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Flavodiiron-mediated O₂ photoreduction links H₂ production with CO₂ fixation during the anaerobic induction of photosynthesis

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One-sentence summary: Flavodiiron proteins recycle large amounts of O₂ during the anaerobic induction of photosynthesis and act as a relay of hydrogenases in priming CO₂ fixation in *Chlamydomonas reinhardtii*.

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Abstract (250)

Some microalgae, such as *Chlamydomonas reinhardtii*, harbor a highly flexible photosynthetic apparatus capable of using different electron acceptors, including carbon dioxide (CO₂), protons, or oxygen (O₂), allowing survival in diverse habitats. During anaerobic induction of photosynthesis, molecular O₂ is produced at photosystem II, while at the photosystem I acceptor side, the reduction of protons into hydrogen (H₂) by the plastidial [FeFe]-hydrogenases primes CO₂ fixation. Although the interaction between H₂ production and CO₂ fixation has been extensively studied, their interplay with O₂ produced by photosynthesis has not been considered. By simultaneously measuring gas exchange and chlorophyll fluorescence, we identified an O₂ photoreduction mechanism that functions during anaerobic dark to light transitions, and demonstrate that flavodiiron proteins (Flvs) are the major players involved in light-dependent O₂ uptake. We further show that Flv-mediated O₂ uptake is critical for the rapid induction of CO₂ fixation, but is not involved in the creation of the micro-oxic niches previously proposed to protect the [FeFe]-hydrogenase from O₂. By studying a mutant lacking both hydrogenases (HYDA1 and HYDA2) and both Flvs (FLVA and FLVB), we show that the induction of photosynthesis is strongly delayed in the absence of both sets of proteins. Based on these data, we propose that Flvs are involved in an important intracellular O₂ recycling process, which acts as a relay between H₂ production and CO₂ fixation.

INTRODUCTION

Chlamydomonas reinhardtii is a unicellular green alga capable of an extraordinary metabolic versatility depending on the environmental conditions it encounters. As a photosynthetic organism, it can grow photoautotrophically, using sunlight as an energy source, by fixing atmospheric carbon dioxide (CO₂) and producing oxygen (O₂) from the photolysis of water. In the presence of organic substrates such as acetate, *C. reinhardtii* may grow either heterotrophically or mixotrophically. Like many soil microorganisms subjected to frequent shifts in O₂ availability, *C. reinhardtii* is a facultative anaerobe, able to rely either on aerobic respiration or anaerobic fermentation to oxidize reduced equivalents produced by glycolysis (Catalanotti et al., 2013). During anaerobic fermentation, *C. reinhardtii* cells express a set of enzymes involved in a complex network of fermentative pathways, resulting in the production of acetate, formate, ethanol and hydrogen (H₂) as major end products (Gfeller and Gibbs, 1984; Mus et al., 2007). Hydrogen can be produced in the dark by fermentation via the pyruvate:formate lyase pathway (Noth et al., 2013), but also in the light due to the coupling of photosynthesis and two plastidial [FeFe]-hydrogenases, HYDA1 and HYDA2, which use reduced ferredoxin as an electron donor for the reduction of protons (Florin et al., 2001). During H₂ photoproduction, photolysis of water at photosystem II (PSII) supplies electrons to the photosynthetic electron transport chain, resulting in the reduction of ferredoxin and production of H₂ (Hemschemeier et al., 2008). This process, often referred to as the direct biophotolysis pathway, is usually transient and limited by the ability of O₂-consuming processes, such as mitochondrial respiration, to maintain an anaerobic environment in the vicinity of the O₂-sensitive [FeFe]-hydrogenases (Melis et al., 2000; Hemschemeier et al., 2008; Burlacot and Peltier, 2018).

When dark-adapted anaerobic cultures of *C. reinhardtii* are exposed to sunlight, their metabolism changes dramatically. During the first minutes of illumination, a transient burst of H₂ occurs (Cournac et al., 2002), then the O₂ produced by photosynthesis initiates a switch from fermentative metabolism to oxidative decarboxylation and oxidative phosphorylation by respiration (Happe et al., 2002), and triggers an irreversible inhibition of the [FeFe]-hydrogenases (Erbes et al., 1979; Stripp et al., 2009). Kessler observed that algal species devoid of hydrogenases experience a delay in the induction of photosynthesis and proposed that H₂ photoproduction would enable algae to oxidize photosynthetic electron acceptors and thereby activate the photosynthetic chain after anaerobic incubation (Kessler, 1973). However, the selective advantage conferred by the presence of O₂-sensitive [FeFe]-hydrogenases in a photosynthetic organism producing O₂ remains to be demonstrated (Ghysels et al., 2013).

During photosynthesis, O₂ can act as an electron acceptor, either through a non-enzymatic process (called the Mehler reaction) or through an enzymatic process involving flavodiiron proteins (Flvs) (Peltier et al., 2010). In cyanobacteria, Flvs have been shown to catalyze the reduction of O₂ into water using NADPH as an electron donor (Helman et al., 2003) and to play a critical role during growth under fluctuating light regimes (Allahverdiyeva et al., 2013). In *C. reinhardtii*, Flvs have been

demonstrated to catalyze a massive and transient O₂ reduction during the induction of photosynthesis (Chaux et al., 2017). Despite their efficiency in reducing O₂ during a light transient (Chaux et al., 2017), Flvs do not compete with CO₂ fixation (Chaux et al., 2017; Wada et al., 2017). It was proposed that Flvs are mainly functioning where the chloroplast stroma reaches a high reducing state (Chaux et al., 2017), conditions which are obtained when illuminating dark anaerobic-adapted cells (Alric, 2010).

In the nitrogen-fixing cyanobacterium *Anabaena* sp. PCC7120, Flv3B was shown to protect the O₂-sensitive nitrogenase (Gallon, 1992) from O₂ attack (Ermakova et al., 2014). Recently, based on the continuous production of H₂ in air-grown *C. reinhardtii*, (Liran et al., 2016) it was concluded that micro-oxic niches allow the [FeFe]-hydrogenases to function in the presence of O₂ and it was proposed that Flvs may be involved in this process. However, O₂-consuming processes have been considered as minor electron sinks under H₂ production conditions (Godaux et al., 2015; Milrad et al., 2018) and, the existence of a possible O₂ reducing process remains to be demonstrated.

In this context, we questioned the role of Flv-mediated O₂ photoreduction during the anaerobic induction of photosynthesis and its possible involvement in the formation of micro-oxic niches. By simultaneously measuring chlorophyll fluorescence and oxygen exchange, we identified an O₂ reduction mechanism during a light transient in anaerobically adapted *C. reinhardtii* cells and further demonstrate the involvement of Flvs in this process. We show that Flv-mediated O₂ reduction is critical for a fast induction of CO₂ fixation, but does not protect the [FeFe]-hydrogenase from O₂ inhibition by creating micro-oxic niches. We propose that Flvs act as a relay of hydrogenases to promote the rapid induction of photosynthesis when O₂ starts to become available as an electron acceptor and the [FeFe]-hydrogenases are inhibited by O₂.

RESULTS

Combined gas exchanges and chlorophyll fluorescence measurements as a tool to study the photosynthetic activity of anaerobically-adapted *C. reinhardtii* cells. During the induction of photosynthesis in anaerobically-acclimated cells, O₂ is produced by PSII while H₂ is produced by photosystem I (PSI). The presence of O₂ inside the chloroplast may lead to O₂ photoreduction at the PSI acceptor side, thus resulting in O₂ recycling. In order to explore the interplay between H₂ and O₂ exchange during the anaerobic induction of photosynthesis, we designed a method to detect the existence of such O₂ recycling in the light. Oxygen uptake in the light has been measured in the past using [¹⁸O]-labeled O₂ and a membrane inlet mass spectrometer (MIMS) (Hoch and Kok, 1963), but this technique cannot be used under anaerobic conditions. However, O₂ uptake rates can be inferred by subtracting the net O₂ evolution from the gross O₂ produced by PSII, the latter determined from chlorophyll fluorescence measurements (Kitajima and Butler, 1975). By performing simultaneous measurements of chlorophyll fluorescence and gas exchange, using a pulse amplitude modulated

(PAM) fluorimeter and a MIMS with [^{18}O]-labeled O_2 , respectively (Cournac et al., 2002) (Supplemental Fig. S1A, B), we first established the relationship under aerobic conditions between the chlorophyll fluorescence parameter $\Delta F/F_M'$ (Supplemental Fig. S2) and the quantum yield of O_2 evolution by PSII. Since our experimental set-up was not designed to measure light absorption in a cell suspension, experiments were performed at similar chlorophyll concentrations, assuming a constant light absorption during the time-course of the experiment. As reported previously in leaves of land plants (Genty et al., 1989), a linear relationship was observed under a wide range of light intensities (Fig. 1A). For the highest quantum yields (measured at low light intensities), a non-linearity was observed which was unaffected by the chlorophyll concentration of the sample (Supplemental Fig. S3), indicating that it likely did not result from a light heterogeneity in the cuvette. Further experiments aiming at determining O_2 production by PSII from chlorophyll fluorescence measurements were performed at light intensities corresponding to the linear range of the relationship (Supplemental Fig. S4).

The PAM-MIMS set-up was then used to determine H_2 and O_2 exchange rates during a dark to light transient in anaerobically-adapted *C. reinhardtii* cells (Fig. 1B). As previously reported (Cournac et al., 2002), net H_2 and O_2 production rates rapidly increased upon illumination, with maximal values being observed after about 1 min of illumination (Fig. 1B). Gross O_2 evolution rates were determined from chlorophyll fluorescence measurements with the assumption that linearity in the relationship established under aerobic conditions (Fig. 1A) was valid under anaerobic conditions. However, PSII absorbance may vary when switching from anaerobic to aerobic conditions due to the movement of light harvesting complex proteins (LHCs) between PSI and PSII, a phenomenon associated with changes in the maximal fluorescence level F_M' (Wollman and Delepelaire, 1984). We were therefore careful to restrict the use of the linear relationship established in Fig. 1A to the first 3 min of illumination, conditions in which no major change in F_M' was observed (Supplemental Fig. S5). From the difference between the gross O_2 evolution rate (determined from chlorophyll fluorescence measurements) and net O_2 evolution (measured by MIMS), we inferred the existence of an O_2 uptake process, starting after about 50 s of illumination and reaching a maximal value after 2 min of illumination, where it was estimated to be about 40% of the gross O_2 production rate (Fig. 1B and C). It is noteworthy that O_2 uptake roughly started when the maximum H_2 and net O_2 production rates were reached (Fig. 1B). We conclude from this experiment that O_2 recycling occurs in *C. reinhardtii* cells upon illumination of anaerobically adapted cells and is concomitant with dramatic changes in the use of electrons at the PSI acceptor side.

Role of Flvs in O_2 recycling. Flvs have been recently identified in *C. reinhardtii* as the main drivers of O_2 photoreduction during the induction of photosynthesis under aerobic conditions (Chaux et al., 2017). To test the possible involvement of Flvs during a light induction in anaerobically adapted cells, we performed similar experiments as in Fig. 1B in two independent *C. reinhardtii flvB* mutants

lacking both FlvB and FlvA under aerobic and anaerobic conditions (Chaux et al., 2017) (Supplemental Fig. S6A). While O₂ uptake started in the wild type (WT) after about 50 s of illumination and reached a maximal value after 2 min, both *flvB-208* and *flvB-308* mutants showed a strongly reduced O₂ uptake during the first 2 min of illumination (Fig. 2A and B). After 3 min of illumination, the O₂ uptake rate in the *flvB* mutants was much lower than in the WT (Fig. 2 B), such a late increase likely reflecting a growing contribution of other O₂ uptake processes such as mitochondrial respiration. We conclude from this experiment that Flvs are the major actors involved in the O₂ recycling occurring upon illumination of anaerobically acclimated cells.

Hydrogen photoproduction is enhanced in *flvB* mutants. Hydrogen photoproduction can be limited *in vivo* by different factors, including: *i.* the O₂-sensitivity of the [FeFe]-hydrogenases (Erbes et al., 1979), O₂ being produced in the light by PSII, and/or, *ii.* competition for electrons at the acceptor side of PSI (Godaux et al., 2015). Flvs could be involved in both aspects, either by competing for electrons on the PSI acceptor side, or by protecting the [FeFe]-hydrogenases from O₂ attack. Indeed, the existence of intracellular micro-oxic niches protecting the [FeFe]-hydrogenases from O₂ has been proposed (Liran et al., 2016), and Flvs have been suggested to be involved (Liran et al., 2016; Burlacot and Peltier, 2018). Hydrogen production rates of WT and *flvB* mutant strains were thus compared during a dark to light transient. Enhanced H₂ photoproduction was observed in *flvB* mutants as compared to the WT between 1 and 3 min of illumination (Fig. 3A and B), and this effect was not due to a difference in initial hydrogenase activities (Supplemental Fig. S7A). The O₂ concentration in the cell suspension was slightly higher in the *flvB* mutants compared to the WT during the first minutes of the light transient (Supplemental Fig. S8). However, no difference in hydrogenase inhibition was observed after 2 min of illumination in a *flvB* mutant as compared to the WT (Supplemental Fig. S7B). We therefore conclude from this experiment that Flv-mediated O₂ uptake competes for electrons with the [FeFe]-hydrogenases. A possible role of Flvs in the protection of the [FeFe]-hydrogenases from O₂ attack could not be evidenced.

Flv-mediated O₂ uptake primes CO₂ fixation during the transition from anaerobiosis to aerobiosis. Hydrogen photoproduction has been reported to prime net photosynthesis and to allow the fast induction of photosynthesis under anaerobic conditions (Ghysels et al., 2013). On the other hand, Flv-mediated O₂ uptake precedes but does not influence CO₂ fixation during the induction of aerobic photosynthesis (Chaux et al., 2017). To determine the extent to which Flv-mediated O₂ reduction participates in the anaerobic induction of photosynthesis, we measured O₂ and CO₂ exchange in *flvB* mutants. While no significant difference in net O₂ evolution and net CO₂ fixation were measured after the first 3 min of illumination, a 30% decrease in both parameters was observed in both *flvB* mutants compared to the WT (Fig. 4A and B; Supplemental Fig. S9). Remarkably, this decrease in net photosynthesis was observed after H₂ production stopped, which occurred after 3-4 min of

illumination (Fig. 3A). We conclude from this experiment that O₂ uptake by Flvs allows a fast induction of photosynthesis in anaerobically acclimated cells and primes CO₂ fixation when H₂ production has ceased.

Flv-mediated O₂ uptake primes CO₂ fixation even in the absence of the hydrogenase. Hydrogen photoproduction has been considered as the main process enabling algae to start photosynthesis following anaerobic incubation (Kessler, 1973). However, hydrogenases need at least 10 min of anaerobiosis to become activated (Cournac et al., 2002) and only a restricted group of microalgae possess a functional hydrogenase (Burlacot and Peltier, 2018). Given that most unicellular green algae harbor Flvs (Supplemental Fig. S10), the involvement of Flvs in the start of photosynthesis after anaerobic incubation in the absence of an active hydrogenase was questioned. A triple mutant (*hydA1/A2 flvB*) lacking both hydrogenases and both Flvs was obtained by crossing two existing mutants: *hydA1/A2* (Meuser et al., 2012) and *flvB* (Chaux et al., 2017); three independent triple mutant progenies (*hydA1/A2 flvB* 1, 2 and 3) were isolated (Supplemental Fig. S6) and further analyzed. As previously shown (Ghysels et al., 2013), the lack of both hydrogenases had a dramatic effect on the induction of photosynthesis upon illumination of anaerobically-acclimated cells (Fig. 5A). While photosynthesis started after a 3 min illumination in the *hydA1/A2* mutant, no change in the PSII yield was detected in triple *hydA1/A2 flvB* mutants during the first 10 min of illumination (Fig. 5A). CO₂ fixation was more severely affected in the triple mutants as compared to the *hydA1/A2* mutant (Fig. 5B). We conclude from this experiment that O₂ uptake by Flvs allows a faster induction of photosynthesis after anaerobic incubation even in the absence of H₂ production.

DISCUSSION

By performing simultaneous gas exchange and chlorophyll fluorescence measurements, we show here that O₂ uptake occurs at the same time as H₂ is produced upon illumination of anaerobic *C. reinhardtii* cells. The study of *flvB* mutants allowed us to show the involvement of Flvs in this O₂ uptake process. While different processes including H₂ production and oxidation (Gaffron and Rubin, 1942), CO₂ fixation, and regulatory mechanisms such as state transitions (Ghysels et al., 2013) or cyclic electron flow (Godaux et al., 2015) have been shown to impact H₂ photoproduction, little attention has been paid to the possible occurrence of O₂ photoreduction, this mechanism being considered as a minor pathway for electrons in these conditions (Finazzi et al., 1999; Godaux et al., 2015). Here, we have shown that Flv-mediated photoreduction can drive up to 40% of the total linear electron flow during the induction of photosynthesis in anaerobic cells (Fig. 1B), supplying experimental evidence for the existence of an important intra-cellular O₂ recycling mechanism during H₂ production.

Flv-mediated O₂ photoreduction primes CO₂ fixation during the transition from anaerobiosis to aerobiosis. Optimal photosynthetic CO₂ fixation requires both ATP and NADPH and a fine tuning of the NADPH/ATP ratio (Kramer and Evans, 2011). By reducing O₂ at the acceptor side of PSI, Flvs divert electrons towards O₂, thus limiting the NADPH supply, and therefore participate in the establishment of a proton motive force which is used to produce ATP. Until now, negative effects observed under fluctuating light regimes in *flv* mutants were considered to result from photoinhibition (Gerotto et al., 2016; Chaux et al., 2017; Shimakawa et al., 2017), suggesting that the Flvs play a role in protecting photosystems from long-term over-reduction that could lead to photoinhibition. However, the loss of Flvs in algae or the expression in angiosperms of Flvs (which do not naturally contain Flvs) had no effect on net photosynthesis during a dark to light transient (Chaux et al., 2017; Wada et al., 2017; Yamamoto et al., 2016). This apparent discrepancy may be due to the fact that the metabolic buffering capacity of cells can compensate for the loss of Flvs during steady state conditions by maintaining a favorable NADPH/ATP ratio for CO₂ fixation, but that repeated light transients induce a progressive imbalance in this ratio, thus resulting in PSI photoinhibition (Chaux et al., 2017). Similar conditions of NADPH/ATP imbalance occur under anaerobic conditions (Clowez et al., 2015), resulting in a delay in the induction of photosynthesis when Flvs are absent (Fig. 4). An alternative explanation of the delayed induction in the *flv* mutants is that over-reduction of PSI acceptors could have resulted in the production of reactive oxygen species (ROS), leading to PSI and/or PSII photoinhibition. However, no difference in PSI and PSII activities was observed between WT and *flv* mutants (Supplemental Fig. S10), showing that if occurring, the production of ROS had no impact on photosystem activity at this time scale. We conclude that rather than protecting photosystems from photoinhibition, Flvs are involved in the priming of photosynthesis likely by rebalancing the NADPH/ATP ratio. By finely tuning the NADPH/ATP ratio, Flvs protect photosystems from photoinhibition in oxic conditions (Chaux et al., 2017), and enable a proper induction of CO₂ fixation in anaerobiosis. This function is particularly important when the buffering capacity of cells is exceeded, conditions which occur after several light transients under aerobiosis, or after a single transient under anaerobiosis.

Flv-mediated O₂ reduction is a relay between H₂ production and CO₂ fixation. During the switch from anaerobiosis to aerobiosis, H₂ photoproduction is a transient process allowing the reoxidation of PSI acceptors and a fast induction of photosynthesis (Ghysels et al., 2013). Differences in the CO₂ fixation rate between a *flvB* mutant and the WT appeared after H₂ production ceased (Supplemental Fig. S9B). H₂ photoproduction therefore compensates the loss of Flvs until inhibition of the [FeFe]-hydrogenase by O₂. Flv-mediated O₂ reduction therefore appears to act as a relay system, supporting the re-oxidation of PSI acceptors when the [FeFe]-hydrogenase is no longer functional. We have also shown that Flvs act as the main drivers for the induction of photosynthesis in mutants devoid of hydrogenase (Fig. 5). Interestingly, although Flvs are present in most green algae (Supplemental Fig.

S11), hydrogenases are restricted to the core Chlorophytes (Meuser et al., 2011; Burlacot and Peltier, 2018). If Flvs alone may allow a transition from anaerobiosis to aerobiosis in the light in most green algae, the presence of hydrogenases may have conferred a selective advantage in specific biotopes from the core chlorophytes by allowing a faster photosynthetic induction when O₂ is not yet available.

Interplay with state transitions. During anaerobiosis, mobile light harvesting antennae are phosphorylated, detach from PSII and become associated with PSI. This process, called state transitions, is reversible and cells in state II (antennae associated with PSI) can return to state I (antennae associated with PSII) in more oxidizing conditions. A transition to state I occurs upon illumination of anaerobically adapted cells (Finazzi et al., 1999) and is of great importance for the re-activation of photosynthesis under these conditions (Ghysels et al., 2013). Forti and Caldiroli (Forti and Caldiroli, 2005) showed that light-driven O₂-dependent processes are involved in this process but could not dissect the molecular contributors. In our study, the increase in F_M' likely due to the transition from state II to state I (Supplemental Fig. S5) occurred after H₂ production (Fig. 2A), and when Flv-dependent O₂ uptake is maximal, highlighting the role of Flv-mediated O₂ reduction in the transition to state I.

Flv-mediated O₂ reduction versus Cyclic Electron Flow (CEF). Since both Flv-mediated O₂ reduction and CEF produce ATP without generating NADPH, they are expected to have similar effects on photosynthesis (Shikanai and Yamamoto, 2017). Recent experiments have shown that these two processes can complement each other in various organisms (Dang et al., 2014; Gerotto et al., 2016; Yamamoto et al., 2016; Wada et al., 2017). However, this question has not been addressed in a H₂-producing context, as O₂ reduction processes have been neglected. Recently, Godaux et al., (2015) observed that induction of photosynthesis in anaerobically-acclimated cells without glucose oxidase was not affected by a defect in Proton Gradient Regulation Like 1 (PGRL1)-dependent CEF, which indicates compensation by other mechanisms such as H₂ production or Flv-dependent O₂ reduction. On the other hand, we have shown in this work that the anaerobic induction of photosynthesis is impaired in *flvB* mutants. However, the maximum capacity of each alternative electron route can account for up to 40% of the linear electron flow (Alric, 2014) (Fig. 1B). Our data thus suggest that Flv-dependent O₂ reduction is the main alternative electron route after H₂ photoproduction upon illumination in anaerobically adapted *C. reinhardtii*, and that in these conditions it exceeds the capacity of CEF in tuning the NADPH/ATP ratio.

Intracellular O₂ recycling. Upon its production by PSII, O₂ can diffuse from thylakoid membranes towards the extra-cellular medium. During the diffusion process, O₂ can be reduced by cellular metabolic pathways, thus resulting in intra-cellular O₂ recycling. This phenomenon has been reported to involve mitochondrial respiration (Lavergne, 1989), and may potentially involve other O₂

photoreduction pathways such as those linked to the Plastid Terminal Oxidase (PTOX) or the enzymatic activities of Flvs. Because a hydrogenase deficient mutant shows PSII activity (Fig. 5A) while not producing O₂ in the medium (not shown), and because this phenomenon depends on the presence of Flvs (absent in a Flv and hydrogenase deficient mutant), we conclude here that Flvs take part in such an intracellular O₂ recycling.

Flvs are not involved in protecting the [FeFe]-hydrogenase in micro-oxic niches. Since the report that some algae can produce H₂ in the presence of atmospheric concentrations of O₂ (Liran et al., 2016), the possibility for intra-cellular micro-oxic niches allowing hydrogenases to be active at high O₂ concentrations has been considered. However, although the molecular players involved in such micro-oxic niches have not been identified so far, Flvs have been proposed as possible candidates (Liran et al., 2016). We have shown here that H₂ production is not negatively impacted and is in fact higher in the absence of Flvs (Fig. 2) and that the activity of the [FeFe]-hydrogenase is not affected (Supplemental Fig. S5). Based on these data we conclude that at least in the experimental conditions that have been tested, Flvs are not involved in the establishment of intracellular micro-oxic niches that would protect the [FeFe]-hydrogenase. We instead showed that the enhancement of H₂ production in *flvB* mutants likely results from an increased electron flux towards [FeFe]-hydrogenases. This directly supports previous data showing that the competition for electrons at the acceptor side of PSI plays a key role in H₂ photoproduction during a light transient (Godaux et al., 2015; Milrad et al., 2018). In aerobic condition, Flvs have their highest activity during light transitions (Chaux et al., 2017), conditions that we have explored in anaerobiosis here. However, no protective effect of the [FeFe]-hydrogenases could be observed under these conditions. The possibility that Flvs might protect the [FeFe]-hydrogenases from O₂ in steady state low light, as proposed by (Liran et al., 2016), seems rather unlikely given their reduced activity during steady state illumination (Chaux et al., 2017). Further investigations will be needed to determine the possible molecular mechanisms involved in the protection of [FeFe]-hydrogenases from O₂ in microalgae.

MATERIALS AND METHODS

***Chlamydomonas* strains and cultures.** The *C. reinhardtii* WT strain CC-4533 and *flvB* mutants (*flvB-208* and *flvB-308*) were previously described (Chaux et al., 2017). Cells were grown photoautotrophically in flasks at 25°C in HSM medium (pH 7.2) under dim light (30-40 μmol photons m⁻² s⁻¹). For some experiments (Figs. 2-4), cells were grown in photobioreactors (Dang et al., 2014; Chaux et al., 2017), under a light intensity of 66 μmol photons m⁻² s⁻¹ by bubbling air in HSM medium (pH 6.2). *C. reinhardtii* double mutants devoid of both hydrogenases (*hydA1/A2*) and triple mutants (*hydA1/A2 flvB*) were grown in TAP medium (Fig. 5.) Cells were cultivated under low light and harvested during the exponential phase. Experiments presented throughout this manuscript were performed on three independent single colony-derived lines for the WT, for each of the two strains

carrying insertions in the *FLVB* gene and for each of the three *hydA1/A2 flvB* strains, thus SDs account for standard errors of biological triplicates calculated with Prism (GraphPad Softwares, USA).

The mutant strain *mt⁻ flvB-308* was back-crossed twice with a 137c strain (Tollete et al., 2011) which is the parental strain of the *hydA1/A2* mutant reference strain (Meuser et al., 2012). The *mt⁻ flvB* mutant obtained was then crossed with the *mt⁺ hydA1/A2* mutant. The progeny of this crossing was selected based on chlorophyll fluorescence to screen for the insertion in the *FLVB* gene and H₂ production to screen for the insertion in *HYDA1* and *HYDA2*. We isolated three independent progenies exhibiting a *flvB*-mutant-like chlorophyll fluorescence transient (Chaux et al., 2017) (Supplemental Fig. S6 B) that did not produce H₂ after 1.5 h of dark anaerobic induction. Absence of the FlvB and FlvA proteins was then checked via immunodetection (Supplemental Fig. S6 B). The three triple mutants obtained were named *hydA1/A2 flvB* (1, 2 and 3 respectively), CC-124 was used as a reference strain for these mutants.

Chlorophyll fluorescence measurements. Chlorophyll fluorescence measurements were performed using a pulse amplitude-modulated fluorimeter (Dual-PAM 100, Walz, Germany), the optic fiber of which was coupled to a Plexiglas optic guide used as a stopper for the measuring chamber (Supplemental Figure S1). Detection pulses (10 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ blue light) were supplied at a 100 Hz frequency. Basal fluorescence (F_0) was measured after a 1.5h dark incubation under anaerobiosis and for aerobic samples after 10 min of dark acclimation. Red saturating flashes (8,000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, 600 ms) were delivered to measure F_M (in dark acclimated samples) and then every 15 s to measure F_M' (upon actinic light exposure). PSII quantum yields were calculated as $(F_M' - F_0)/F_M'$ according to (Genty et al., 1989). An actinic green light was chosen in order to limit light heterogeneity in the measuring chamber, delivered by three LEDs surrounding the measuring chamber and powered by a stabilized voltage generator. Anaerobic gross O₂ production was calculated assuming, like in aerobic conditions, that there is a linear relationship between the quantum yield of O₂ evolution and the F_V/F_M ratio (Fig. 1A). Gross O₂ evolution was then deduced linearly from the F_V/F_M ratio and was minimized by the MIMS-measured net O₂ evolution to overcome any initial difference in PSII absorbance.

Membrane Inlet Mass Spectrometry (MIMS) measurements. Gas exchanges were monitored inside a water-jacketed and thermoregulated (25°C) measuring chamber (modified Hansatech O₂ electrode chamber) containing 1.5 mL of cell suspension. The bottom of the chamber was sealed by a Teflon membrane (13 μm thickness) allowing dissolved gases to be introduced into the ion source of the mass spectrometer (model Prima δB , Thermo-Fischer) through a vacuum line containing a water trap cooled at -65°C. Cells were harvested from stabilized (at least 48 h) turbidostatic cultures in photobioreactors or exponential growth phase cultures in flasks, then centrifuged at 450 x g for 5 min and resuspended in fresh HSM medium (pH 6.2) or fresh TAP medium (pH 7.2) at a final

concentration of 20 $\mu\text{g chl mL}^{-1}$. The cell suspension was introduced into the measuring chamber under constant stirring and the cuvette was closed with the optic guide stopper. Actinic green light was supplied by three green LEDs. For anaerobic measurements, self-anaerobiosis was reached inside the measuring chamber and cells were incubated for 1.5 h or 45 min in anaerobiosis. For aerobic measurements, [^{18}O]-enriched O_2 (99% $^{18}\text{O}_2$ isotope content; Euriso-Top) was bubbled into the cell suspension until approximately equal concentrations of $^{16}\text{O}_2$ and $^{18}\text{O}_2$ were reached. The mass spectrometer sequentially scanned gas abundances (H_2 , N_2 , $^{16}\text{O}_2$, $^{18}\text{O}_2$ and CO_2) by automatically adjusting the magnet current to the corresponding mass peaks ($m/z = 2; 28; 32; 36; 44$, respectively). The amperometric signal collected by the spectrometer was calibrated by bubbling pure gases in a cell-free solution. Gas exchange rates were determined after correction for the gas consumption by the mass spectrometer assuming a first order kinetic, and normalizing H_2 , O_2 and CO_2 concentrations to the N_2 concentration used as an internal reference. O_2 exchange rates were measured according to (Peltier and Thibault, 1985). The apparent quantum yield of O_2 evolution was determined by dividing gross O_2 evolution rates by the incident light intensity integrated over the measuring chamber.

For H/D exchange rate measurements, D_2 was bubbled into the cell suspension until saturation. Then, the cuvette was closed and gas abundances (H_2 , HD, D_2 and N_2) were recorded ($m/z=2; 3; 4; 28$; respectively). The H/D exchange rate was determined according to (Jouanneau et al., 1980).

Accession Numbers

Genes studied on this articles can be found on <https://phytozome.jgi.doe.gov/> under the loci Cre03.g199800 (HYDA1), Cre09.g396600 (HYDA2), Cre12.g531900 (FLVA) and Cre16.g691800 (FLVB).

Sequence data from this article can be found in the GenBank data library under accession numbers XM_001693324.1 (HYDA1), XM_001694451.1 (HYDA2), XM_001699293.1 (FLVA) and XM_001692864.1 (FLVB).

Supplemental Data

Supplemental Figure S1. Experimental device used for combined measurements of gas exchange by using MIMS and chlorophyll fluorescence with a PAM.

Supplemental Figure S2. Representative chlorophyll fluorescence recordings obtained during a dark to light transient in anaerobically acclimated *C. reinhardtii* cells.

Supplemental Figure S3. Relationship between quantum yield of O_2 evolution and $\Delta F/F_M$ in aerobiosis at a low chlorophyll concentration.

Supplemental Figure S4. Saturation curve of net photosynthesis with green light.

Supplemental Figure S5. Maximum fluorescence yield (F_M') upon a shift from dark anaerobiosis to light as a control for assessing PSII absorbance changes.

Supplemental S6. Characterization of the mutant *Chlamydomonas* cells.

Supplemental Figure S7. Hydrogenase activity assayed *in vivo* by H/D exchange.

Supplemental Figure S8. Differences in O₂ concentration present in the MIMS chamber containing *flvB* mutants compared to the WT during a dark to light transient in anaerobically acclimated *C. reinhardtii*.

Supplemental Figure S9. Net O₂ and CO₂ exchange rates in the light in *C. reinhardtii* wild-type and two *flvB* mutants.

Supplemental Figure S10. PSI and PSII activities as measured by ElectroChromic Shift (ECS) upon illumination of anaerobically-acclimated *Chlamydomonas* cells.

Supplemental S11. Repartition of Flavodiiron proteins (Flv) and [Fe-Fe] hydrogenase on the evolutionary tree of eukaryotic microalgae.

Supplemental Table S1. List of algae exhibiting a Flv in their genome.

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Figure legends

Figure 1. Combined measurements of gas exchange and chlorophyll fluorescence show the existence of O₂ recycling during the first minutes of illumination of anaerobically acclimated *C. reinhardtii*. (A) Relationship between the chlorophyll fluorescence parameter $\Delta F/F_M$ and the apparent quantum yield of gross O₂ evolution established from simultaneous measurements of chlorophyll fluorescence by a PAM fluorimeter, and O₂ exchange by MIMS in the presence of [¹⁸O]-enriched O₂. The red line shows the linear regression for the $\Delta F/F_M$ values lower than 0.58. The chlorophyll concentration was 20 $\mu\text{g Chl mL}^{-1}$. Data were recorded after 10 minutes dark adaptation. (B) Combined measurements of H₂ and O₂ exchange during a dark to light (660 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ green light) transient in 1.5h anaerobically acclimated WT cells. Measured net O₂ and H₂ evolution rates are shown as filled circles (blue and green, respectively). Gross O₂ evolution rates (black empty circles) were determined from $\Delta F/F_M$ measurements based on the relationship established in (A), and O₂ uptake rates (red empty circles) from the difference between gross and net O₂ evolution rates. Right panel: box plots show gross O₂ evolution and O₂ uptake rates determined after 3 min of illumination (mean \pm SD, n=5 biological replicates).

Figure 2. Determination of oxygen uptake rates during the first minutes of illumination of anaerobically-adapted cells. After 1.5h of dark anaerobic acclimation, algal suspensions of wild type

(WT, red) and two *flvB* mutants (*flvB-208* in black, and *flvB-308* in grey) were illuminated (660 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ green light). Gas exchange was measured using a MIMS and PSII yields were determined using a PAM. Gross oxygen evolution was determined from PSII yields. The minimal gross oxygen uptake was calculated by subtracting the net oxygen evolution to the calculated gross oxygen evolution. (A) Representative traces of minimal O_2 uptake in the WT and mutant strains. (B) Mean values of minimal O_2 uptake after 1 min, 2 min and 2.5 min illumination. Shown are mean values (\pm -SD, $n=3$ for the mutants, $n=6$ for the WT). A star marks significant differences (Pvalue <0.05) based on ANOVA analysis (Tukey adjusted P value).

Figure 3. Hydrogen photoproduction in *C. reinhardtii* wild-type and two *flvB* mutants. After 1.5h of dark anaerobic acclimation, wild-type (WT, red) and two *flvB* mutants (*flvB-208* in black, and *flvB-308* in grey) cell suspensions were illuminated (660 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ green light) and H_2 production was recorded with a MIMS. (A) Representative traces of H_2 production. (B) H_2 production rates during the first three minutes of illumination. Due to some variability in absolute gas exchange values between biological replicates, shown data are expressed relative to the WT mean level (\pm -SD, $n=3$ for the mutants, $n=6$ for the WT). Stars indicate significant difference between WT and mutant strains (P-value < 0.05) based on ANOVA analysis (Tukey adjusted P value).

Figure 4. Net O_2 and CO_2 exchange rates during a light transient in *C. reinhardtii* wild-type and two *flvB* mutants. After 1.5h of dark anaerobic acclimation, wild-type (WT, in red) and two independent *flvB* mutants (*flvB-208* in black, and *flvB-308* in grey) cell suspensions were illuminated (660 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ green light). O_2 and CO_2 gas exchange were measured using a MIMS. (A, B) Representative traces of net O_2 evolution and net CO_2 evolution. (C, D) Mean values of net O_2 evolution (C) and net CO_2 uptake (D) rates measured at 3 min and 9 min of illumination. Due to some variability in absolute gas exchange values between biological replicates, shown data are expressed relative to the WT mean level (\pm -SD, $n=3$ for the mutants, $n=6$ for the WT). Stars indicate significant differences between the wild-type and the mutant strains (P-value < 0.05) based on ANOVA analysis (Tukey adjusted P value).

Figure 5. Induction of photosynthesis in anaerobically adapted *C. reinhardtii* cells devoid of hydrogenases and Flvs. Chlorophyll fluorescence and gas exchange rates were recorded under illumination (660 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). (A) Operating PSII quantum yield. (B) CO_2 fixation rates measured after 9 min of illumination. Anaerobic acclimation was performed during 90 min for hydrogenase containing strains and 45 min for hydrogenase deficient strains. Shown are mean (\pm -SD, $n=3$), a star marks a significant difference between WT and *flvB* (on the left) and triple mutants and the *hya1/A2* mutant (on the right) based on ANOVA analysis (Tukey adjusted P value).

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