobically in the presence of limiting nitrate concentration to the onset of the stationary

Table 1. Comparative characterization of *G. alpicola* cultures grown under nitrogen-sufficient and nitrogen-limited conditions.

| Parameter | $NO_3$-sufficient cells | $NO_3$-starved cells |
|---|---|---|
| Photosynthetic $O_2$ evolution, $\mu$mol mg$^{-1}$ Chl *a* h$^{-1}$ | 150 | −12 |
| Photosynthetic $CO_2$ fixation, $\mu$mol mg$^{-1}$ Chl *a* h$^{-1}$ | 38 | 9.0 |
| Glycogen content, % to dry weight | 10 | 30–50 |
| Hydrogenase activity as MV-dependent $H_2$ evolution, nmol mg$^{-1}$ protein h$^{-1}$ | 890 | 2860 |
| *In vivo* light-dependent $H_2$ uptake, nmol mg$^{-1}$ protein h$^{-1}$ | | |
| no addition | 240 | 2820 |
| DCMU (10 $\mu$mol) | 38 | 2800 |
| DBMIB (5 $\mu$mol) | < 1.0 | < 1.0 |
| *In vivo* $H_2$ evolution in dark and anaerobic conditions, nmol mg$^{-1}$ protein h$^{-1}$ | 57 | 300 |

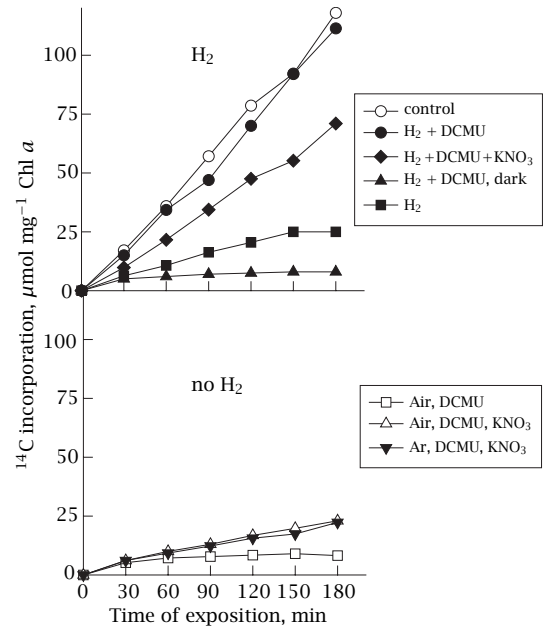


Figure 2. *Kinetics of* $CO_2$ *photoassimilation in cell suspensios of nitrogen starved G. alpicola under different conditions. For comparison an aerobic* $CO_2$ *photoassimilation in nitrogen-sufficient cells (control) is present.* DCMU *and* $KNO_3$ *were added in final concentration* 10 $\mu$*M and* 5 *mM, respectively.*

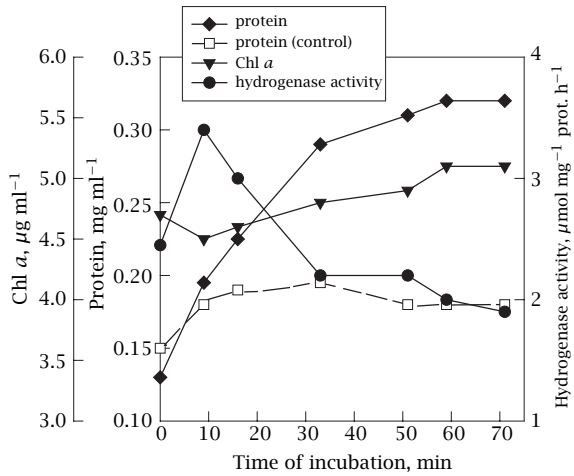


Figure 3. *Time cource of protein concentration, chlorophyll a concentration and hydrogenase activity in batch culture of G. alpicola during incubation under anaerobic conditions in the presence of* $H_2$. *As a control the time course of protein concentration in the absence of* $H_2$ *is present. Conditions of incubation:* 30 °*C;* 70 *mE* $m^{-2}$ $s^{-1}$, *sparging gas (*120 *ml* $min^{-1}$*)* Ar + 1%$CO_2$ + 30%$H_2$*; in time* 0 $KNO_3$ *(*10 *mM) and* DCMU *(*20 $\mu$*M) were added.*

phase, when hydrogenase activity reached high levels. Then, the cultures were diluted by $O_2$-free culture medium and incubated anaerobically (Ar, $CO_2$) in the dark for 15 min, when DCMU (final concentration of 20 $\mu$M), $KNO_3$ (10 mM) and $H_2$ (30%) were added and the incubation was continued under illumination. As a result, the increase of cellular protein was observed, while the chlorophyll *a* concentration increased insignificantly (Figure 3). The doubling time on the protein basis during anoxygenic growth was similar to the oxygenic one, which was 12 hours. Interestingly, it was possible to observe that the initially yellow culture became green during incubation, evidencing that the phycobiliprotein synthesis seems to take place. It was also detected on absorbance spectrum (Figure 1, (C)). Most likely, the intensive phycobiliprotein synthesis was caused by aspiration "to repair" the potential for constitutive oxygenic photosynthesis which was destroyed by nitrogen deprivation.

The primary step of use of $H_2$ as electron donor for anoxygenic photosynthesis is catalyzed by the hydrogenase. *G. alpicola* possesses a hydrogenase of the reversible type, which has a high affinity to molecular hydrogen, $K_m^{H_2}$=38 $\mu$mol. The genes encoding for this enzyme have been characterized for several cyanobacteria [19–22]. They are highly homologous to those coding for the NAD-reducing hydrogenase of

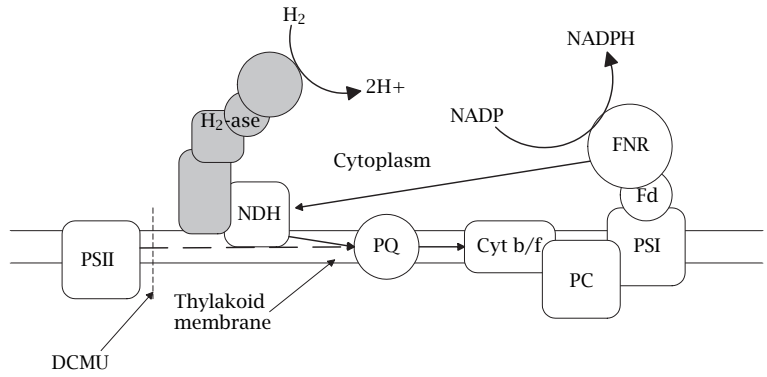


Figure 4. *Hypothetical electron-transport pathway at* $H_2$*-dependent anoxygenic photosynthesis by G. alpicola. The hydrogenase interacts with photosynthetic electron-transport chain via complex I* (NDH) *passing electrons from* $H_2$ *to the plastoquinone pool* (PQ). Cyt, *cytochrome;* Fd, *ferredoxin;* FNR, *ferredoxin*-NADP *reductase;* PC, *plastocyanine.*

*Alcaligenes eutrophus* [23] and NADP-dependent hydrogenase of *Desulfovibrio fructosovorans* [24]. Sequence comparisons indicated that cyanobacterial enzyme is composed of the $H_2$-cleaving dimer Hox YH and the diaphorase moiety HoxFU transferring electrons to NAD(P). These molecular data are confirmed by physiological reactions of NAD(P)-dependent hydrogenase activity in *G. alpicola* (Table 2). Cell-free extracts were able to evolve molecular hydrogen in the presence of NADH or NADPH. Therefore it may be suggested that the hydrogenase interacts with the photosynthetic electron-transport chain on the plastoquinone level. Accordingly, in the case of inactive PSII and in the presence of molecular hydrogen, a plastoquinone pool can be provided for electrons from $H_2$ via hydrogenase and NAD(P)H-dehydrogenase (complex I). Figure 4 demonstrates the hypothetical model for electron transport at $H_2$-dependent anoxygenic photosynthesis in *G. alpicola* cells.

Table 2. Hydrogen evolution by cell-free extracts of *G. alpicola.*

| Reductant | Relative value of $H_2$ evolution, % |
|---|---|
| None | n.d. |
| Methyl viologen 1 mM+ $Na_2S_2O_4$ 5 mM | 100 |
| NADH 3 mM + $Na_2S_2O_4$ 5 mM | 3.4 |
| NADPH 3 mM + $Na_2S_2O_4$ 5 mM | 1.7 |
| $Na_2S_2O_4$ 5 mM alone | 0.6 |

The reaction mixture contained 1.2 mg protein per ml. 100% $H_2$ evolution was equivalent to 315 nmol $min^{-1}$ $mg^{-1}$ protein.

## 4. CONCLUSIONS

The results obtained evidence that *G. alpicola* is capable to perform facultative anoxygenic photosynthesis using molecular hydrogen as an electron donor, at a level sufficient to support at least anaerobic photoautotrophic protein synthesis in this cyanobacterium. It is unlikely that conditions favorable for $H_2$-dependent anoxygenic photosynthesis in cyanobacteria may exist in natural ecosystems. However this study may be important for the understanding of the place of cyanobacteria in evolution of the phototrophs.

## ACKNOWLEDGEMENTS

This work was kindly supported by Russian Foundation of Basic Research (01-04-97003; 01-04-48502).

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