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Hydrogen photoproduction in green algae
Chlamydomonas reinhardtii under Magnesium deprivation

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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.

Molecular hydrogen can photoproduce molecular hydrogen (H₂) [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 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 culture of Chlamydomonas 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, O₂-producing protein complex [8,9]. The remaining PSII centers continue to serve as electron donor to hydrogenase with the residual O₂ 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 Q₀-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 H₂ 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 Chlamydomonas reinhardtii can photoproduce considerable amounts of H₂ gas under the Mg-D conditions. H₂ evolution takes place over the prolonged period of time of more than 7 days. This is a new alternative approach to the photobiological H₂ production in green algae for the generation of H₂ based solar fuels.

Results and discussion
Mg-D results in significant metabolic changes in Chlamydomonas 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 photosynthetic activity, increased respiration and accumulation of starch. After 35 hours anaerobic conditions were established and sustained H₂ evolution (> 7 days) was detected.

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 H₂ evolution (> 7 days) was detected.

Table 1 Effect of initial pH on the physiological characteristics of aerobic Chlamydomonas reinhardtii culture. Cells were grown 7 days in the complete (TAP) and Mg-free (TAP-Mg) medium. Photosynthesis and respiration are shown in µmoles O₂ (mg Chl)⁻¹h⁻¹. Data in parenthesis were measured in the presence of 1 mM DCBQ as an electron acceptor.
RH2 forms were observed during Mg-D was reported for functional PSII centers by LHCII during Mg-D [34]. Photosynthetic activity measured as $FV/FM$ state faster in the closed cultures.

- Photosynthesis/respiration ratio in order to achieve the anaerobic conditions. In a few hours after the onset of anaerobiosis, the H2 evolution by days in open cultures, was found to be necessary in order to observe 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 H2 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)$^{-1}\times$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)$^{-1}\times$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±2.1 µmoles $H_2$ (mg Chl)$^{-1}\times$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)$^{-1}\times$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 $H_2$ (100 cells)$^{-1}\times$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 $H_2$ evolution. The rate of $H_2$ 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. 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.](image-url)

- LHCII during Mg-D [34]. Photosynthetic activity measured as $FV/FM$ 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 CO2 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 $Fv/Fm$ 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).
The kinetic traces recorded in the H₂ 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 O₂ 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 O₂ 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 algae 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 O₂ 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-Qo-reducing PSII centers increased during the O₂ consumption and anaerobic phases. Importantly, all fluorescence transients were restored to the control level when the PQ-pool was re-oxidized by introduction of O₂ to the cell culture (Fig. 3, trace f).

More reduced environment and anaerobiosis are necessary preconditions for the potential H₂ formation in the algal cells. Indeed, this was the case during the Mg-D treatment. We were able to detect significant amount of the H₂ gas formation by the Mg-D cultures of *C. reinhardtii* (Fig. 1). H₂ production was preceded by decline in the O₂ evolution rate and increase in the rate of respiration and, finally after ~30 hrs, establishment of the anaerobic conditions (Table 1, Fig. 1). The H₂ 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 H₂ formation at the time point when the anaerobic conditions were 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 H₂ formation under the Mg-D conditions.

Our results show that the light-induced H₂ formation can be observed under the Mg-deprived cultivation of green algae *C.
reinhardtii. The H₂ production was 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 H₂ 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 phototrophically in TAP medium, pH 7.0, in Erlenmeyer flasks at 25 °C under continuous illumination (100 µE m⁻² s⁻¹) and constant mixing. After reaching the late-logarithmic phase (9×10⁶ cells/ml), 10 ml aliquots of the culture was centrifuged at 3000 g for 3 min, the pellet was resuspended in TAP medium at a density of 1×10⁶ 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 MgSO₄ by Na₂SO₄ at the same concentration. For the H₂ production experiments, the Mg-D culture (pH 7.7) was collected by centrifugation (3000g, 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 1×10⁶ 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 MgSO₄ by Na₂SO₄ at the same concentration.

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 OJIP transients. EA}