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Pathways of hydrogen photoproduction by immobilized *Chlamydomonas reinhardtii* cells deprived of sulfur

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ABSTRACT

The green algae *Chlamydomonas reinhardtii* entrapped in a thin alginate film have been shown to sustain elevated rates of hydrogen photoproduction under anaerobic incubation in sulfur/phosphorus depleted tris-acetate medium. In the present work we studied mechanisms, underlying hydrogen photoproduction by the immobilized culture, particularly, the roles of PSII and starch accumulation/breakdown. DCMU, a specific inhibitor of electron transport in PSII, is known to suppress hydrogen evolution by circa 80% in suspension cultures of S-deprived *C. reinhardtii*. In immobilized cells DCMU caused successive stimulatory and inhibitory effects on hydrogen photoproduction, both depending on the deprivation status of the algal cell. The inhibitory effect of DCMU was 25% at 70 h of S deficiency when maximal rates of hydrogen photoproduction were observed. Measurements of the light-induced prompt and delayed chlorophyll fluorescence transients and reflectance at 820 nm (P_{700} redox transitions) revealed very rapid decline of PSII activity in the entrapped S-deprived cells as compared with the suspension culture, whereas PSI suffered less. The immobilized culture showed a high capacity to accumulate starch during early stages of S deprivation and relatively high rates of anaerobic starch degradation during the following hydrogen evolution period. DCMU partly inhibited starch breakdown. Results of the present work brought us to the conclusion that PSII-independent pathway of hydrogen evolution is elevated in the immobilized S-deprived cells rather due to the rapid inactivation of PSII, efficient starch catabolism and non-photochemical PQ reduction.

Abbreviations: PS, photosystem; H_2ase, hydrogenase; S, sulfur; *C. reinhardtii*, *Chlamydomonas reinhardtii*; Chl, chlorophyll; PF, prompt fluorescence; DF, delayed fluorescence; MR, modulated reflectance at 820 nm; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; TAP, tris-acetate-phosphate medium; PPFD, photosynthetic photon flux density.

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Introduction

Hydrogen is considered as a promising clean fuel in future because its oxidation by molecular oxygen releases a lot of energy with water as the only by-product. Some green algae can use solar energy to perform the endergonic process of water biophotolysis, or generation of hydrogen and oxygen from water [1,2]. This process requires water splitting in photosystem (PS) II and photosynthetic electron flow via PS I to ferredoxin, an electron donor for many redox reactions in the chloroplast stroma, including molecular hydrogen synthesis; the latter is mediated by the highly efficient algal [FeFe]-hydrogenase (H_2ase) enzymes (reviewed in Refs. [3,4]). The activity of algal H_2ases is strongly repressed by molecular oxygen generated by PSII upon illumination. Therefore, hydrogen evolution in the light is normally a transient process observed shortly, e.g. after the transition of algal cells from the dark anaerobic conditions to the light. The problem of incompatibility between water splitting and H_2ase reaction has been overcome by exposure of green algae to nutrient deficiency stress [5,6]. Melis and co-authors [5] proposed a temporal separation of a photosynthetic (aerobic) stage of oxygen production from hydrogen synthesis (anaerobic) stage by incubating *Chlamydomonas reinhardtii* in S-depleted medium under constant illumination. Since in nutrient deprived cells the consumption of photosynthetic products is reduced due to the impaired cell division, starch is accumulated during the initial stage of S depletion. During the subsequent anaerobic period, energy stored in starch is released due to starch catabolism and used to sustain hydrogen evolution for several days. However, it has been established by using PSII inhibitors that up to 80% of hydrogen evolution is coupled to the residual water splitting activity in PSII, indicating that the major part of electrons for hydrogen synthesis originates from water [7,8]. The rate of hydrogen evolution is higher in strains with a higher residual PSII activity [9]. It is only the rest of electrons that is believed to inflow to the photosynthetic electron transport chain from the stromal reductants at the level of plastoquinones (PQs).

Efficiencies of light conversion to hydrogen in the suspension culture of S-deprived *C. reinhardtii* are only about 0.3%. Different approaches have been proposed to increase hydrogen production capacity (reviewed in Refs. [10,11]). One way to improve hydrogen photoproduction is to transfer algal cells from S deficient medium (hydrogen production conditions) to S replete medium (culture recovery conditions) and *vice versa*. Another way is to increase light absorption of algal cells at high cell concentration because restricted light absorption by deeper layers decreases the overall hydrogen yield. Cell immobilization techniques have been developed to alleviate these difficulties. Immobilization of *C. reinhardtii* on glass fibers [12] has proven to prolong hydrogen production. Furthermore, hydrogen photoproduction with an improved by several times light conversion efficiencies has been reported for S-deprived *C. reinhardtii* cells entrapped in Ca-alginate which is a non-toxic natural polymer [13]. However, the mechanisms by which immobilization improves hydrogen evolution have not been studied in detail.

The aim of the present work was to elucidate the relationships between hydrogen photoproduction, photosynthetic electron transport pathways, and starch biosynthesis/breakdown in immobilized *C. reinhardtii* cells incubated photoheterotrophically in S-depleted medium under constant illumination. In order to achieve this goal, we tested the effect of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) on hydrogen photoproduction, estimated starch and chlorophyll content at different stages of S deficiency, and probed photosynthetic reactions with a newly designed Multi-function Plant Efficiency Analyser (M-PEA-2). This instrument is designed to gain a comprehensive picture of primary photosynthetic events by simultaneous recording prompt and delayed fluorescence kinetics and reflectance at 820 nm at high temporal resolution. Here we introduce the results of the first attempt to evaluate photosynthetic characteristics of algal cells using M-PEA-2.

Methods

Strains and growth conditions

Stock cultures of *C. reinhardtii*, strains CC-124 mt^- and CC-4147 FUD7 mt^+ were pre-grown photoheterotrophically in 250 ml Erlenmeyer flasks, containing 50 ml of tris-acetate-phosphate (TAP) medium (pH 7.2) at 26 °C. The flasks were placed on a shaker (~100 rpm) and illuminated from the top with cool-white fluorescence lamps at photosynthetic photon flux density (PPFD) ~20 μmol photon m^{-2} s^{-1}. The stock cultures were maintained by weekly dilutions with fresh TAP medium. Before immobilization, algal cells of CC-124 strain were inoculated into 1.5-L flat glass bottles containing 700 ml of TAP medium. Thereafter algal cultures were grown photomixotrophically for three days at 26 °C under continuous illumination from one side at PPFD ~100 μmol photon m^{-2} s^{-1} of cool-white fluorescence light. During growth, the cultures were continuously bubbled with sterile 2% CO_2 in air using 0.2 μm pore-size membrane filters (Acro 37 TF, Gelman Sciences, Inc., Ann Arbor, MI). The CC-4147 cultures were grown under the same conditions, but low light intensity of around 40 μmol photon m^{-2} s^{-1} was applied.

Cell immobilization and hydrogen photoproduction experiments

Mature algal cultures (~20–25 μg Chl mL^{-1}) were washed once in TAP medium depleted of sulfur and collected by centrifugation at 2000 *g* for 3 min. Thereafter cells were immobilized in Ca^{2+}-alginate films as described before [13]. Shortly, films were prepared using a formulation ratio of 1 g of wet algal cell biomass, 0.5 ml Milli-Q water, and 1 ml 4% sodium alginate. The mixture was pipetted onto a template consisting of Scotch tape attached to plastic screen and drawn down by hand with a sterile plastic rod. The alginate films were polymerized by spraying a 50 mM $CaCl_2$ solution over the mixture. After fabrication, the alginate film strips (1 cm × 6 cm) were transferred into 75-ml glass vials containing 10 ml of tris-acetate medium without phosphate buffer, containing sulfur, (+S or