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Hydrogen photoproduction in green algae *Chlamydomonas reinhardtii* under Magnesium deprivation

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The effect of Mg-deprivation on green algae *Chlamydomonas reinhardtii* was studied. It resulted in the decrease of photosynthetic activity, increased respiration and accumulation of starch. After 35 hours anaerobic conditions were established and sustained H_2 evolution (> 7 days) was detected.

Introduction

Quick development and technological shift to clean renewable energy sources is a global challenge. An attractive solution is the development of solar fuels as alternative energy carriers. Molecular hydrogen is one of the most promising clean fuels of the future. Oxygenic photosynthetic organisms are the most advanced users of solar energy among biological species. The remarkable evolutionary achievement of the oxygenic photosynthesis is that the reductive electrons, which are necessary for growth and reproduction, are obtained from water, a ubiquitous source of electrons and an attractive substrate for the solar fuel production.

Among oxygenic species cyanobacteria and green algae can photoproduce molecular hydrogen (H_2) [1-3]. The reaction is catalyzed by hydrogenase enzyme [4] and is ultimately connected to the photosynthetic electron transport chain [3,5]. Green algae possess fastest [FeFe]-type of hydrogenase which is irreversibly inhibited by oxygen [1,4]. Sulfur (S)-deprivation is one of the commonly used methods to induce anaerobic conditions in the liquid cultures of *C. reinhardtii* placed in the gas-tight bioreactors [6,7]. Under these conditions, decrease of photosynthetic activity and establishment of anaerobiosis are caused mainly by decrease of the amount of PSII [†], O_2 producing protein complex [8,9]. The remaining PSII centers continue to serve as electron donor to hydrogenase with the residual O_2 evolution that is fully consumed by respiration [8,9].

Magnesium (Mg) is a major element in any culture medium along with potassium, sulfate and phosphate and plays a key role in many physiological and metabolic processes [10-17, and references therein]. Photosynthetic reactions have an absolute requirement for this element because Mg occupies the crucial position in the photosynthetic apparatus as a constituent of the Chl molecule. Lack of Mg significantly alters photosynthetic reactions and structure of photosynthetic apparatus [13,14,18-24]. Mg-deprivation (Mg-D) results in decrease of the photosynthetic activity, which is accompanied by decrease in the electron transport rates, increase in the amount of non Q_B-reducing centers [25] and reduction of the PQ-pool [24]. It results also in changes in photosynthetic antenna and fluorescence characteristics on both PSII and PSI [20,26,27]. The total amount of Chl always decreases under Mg-D [19-21,23,25,28,29].

High rate of respiration and small antenna are important factors to consider the potential H_2 production under the Mg-D conditions. The first one is a prerequisite for creation of the anaerobic state and expression of hydrogenase and the second one could increase the light utilization efficiency by increasing the light penetration in the dense cultures [30-33]. In this study, we show for the first time, that cells of *C. reinhardtii* can photoproduce considerable amounts of H_2 gas under the Mg-D conditions. H_2 evolution takes place over the prolonged period of time of more than 7 days. This is new alternative approach to the photobiological H_2 production in green algae for the generation of H_2 based solar fuels.

Results and discussion

Mg-D results in significant metabolic changes in *C. reinhardtii*. 7 days in Mg-D media resulted in substantial decrease of total Chl per cell (by 60%) and increase of Chl *a/b* ratio (from 2.5 to 3.2, Table 1). The lower Chl *b* content is indicative of the decrease of

Parameters	Growth medium			
	TAP		TAP-Mg	
	pH 7.0	pH 7.7	pH 7.0	pH 7.7
pH after 7 days	7.89±0.03	7.91±0.01	7.86±0.04	7.90±0.03
Chl pg/cell	4.93±0.51	4.87±0.32	1.99±0.24	1.91±0.27
Chl *a/b*	2.49±0.1	2.41±0.1	3.16±0.4	3.14±0.4
Cells, 10^6/ml	8.21±0.2	6.24±0.1	4.28±0.5	3.01±0.4
F_V/F_M	0.75±0.11	0.77±0.10	0.57±0.31	0.58±0.20
Photosynthesis	84±4 (349±14)	80±2 (328±14)	47±1 (279±20)	39±1 (262±11)
Respiration	11.2±0.9	12.6±0.5	12.5±0.4	16.9±0.5

Table 1 Effect of initial pH on the physiological characteristics of aerobic *C. reinhardtii* culture. Cells were grown 7 days in the complete (TAP) and Mg-free (TAP-Mg) medium. Photosynthesis and respiration are shown in μmoles O_2 (mg Chl)$^{-1}$×h^{-1}. Data in parenthesis were measured in the presence of 1 mM DCBQ as an electron acceptor.

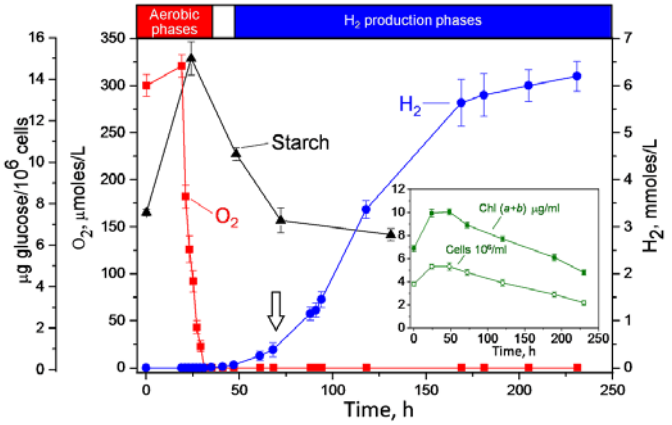

Fig. 1 Changes in O_2 and H_2 concentrations in the gas phase and in the cell starch concentration during Mg-D of *C. reinhardtii* in gas-sealed bioreactor. White arrow indicates the time point at which aliquots of the cell culture were taken for the experiments in the presence of DCMU (see Fig. 2). The inset shows changes in the cell and Chl concentrations.

LHCII during Mg-D [34]. Photosynthetic activity measured as F_V/F_M ratio from PSII decreased by 24% during the Mg-deprivation if compared to the control. This result is consistent with drop in the photosynthetic O_2 evolution, measured in the presence of electron acceptors. After seven days the rate of O_2 evolution in Mg-D cells declined by 19% (Table 1). These data show decrease in the amount of functional PSII centers by ~20%. Similar repression of the photosynthetic activity during Mg-D was reported for *Chlorella vulgaris* [18] and reflects a combined result of the decreased activity of enzymes involved in CO_2 fixation [14,22] and repair cycle of PSII [13,14,35]. The rate of respiration, measured as O_2 consumption slightly increased by 11% after 7 days of Mg-D (Table 1). This effect is similar to the S-deprivation and is result of the lower photosynthetic activity and accumulation of the endogenous substrate (see below).

Elevated pH of TAP medium was shown to yield in higher H_2 production under S-deprivation [36]. At pH 7.7, response of photosynthetic parameters of cells to Mg-D was quite similar (Table 1). However, the respiration rate was increased by >30%. Interestingly, the O_2 evolution rate measured in the absence of electron acceptors was decreased by ~50% at both pH values, indicating significant limitation of the electron transport after PSII (Table 1). Our flash induced fluorescence and thermoluminescence measurements show significant reduction of the PQ pool after 7 days of Mg-D in *C. reinhardtii*, see ESI.

It should be mentioned, that preceding Mg-D incubation for 7 days in open cultures, was found to be necessary in order to observe the H_2 evolution by *C. reinhardtii* in gas tight bioreactors on the later stage. If the Mg-D culture was transferred to the bioreactors directly without the preliminary incubation, no H_2 formation was observed. The reason for this is that the Mg-D procedure is milder treatment if compared to others reported in the literature and does not inhibit photosynthesis fast enough. 7 days of the preliminary incubation was found to be optimal with respect to changes in the F_V/F_M and photosynthesis/respiration ratio in order to achieve the anaerobic state faster in the closed cultures.

When incubated in the gas-tight bioreactors, Mg-D *C. reinhardtii* cells undergo dramatic changes in the photosynthetic activity, and consume all produced and dissolved O_2, thus, creating anaerobic conditions. In a few hours after the onset of anaerobiosis, the H_2 production is observed under the Mg-D conditions (Fig. 1). Not surprisingly, the Mg-D culture passed through the same distinct physiological stages that were found for the S-deprived cells: the O_2 evolution phase, the O_2 consumption phase, the anaerobic phase and the H_2 production phase, Fig. 1, [9,36].

During the O_2 evolution phase, the concentration of O_2 in the gas phase was increased in the first 20 hrs, indicating that cells were still photosynthetically active (Fig. 1). After that the concentration of O_2 was quickly decreased (O_2 consumption phase), reaching zero level at ~30 h and remaining at zero level until the end of the entire experiment (up to 231 hrs, Fig. 1). The reason for this was that the decreased O_2 evolution was completely overtaken by the O_2 consumption. The rate of O_2 evolution in the Mg-D cells after 24 hrs incubation in the gas-tight vials decreased by two fold (~19 μmol O_2 (mg Chl)$^{-1}$×h^{-1}) if compared with the values obtained at the beginning of the experiment. The rate of respiration changed little and remained at ca 15 μmol O_2 (mg Chl)$^{-1}$×h^{-1}.

The appearance of H_2 in the gas phase of the glass vial was detected ~5 hrs after establishment of anaerobiosis and reached the final yield of 6.3 mmol/L of culture after about 200 hrs of the experiment. The Chl concentration at the beginning of experiment was ~7 μg Chl ml^{-1} (Fig. 1, inset). Cells produced H_2 with the increasing rate up to ~70 hrs (100 hrs from the beginning of experiment), and then the production declined gradually thereafter. After 7-8 days the amount of produced H_2 stopped to increase and the evolution rate became negligible. Nevertheless, the maximum rate of H_2 evolution during the active phase of production, calculated on the Chl basis, was 17.2±2.1 μmoles H_2 (mg Chl)$^{-1}$×h^{-1}. It is nearly two times higher if compared to the H_2 evolution by the S-deprived cells (7.3±1.2 μmoles H_2 (mg Chl)$^{-1}$×h^{-1}, Fig. 2A). When Chl loss during Mg-D was taken into account (or comparison is made on the cell basis) the difference was not so significant (20 vs 16 μmoles H_2 (10^6 cells)$^{-1}$×h^{-1}, Fig. 2B). More importantly, the Mg-D cells were able to produce twice the amount of H_2 and to sustain the production for much longer than the S-deprived cells (Fig. 2B and C).

We have also tested how this H_2 evolution, induced by Mg-D is dependent on the PSII activity. It was shown that in the case of S-deprivation, the contribution of PSII induced electrons (i.e. electrons obtained from the water oxidation) constitute up to 80% of electrons necessary for the hydrogenase activity [9 and references therein]. We tested this by addition of DCMU, known PSII inhibitor, to the H_2 cultures after 30 hrs of anaerobiosis (white arrow in Fig. 1). Addition of DCMU inhibited the H_2 evolution by 74% in the Mg-D culture (Fig. 2A), similar to the 80% inhibition reported for the S-deprived culture. Thus, PSII is indispensable for the H_2 formation in *C. reinhardtii* also under the Mg-D conditions.

Another important source of electrons to hydrogenase is fermentation process of the endogenous substrate [5]. The concentration of starch, measured as glucose equivalents, in cells

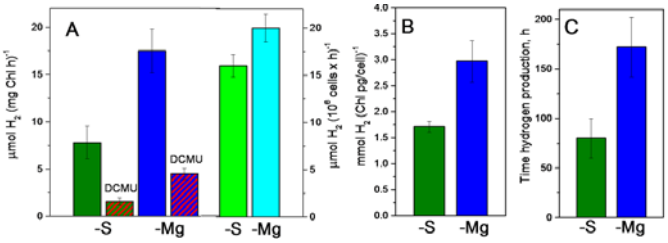

Fig. 2 Effect of Mg- and S-deprivation on the H_2 production by *C. reinhardtii*. The maximum rate of H_2 evolution is shown on the Chl basis in the absence and presence of 20 μM DCMU (at the time point indicated in Fig. 1) and on the cell basis (A). The normalized total yield of the H_2 production (B) and time from the beginning of the H_2 production until reaching the steady state level (C).

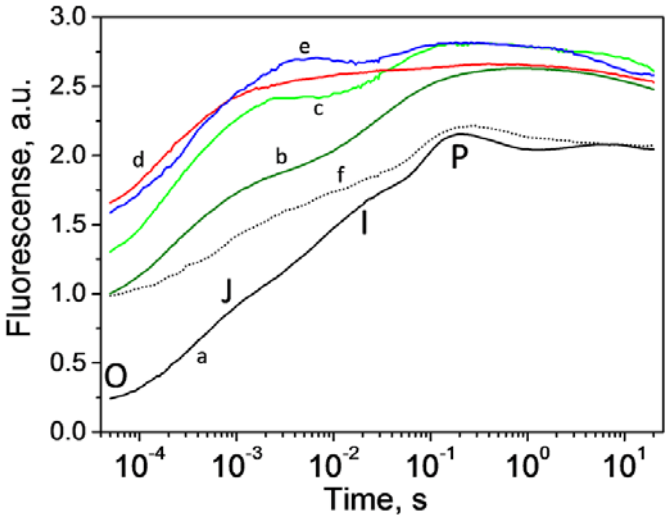

Fig. 3. Effect of Mg-D on Chl fluorescence induction curves measured from *C. reinhardtii* cells. OJIP transients were recorded in control (a, TAP medium) and Mg-D (b, c, d, e, and f, TAP-Mg medium) cells at pH 7.7. Samples were measured aerobically in a and b (O_2 evolution phase, 3 hrs) and anaerobically in c (O_2 consumption phase, 21 hrs), d (anaerobic phase, 30 hrs) and e (H_2 evolution phase, 50 hrs), see Fig. 1. Trace f was obtained after aeration of sample from the anaerobic phase (d) for 5 min.

grown in the complete medium (TAP) was around 1.6 µg glucose $(10^6$ cells$)^{-1}$. In our experiments, cells were grown in the TAP-Mg medium for a week before they have been placed in the gas-tight bioreactors (see Materials and methods). By this time (Fig. 1, t = 0 hrs) the concentration of starch in the cells increased to 7.6 µg $(10^6$ cells$)^{-1}$. During the O_2 consumption phase (Fig. 1, t = 24 hrs) the concentration of starch has reached the maximum of 15 µg $(10^6$ cells$)^{-1}$ and started to decrease. Subsequently, during the anaerobic and H_2 production phases, the starch content continued to decline to 10.4 µg $(10^6$ cells$)^{-1}$ at 48 hrs and 7.2 µg $(10^6$ cells$)^{-1}$ at 72 hrs, respectively (Fig. 1). Thus, it is clear that starch catabolism also contribute to the H_2 production under the Mg-D conditions at least in the beginning of the H_2 production phase. We can estimate this contribution to be about 25% since this was the fraction of H_2 formation which was not inhibited by the DCMU addition (Fig. 2A).

Effect of DCMU addition on the H_2 evolution under Mg-D shows that the main flow of electrons for hydrogenase activity originates from the water splitting activity by PSII [9]. In order to access changes in the functional properties of PSII, fluorescence induction kinetics (OJIP transients) were recorded from cells removed from the bioreactor during different phases of Mg-D (Fig. 3). The kinetic traces measured in the control cells (trace a) demonstrated three well-known transients of the fluorescence changes of the active PSII [37,38]. The first transient (OJ), reflects the reduction of Q_A while the following JIP transition reflects the subsequent reduction of the PQ-pool [37,38]. Lack of Mg in the medium led to increase in both F_0 (transient O) and F_M (transient P) levels and significant changes in the shape of the induction kinetics (Fig. 3). The kinetic trace recorded in the Mg-D cells in the O_2 evolution and O_2 consumption phases (Fig. 3, traces b, c) displayed four-fold and five-fold increased O value if compared to the control. The P level was increased by 20% and 30%, respectively. The amplitude of the JIP phase, which represents the reduction of the PQ-pool [38], was decreased by 30% and 70% in the O_2 evolution and O_2 consumption phases, respectively. In the anaerobic phase this value didn't exceed 14% if compared to control (Fig. 3). The shape of the induction kinetics recorded in the anaerobic phase (Fig. 3, trace d) was very similar to the kinetics measured in the presence of DCMU with JIP transients practically absent, indicating a complete block in the electron transport from Q_A^- to Q_B [37,38]. The kinetic trace recorded in the H_2 evolution phase (Fig. 3, trace e) displayed reappearance of the JIP transition indicating partial re-oxidation of PSII acceptor side and the PQ-pool by the electron flow to the activated hydrogenase.

Interestingly, introduction of O_2 to the anaerobic culture (trace f) led to restoration of the typical OJIP transients, similar to that of the control sample (Fig. 1, trace a) within 5 min. This is another confirmation that Mg-D results in the complete reduction of the PQ-pool which is reversible when some kind of the oxidant (hydrogenase) becomes available. Presence of O_2 is known to keep the redox state of the PQ-pool in the mostly oxidized state [39].

Conclusions

Mg-D exerts a major effect on metabolic, physiological and biochemical processes in alga and plants affecting cell growth and development. Our study demonstrates that Mg-D alters photosynthetic activity, antenna composition and starch accumulation in *C. reinhardtii*. These were accompanied with the decrease in the amount of functional PSII by about 20% (Table 1) which is much less if compared to the effect of the S-deprivation where 80% decrease of the PSII centers was reported [9].

Analysis of the functional status of these remaining after Mg-D PSII centers with the flash-induced fluorescence decay and thermoluminescence measurements (see ESI) showed that decline in the electron transport occurred due to the limitations on the acceptor side of PSII. This conclusion is supported by the decrease in the O_2 evolution when measured in the absence of the exogenous electron acceptor (Table 1). Taken together, these data indicate to the electron transfer block at the level of PQ-pool which was induced by Mg-D already in the aerobic phase (Fig. 1).

The over-reduction of the PQ-pool was also confirmed by the OJIP transient measurements in cells taken from bioreactors. The number of non-Q_B-reducing PSII centers increased during the O_2 consumption and anaerobic phases Importantly, all fluorescence transients were restored to the control level when the PQ-pool was re-oxidized by introduction of O_2 to the cell culture (Fig. 3, trace f).

More reduced environment and anaerobiosis are necessary preconditions for the potential H_2 formation in the algal cells. Indeed, this was the case during the Mg-D treatment. We were able to detect significant amount of the H_2 gas formation by the Mg-D cultures of *C. reinhardtii* (Fig. 1). H_2 production was preceded by decline in the O_2 evolution rate and increase in the rate of respiration and, finally after ~30 hrs, establishment of the anaerobic conditions (Table 1, Fig. 1). The H_2 production under Mg-D was more efficient and longstanding (>7 days) if compared to other procedures (Fig. 2C).

The reason for this could be the amount of PSII centers still present when anaerobic conditions were created in the culture [9]. The Mg-D procedure preserves more functional PSII centers than it has been demonstrated for the S- and N-deprivation [8,9,40,41]. More PSII centers imply that there are more electrons available to hydrogenase for the H_2 formation at the time point when the anaerobic conditions are created in the cell culture. This is also confirmed by experiments with the DCMU addition (Fig. 3A) which showed that 75% of the electrons, utilized by hydrogenase, were originated in the water oxidation process in PSII. Thus, we conclude that PSII is indispensable for the H_2 formation under the Mg-D conditions.

Our results show that the light-induced H_2 formation can be observed under the Mg-deprived cultivation of green algae *C.*

reinhardtii. The H_2 production is sustained for over seven days under these conditions, which is significantly longer than under any other treatment reported so far. We also report significant involvement of PSII, a donor of electrons obtained from water, in the H_2 formation. This is new, potentially very promising approach to the photobiological solar fuel formation in green algae.

Materials and methods

C. reinhardtii wild type strain Dang 137^+ was grown photoheterotrophically in TAP medium, pH 7.0, in Erlenmeyer flasks at 25 °C under continuous illumination (100 μE $m^{-2}\times s^{-1}$) and constant mixing. After reaching the late-logarithmic phase (9-10×10^6 cells/ml), 10 ml aliquots of the culture was centrifuged at 3000×g for 3 min, the pellet was resuspended in TAP medium without manganese (TAP-Mg, pH 7.0 or 7.7) to a final concentration of 1×10^6 cells/ml. This was followed by 7 days of aerobic incubation in Erlenmeyer flasks at 25 °C under the same light and mixing conditions. TAP-Mg medium was modified from the standard TAP medium by replacing $MgSO_4$ to Na_2SO_4 at the same concentration. For the H_2 production experiments, the Mg-D aerobic culture (pH 7.7) was collected by centrifugation (3000×g, 3 min), the pellet was resuspended in fresh TAP-Mg (pH 7.7) and placed into 14 ml gas-tight glass vials (bioreactors, Ø 22 mm) to a final concentration of about 4×10^6 cells/ml (~7 μg Chl ml^{-1}). The culture was incubated at 25 °C under the light intensity of 100 μE $m^{-2}\times s^{-1}$ and constant mixing for the indicated period of time. Cell concentration and Chl content was determined as in [9]. Starch content measurement was performed according to [42]. Rates of respiration and photosynthetic O_2 evolution was measured as described in [9].

The Chl fluorescence induction (OJIP transients) was measured using Plant Efficiency Analyzer (Hansatech, UK). Red light at 650 nm at the intensity of 2000 μE m^{-2} s^{-1} was used as an excitation light. The fluorescence data were sampled at intervals of 0.1 ms for the first 2 ms, 1 ms in the range between 2 ms and 1 s, and 100 ms thereafter. The fluorescence signal at 50 ms, being the earliest measuring point free of the electronic artifacts was considered as F_0.

The amount of H_2 in the gas phase was measured using gas chromatograph Gazokhrom-2000 (Zchrom, Moscow, Russia) completed with the detector of thermoconductivity with argon as a carrier gas. 200 μl of gas sample was taken for the measurements at the indicated time points. The effect of DCMU addition (20 μM) on the H_2 production was measured as described in [9] after 30 hrs after the anaerobic conditions have been established.

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† Abbreviations: Chl, chlorophyll; DCMU, 3-(34-dichlorophenyl)-11-dimethylurea; DCBQ, 26-dichloro-p-benzoquinone; LHC, light harvesting complex; Mg-D, Mg-deprivation; PQ, plastoquinone; PSII, photosystem II; Q_A and Q_B, primary and secondary plastoquinone acceptors in PSII; TAP, tris-acetate-phosphate.

Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here].

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