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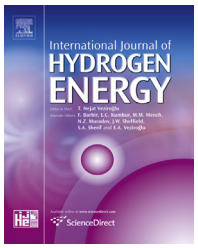

Metabolomics of photobiological hydrogen production induced by CCCP in *Chlamydomonas reinhardtii*

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ABSTRACT

The green alga *Chlamydomonas reinhardtii* can produce hydrogen gas (H_2) in the presence of the proton uncoupler carbonyl cyanide m-chlorophenyl hydrazone (CCCP). The addition of 15 μM CCCP to the algal cultures led to 13-fold increase in H_2 photoproduction compared to the control cultures without CCCP treatment. CCCP completely inhibited the photochemical activity of photosystem (PS) II under illumination. In order to better understand metabolic conditions necessary for sustained H_2 production, we have used gas chromatography coupled to time-of-flight mass spectrometry (GC-TOF) for metabolomics analysis that is independent of nutritional stress, specifically, sulfur deprivation, which had been used previously to induce H_2 photoproduction. Even 10 min after addition of CCCP, metabolites from many metabolic modules were found drastically decreased, including levels of free amino acids, unsaturated free fatty acids and nucleotides. During prolonged CCCP exposure H_2 production was found to be stable for at least 12 h with a continued increase in levels of free fatty acids. These results indicate that CCCP might become a useful treatment for production of biohydrogen in reactors. The increase in fatty acid production might then be a useful addition for production of carbon-derived biofuels.

1. Introduction

Biofuel from algae [1] (bioethanol [2], methane [3] and biohydrogen [4]) has the potential to exist as a sustainable energy resource through providing an economically viable and low emissions fuel source. Among biofuels, hydrogen (H_2) can serve as an ideal transportation fuel because it is the cleanest energy source and releases a large amount of energy per unit mass [5]. Unicellular green algae, especially *Chlamydomonas reinhardtii*, a soil-dwelling microalgae, can use sunlight to produce hydrogen upon illumination conditions [6].

However, when exposed to light, hydrogen production by green algae is a temporary phenomenon because the reversible hydrogenase becomes inactive when exposed to oxygen;

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even partial pressures 2% oxygen can inactivate hydrogenase [7]. Therefore, anoxia is an essential condition for hydrogen production. A striking breakthrough in photobiological hydrogen production by *C. reinhardtii* was developed by Melis et al. [8,9] via proposing an indirect two-stage approach based on sulfur (S) deprivation for long-term H_2 production. Under sulfur deprivation conditions, the Photosystem II (PS II) activity of *C. reinhardtii* decreases and causes relatively high respiration rates, resulting in the cultures to become anaerobic. Hydrogenase can then be induced to catalyze hydrogen production [8]. In the algae, two pathways provide electrons and protons to hydrogenase in H_2 photoproduction: PS II-dependent and -independent pathways [10,11]. The first of the photoproduction pathways is achieved by the reversible hydrogenase depending on electrons from water photolysis by PS II. Electrons then transfer to an [Fe–Fe]-hydrogenase active site through the electron transport chain [5,10]. For the PS II-independent pathway, the electrons originate from the degradation of endogenous substrate, such as starch [4,5,12,13].

Carbonyl cyanide m-chlorophenyl hydrazone (CCCP), a protonophore uncoupler of photophosphorylation, has been used for photobiological hydrogen production in green alga *C. reinhardtii* [14], *Tetraselmis subcordiformis* [15,16], and *Platymonas helgolandica var. tsingtaoensis* [11]. CCCP can decrease oxygen production by inhibiting the PS II activity without affecting mitochondrial oxidative respiration severely. This reaction resulted in anaerobic condition and hydrogenase expression in the algal cultures. CCCP can also increase dissipation of the proton gradient across thylakoid membrane to enhance H_2 production, because hydrogenase and ferredoxin are located in chloroplast stroma in algae [17,18]. It was also reported that there had been considerable H_2 production even in the PS II-deficient mutant of *Chlamydomonas* FUD7 by addition of the uncoupler carbonyl cyanide p-trifluoromethoxyphenyl hydrazone (FCCP). This production meant PS II-independent electron transport activity is limited by a trans-thylakoid proton gradient [19].

The metabolism of *C. reinhardtii* during sulfur deprivation-induced H_2 production has been studied [20,21], concluding that the decrease of H_2 production at prolonged time points was not due to lack of energy reserves but might be due to either accumulation of other metabolites or simple lack of sulfur reserves for protein biosynthesis. We therefore studied the metabolism of *C. reinhardtii* under conditions of H_2 production in a comprehensive manner for thermostabile compounds using GC-TOF mass spectrometry [22]. Uncoupling H_2 production from stress conditions concomitant with metabolomic analysis might help to elucidate mechanisms of substrate supply to hydrogenase during CCCP-induced H_2 production and be propitious to conduct further metabolic engineering approaches.

2. Material and methods

2.1. Alga strain and cultivation

The *C. reinhardtii* strain CC-124 was used for this study. The alga was cultivated in tris acetate phosphate (TAP) medium [23] at 23 °C under constant illumination with cool white fluorescent bulbs at a fluorescence rate of 60 μmol m^{-2} s^{-1} and with continuous shaking.

2.2. H_2 photoproduction procedure

Cells at the mid-log phase (2–5 × 10^6 cells/mL) were harvested by centrifugation (4000× *g* for 5 min), and resuspended in fresh TAP medium to a final concentration of around 6–10 μg Chl/mL. Approximately 120 mL of algal cell suspensions were placed in 130 mL anaerobic jars sealed with butyl rubber plugs and retroflexed stoppers. The algal cells were first flushed with pure N_2 for 10 min. CCCP was then added to a final concentration of 15 μmol/L. After 10 min of induction in the dark, the culture was placed under 150 μmol photons/(m^2·s) illumination with magnetic stirring at 25 °C for 12 h. The amount of H_2 produced by the culture was measured by a gas chromatograph equipped with a 5 Å molecular sieve column and a thermal conductivity detector.

2.3. Harvest of samples for metabolomics analysis

During the hydrogen production period, six replicates of 1 mL sample of algae with CCCP treatment, and the control without CCCP treatment, were harvested at 0 h (10 min after by addition of CCCP), 3 h, 6 h, 9 h and 12 h, respectively. Sample preparation for the metabolomics analysis was similar to previous published studies [24–26]. 1 mL cell suspensions were injected into 1 mL of −70 °C cold quenching solution composed of 70% methanol in water using a thermo block above dry ice. The pellet was flash frozen in liquid nitrogen and lyophilized at −50 °C in a 2 mL round bottom Eppendorf tube.

Lyophilized cells were disrupted using the ball mill MM 301 (Retsch GmbH & Co., Germany) with a single 5 mm i.d. steel ball concomitant with 0.5 mL extraction solvent Methanol:-chloroform:water (10:3:1). Solvent ratios are given as volumetric measures. Solvent was degassed by directing a gentle stream of nitrogen through the solvent for 5 min and used pre-chilled to −20 °C prior to extraction. After 3 min centrifugation at 16,100 rcf, the supernatants were removed, followed by a secondary extraction step using an additional 800 μL extraction solvent, centrifugation and adding the supernatant to the first aliquot. Samples were dried in a vacuum concentrator and kept at −80 °C before derivatization by methoximation and trimethylsilylation and subsequent metabolomic data acquisition by GC-TOF mass spectrometry.

2.4. Chlorophyll (Chl) fluorescence measurements

Chlorophyll fluorescence measurements were performed out using a Dual-PAM-100 measuring system. Following 10 min of adaptation in the dark, 1 mL of algal cell suspension was placed in the measurement chamber. The ratio of variable to maximal fluorescence (Fv/Fm) was then measured as an indicator of photosynthetic efficiency of PS II. The fluorescence parameters are defined as follows: Fv, variable chlorophyll fluorescence; and Fm, maximum chlorophyll fluorescence.

2.5. Determination of chlorophyll content and pH

Pigments were extracted from the algal cells with 95% ethanol. The ethanol extract was subjected to the spectrophotometric determination of Chlorophyll a and b contents. The extracellular pH of the cell cultures was measured using a pH meter.

2.6. Sample derivatization

A mixture of internal retention index (RI) markers were prepared using fatty acid methyl esters of C8 to C30 carbon chain lengths that dissolved in chloroform at a concentration of 0.8 mg mL^{-1} (C8–C16) and 0.4 mg mL^{-1} (C18–C30). 10 μL of solution, consisting of 40 mg mL^{-1} of 98% pure methoxyamine hydrochloride (Sigma, St. Louis, MO) in pyridine (Acros Organics), was added to the dried algal extracts and shaken at 30 °C for 90 min to protect aldehyde and ketone groups. 10 μL of FAME marker and vortex was added to 1 mL of MSTFA (Aldrich) for 10S. Afterward, 91 μL of MSTFA and FAME mixture were added, for trimethylsilylation of acidic protons, and shaken at 37 °C for 30 min. Reaction mixtures were transferred to 2 mL clear glass autosampler vials, with microinserts, (Agilent, Santa Clara, CA) and closed by 11 mm T/S/T crimp caps (MicroLiter, Suwanee, GA).

2.7. GC-TOF measurement

The GC–MS analysis was performed using an Agilent 6890 gas chromatograph (Santa Clara, CA) with a LECO Pegasus IV time-of-flight (TOF) mass spectrometer (St. Joseph, MI), and was controlled by the LECO ChromaTOF software (St. Joseph, MI). A 30 m long, 0.25 mm i.d. Rtx-5Sil MS column with 0.25 μm 95% dimethyl 5% diphenylpolysiloxane film was used (Restek, Bellefonte, PA). Helium gas acted as a carrier gas with a constant flow of 1 mL/min.

0.5 μL derivatized sample was injected at splitless mode by a Multipurpose Sampler MPS2 (Gerstel, MD). The temperature program was started after a solvent delay of 350 s at 50 °C for 1 min. The temperature was ramped to 330 °C at 20 °C min^{-1}, and then held at 330 °C for 5 min. The ionization source temperature was set to 250 °C. The inlet temperature was set to 250 °C, and the transfer line temperature from the GC to mass spectrometer was set to 280 °C. Ions were generated by −70 eV electrons and detected under full scan monitoring mode, in the mass range of 85–500 *m/z* at 17 spectra s^{-1} and 1750 V detector voltage, without turning on the mass defect option.

2.8. Data analysis

ChromaTOF was used for data pre-processing. ChromaTOF settings were: no smoothing, 3 s peak width, baseline subtraction just above the noise level, and automatic mass spectral deconvolution and peak detection at signal/noise (s/n) levels of 5:1 throughout the chromatogram. Result files were exported to a data server with absolute spectra intensities and further processed by the BinBase algorithm [27]. As a result, 252 chromatographic peaks could be detected with 100 compounds being successfully identified (Supplement 1).

Statistical analyses were performed by the Statistica9 (StatSoft, Tulsa, OK). Univariate statistics for multiple study design classes was performed by breakdown and one-way ANOVA. F-statistics and *p*-values were generated for all metabolites. Data distributions were displayed by box-whisker plats, giving the arithmetic mean value for each category, the standard error as box, and whiskers for 1.96 times the category standard error to indicate the 95% confidence intervals, assuming normal distributions.

3. Results and discussion

3.1. Increased hydrogen production in CCCP-treated C. reinhardtii

Previous studies [11,14–16] have shown that H_2 photoproduction in various green algae could be significantly increased due to the addition of CCCP in algal culture. Similarly, in the present study, we show that CCCP-induced H_2 production in *C. reinhardtii* (Fig. 1A). A maximum H_2 yield of 17.8 μmol/mg Chl was obtained from CCCP-treated *C. reinhardtii* cells at 6 h and hydrogen can be produced continuously for 12 h. H_2 production increased quickly within 3 h leading to a 13-fold increase, compared to the control, and kept constant from 3 h to 12 h. The chlorophyll content was decreased from 7.55 to 6.90 μg/mL in CCCP-treated cultures; in contrast, in control cultures the Chl content was increased from 7.62 to 13.52 μg/mL at 9 h and remained around 13 μg/mL until 12 h (Fig. 1B). These findings reflect the growth in overall algae biomass under control conditions whereas CCCP-treated cultures lack ATP-supply and therefore stop cell division. This decrease in chlorophyll contents has also previously been observed [11]. The pH of control cultures increased from an initial pH 7.1 to a maximum pH 7.8, whereas the pH of CCCP-treated cultures remained at nearly 7.0 during H_2 production (Fig. 1C), indicating that the stop in biomass production is unrelated to potential accumulation of acids, unlike observed under sulfur deprivation [20]. Conversely, the increase in pH in control cultures can be interpreted as due to photosynthetic consumption of dissolved CO_2 and the utilization of organic acids [28]. CCCP is a proton gradient uncoupler, inactivating the water-splitting enzyme system and therefore the overall activity of PS II, including oxygen production [14]. Lack of oxygen is a prerequisite for hydrogen production [11,14], and consequently, CCCP enables the reduction in PS II photochemical activity (Fig. 1D) by measuring the ratio of Fv/Fm [29]. In our cultures, PS II photochemical activity decreased rapidly from 0.23 to 0 for CCCP-treated cultures within 3 h (Fig. 1D). Unlike to a lowered PS II activity in the marine green algae *Tetraselmis subcordiformis* under CCCP treatment [15,16], we found the PS II activity in *C. reinhardtii* to be completely inhibited. PS II activity remained at a high level (~0.6 Fv/Fm ratio) in the control cultures under illumination for 12 h. This result indicated that the electrons and protons for H_2 production did not originate from the water splitting in PS II. Therefore, we hypothesized that the electrons and protons originated in catabolism of endogenous metabolites.

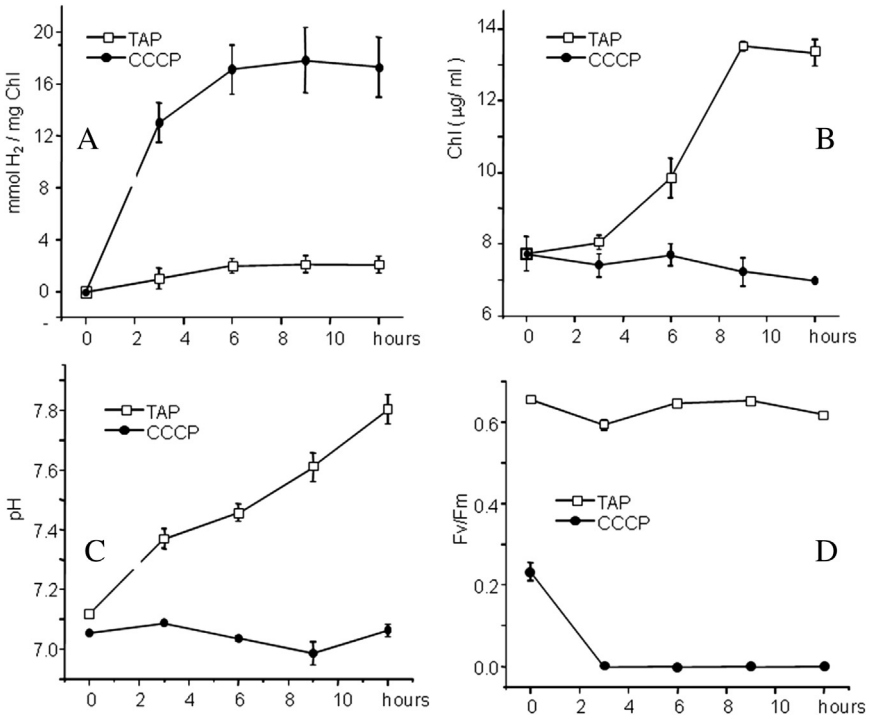

Fig. 1 – Physiological changes in *C. reinhardtii* during CCCP-induced H_2 production. The values are averages ± SD (standard deviation) for six replicate culture flasks. A: Time course of H_2 production by *C. reinhardtii* with and without CCCP treatment under 150 μmol photons/(m^2 s) illumination. B: Effect of CCCP on PS II photochemical activity. C: Time course of pH in cell cultures. D: Chlorophyll contents of algal cells during H_2 production phase.

3.2. *Glycolytic pathways are blocked by CCCP treatment*

Using the metabolomics data of 100 structurally identified compounds, we investigated the impact of CCCP treatment on algal metabolism. Partial least square multivariate statistics showed that metabolic phenotypes of the algae cultures showed the greatest differences at the 12 h time point when comparing the CCCP to TAP-control cultures (Supplement 2), but that for both cultures, clear metabolic differences were observed along the 0 h to 12 h time trajectories. Use of ChlamyCyc [30] and MetaMapp [31] enabled a classification of metabolites on pathway and metabolic module level (Supplement 3). These overall metabolic differences are mechanistically interpreted as given below.

As proton uncoupler, CCCP directly inhibits the activity of ATP synthase [32,33] (Fig. 2), consequently also affecting the use of NADH and $FADH_2$ and the oxidation of metabolic substrates [32]. Our results show that both adenosine and adenosine-5-phosphate (AMP) are rapidly decreased under CCCP treatment (Fig. 2). Since adenosine kinase activity requires ATP, we conclude that CCCP not only inhibits ATP production by uncoupling the proton gradient but also by decreasing the amount of metabolic precursors adenosine and AMP. Even without a functional PS II system, electrons and protons are needed for hydrogen production. We have therefore used metabolomics to assess differences in metabolic substrates under CCCP treatment.

Under CCCP treatment, glycolytic intermediates, mainly hexose phosphates (Fig. 3), were found rapidly decreased. This decrease is likely due to the lack of initiating ATP, needed in the first step of phosphorylation of glucose by hexokinase or glucokinase to form glucose-6-phosphate. Algae can utilize surplus carbon assimilation in form of starch [34] which might subsequently be utilized for energy production. Starch degradation leads to intermediate formation of maltose and subsequently, glucose reservoirs that are independent from photosynthesis. At 12 h of treatment, maltose and glucose as well as the intermediate glucose-1-phosphate were found to be drastically reduced as well (Fig. 3). We conclude that carbohydrate pools and glycolysis can be disregarded as source of reductive energy for hydrogen production under CCCP treatment of algae.

3.3. *TCA cycle is maintained under CCCP treatment*

The largely decreased levels of carbohydrates and the lower activity of glycolysis reduced the overall carbon flux into the TCA cycle, using pyruvate dehydrogenase to generate acetyl-CoA. Surprisingly, however, the levels of TCA cycle intermediates in CCCP-treated cultures including succinate, fumarate, and malate (Fig. 4), were similar during all time points while citric acid remained below detection limits. The levels of the TCA intermediates might therefore be replenished by anaplerotic reactions from other carbon sources, either from protein degradation and release of amino acids, or from acetate, which is found plentiful in our culture TAP medium. Because the TCA cycle does not depend on ATP levels, CCCP treatment cannot block this cycle and its

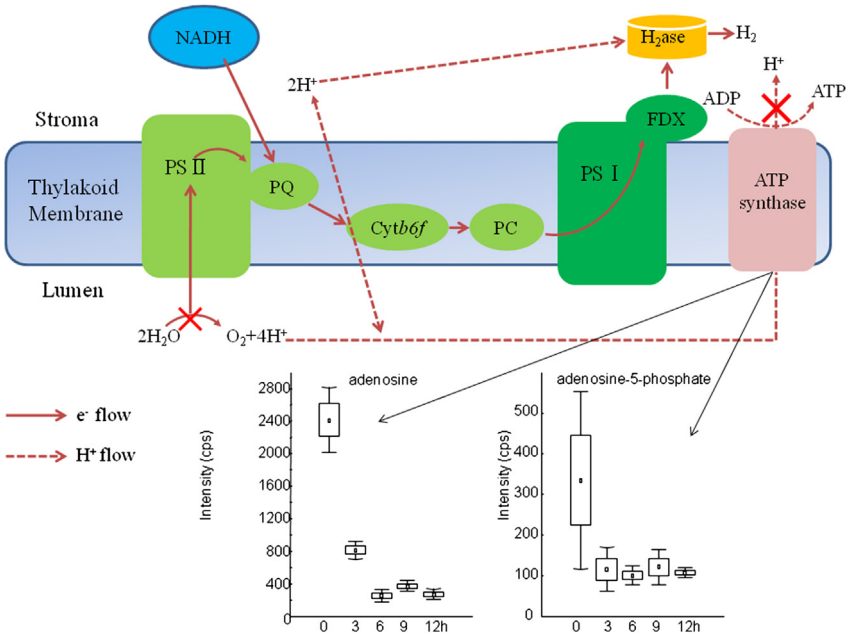

Fig. 2 – Illustrate of hydrogen production process induced by CCCP in *C. reinhardtii*. Effect of CCCP on PS II and ATP synthesis was labeled with ×. Changes of ATP precursors, adenosine, adenosine-5-phosphate, were also shown in this figure. PQ = plastoquinone; Cyt*b6f* = cytochrome *b6f*; PC = plastocyanine; FD = ferredoxin.

substrates could potentially be used to produce reducing equivalents (NADH and $FADH_2$). More detailed flux studies would be required to confirm the source of carbon in the maintained steady-state levels of TCA cycle intermediates that we observed here, and its implication as potential source of substrates for production of electron equivalents and H_2 production under anaerobic condition.

As we mentioned in the introduction, there are two pathways that can be used to provide electrons to hydrogenase in H_2 photoproduction: PS II-dependent and -independent pathways. It was previously reported that starch accumulates during the first 24 h after transfer into sulfur-depleted medium, and subsequently appears to serve as a substrate source for H_2 production [20,21]. However, CCCP-induced hydrogen production depends on non-carbohydrates substrates. Since PS II is inhibited by CCCP, starch was not produced by algae; additionally, no electron was transferred from water to hydrogenase enzyme. Since wild type *C. reinhardtii* only has

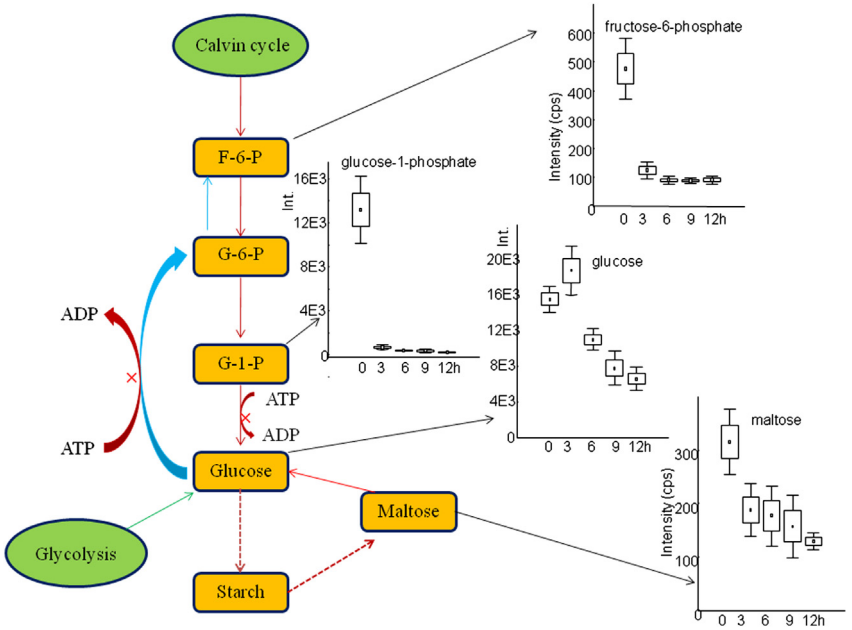

Fig. 3 – Effect of CCCP on glycolysis and starch biosynthesis pathways in *C. reinhardtii* during CCCP-induced H_2 production. F-6-P: fructose-6-phosphate; G-6-p: glucose-6-phosphate; G-1-P: glucose-1-phosphate.

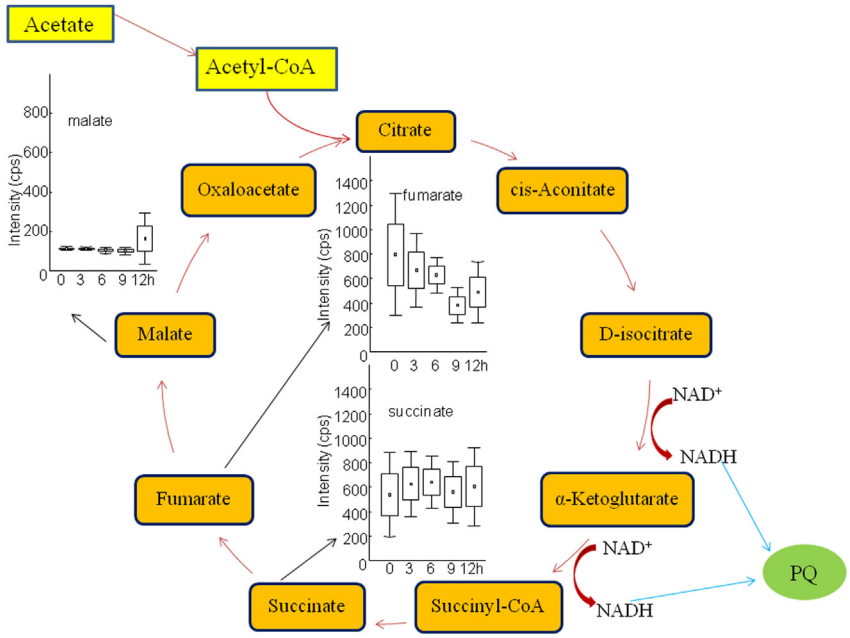

Fig. 4 – Effect of CCCP on TCA cycle in *C. reinhardtii* during CCCP-induced H_2 production. Time course of succinate, fumarate and malate showed no significant changes in TCA cycle intermediates during the H_2 production phase.

negligible amount of starch [35], our CCCP treatment approach to use non-sugar substrates can be very efficient for hydrogen production by *C. reinhardtii*. Non-sugars substrates can be utilized for the production of various biofuel contents [36]. It has been reported that H_2 production also occurs in the absence of PS II, which may involve the existence of a non-photochemical reduction pathway of plastoquinone (PQ) [37,38]. In this pathway, NADH or NADPH is transferred to thylakoid membranes in *C. reinhardtii*, thereafter, via PS I and the chloroplast ferredoxin to the hydrogenase (Fig. 2). Based on these findings, Matthew et al. [20] proposed the hypothesis that blocking NAD(P)H oxidation can increase H_2 production. Our results support the hypothesis that TCA cycle generated NAD(P)H are transferred to PQ pool, cytochrome *b6f* (Cyt*b6f*), plastocyanine (PC), and then, via PS I, from chloroplast ferredoxin to the hydrogenase to induce H_2 production. The TCA generated NADH/$FADH_2$ are major electron sources during CCCP-induced H_2 production (Fig. 2). It is further supported by low activity of glycolysis in algae.

3.4. *Increased production of fatty acids by CCCP-treated* C. reinhardtii

In stressful conditions, fatty acid biosynthesis is up-regulated [26,39] which has also been reported for sulfur deprivation [20,21] before H_2 production, but not during generation of

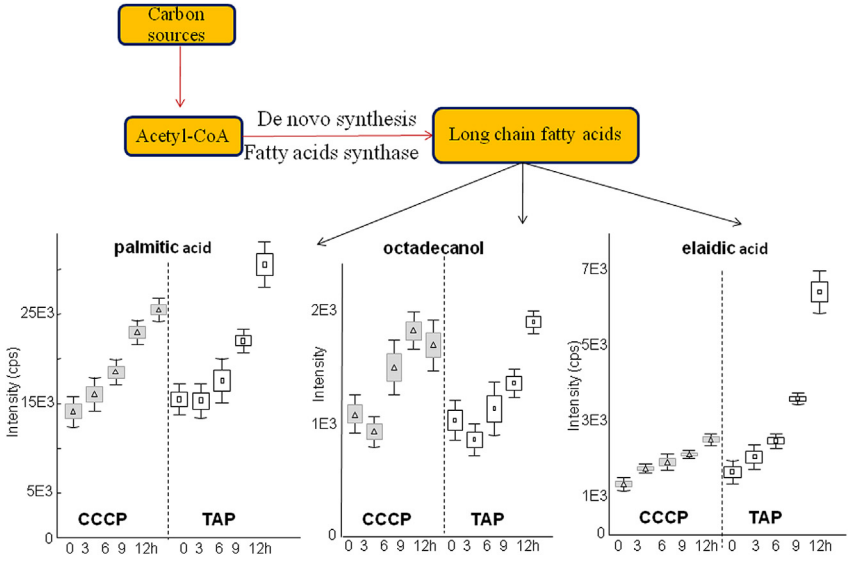

Fig. 5 – Box plots of free fatty acid products comparing CCCP-induced conditions (gray boxes) with TAP-control conditions.

hydrogen. We here find that under continued CCCP treatment and even under sustained production of hydrogen, we also see an increase in levels of free fatty acids, including palmitic acid, oleic acid and elaidic acid (Fig. 5). Acetyl-CoA as immediate precursor for fatty acid production could either be generated through glycolysis, through citrate lyase or through activation of acetate import from the media. While only stable-isotope based flux studies could reveal the contributions of each pathway, the immediate decrease of citric acid levels below the detection limit might hint towards higher activity of citrate lyase, which had been reported before for mammalian cell cultures under anaerobic condition [40]. Reducing power for fatty acid biosynthesis (NADPH) might be attributed to malate dehydrogenase activity.

3.5. *Amino acid metabolism of* C. reinhardtii *during CCCP-induced H_2 production*

One of the most dramatic changes in overall metabolic phenotypes in CCCP-treated cells was a drastic and immediate decrease in the levels of most amino acids including alanine, serine, valine, tyrosine, leucine, isoleucine, phenylalanine, threonine, glycine, lysine, methionine, ornithine and glutamine (Fig. 6). Even at 10 min after addition of CCCP in algal cultures, the content of amino acids had been decreased in CCCP-treated cells compared to the control (Supplemental 3). These findings can be interpreted in two ways. First, the blockage of glycolysis may have resulted in a shortage of starting substrates for amino acid synthesis. However, precursors for most amino acids are actually coming from TCA intermediates which we did not find to be reduced. We rather interpret this finding in the opposite manner: Low levels of ATP specifically lead to an arrest of the cell cycle, concomitant with less need for protein biosynthesis. In order to maintain flux towards the TCA cycle, protein breakdown would deliver precursors for oxidative deamination, delivering carbon backbones towards the TCA cycle into oxaloacetate and alpha-ketoglutarate. Our finding of lowered levels of amino acids would indicate a high need of these precursors for maintaining the TCA flux in addition to lowered need for protein biosynthesis. Overall, however, the role of amino acid levels in algal H_2 production is unclear yet [20] and would also require further flux analysis.

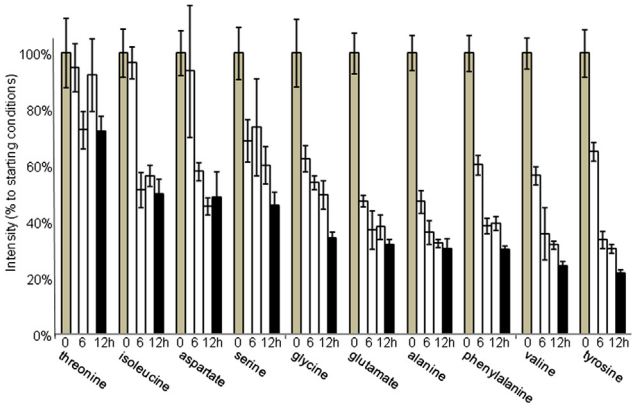

Fig. 6 – Changes of the top-10 most abundant amino acids in *C. reinhardtii* during H_2 production phase.

4. Conclusion

C. reinhardtii can produce H_2 in the presence of CCCP. We investigated metabolic changes along with induction of H_2 production under CCCP treatment in order to avoid pleiotropic metabolic changes that are merely due to general nutritional starvation. We found that in *C. reinhardtii*, the photochemical activity of PS II was completely inhibited in the presence of CCCP under illumination. These results indicated that the electrons for H_2 production did not come from the water splitting in PS II, but from the oxidation of endogenous substrate. We characterized the metabolic profiles of *C. reinhardtii* during different time points in the H_2 production process, induced by CCCP, to identify potential crucial components for the mechanism of substrate supply to hydrogenase. Using a metabolomics approach, we found large shifts in carbohydrate, fatty acid, amino acid and nucleotide metabolism (adenosine, AMP and guanosine) in the CCCP-treated algae. Specifically, glycolysis and starch biosynthesis was rapidly blocked while the TCA cycle was not affected with CCCP treatment, and remained functional throughout H_2 production process. Functional TCA cycle was probably a major electron source to H_2 production and also a reason of elevation in fatty acids. These findings can provide a new and detailed picture of H_2 production in algae that are propitious to conduct further metabolic engineering approaches to improve H_2 production. We also showed an increase in fatty acid biosynthesis that might add economic value in case such process would be used for production of biofuel. We utilized batch culture techniques, but if the cultures are grown continuously, it is possible that a large amount of substrates can be converted into hydrogen by the catabolic pathways of *C. reinhardtii*. The metabolic organization inferred in this study laid the foundations for innovative metabolic engineering of algal species in the hope of producing non-conventional energy on a large scale.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijhydene.2013.09.116.

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