Ascorbate accumulation during sulphur deprivation and its effects on photosystem II activity and H_2 production of the green alga *Chlamydomonas reinhardtii*

Running title: Ascorbate modulates photobiological H_2 production

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Summary

In nature, H_2 production in *Chlamydomonas reinhardtii* serves as a safety valve during the induction of photosynthesis in anoxia and it prevents the over-reduction of the photosynthetic electron transport chain. Sulphur deprivation of *C. reinhardtii* also triggers a complex metabolic response resulting in the induction of various stress-related genes, downregulation of photosynthesis, the establishment of anaerobiosis and expression of active hydrogenase. Photosystem II (PSII) plays dual role in H_2 production because it supplies electrons but the evolved O_2 inhibits the hydrogenase. Here we show that upon sulphur deprivation the ascorbate content in *C. reinhardtii* increases about 100-fold, reaching the mM range; at this concentration ascorbate inactivates the Mn-cluster of PSII and afterwards it can donate electrons to tyrozin Z^+ at a slow rate. This stage is followed by donor-side induced photoinhibition, leading to the loss of charge separation activity in PSII and reaction center degradation. The time point at which maximum ascorbate concentration is reached in the cell is critical for the establishment of anaerobiosis and initiation of H_2 production. We also show that ascorbate influenced H_2 evolution via altering the photosynthetic electron transport rather than hydrogenase activity and starch degradation.

Keywords: ascorbate, *Chlamydomonas reinhardtii*, hydrogenase, oxygen evolution, photosystem II, sulphur deprivation

Summary statement

Sulphur deprivation of sealed *Chlamydomonas reinhardtii* cultures results in the downregulation of photosynthesis, establishment of anaerobiosis and expression of hydrogenases. Photosystem II has a determining role in H_2 production because it supplies electrons but the evolved O_2 inhibits the hydrogenase. Here we show that upon sulphur deprivation the ascorbate content in *C. reinhardtii* increases dramatically and in the mM range it inactivates the oxygen-evolving complex. Therefore, we propose that photosystem II inactivation upon sulphur deprivation is initiated by a strong ascorbate accumulation and it occurs via donor-side induced photoinhibition.

Introduction

Solar energy-driven H_2 production by photosynthetic microorganisms may become an alternative method to complement the proposed chemical technologies to produce H_2 gas. For research on the photoproduction of H_2, the unicellular green alga *Chlamydomonas reinhardtii* is one of the most popular organisms. *C. reinhardtii* has two [Fe-Fe]-type hydrogenases, called HYDA1 and HYDA2. They are located in the chloroplasts stroma and receive electrons from photosystem I (PSI) via ferredoxin. H_2 production is considered an evolutionary relic that may serve e.g. under the induction of photosynthesis in anoxia as a safety valve fine tuning the ATP to NADPH ratio and accelerating the light-induced increase in stromal pH that triggers activation of CO_2 fixing reactions; by this means the risk of over-reduction of the electron transport chain and photodamage is decreased (Ghysels et al., 2013, Godaux et al., in press).

The hydrogenases of *C. reinhardtii* are highly efficient; their turnover rate is several thousands per second, about 100-fold higher than that of other types of hydrogenases (Rousset and Liebgott 2014). However, in the presence of O_2, hydrogenase expression is inhibited (Eivazova and Markov 2012) and O_2 also reacts with the 2Fe subcluster of HYDA1, leading to its degradation and leaving an inactive [4Fe-4S] subcluster state (Swanson et al., 2015). There are attempts to engineer the hydrogenase enzyme to reduce its sensitivity to O_2 (e.g. King et al., 2009). However, a constitutively high expression of an O_2-resistant enzyme may be potentially disadvantageous because it could compete with the Calvin-Benson cycle and as a result, it could lead to a decreased autotrophic growth. As a matter of fact, even when the native HYDA1 was expressed in the chloroplast of *C. reinhardti*, slower growth and increased stress sensitivity was observed (Reifschneider-Wegner et al., 2014).

In normal, actively photosynthesizing cultures of *C. reinhardtii* H_2 production occurs only transiently. H_2 production upon anaerobic induction, which consists of a relatively long dark-adaptation and a continuous illumination period, may last for a few hours (Degrenne et al., 2011). For maintaining H_2 production a balance between the O_2 evolved and respiration consuming O_2 needs to be established (Scoma et al., 2014). Alternatively, bacterial partners may be also used to eliminate the evolved O_2 in the medium (Lakatos et al., 2014), in a similar way as microbial community consumes the available O_2 in the soil.

There is also a strategy to decrease photosystem II (PSII) activity below a certain threshold value, under which the O_2 produced by PSII would not be inhibitory anymore for the hydrogenase. This can be achieved by photoinhibition (Markov et al., 2006) and inducible

gene expression to downregulate the expression of the reaction center protein PsbD (Surzicky et al. 2007). Sulphur deprivation, the most frequently used method to induce photobiological H_2 production (Melis et al., 2000, Zhang et al., 2002) is also supposed to act by a similar mechanism.

When *C. reinhardtii* cultures are deprived of sulphur, cells start to accumulate starch within a few hours, which is followed by the down-regulation of photosynthesis and the induction of the hydrogenases. The amount of Rubisco is strongly reduced during the first 24 h and photosynthetic electron transport is also inhibited, which is mostly associated with decrease in PSII activity (reviewed by Burgess et al., 2011, Torzillo et al., 2013). During sulphur deprivation cellular respiration is maintained or even increased, contributing to the establishment of anaerobiosis.

Metabolism of sugars derived from starch via glycolysis provides electrons to the PQ-pool via the plastidial type II NAD(P)H dehydrogenase (NDA2) complex and thereby supplies a significant amount of electrons for the expressed hydrogenase (Mignolet et al., 2012, Volgusheva et al., 2013). It was shown that by overexpressing NDA2, nonphotochemical reduction of the PQ-pool increased and the contribution of the indirect pathway to the H_2 production could be improved (Baltz et al., 2014). Increased H_2 production was achieved also in a *pgrl1* mutant with impaired PSI cyclic electron flow, which is in competition for electrons with the hydrogenase (Tolleter et al., 2011). Using truncated antenna mutants immobilized on alginate films to improve light utilization promoted the H_2 evolution as well (Kosourov et al., 2011).

The main sources of H_2 production are PSII activity and linear electron flow. Using *PsbA* mutants differing in their PSII activity, it has been demonstrated that the loss of PSII activity leads to a faster induction of anaerobiosis during sulphur deprivation, but less starch is accumulated and less H_2 is produced and below a certain PSII activity, there is no H_2-production observed (Makarova et al. 2007). The importance of the linear electron flow was more recently demonstrated by the *state transition mutant 6* (*Stm6*) mutant of *C. reinhardtii* subjected to sulphur deprivation (Volgusheva et al., 2013).

It has been proposed that the inactivation of PSII results from an imbalanced photoinhibition and repair of the PsbA protein due to the lack of sulphur (Zhang 2002). However, changes in gene expression occur within hours, and there is a very complex adaptation process to sulphur deprivation (González-Ballester et al., 2008, Toepel et al., 2013); therefore the question may be raised if it is directly the lack of sulphur that hinders the turnover of the PsbA protein, especially if we take into account that cells division is ceased

upon the transfer of *C. reinhardtii* to sulphur-free conditions (Zhang et al., 2002). Therefore, in this study we aimed at better understanding the mechanism by which PSII gets inactivated, which may be essential for the improvement of the energy conversion efficiency of this promising renewable energy source.

Materials and Methods

Algal strains

Six different *C. reinhardtii* strains were used in this work. S-01 is our laboratory strain, used earlier (Nagy et al., 2012; Corrigendum: Nagy et al., 2015). Besides, we used the strains CC124, CC125, CC400, CC409 and CC849, which were used in earlier studies as wild-type, obtained from the Chlamydomonas Resource Center (http://chlamycollection.org/).

Algal growth conditions

C. reinhardtii cultures were grown in Tris-acetate-phosphate (TAP) medium at a light intensity of 80-90 µmol photons m^{-2} s^{-1} and 24-25 ºC in an algal growth chamber. The 250 ml flasks containing 50 ml TAP medium were shaken at 120 rpm and the cultures were grown for three days in sulphur-containing medium. After three days of cultivation, the cells were washed five times with sulphur-free TAP medium (centrifugation at 1000 g, at 24 ºC for 5 min). For the H_2 production experiments the Chl content was set at 8 µg chl/ml (based on Porra et al., 1989) and 30 ml culture in sulphur-free TAP medium was placed into 125 ml serum vials and sealed off with rubber septa. All steps were carried out under sterile conditions. The gas phase of the bottle was flushed with N_2 gas for 10 min and the cultures were kept in the algal growth chamber, under the same conditions as indicated above. The Asc treatment (10 mM Na-Asc) was carried out at the start of sulphur deprivation (day 0).

Determination of H_2 and O_2 accumulation by gas chromatography (GC)

The daily amount of H_2 and O_2 accumulated by the cultures was determined by taking 500 µl aliquot from the gas phase of the cultures with a gas tight syringe. These samples were injected manually into an Agilent 6890 gas chromatograph equipped with a HP-Molesieve 5Å column (30 m x 0.53 mm x 25 µm) and a TCD detector. The oven temperature was 60 ºC. The carrier gas was argon, and a linear velocity of 115 cm/s was used. The bottle was flushed with N_2 gas daily after the determination of the gas accumulation.

Ascorbate content determination

At each time point, 6 ml of culture was collected, spun-down for removal of the supernatant (collected by centrifugation at 11,500 g, 24 ºC, for 1 min), washed twice with distilled water, and the Asc was extracted by re-suspending the cells in 200 µl of extraction buffer (2 mM EDTA, 5 mM DTT, 5 % orthophosphoric acid) and vigorously vortexing with glass beads (Sigma, 212-300 µm) for 30 s. This was followed by centrifugation at 11,500 g, 4 ºC, for 30 min and the supernatant was collected. Quantification of Asc was performed by HPLC using an Agilent 1100 Series HPLC system with a diode array detection unit (Agilent, Waldbronn, Germany) set to 245 nm. The Asc content was quantified using standards. For all separations, an YMC ODS-A 250 x 4.6 mm column was used with a particle size of 5 µm. The running was performed using 100% solvent A (50 mM KH_2PO_4, pH 2.5), with a flow rate of 1 ml/min; the column was kept at 30 ºC and the samples were stored at 4 ºC. The column was allowed to re-equilibrate in 60 % solvent A and 40 % solvent B (acetonitrile) for 15 min prior to the next run.

Thermoluminescence (TL) measurements

For TL measurements, cell suspension (300 µl, 8 µg Chl/ml) was placed on a copper sample holder, connected to a cold finger immersed in liquid N_2. A heater coil, placed under the sample holder, ensured the desired temperature of the sample during the measurement. Dark adapted samples were illuminated at 5 ºC by two saturating single-turnover flashes, and glow curves were recorded while heating the sample to 70 ºC in darkness with a heating rate of 20 ºC/min. The emitted TL was measured with a Hamamatsu end-window photomultiplier.

Fast Chl a fluorescence (OJIP) measurements

Chl *a* fluorescence measurements were carried out at room temperature with a Handy-PEA instrument (Hansatech Instruments Ltd, UK). *C. reinhardtii* cultures were dark-adapted for 15 min and then 5 ml of cell suspension (8 µg Chl/ml) was filtered onto a Whatman glass microfibre filter (GF/C) that was placed in a Handy-PEA leaf clip. The alga sample was illuminated with continuous red light (3500 µmol photons m^{-2} s^{-1}, 650 nm peak wavelength; the spectral half-width was 22 nm; the light emitted by the LEDs is cut off at 700 nm by a NIR short-pass filter). The light was provided by an array of three light-emitting diodes focused on a circle of 5 mm diameter of the sample surface. The first reliably measured point of the fluorescence transient is at 20 µs, which was taken as F_0. The length of the measurements was 5 s.

Measurement of oxygen evolution

Oxygen evolving capacity of *C. reinhardtii* cultures (8 µg Chl/ml) was measured with a Clark-type oxygen electrode (Hansatech Instruments Ltd, Norfolk, UK) in a temperature controlled cell at 25°C under saturating light intensities (1800 µmol photons m^{-2} s^{-1}) for 2 min. The measurements were carried out in the presence of 500 µM phenyl-p-benzoquinone (PPBQ) as an electron acceptor of PSII and the dark O_2 consumption was subtracted. Because Asc reduces PPBQ, the Asc-treated samples could not be reliably measured; therefore those data are not presented.

Western blot analysis

At each time point, 6 ml of culture were collected, spun-down for removal of the supernatant and frozen in liquid N_2. The samples were then solubilized with 500 µl of protein extraction buffer (50 mM Tris/HCl [pH 8.3], 0.25 % Triton X-100, 1 mM dithiothreitol and 1x cOmplete Protease Inhibitor Cocktail [Roche]), incubated in the dark at 4 ºC for 30 min with vigorous shaking, and then centrifuged at 20,800 g, 4 ºC, for 10 min. The supernatant was collected into a new Eppendorf tube and the protein content determined by the Bradford method. An amount equivalent to 1 or 2 µg protein was then mixed with 4x Laemmli buffer and incubated at 43 ºC for 30 min. Proteins separated by SDS-PAGE (Perfect Blue Twin Gel System, Peqlab) were transferred to a polyvinylidene difluoride membrane (Hybond P) using a tank blotting system (Cleaver Scientific Ltd). Specific polyclonal antibodies (produced in rabbits) against PsbA was purchased from Agrisera AB, and antibodies against the PSBO and ATPD proteins were obtained from AntiProt GmbH. As secondary antibody, an anti-rabbit IgG peroxidase conjugate was used (Sigma-Aldrich). Immunochemical detection was carried out with the ECL Prime System (GE Healthcare), according to the instructions of the manufacturer.

Measurement of the oxidation-reduction kinetics of P_{700}

The light-induced absorbance changes at 830 nm reflecting changes in the redox state of P_{700} and PC were recorded by a Dual-PAM-100 instrument (Heinz Walz GmbH, Germany) in a dual wavelength (860-810 nm) mode in continuous red light of about 2000 µmol photons m^{-2} s^{-1} and in the dark, with a time resolution of 1 ms. *C. reinhardtii* cells were dark-adapted for 15 min and then 5 ml of cell suspension (8 µg Chl/ml) was filtered onto a Whatman glass

microfibre filter (GF/C) that was placed in between two microscopy cover slips for the measurement of the 830 nm absorbance transient.

3-(3',4'-dichlorophenyl)-1,1-dimethylurea (DCMU) and dibromothymoquinone (DBMIB) were added about 15 min before the measurements. DCMU was dissolved in dimethyl sulfoxide (100 mM stock solution) and its final concentration was 20 µM; DMBIB was dissolved in ethanol (100 mM stock solution) and its final concentration was 5 µM.

Determination of starch content

1 ml *C. reinhardtii* culture was spun down (at 12,000 g, 4 min), and re-suspended twice in 1 ml methanol to solubilize the pigments. The sample was spun down again (at 12,000 g, 4 min) after washing it with 1 ml sodium acetate buffer (0.1 M, pH 4.5). The sample was then re-suspended in a mixture of sodium acetate buffer and glass beads (1:1), and submitted to a 4-min cycle in a Mini Bead Beater. A volume of 0.3 ml of the supernatant was incubated in a boiling water bath for 15 min, and after cooling, 3 U amyloglucosidase were added and the starch was hydrolysed overnight at 55 °C. Glucose in the sample was measured enzymatically with Fluitest® GLU kit (Analyticon® Biotechnologies AG).

In vitro hydrogenase activity assay

The 13.5-ml serum vials in which the assay was carried out contained 20 µl of 1 M oxidized methylviologen, 380 µl water, 1.5 ml 100 mM KH_2PO_4 buffer (pH 6.5) and 100 µl 10% Triton X100. 1.6-ml cell suspension samples were taken anaerobically from the batch samples and injected into sealed and N_2-purged vials. The reaction was started by the addition of 400 µl of anaerobic, 1 M Na-dithionite. The assay was performed at 37°C in darkness. H_2 was determined 4 times during the 90 minutes long assay (Kosorouv et al., 2003).

Statistics

The presented data are based on at least three independent experiments. When applicable, averages and standard errors (SE) were calculated. Statistical significance was analysed using Student's t-test and the significance level are presented as: * $p<0.05$; ** $p<0.01$;*** $p<0.001$ (in Fig. 1, analysis between the Asc-treated and untreated samples).

Results

The effects of externally provided Asc on the H_2 production in several C. reinhardtii strains

Previously we showed that during sulphur deprivation externally supplied Asc stimulated H_2 production, and anaerobiosis was reached earlier in the *C. reinhardtii* strain S-01 (Nagy et al., 2012, Nagy et al., 2015). To investigate whether it is a general phenomenon, six different *C. reinhardtii* strains, used in earlier studies on photobiological H_2 production, were treated with Asc and their H_2 production yields were compared.

Upon sulphur deprivation, similarly high H_2 production was achieved in the CC124 and CC125 strains, about 50 µl/ml in four days, which is in agreement with literature data (Fig. 1A, Ghirardi et al., 2000, Torzillo et al., 2009). In the other strains, namely in CC400 (Sun et al., 2013), S-01 (Nagy et al., 2012), CC409 (Torzillo et al., 2009) and CC849 (Wu et al. 2010), H_2 production was moderate, on average about 5 times less than in CC124 and CC125 after four days of sulphur deprivation (Fig. 1A).

The addition of 10 mM Asc at the beginning of sulphur deprivation led to an increase in H_2 production in the strain S-01 (Nagy et al., 2012). Similar enhancement was observed in CC849, and a moderate effect was detected in the CC400 and CC409 strains (Fig.1A). In the CC124 and CC125 strains externally supplied Asc had strong adverse effects on the photoproduction of H_2 (Fig. 1A). In general, the addition of 10 mM Asc led to similar H_2 production in all the strains: approximately 10 to 15 µl H_2 gas/ml culture was produced during the four days of sulphur deprivation. On the other hand, the amount of O_2 accumulated in the headspace of the serum bottles was strongly and equally reduced in all the strains upon the Asc treatment (Fig. 1B).

In terms of H_2 production, the largest differences were observed between CC124 and S-01; therefore we decided to focus on these two strains. In Fig. 2 the daily H_2 and O_2 accumulation of the two strains are shown until day 6, which includes the final, so-called termination phase of sulphur deprivation as well. In S-01 H_2 production peaked after four days (Fig. 2A), whereas the maximum was observed on days 2 and 3 in the case of CC124 (Fig. 2C). Fig. 2B shows that in the S-01 strain Asc enhanced the H_2 production during the first three days, whereas H_2 production was lower in the presence of Asc during the termination phase. Upon Asc treatment of the CC124 strain H_2 production was strongly decreased throughout the experiment, to the level of the Asc-treated S-01 strain (Figs. 2C and 2D). In the strain S-01 anaerobiosis was reached by day 5, whereas in the strain CC124 it was

reached much earlier, within 48 hrs (Figs. 2A and C). Upon Asc-treatment there was no detectable O_2 by the second day of sulphur deprivation in either strain (Figs. 2B and D).

Cellular Asc concentration during sulphur deprivation

Ascorbate has essential roles in cellular metabolism and stress defense (Tóth et al., 2013, Zhang 2013) and it acts mostly as a reductant. Under normal physiological conditions the Asc concentration of *C. reinhardtii* cells is approx. 100 times lower than in plant cells (Gest et al., 2013, Wheeler et al., 2015), but upon oxidative stress there is a rapid, several-fold increase in the Asc level (Urzica et al., 2012). Transcriptomic data show that the expression of various stress-related genes increase upon sulphur deprivation, just as well as the expression of the *VTC* gene (Toepel et al., 2013), encoding GDP-l-galactose phosphorylase, a central enzyme in Asc biosynthesis (Urzica et al., 2012).

Fig. 2 shows temporal Asc concentration profiles of the two strains. As a result of sulphur deprivation, there was a dramatic increase in Asc concentrations: in control, sulphur-replete S-01 cultures it is about 1.8 pmol/µg Chl, and after four days of sulphur deprivation it increases to about 100 pmol/µg Chl (approx. 1 mM, calculated by assuming a cell volume of 140 femtoliters, Urzica et al., 2012). In CC124 the increase is even stronger (from about 5.1 to 160 pmol/µg Chl, i.e. to approx. 3 mM) and this occurs within 48 hrs of sulphur deprivation.

Upon the addition of 10 mM Asc, the Asc content of cells increased steeply, and within 24 h, similar values were reached as in both strains (approx. 90 and 110 pmol/µg Chl in S-01 and CC124, respectively). These values were in the same range as those obtained for the Asc non-treated samples, but the maximal Asc concentration was reached earlier.

The very strong Asc accumulation during sulphur deprivation and the observation that externally supplied Asc promoted the establishment of anaerobiosis indicate that Asc may modulate photobiological H_2 production, either by i) affecting the activity of the oxygen-evolving complex (OEC) and thereby the photosynthetic electron transport, ii) the activity of the hydrogenase or iii) starch degradation. In the following sections, these various possibilities are explored.

The effects of Asc on PSII activity

Early *in vitro* studies on isolated higher plant PSII membranes demonstrated that Asc may over-reduce the Mn-cluster if the extrinsic proteins of the OEC are removed (Tamura et al., 1990). On the other hand, we showed that in sulphur-deprived *C. reinhardtii* cultures Asc can

act as an alternative electron donor of PSII (Nagy et al., 2012), similarly to heat stress conditions (Tóth et al., 2009). These earlier findings prompted us to thoroughly investigate the effect of Asc on PSII in *C. reinhardtii* under sulphur deprivation.

Figs. 4A and C show that the B thermoluminescence (TL) band, resulting from charge recombination between S_2/S_3 states of the OEC and Q_B^- (Ducruet and Vass 2009), gradually decreased in both strains and it was eliminated after 96 and 72 hrs of sulphur deprivation in the S-01 and the CC124 strains, respectively. Beside the decrease in amplitude, a slight shift to lower temperatures could be also observed, which is most likely due to the interaction of Asc with the S2 and possibly S0 and S1 states of the Mn-complex (Tamura et al., 1990). The loss of the B band indicated OEC inactivation, which was confirmed by O_2 evolution measurements using PPBQ as an electron acceptor (Suppl. Fig. 1). Upon the addition of 10 mM Asc the decrease of the B band became much faster and already after 2 hrs of incubation in the light, there was an approx. 70% reduction in its amplitude; after 8 hrs of sulphur depletion, the B band disappeared (Figs. 4B and D). The B band was shifted and its amplitude decreased also in sulphur-deprived cultures that were unsealed (Fig. 4E). Moreover, Asc addition reduced the intensity of the B band in sulphur-replete cultures as well, but it recovered after a few hours (Fig. 4F).

Fast Chl *a* fluorescence (OJIP) transients were used to gain further information on PSII activity. Upon strong illumination (in this case 3500 µmol photons m^{-2} s^{-1}) Chl *a* fluorescence rises from a basic F_0 level to a maximum, F_M, in about 300 ms. The OJ phase (0-3 ms) is called the photochemical phase because of its strong light-dependence, the JI phase (approximately 3-30 ms) parallels the reduction of the PQ-pool, and the IP phase (approximately 30-300 ms) is correlated with the reduction of ferredoxin in the presence of inactive ferredoxin:$NADP^+$ oxidoreductase (reviewed by Schansker et al., 2014). It is to be noted that in *C. reinhardtii* the I step is less pronounced as in higher plants (Fig. 5). During sulphur deprivation the F_0 and J levels increased, particularly in the CC124 strain, indicating a reduced PQ-pool (Tóth et al., 2006). Upon Asc treatment similar effects were observed, but at a later stage the F_M values also decreased and variable fluorescence (F_V) was lost, indicating PSII reaction center inactivation.

The comparison of the kinetics reveals that the decrease of the B band occurs later in the S-01 strain than in CC124 (Figs. 6A and C). On the other hand, the loss of the B band precedes significantly that of the F_V/F_M value, especially in the Asc-treated samples (Fig. 6). This suggests that the inactivation of the OEC occurs before the loss of charge separating activity of PSII. In the non-Asc-treated CC124 strain the time difference between the loss of

the B band and the F_V/F_M was less pronounced (Fig. 6C), but this may be due to the strongly reduced PQ-pool, as indicated by the particularly high F_0 and J values (Fig. 5).

The Chl contents were rather stable during the first three days of sulphur deprivation and after that it decreased by about 40% both in the S-01 and the CC124 strains. The decrease in Chl contents was slightly enhanced by the Asc treatment (Suppl. Fig. 2).

Western blot analysis showed that in the S-01 strain both PsbA and PSBO protein contents decreased slowly; by the 72nd h, both decreased to about 50% of the initial amount (Figs. 7A and B). In the CC124 strain the PsbA and PSBO showed some moderate reduction already by the 24th h, and less than 50% was detected by the 48th h (Figs. 7D and E). This faster losses of PsbA and PSBO are in agreement with the TL and Chl *a* fluorescence data (Figs. 4 to 6), showing that PSII activity was lost earlier in the CC124 strain. Interestingly, upon the Asc treatment, the losses of PsbA and PSBO proteins were slower in both strains (Figs. 7C and 7F).

In order to monitor electron transport through PSI, 830 nm transmission measurements, reflecting the redox state of P700 and PC (e.g. Klughammer and Schreiber, 1994) were carried out, after 72 h of sulphur deprivation. At this stage, the activity of the OEC is very low in both strains (Fig. 4), but the F_V/F_M is relatively high (Fig. 6) and the PsbA protein is still well-detectable in the S-01 strain, whereas in the CC124 strain the PsbA protein is lost (Fig. 7).

In the S-01 strain oxidation and partial re-reduction of PC and P700 can be observed in continuous red light (Fig. 8A). In the presence of DCMU, which displaces Q_B from its binding site in PSII, there is no re-reduction, showing that the electrons originated from PSII, either from the remaining OEC activity or from Asc as an alternative PSII donor (Tóth et al., 2009, Nagy et al., 2012). *In vitro* studies showed that Asc may be an electron donor to PSI as well, but this seems to be insignificant in intact plants (Tóth et al., 2009). In the presence of DBMIB, an artificial quinone, which inhibits the re-oxidation of PQH_2 molecules by the $cytb_6f$ complex, P700 oxidation occurred with very similar kinetics as with DCMU.

In the case of CC124, a more complete oxidation of P700 and PC was observed, which was only moderately affected by DCMU or DBMIB (Fig. 8C). This is in agreement with the TL, Chl *a* fluorescence and western blot data, showing that PSII reaction centers are mostly inactive at 72 hrs of sulphur deprivation (Figs. 4 to 7) and therefore Asc could not donate significant amounts of electrons to PSII in the CC124 strain after 72 h of sulphur deprivation.

After the red light illumination, the re-reduction of PC^+ and $P700^+$ was followed in the dark. Fig. 8B shows that it is DCMU- and DBMIB-sensitive in the S-01 strain confirming

that electrons at PSI arrived mostly from PSII and possibly from Asc as an alternative electron donor (Nagy et al., 2012). However, in the CC124 strain the rate of re-reduction was mostly independent of the presence of DCMU, while it was remarkably decelerated in the DBMIB-treated samples (Fig. 8D). These results confirm that in CC124 after 72 h of sulphur deprivation, PSII was mostly inactive and electrons arrived at PSI via alternative routes, e.g. from starch degradation and perhaps PSI cyclic electron flow. It is to be noted that depending on its concentration, DBMIB can donate electrons directly to PC^+ and $P700^+$ (Schansker et al., 2005), which may explain why there is a slow re-reduction upon the DBMIB-treatment in the dark.

Starch accumulation and degradation during sulphur deprivation

It is well established that at the beginning of sulphur deprivation starch rapidly accumulates and later starch degradation will contribute significantly to the H_2 production and to the maintenance of anaerobiosis (Zhang et al., 2002). Fig. 9 shows that the amount of accumulated starch was about 25% lower in the S-01 strain than in CC124 after 24 hrs of sulphur deprivation. The amount of starch is about 15% lower in the Asc-treated samples both in the S-01 and the CC124 strains, which may be explained by the inhibition of OEC activity during the first 24 h (Fig. 5).

The rate of starch degradation is much lower in the S-01 strain than in CC124: by the fourth day of sulphur deprivation only approximately 10% is degraded in S-01, whereas in CC124 all the accumulated starch is consumed (Fig. 9). In S-01 starch consumption was increased by Asc during the termination phase, i.e. from day 4 to 6, whereas in CC124, starch degradation rates were very similar in the presence or absence of externally added Asc during the entire experiment.

The effects of Asc on in vitro hydrogenase activity

In order to test if Asc directly affects the activity or the amount of the hydrogenase enzyme accumulated, *in vitro* H_2 production measurements were carried out. H_2 production peaks on day 2 in the strain CC124; in the strain S-01 H_2 production is yet moderate and there is significant O_2 accumulation. The data of Suppl. Fig. 3 show that the H_2 producing capacity was about 6-fold higher in the CC124 than in the S-01 strain (approximately 1 and 0.17 µl H_2/min, respectively) and that the supplied Asc did not have any effect on these values.

Discussion

It is a widespread view that the loss of PSII activity during sulphur deprivation is due to the inhibition of PSII repair, since the repair of the photoinhibited reaction centers requires *de novo* protein synthesis, which is halted by the lack of sulphur and by the inability of the cells to synthesize the required amino acids (Zhang et al., 2002). However, cell division and Chl biosynthesis are stopped shortly after the initiation of sulphur deprivation (Zhang et al., 2002 and Suppl. Fig. 2), thus the sulphur content within the cells is not expected to change drastically. Moreover, algal cells also have sulphur reserves in the form of cysteine and methionine, which are used up following transfer to sulphur-free medium (Matthew et al., 2009). The loss of sulphur from the media itself also cannot explain the differences between the *C. reinhardtii* genotypes in terms of PsbA content decrease nor that externally provided Asc slows down the degradation of PsbA (Fig. 7). Therefore, it is unlikely that the lack of sulphur would hinder the repair of PsbA leading to a loss of PSII activity.

Indeed, gene expression analyses suggest that the picture is very complex. Regulatory elements controlling sulphur deprivation responses have been identified, such as the sulphur acclimation gene *SAC1* (Wykoff et al., 1998, Zhang et al., 2004), and the SNRK2.1 and SNRK2.2 kinases, which are responsible for repression of sulphur-inducible genes and repression of chloroplast transcription (Irihimovitch and Stern, 2006; González-Ballester et al., 2008; González-Ballester et al., 2010).

Response in gene expression occurs already after a few hours of sulphur deprivation (González-Ballester et al., 2008), during which most photosynthetic genes, Rubisco and antenna proteins genes are down-regulated (see also Toepel et al., 2013). In parallel, there is a strong up-regulation of specific LHCBM and LHCSR genes, which play a role in photoprotection and scavenging reactive oxygen species (Nguyen et al., 2011, Grewe et al., 2014). Genes involved the mobilization and relocalisation of sulphur are also upregulated, all suggesting that there is a rapid and complex, "whole-cell" adaptation process involved in the initiation of H_2 production (Aksoy et al., 2013, Toepel et al., 2013). These processes occur much faster than the actual loss of the PsbA protein (Zhang et al., 2002, Fig. 7 of this paper), supporting our view that sulphur starvation affects PSII activity in a different way than hindering the repair of PsbA.

Ascorbate accumulation and its effects on PSII

Ascorbate is a metabolite with various functions in eukaryotic cells and most commonly it acts as a reductant. Its role in scavenging reactive oxygen species in plants is widely studied (reviewed by e.g. Foyer and Shigeoka 2011), but Asc also plays roles in cell division and cell wall synthesis and it modulates the synthesis of several signaling molecules. Ascorbate also modulates the expression of specific sets of photosynthesis and defense genes (reviewed by Smirnoff 2011) and recently an epigenetic role exerted as a cofactor for DNA and histone demethylases in the nucleus was demonstrated (Young et al., 2015).

Cyanobacteria, algae and bryophytes contain much lower concentrations of Asc than higher plants (reviewed by Gest et al., 2013). The signaling pathways leading to Asc accumulation in plants are poorly understood. However, it has been demonstrated that in higher plants Asc levels are dependent of photosynthetic electron transport (Yabuta et al., 2007), Asc biosynthesis responds to changes in light intensity, it is under circadian control (Page et al., 2012, Kiyota et al. 2006) and it is subject to feedback inhibition by Asc (Pallanca and Smirnoff, 2000). In *C. reinhardtii* these processes have not been studied, although it has been demonstrated that Asc biosynthesis responds quickly to H_2O_2 treatment and the *VTC* gene is upregulated (Urzica et al., 2012), which plays a central role in regulating the Asc contents in higher plants as well (Dowdle et al., 2007). It was shown recently that in the green alga, *Chlorella sorokiniana* sulphur deficiency causes oxidative perturbation resulting in a sudden increase in H_2O_2 concentration and Asc accumulation (Salbitani et al., 2015). On the other hand, transcriptomics data by Toepel et al. (2013) show that the expression of the *VTC* gene strongly increases upon sulphur deprivation as well. In line with these results, we here found that Asc biosynthesis in *C. reinhardtii* is strongly induced by sulphur deprivation (Fig. 3). The addition of 10 mM Asc to the cultures upon the start of sulphur deprivation led to a rapid Asc content increase in the cells and similarly high Asc concentration was reached as in the non-Asc-treated samples (Fig. 3). Unexpectedly, the Asc treatment equalized the H_2 production in all the strains (Fig. 1). As also seen in Figs. 1 and 2 there is much less O_2 accumulated upon Asc addition; our results show that it is not due to stimulated starch consumption (Fig. 9), instead Asc inactivated the OEC as shown by TL measurements (Figs. 4 and 6). Similarly, in sulphur-replete cultures, the OEC became partially inactivated when supplied with 10 mM Asc, although the cells recovered within a few hours, possibly due to the oxidation or metabolization of Asc (Fig. 4F); this shows that the OEC of *C. reinhardtii* may be susceptible to the reducing effect of Asc under normal growth conditions as well.

Earlier *in vitro* data indicate that Asc inactivates the Mn-cluster in higher plant PSII membrane preparations of which the extrinsic OEC proteins were removed by chemical treatments (Tamura et al., 1990). The redox potential of Asc (approximately +54 mV) enables both the over-reduction of the Mn-cluster and also the support of a continuous electron flow to Tyr_Z^+; there are various chemicals with such properties of which hydroxylamine is the best studied example (Kuntzleman and Yocum 2005). The observation that in higher plants Asc over-reduces the Mn-cluster only in the absence of the extrinsic OEC proteins suggests that they have a role in shielding the Mn-cluster from luminal reductants, such as Asc. In *C. reinhardtii* the situation seems to be different: under 'normal', non-stress conditions, the Asc content is two to three orders of magnitude lower than in higher plant chloroplasts (Gest et al., 2013, Zechmann et al., 2011); when 10 mM Asc is externally supplied (both in sulphur-replete and depleted cultures), it inactivates the Mn-complex (Fig. 4). Due to the sulphur deprivation, the Asc concentration increases strongly within the cell, which coincides with the inactivation of the OEC in both strains (Figs. 2, 3 and 6); this strongly suggest that under sulphur deprivation the naturally accumulating cellular Asc inactivates the Mn-complex of PSII.

When the Mn-cluster is destroyed, charge separation may still occur in PSII and Asc can donate electrons to Tyr_Z^+ with a halftime of 20-50 ms, depending on the Asc concentration within the cell (Tóth et al., 2009, Nagy et al., 2012). However, this electron donation by Asc is relatively slow compared to that from water oxidation and does not prevent completely the formation of strongly oxidizing compounds, such as Tyr_Z^+ and $P680^+$ (Tóth et al., 2011). Their accumulation leads to the so-called donor-side induced photoinhibition, i.e. inactivation of the charge separation activity of PSII (Chen et al., 1995, Jegerschöld and Stryring 1996) and rapid losses of the PSBO, PsbA and CP43 proteins (Tóth et al., 2011). Upon sulphur deprivation, the inactivation of the Mn-cluster was followed by the inactivation of PSII reaction centers, as shown by the complete elimination of variable fluorescence (Fig. 5) and the amounts of PsbA and PSBO proteins were also strongly reduced (Fig. 7), indicating that donor-side induced photoinhibition occurred. The inactivation of PSII and degradation of PsbA and PSBO happened earlier in the CC124 strain, which is characterized by a faster accumulation of cellular Asc and earlier OEC inactivation compared to the S-01 strain.

The overall effect of ascorbate on the photoproduction of H_2

When Asc was supplied externally, it had a positive effect on the total H_2 production of the weak-performing strains (e.g. S-01) and a strong negative effect on the good H_2-producing strains (e.g. CC124, Fig. 1). The CC124 strain has a high respiration rate (Rühle et al., 2008) and efficient starch degradation (Fig. 9), which can compensate for a relatively large O_2 evolution and the electrons released by the OEC may be efficiently used for H_2 production. When Asc is added, it suddenly decreases the OEC activity and -since the electron donation by Asc is relatively slow compared to that of the OEC (Tóth et al., 2009)-, much less electrons will become available for H_2 production. On the other hand, in a strain with slow starch degradation (S-01), the inactivation of the OEC is beneficial, since the inhibitory effect of O_2 on the hydrogenase may be eliminated and the electron supply to PSII by Asc (resulting in no O_2 evolution) will increase H_2 production, to the level of the Asc-treated CC124 strain (Fig. 1). It is also important to note that not only the rate of starch degradation, but also the hydrogenase activity of the CC124 and the S-01 strains differ significantly after three days of sulphur deprivation (Fig. 9) and it is independent of the addition of Asc. Equal H_2 production was measured in the Asc-treated CC124 and S-01 strains (Fig. 1), which underlines the importance of linear electron flux in the process of photobiological H_2 production. In other words, when linear electron transport is limited, neither efficient starch degradation nor high hydrogenase activity would result in strong H_2 production.

In summary, we propose a novel mechanism for PSII inactivation during sulphur deprivation. Ascorbate accumulates dramatically upon sulphur deprivation (which is probably induced by oxidative stress conditions) and when it reaches the mM range in the cell, it inactivates the Mn-cluster due to its reducing capacities. There is a slow electron donation by Asc to PSII, but donor-side induced photoinhibition may still take place, causing a loss of the charge separating activity of PSII. Meanwhile, anaerobiosis is also established, which is essential for the initiation of H_2 production. Thus, Asc seems to have an important modulatory effect on photobiological H_2 production in *C. reinhardtii.*

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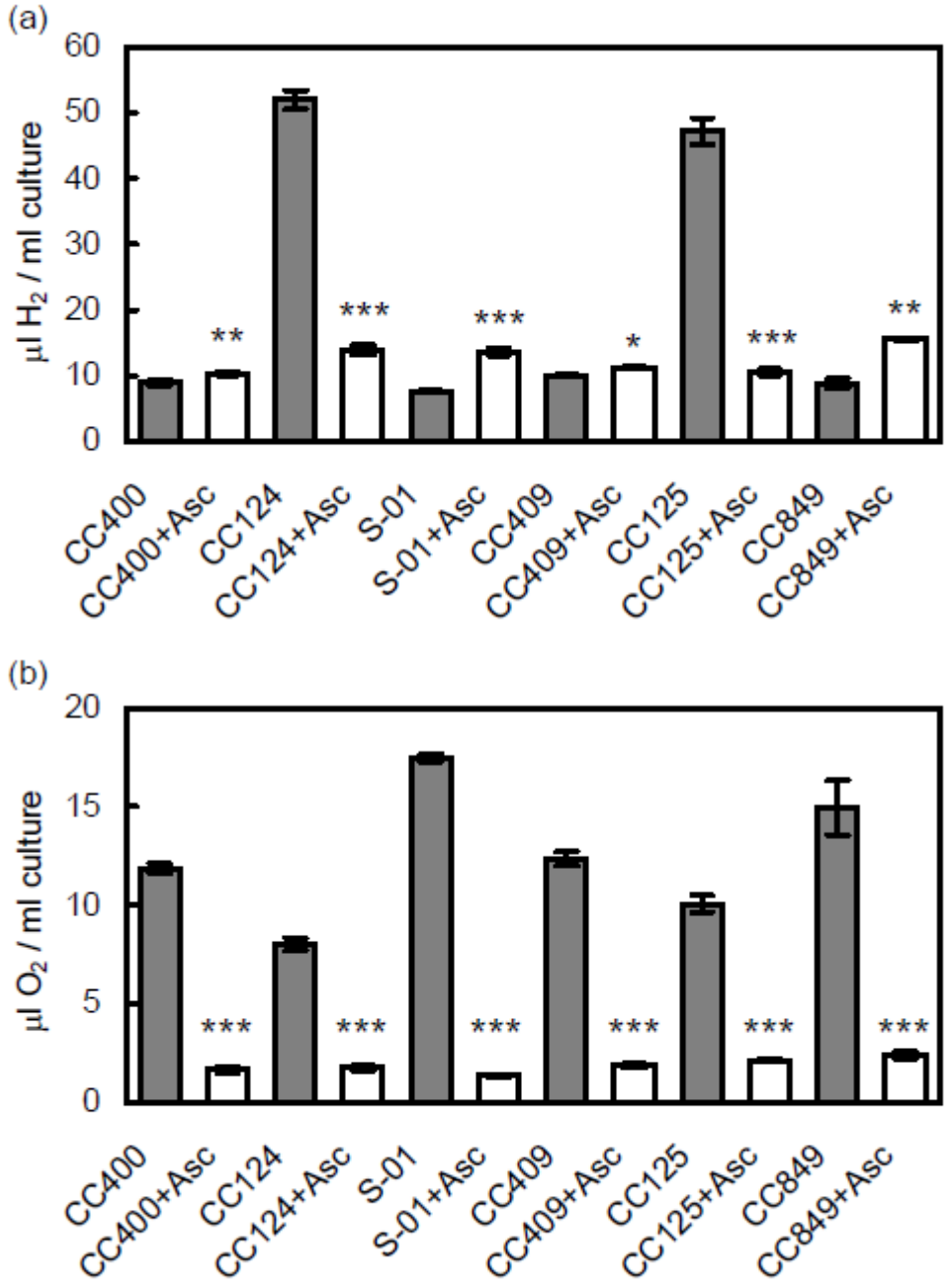

Figure 1. H_2 (A) and O_2 (B) accumulation by several *C. reinhardtii* strains without or with 10 mM Na-Asc added to the cultures at the start of sulphur deprivation. The amounts of H_2 and O_2 were determined daily by GC and the accumulated gases were removed by N_2 flushing after the measurements each day. Averages of the total H_2 and O_2 gases accumulated during four days, in three independent experiments are presented. Error bars represent standard errors and the significance level between the Asc-treated and untreated samples are presented as: * $p<0.05$; ** $p<0.01$;*** $p<0.001$.

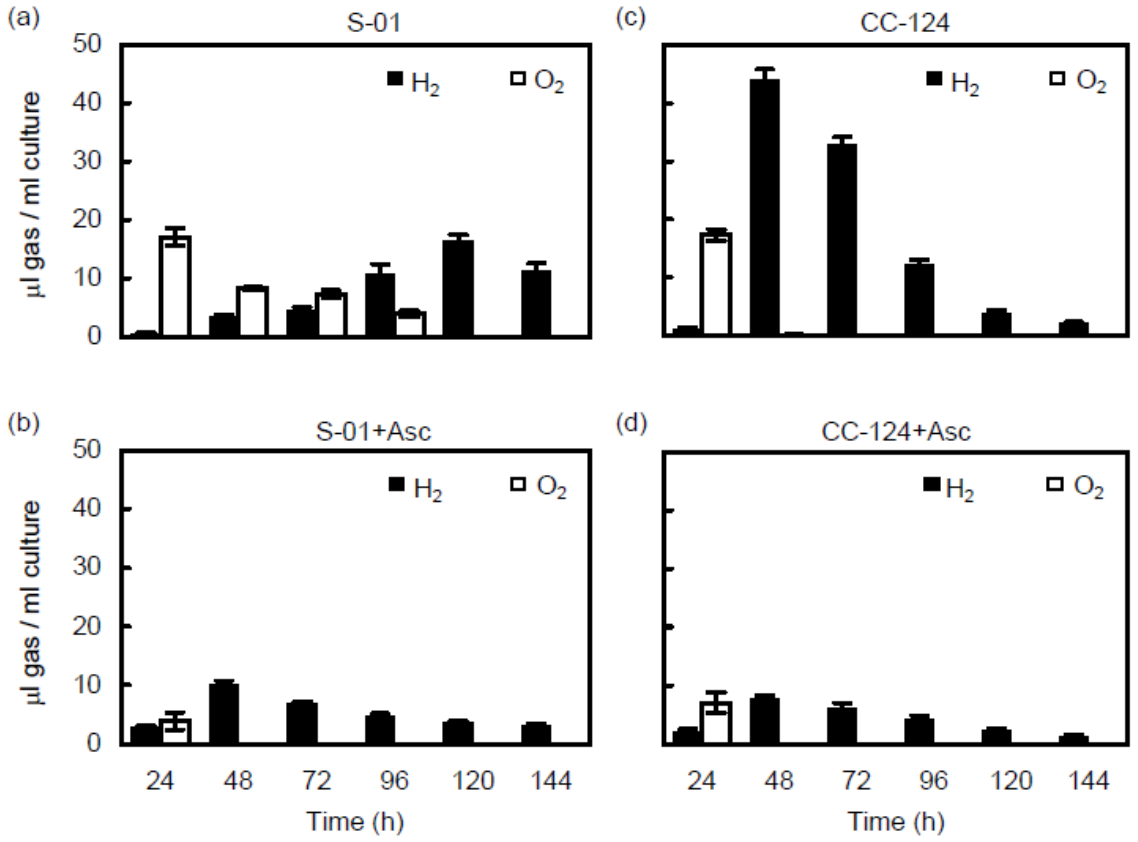

Figure 2. H_2 and O_2 accumulation by S-01 (A, B) and CC124 (C,D) strains of *C. reinhardtii* without (A,C) or with 10 mM Na-Asc added to the cultures (B,D) at the start of sulphur deprivation. The amounts of H_2 and O_2 were determined daily by GC and the accumulated gases were removed by N_2 flushing after the measurements each day. Averages of the H_2 and O_2 gases accumulated during six days, in eight independent experiment are presented. Error bars represent standard errors.

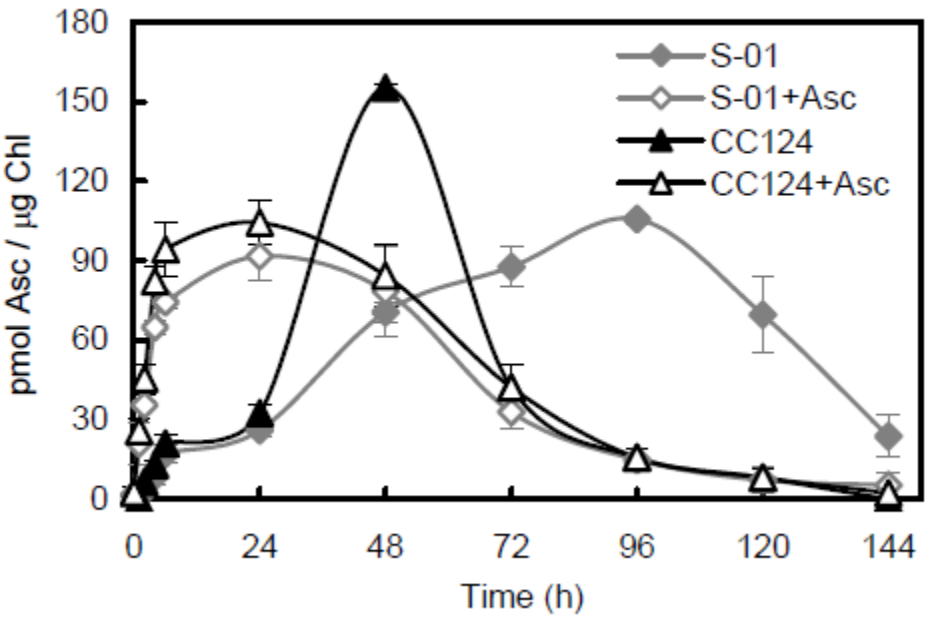

Figure 3. Asc accumulation in S-01 and CC124 *C. reinhardtii* cells without or with 10 mM Na-Asc added to the cultures at the start of sulphur deprivation. The presented values are derived from three independent experiments, shown with their standard errors.

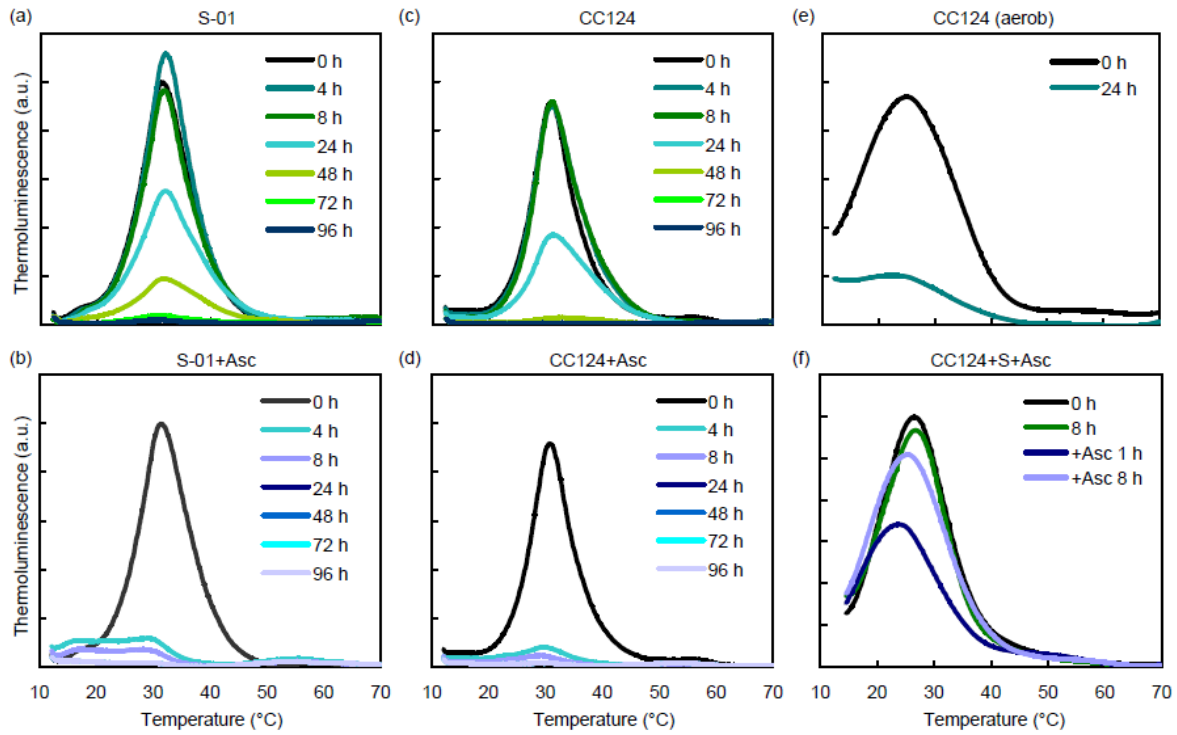

Figure 4. Thermoluminescence emission of the S-01 (A, B) and CC124 (C, D) *C. reinhardtii* strains, in the absence (A, C) and presence (B, D) of 10 mM Na-Asc added at the beginning of sulphur deprivation. E: TL emission of sulphur-deprived but unsealed CC124 cultures, without Asc addition, F: TL emission from Asc-treated (10 mM Na-Asc) sulphur-replete and unsealed CC124 cultures.

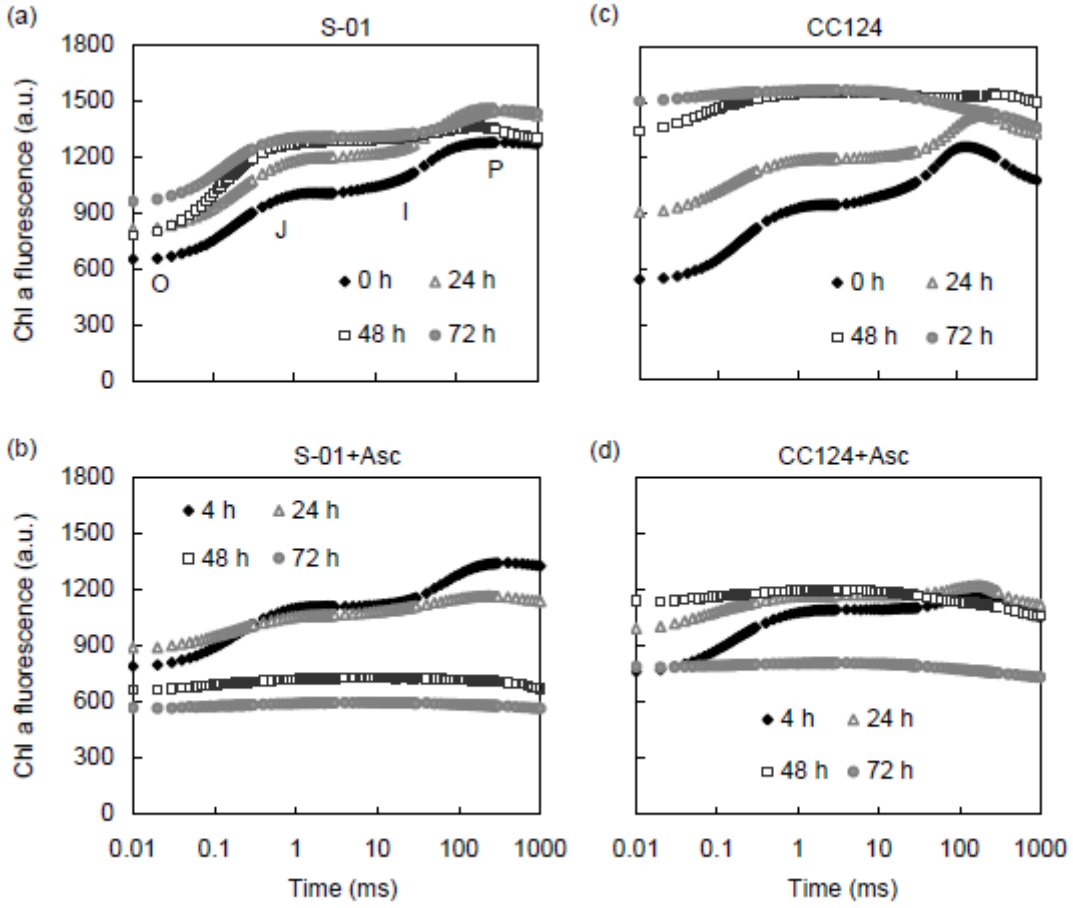

Figure 5. Chl *a* fluorescence (OJIP) transients of H_2-producing S-01 (A, B) and CC124 (C, D) *C. reinhardtii* strains, in the absence (B, D) or presence (A, C) of 10 mM Na-Asc added at the beginning of sulphur deprivation. The approximate positions of the different steps of the OJIP transients are indicated.

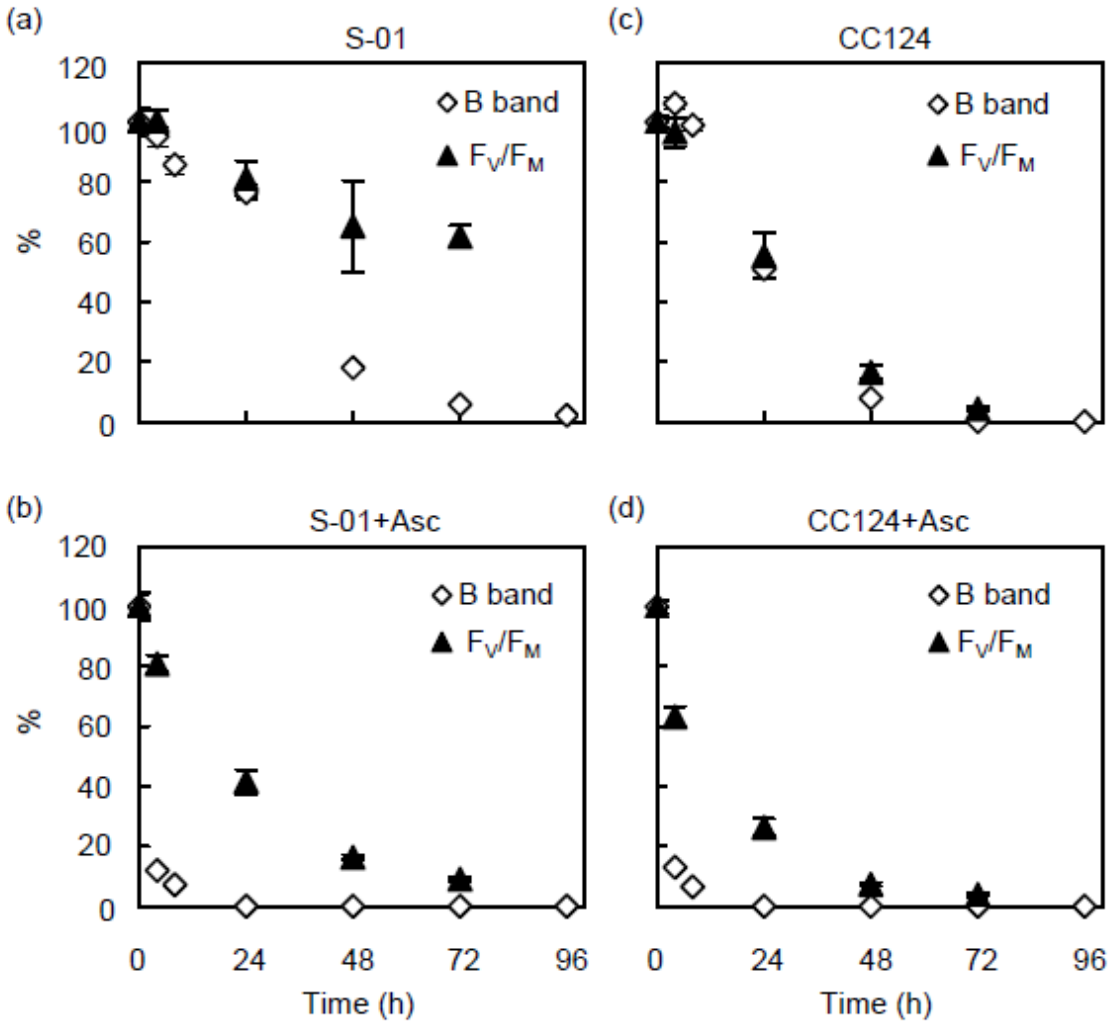

Figure 6. Time courses of the decrease of B the thermoluminescence band and the F_V/F_M value during sulphur deprivation of S-01 (A, B) and CC124 (C, D) cultures, without (A and C) and with (B and D) the addition of 10 mM Na-Asc. The data are derived from at least three independent experiments and are shown with their standard errors.

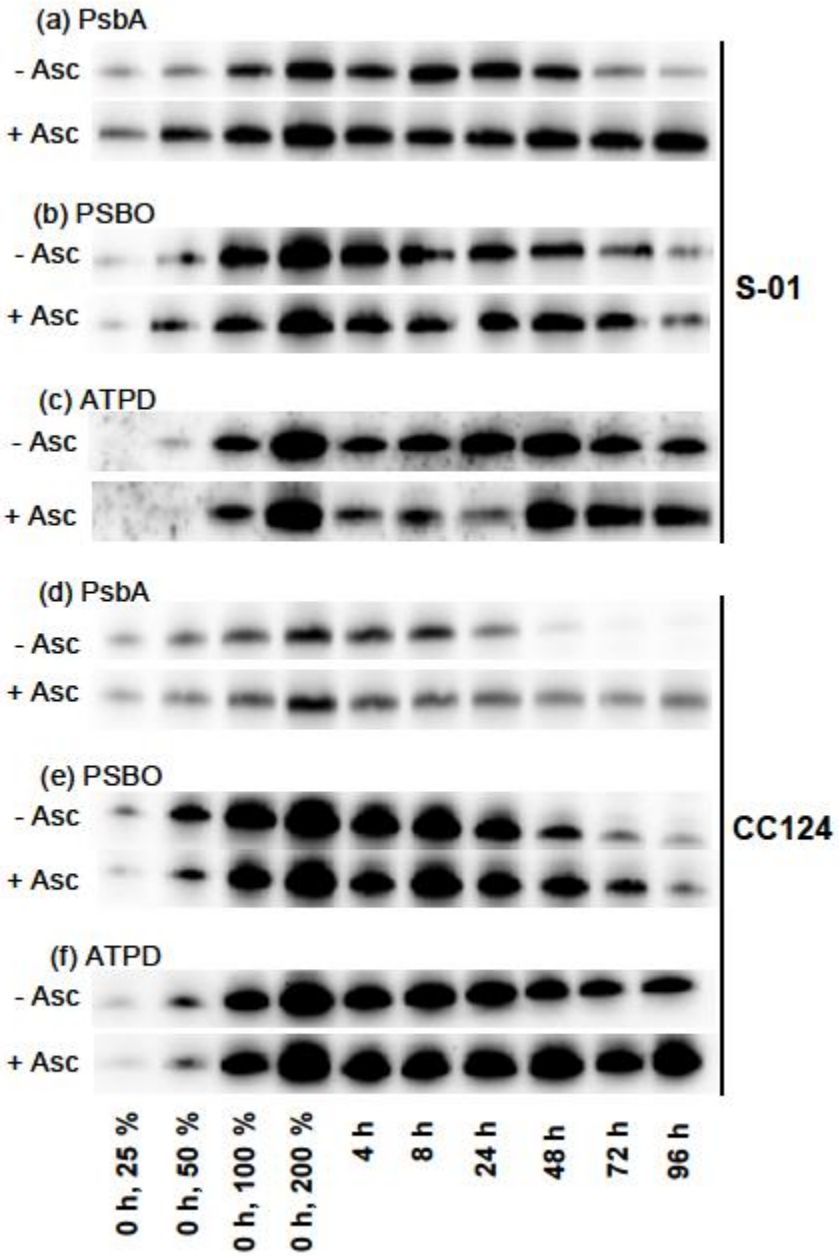

Figure 7. Western blot analysis for the PsbA (A, D), and PSBO (B, E) and ATPD (used as loading control; C, F) proteins of S-01 (A, B, C) and CC124 (C, D, E) cultures, deprived of sulphur without or with 10 mM Na-Asc added. The first four lanes (25%, 50%, 100% and 200% of 0 h sulphur-deprived cultures) are included for approximate quantification of the proteins.

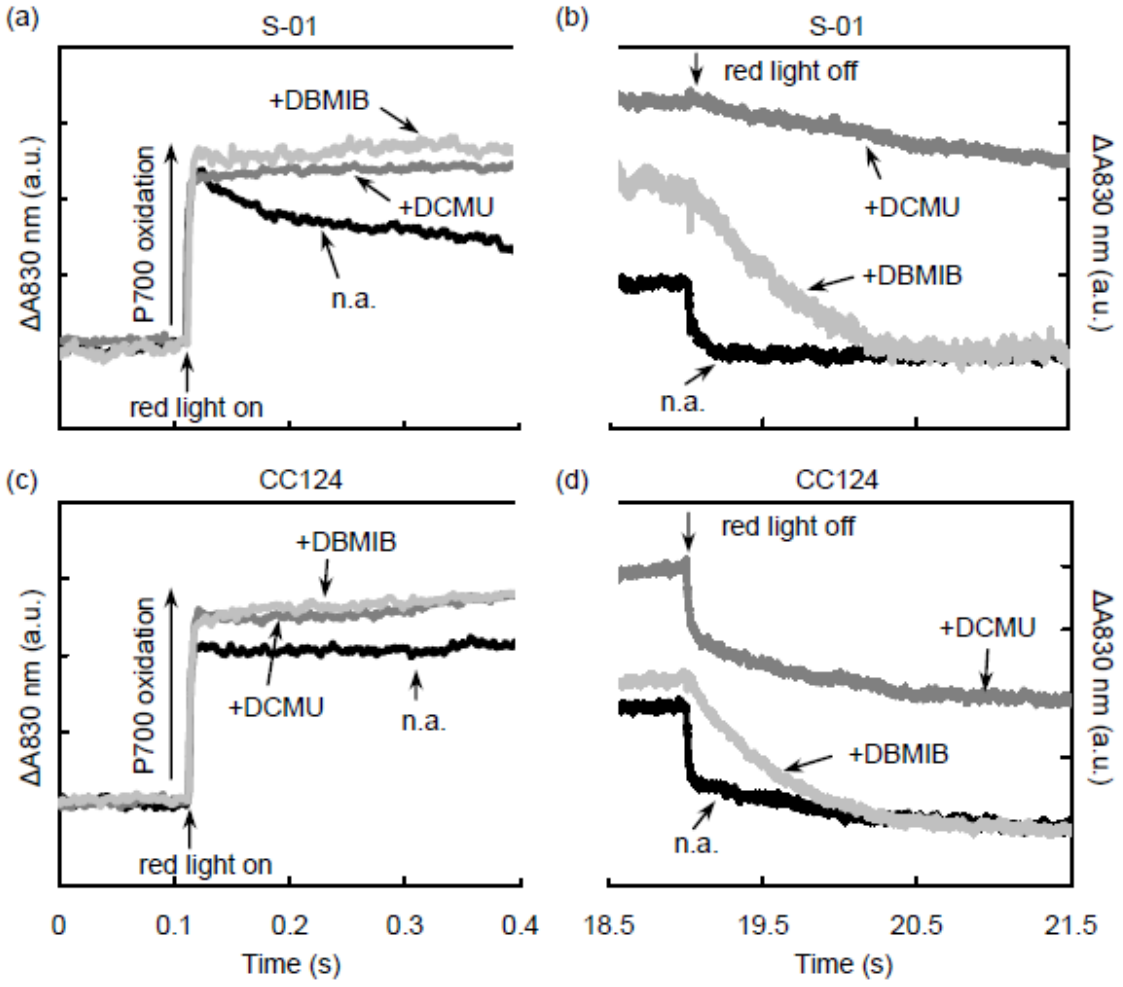

Figure 8. Effects of DCMU (20 µM) and DBMIB (5 µM) on the light-induced 830 nm absorbance transients in S-01 (A, C) and CC124 (B, D) *C. reinhardtii* cultures deprived of sulphur for 72 hrs. After the addition of DCMU or DBMIB, the cells were dark-adapted for 15 min and then 5 ml of cells suspension (8 µg Chl/ml) was filtered onto a Whatman glass microfibre filter (GF/C). The kinetics were measured during continuous illumination with red light of about 2000 µmol m^{-2} s^{-1} photon flux density (A, B); after 20 s, the light was switched off and the re-reduction kinetics was measured in the dark (C, D). The traces are averages of 4-6 measurements. n.a., no addition.

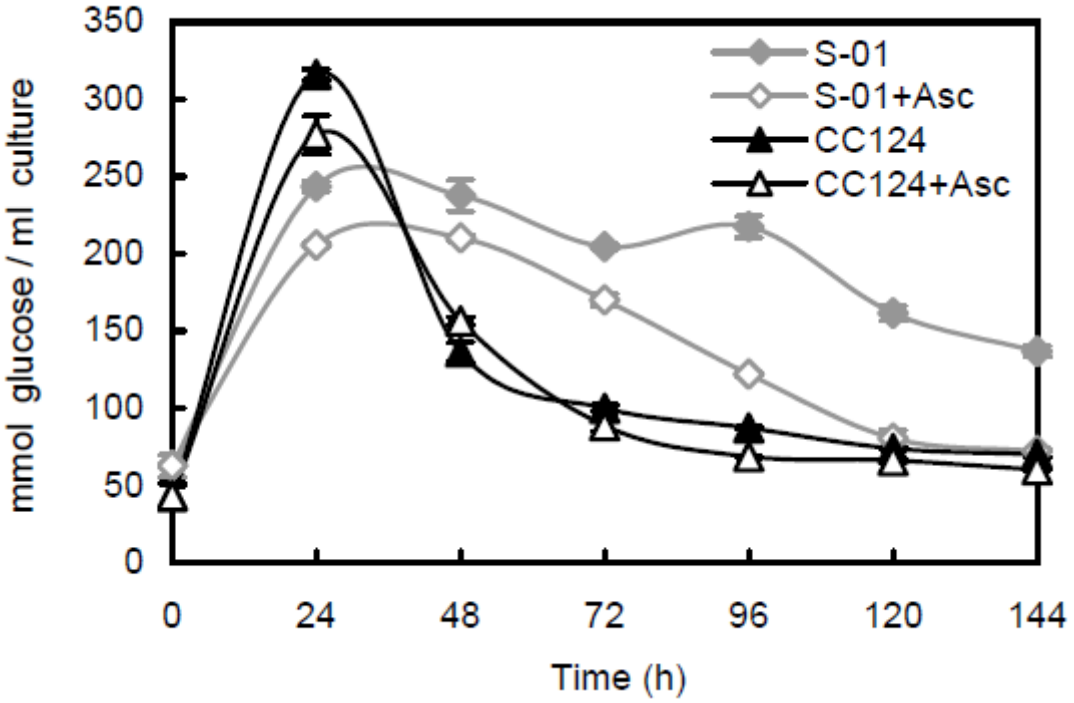

Figure 9. Starch accumulation and degradation measured as glucose equivalents during sulphur deprivation in the S-01 and CC124 strains without and with 10 mM Na-Asc added. The data are derived from three independent experiments and are shown with their standard errors.