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The hydrogen metabolism of sulfur deprived *Chlamydomonas reinhardtii* cells involves hydrogen uptake activities

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ABSTRACT

Several species of unicellular microalgae such as the model species *Chlamydomonas reinhardtii* possess plastid-localized [FeFe]-hydrogenases which, via ferredoxin, can accept electrons from photosynthetic electron transport. Thereby, under specific conditions, these algae light-dependently produce molecular hydrogen (H_2), which offers a sustainable way to generate a "green" and efficient fuel. Until today, the most common way to induce sustained H_2 production is to deprive *Chlamydomonas* of macronutrients such as sulfur (S) which results in a downregulation of photosynthetic production of molecular oxygen (O_2) and of assimilatory processes. These acclimation responses allow the O_2 sensitive algal [FeFe]-hydrogenases to become active and serve as an alternative electron sink of photosynthesis. Despite much progress in the field and a general understanding of the underlying mechanisms, many basic and applied aspects of the photosynthetic H_2 metabolism of eukaryotic algae remain to be elucidated. One rarely investigated factor is that microalgae have also been reported to consume H_2, especially as a response to high H_2 concentrations. Here, we analyzed the H_2 uptake activities of S-deprived *Chlamydomonas* cells incubated in different PBRs providing different gas phase volumes, either in continuous light or in the dark. We show that H_2 uptake occurs after prolonged incubation in the light as well as in sudden darkness. Dark-induced H_2 uptake can be delayed adding the phosphoribulose kinase inhibitor glycolaldehyde, suggesting a connection to carbohydrate metabolism. The results indicate that PBR setups as well as envisioned outdoor cultivation systems with natural light-dark cycles have to be carefully designed to prevent efficiency losses.

1. Introduction

The capability of certain species of unicellular microalgae to generate molecular hydrogen (H_2) using electrons derived from photosynthetic electron transport (PET) is a promising way to sustainably generate this clean and efficient fuel [1]. In the model green alga *Chlamydomonas reinhardtii* (*Chlamydomonas* in the following) chloroplast localized [FeFe]-hydrogenases accept electrons directly from the photosynthetic ferredoxin PetF [2]. High H_2 yields are only observed in the light (e.g., [3]), and photosynthetic electron supply occurs via two main pathways, termed direct and indirect. In the direct pathway, electrons originate from water oxidation at photosystem II (PSII) [4–6]. The indirect pathway utilizes electrons derived from NAD(P)H oxidation by the plastidic NAD(P)H-plastoquinone oxidoreductase Nda2 [7–9] and thus ultimately from the oxidation of organic reserves such as starch or proteins [4,10]. *Chlamydomonas* can also produce low amounts of H_2 in the dark, probably through PetF reduction by the plastidic fermentative enzyme pyruvate ferredoxin oxidoreductase (PFR) [11,12]. Because of the severe sensitivity of [FeFe]-hydrogenases towards molecular oxygen (O_2) [13,14], a H_2 metabolism is only established under anoxic or hypoxic conditions. Photoevolution of H_2 can be observed in *Chlamydomonas* cells pre-incubated under anoxic conditions in the dark and then abruptly shifted to illumination (e.g., [15]). Although vital for the reactivation of photosynthesis [16,17], H_2 production under this condition is only short-lived due to the accumulation of O_2 and the induction of the Calvin-Benson-Bassham (CBB) cycle. The latter is a major competitor of H_2 photoproduction [6,18,19], because it consumes the NADPH produced by ferredoxin NADPH reductase (FNR) and thus ultimately competes with electrons delivered by PetF [20,21]. Long-term H_2 production can be achieved in dense *Chlamydomonas*

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Nomenclature

(PET) photosynthetic electron transport
(PSII) photosystem II
(PFR) pyruvate ferredoxin oxidoreductase
(CBB cycle) Calvin-Benson-Bassham cycle
(FNR) ferredoxin NADPH reductase
(S) sulfur
(PBRs) photobioreactors
(PTOX) plastid terminal oxidase
(GA) glycolaldehyde
(Chl) chlorophyll *a* + b
(TAP-S) sulfur-deprived TAP medium

cultures at low light intensities resulting in balanced photosynthesis and respiration, thereby establishing hypoxia [22]. These conditions favor the accumulation of excess reducing power by the cell, which eventually triggers long-lasting H_2 production at low rates, which is primarily based on a direct contribution from PSII [23]. Sustained and prolonged H_2 production in the light can also be induced by depriving the cells of macronutrients. For the first time demonstrated by Melis et al. [10] in case of sulfur (S) deprived cells, following studies showed that also nitrogen, phosphorous and magnesium deprivation induce a sustained H_2 metabolism [24–26]. All of these approaches have in common that they result in a decreased PET activity and to impaired or ceased growth. Thereby, two important requirements for H_2 production are fulfilled, that is decreased O_2 concentrations and reduced electron consumption in assimilatory processes. In the first one or two days, macronutrient deprivation stimulates the accumulation of organic reserves such as starch and lipids [10,24–27], and subsequent H_2 photoevolution is typically driven by electrons derived both from the direct and indirect pathways [4,5,9,28,29].

Although H_2 photoproduction by microalgae represents a sustainable way of generating this fuel gas, it has not been economically established yet. Its theoretical maximum light conversion efficiency is about 10% [1,30], but this has yet to be achieved, although several *Chlamydomonas* mutant strains with significantly enhanced H_2 production capabilities have been described [1,30,31]. Technical issues are related to suitable photobioreactors (PBRs), regarding both design and operating principles, and the light source [32,33]. For example, an inhibiting influence of the H_2 partial pressure has been shown [34]. The build-up of a H_2 atmosphere is, amongst others, dependent on the bioreactor geometry in that a higher ratio of the gas phase to the cell suspension results in a more dilute gas. It has been discussed that H_2 uptake mechanisms are activated in *Chlamydomonas* cells at H_2 partial pressures > 5% [34]. Microalgae have been reported to consume H_2 and utilize it either for the photoreduction of carbon dioxide (CO_2) in low light or for the so-called oxy-hydrogen reaction in the dark [35–39]. Although the exact pathways are yet unknown, both reactions are proposed to involve the reduction of ferredoxin through the H_2 oxidizing reaction of the hydrogenases and subsequent NADPH formation via FNR. During photoreduction, NADPH is probably consumed by the CBB cycle, while during the oxy-hydrogen reaction, NADPH or reduced ferredoxin are assumed to be re-oxidized ultimately by the plastoquinone pool. The latter can be oxidized by plastid terminal oxidase (PTOX) upon the reduction of O_2 to H_2O ([40] and references therein). However, also the oxy-hydrogen reduction is accompanied by a low rate of CO_2 fixation [35,36,39]. An additional issue of large-scale applications for photobiological H_2 production by microalgae is that the use of sunlight as a light source would be most desirable. However, analyses of outdoor H_2 photoproduction using *Chlamydomonas* resulted in much lower H_2 yields than in the laboratory, and photoinhibition due to the extremely high intensity of sunlight was discussed as one reason [33,41]. Another factor is that cultivation under natural conditions would also involve a phase of darkness in the night which might result in efficiency losses similar to reported biomass decay rates [42]. In the present study, we addressed H_2 uptake phenomena and their implications for the biotechnological application of *Chlamydomonas* subjected to S deprivation both after prolonged incubation in the light and upon sudden darkness, and show that S-deprived cells retain H_2 oxidation capacity even after seven days. The application of larger PBR headspaces delays the establishment of a net H_2 consumption activity. In the dark, addition of glycolaldehyde (GA), an inhibitor of the CBB cycle [43], delays the onset of H_2 consumption activity, suggesting a cross-talk with CO_2 fixation.

2. Materials and methods

2.1. Algal strains and growth conditions

Pre-cultures of *Chlamydomonas reinhardtii* strain CC-124 were grown photomixotrophically in standard TAP medium, pH 7.2 [44] in Erlenmeyer flasks on a rotary (Bologna) or reciprocating (Bochum) shaker. Light of 100 µmol photons·m^{-2}·s^{-1} was provided from the top (Bologna) or from the bottom (Bochum) by cool white fluorescent lamps (Osram, Dulux L). Incident light was measured with a flat quantum radio-photometer (Skye Instruments Ltd). Cultures were kept at 20 °C (Bologna) or at 18 °C (Bochum). A mixture of air and CO_2 (98.5:1.5, v:v) was provided during growth in Bologna.

2.2. H_2 production and consumption experiments and measurements

Cultures were collected by centrifugation (6000 *g*, 20 °C, 10 min) at the late exponential phase of growth (20 mg chlorophyll *a* + *b* [Chl]·L^{-1}), washed five times in S-free medium (TAP-S; TAP medium in which all sulfate salts were replaced by chloride salts; pH 7.2) and finally resuspended in TAP-S at an initial Chl concentration of 20 ± 2 mg·L^{-1}. Light was provided on two sides at 65 ± 5 µmol photons·m^{-2}·s^{-1}. The PBRs (see below) were kept at 20 °C.

Experiments at low gas: liquid phase ratio were conducted in 1.2 L Roux-flask PBRs with a light path of 5 cm (depicted in [45]) (see Supplemental Fig. 1A). The liquid volume was 1.1 L and the gas phase volume was 0.1 L (i.e., a gas:liquid ratio of 0.09). Cultures were mixed using either a stir bar or an impeller. The latter enhances the photosynthetic performance of algal cultures also in S deprivation [45]. In the beginning of the incubation (0 h) the headspace of the PBR including the tubing connecting the gas collection system were flushed with N_2 for 5 min. The rates of H_2 gas accumulation were measured using the water displacement method described by Melis et al. [10], i.e. recording the volume of gas that accumulated over a given time period (every 4 h, three times a day). Note that these measurements of determining H_2 production (termed "H_2 accumulation rates" in the following) differ from the small-scale tests of algae withdrawn from the PBRs and incubated in independent vessels described below (termed "in vivo H_2 production rates" in the following). Similarly, H_2 consumption rates were measured considering the amount of water drawn back in the gas-trap over a given time period (every 4 h, three times a day, except for specific time frames when it was recorded every 1 or 2 h), the latter being also evidence of a gas-tight collection system. The composition of the gas (H_2 and, if applicable, O_2) was analyzed by withdrawing gas samples from the headspace of the reactor and injecting them into a gas chromatograph (GC) (see below).

Experiments at a high gas: liquid phase ratio were conducted in 325 mL square glass bottles with a light path of 5 cm sealed with gas-tight rubber stoppers (Suba Seals® 37, Sigma-Aldrich, www.sigmaaldrich.com) (described in [25]) (see Supplemental Fig. 1B).