Photosynthetic electron transfer pathways during hydrogen photoproduction in green algae mechanisms and limitations

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Chapter 8

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Summary

Some microalgal species produce hydrogen in the light owing to a coupling between the photosynthetic electron transfer chain and a [FeFe]-hydrogenase. This reaction, which represents a waste of energy for algal cells, avoids over-reduction of photosynthetic electron carriers particularly during the anaerobic induction of photosynthesis. Algal hydrogen photoproduction is promising for biotechnological applications, but represents a minor route for electrons in most situations. The first pathway limitation relates to the sensitivity of the [FeFe]-hydrogenase to oxygen, which is produced in the light by photosystem II (PSII). The second limitation relates to the supply of electrons to the [FeFe]-hydrogenase, since electron transfer reactions of oxygenic photosynthesis are highly regulated. Multiple pathways operate during oxygenic photosynthesis, including the linear pathway (the so-called "Z" scheme) oxidizing water at PSII and reducing $NADP^+$ at PSI, cyclic pathways recycling electrons around PSI, and electron pathways reducing molecular oxygen. The last occur either in the chloroplasts (using the plastid terminal oxidase PTOX or flavodiiron proteins, Flv) or in mitochondria thanks to the operation of metabolic shuttles. We will describe in this chapter how these different pathways interact to supply electrons to the [FeFe]-hydrogenase during the process of hydrogen photoproduction and discuss future possible biotechnological improvements.

8.1. Introduction

Since the early discovery by Gaffron and Rubin that microalgae can produce hydrogen in the light for short periods of time[1], extensive research has covered multiple aspects of the biological mechanisms involved in H_2 production. They include the nature, structure, and function of the [FeFe]-

hydrogenase; its reactivity and sensitivity to oxygen; and metabolic aspects related to H_2 production. The [FeFe]-hydrogenase requires reducing power to produce H_2, which can be supplied in the dark by the fermentative metabolism or in the light by coupling with the photosynthetic electron transfer reactions. During photosynthesis, the reducing power originates from the photolysis of water, which takes place at photosystem II (PSII) (see Chapter 1). PSII activity results in the reduction of the primary PSII electron acceptor, Q_A, and subsequently to the reduction of the membrane pool of plastoquinones. Electrons are then transferred to the cytochrome *b_6/f* complex; to plastocyanin, a soluble electron carrier located in the lumen; and then on to the photosystem I (PSI) donor side. PSI activity reduces stromal ferredoxin at its acceptor side, and reduced ferredoxin is used for multiple metabolic reactions, including synthesis of NADPH by the ferredoxin-$NADP^+$ reductase (FNR) and CO_2 fixation. Reduced ferredoxin also supplies electrons to the [FeFe]-hydrogenase[2], which combines electrons and protons to produce H_2. In addition to this set of electron transfer reactions occurring from PSII to PSI (the "Z" scheme), other pathways have been described, such as the cyclic electron flow (CEF) around PSI, which recycles electrons from reduced ferredoxin to the plastoquinone pool (Figure 1).

Hydrogen photoproduction has received strong interest since it allows transforming light energy into molecular H_2, which can be used as an energy vector for multiple applications. It was recognized early that producing H_2 in the light by an enzyme highly sensitive to O_2 is a major limitation, since molecular O_2 is produced by PSII[3]. Different strategies have been developed to overcome this limitation, based on the anaerobic induction of hydrogenases and either on a temporal-based separation of oxygenic and anaerobic phases or on a spatial separation related to the existence of anoxic micro-domains at the enzyme vicinity. Such strategies are mainly based on the flexibility of the photosynthetic electron transfer pathways. Indeed, in the case of a temporal-based separation of the O_2 and H_2 production phases, the reducing power generated during the aerobic phase is transiently stored as a reserve compound (mainly starch[4]) and subsequently re-used under anoxic conditions to produce H_2[5]. In the case of a spatial separation in the vicinity of the enzyme, part of the photosynthetic electron flow (producing oxygen at PSII) is diverted to O_2 consuming pathways to protect the hydrogenase against O_2.

In this chapter we will focus on the photosynthetic electron transfer reactions involved in H_2 photoproduction in unicellular green algae. We will particularly discuss the nature and regulation of electron transfer pathways involved in the supply of reductant to the [FeFe]-hydrogenase, and pinpoint conditions under which electron transfer reactions may limit H_2 photoproduction.

8.2 Electron pathways involved in hydrogen photoproduction

Following the initial discovery of a light-dependent H_2 production by algae[1], Spruit first showed that H_2 and O_2 are simultaneously produced in the light with a 2/1 ratio[6]. By using PSII inhibitors, Bishop and Gaffron[7] concluded that PSII is required for H_2 photoproduction, but later on Stuart and Gaffron showed that PSII is not needed in some algal species[8]. Actually, the PSII contribution to H_2 photoproduction was found to vary according to species and culture conditions[9]. The existence of two pathways, a PSII-dependent and a PSII-independent pathway was finally proposed[10].

The requirement of PSI has also been challenged when oxygenic photosynthesis and H_2 photoproduction were claimed to be possible in the absence of functional PSI[11]. However, it turned out that the "PSI-deficient" *Chlamydomonas* mutant strains used in this work contained low amounts of PSI, and that PSI is indeed required for oxygenic photosynthesis[12] and H_2 photoproduction[13]. Like PSI, the plastoquinone pool and the cytochrome b_6/f complex are common to both PSII-dependent and PSII-independent pathways[2].

8.2.1 Direct biophotolysis

The H_2 photoproduction pathway using bio-photolysis of water as a direct source of electrons, was called "direct biophotolysis"[14]. This pathway utilizes the whole photosynthetic chain from PSII to PSI, and to ferredoxin (PetF), the electron donor to the [Fe-Fe] hydrogenase[2]. Reduced ferredoxin reversibly binds the algal [Fe-Fe] hydrogenase, thus providing an electron path to the catalytic site for the reduction of protons[15]. The direct pathway of H_2 photoproduction is the simplest way to convert solar energy and water into molecular H_2 using a biological process, and has therefore considerable biotechnological potential. However, since it is producing molecular oxygen with an O_2/H_2 ratio of 1/2, the potential of this pathway is considerably limited due to the rapid and irreversible inactivation of the [FeFe]-hydrogenase by molecular O_2[3,16,17]. Consequently, most of the research efforts to optimize the direct pathway currently aim at protecting the [FeFe]-hydrogenase from O_2 either by engineering the [FeFe]-hydrogenase itself (see Chapters 3, 5, and 6) or by creating micro-oxic zones in the vicinity of the [FeFe]-hydrogenase (see § 8.4; Chapter 15). The concept of "direct biophotolysis" should be distinguished from the concept of a "PSII-dependent" pathway since both direct and indirect processes actually require PSII, PSII being needed during the aerobic phase of indirect biophotolysis for the synthesis of reserve compounds (see §8.2.2).

8.2.2 Indirect biophotolysis

The concept of "indirect biophotolysis" was introduced to refer to a separation (either spatial or temporal) of aerobic stages, where photosynthesis is active and produces O_2 at PSII and anaerobic stages where the hydrogenase is induced and H_2 is produced[14]. Spatial separation between both

processes is achieved in nitrogen-fixing cyanobacteria, where H_2 is produced in heterocyst cells, specialized cells that protect their contents from O_2, and where the O_2-sensitive nitrogenase produces H_2 as a side reaction[18,19]. It can also be achieved in single-cell organisms like microalgae on a temporal-based manner, due the flexibility of the electron transfer and metabolic reactions. In microalgae indirect biophotolysis relies on two successive processes, storage of photosynthetic compounds (mainly as starch reserves) during an aerobic phase, and their subsequent conversion either in the dark by fermentation or in the light by photo-fermentation[14]. Dark fermentation is a relatively inefficient process for the production of H_2, and photofermentation is considered as more efficient and promising[20]. Algal photo-fermentation relies on different factors: *i.*, down-regulation of PSII to limit O_2 production to maintain anaerobiosis and [FeFe]-hydrogenase active, *ii.*; flexibility of the electron transfer pathways, in order to supply electrons originating from starch metabolism to PSI and then to the [FeFe]-hydrogenase in the absence of PSII, and *iii.*; significant accumulation of starch reserves. Such two-stage H_2 production was nicely illustrated by experiments carried out in closed flasks under conditions of sulfur deprivation[21]. During the aerobic phase, sulfur deprivation triggers a progressive decrease in PSII activity and massive starch accumulation[4]. When PSII activity drops below the level of respiration, the [FeFe]-hydrogenase is induced and H_2 photoproduction proceeds[21]. The study of *C. reinhardtii* mutants affected in starch metabolism established that the PSII-independent pathway, which is involved in the indirect pathway, is essentially fueled by starch catabolism[22].

During indirect biophotolysis, electrons are first injected into the intersystem electron transport chain at the level of the plastoquinone pool, and then to the [FeFe]-hydrogenase through PSI (Figure 2). The existence of an electron transport pathway capable of reducing the intersystem chain at the expense of soluble stromal pools was initially considered possible due to the existence of a respiratory chain called chlororespiration in the chloroplast of algae[23,24]. While land plants, particularly angiosperms, contain a multi-subunit NDH-1 complex involved in the non-photochemical reduction of the plastoquinone pool from stromal NAD(P)H, microalgae are notably devoid of such a complex[25,26]. Microalgae were proposed to harbor a non-electrogenic type II dehydrogenase (NDH-2) capable of reducing plastoquinones in a non-photochemical manner[25,27]. The single subunit plastidial NDH-2 (called Nda2) was characterized enzymatically[28], and the role of this enzyme in the non-photochemical reduction of plastoquinones and H_2 production was demonstrated by the study of Nda2-deficient mutants[29]. Indirect biophotolysis was considered as having great potential for H_2 photoproduction[22,30]. On one hand, it avoids inhibition of the [FeFe]-hydrogenase by O_2. On the other hand, the use of a two-stage process, in which the second stage has a lower quantum requirement (the indirect pathway requires only PSI during the anaerobic stage)

makes it possible to carry out the aerobic phase in low-cost production systems such as open ponds and the anaerobic phase of H_2 production in closed photobioreactors of smaller volume[30]. However, even if the rate of H_2 production by the indirect pathway measured by using PSII inhibitors increases during sulfur-deprivation, it is still much lower than by the direct pathway {Cournac, 2002 #608}[31,32], showing that it suffers from limitations, and requires biotechnological improvement (see §8.3.2).

8.2.3 Effect of nutrient deprivation and role of starch reserves

Both pathways for H_2 photoproduction have limiting steps that preclude sustained H_2 photoproduction for days, and a great advance for surmounting these limitations was made through the development of experimental protocols based on nutrient deprivation, especially sulfur deprivation[21]. Sulfur depletion has been shown to down-regulate photosynthetic capacity by selectively inhibiting PSII activity[33]. During sulfur deprivation, O_2 evolution by PSII decreased by more than 90% after 24 hours, while dark respiration rate declines significantly less and remains stable after 24 h[21,34]. When placed in sealed flasks, sulfur-deprived cells reach anaerobiosis as soon as photosynthetic O_2 production by PSII drops below the level of respiration, thus resulting in a net consumption of oxygen[21,35]. When anaerobiosis is reached, the [FeFe]-hydrogenase is rapidly induced, resulting in sustained H_2 photoproduction[21]. Sulfur deprivation also induces the degradation of Rubisco[36], which is the first enzyme of the Calvin-Benson cycle responsible for CO_2 fixation. Therefore, sulfur deprivation surmounts two major limitations of H_2 photoproduction, the sensitivity of the [FeFe]-hydrogenase to O_2 and the competition between CO_2 fixation and H_2 production for electrons produced by PSII.

From the early sulfur deprivation experiments, it was noticed that the intracellular starch content widely fluctuates[36,37]. The role of starch reserves during H_2 photoproduction was first evidenced by the isolation of a *C. reinhardtii* mutant affected in H_2 photoproduction, which turned out to be impaired in starch biosynthesis[5]. Actually, the massive accumulation of starch is part of a general cellular response to nutrient starvation. Under conditions of sulfur deprivation, PSII activity is decreased, but the remaining PSII activity is required for both the accumulation of starch[4,37] and direct electron transport from water to the [FeFe]-hydrogenase after anaerobiosis[38]. Subsequent starch breakdown is used for two different purposes during the process of H_2 production to, *i.* fuel mitochondrial respiration that allows establishing and maintaining anaerobiosis, and *ii*. supply reductants for the indirect pathway. Under mixotrophic conditions (i.e., in the presence of an external acetate supply) intracellular starch accumulates during the first phase of sulfur deprivation, is then degraded when H_2 production starts, and keeps being degraded throughout the H_2-production phase[34,37]. Under fully photoautotrophic conditions, starch breakdown starts during the aerobic phase

likely to fuel mitochondrial respiration to help establish anaerobiosis[39]. Based on the effect of the PSII inhibitor DCMU on starch mutants, Chochois et al.[40] established that during sulfur deprivation starch is essential for the indirect pathway, but is dispensable during the direct pathway. If available, acetate can be used to help establishing anaerobiosis under mixotrophic conditions, is no longer consumed after the onset of H_2 production[31], but can be dispensable and replaced by starch under fully autotrophic conditions[39,40]. Thus during temporal separation of O_2 and H_2 production (e.g., during sulfur deprivation), starch can be used by both indirect and direct pathways (Figures 2 & 3).

8.3 Limitation of hydrogen photoproduction related to the electron supply

Under most physiological conditions H_2 photoproduction is a minor pathway, used as a temporary sink to avoid harmful over-reduction of photosynthetic electron carriers. Indeed, H_2 photoproduction is a waste of energy for the algal cell, and photosynthetic electron transfer pathways have been optimized to limit such waste. Understanding regulatory mechanisms controlling electron transfer reactions and identifying limitations in the supply of electrons to the [FeFe]-hydrogenase are important points to consider for catalyzing further biotechnological improvements.

8.3.1 Competition with the photosynthetic carbon reduction cycle

The photosynthetic carbon reduction cycle is the major electron sink during oxygenic photosynthesis and was recognized early as a major potential competitor for H_2 photoproduction[41]. Increased H_2 photoproduction was reported in Rubisco deficient mutants, photoproduction being observed even in the presence of sulfur likely due to lower production of PSII O_2-evolution activity[34,42]. Actually, the competition between H_2 production and CO_2 fixation takes place at the level of the ferredoxin-$NADP^+$ reductase (FNR), since both $NADP^+$ and [FeFe]-hydrogenase use ferredoxin as an electron donor (Figure 1). Furthermore, genetic engineering has been used to preferentially redirect electrons towards the [FeFe]-hydrogenase. Replacement of the [Fe-Fe]-hydrogenase with a fusion protein between ferredoxin and [FeFe]-hydrogenase was shown to switch the bias of electron transfer from FNR to the [FeFe]-hydrogenase, resulting in an increased rate of H_2 production[43,44]. More recently, the engineering of ferredoxin showed that it is possible to suppress FNR binding and redirect electrons towards the [FeFe]-hydrogenase, thus enhancing H_2 production *in vivo*[45]. These engineering strategies have demonstrated the possibility of channeling electrons towards the [FeFe]-hydrogenase, thus paving a new path for the creation of optimized H_2-producing organisms that are less limited by competitive competition on the acceptor side of PSI.

8.3.2 Down-regulation by the proton gradient and contribution of cyclic electron flow

CEF, by recycling electrons around PSI, generates a component of the proton gradient, which in addition to the proton gradient generated by the "Z" scheme, is used for ATP synthesis. Thus CEF participates in re-equilibrating the balance between ATP and NADPH during oxygenic photosynthesis[46]. In *C. reinhardtii*, two main pathways of CEF have been identified based on their sensitivity to inhibitors[47]. The antimycin A-sensitive pathway involves PGRL1[48,49] and PGR5[50], while the antimycin A-insensitive pathway involves Nda2[29]. A link between H_2 photoproduction and cyclic electron flow around PSI has been proposed, based on the high H_2-production capacity of a mutant affected in state transition and showing lower cyclic electron flow activity[51]. It was initially suggested that H_2 photoproduction is enhanced in this mutant due to rerouting of electrons from cyclic electron flow to the [FeFe]-hydrogenase[51]. Such an explanation seems unlikely, however, since cyclic electron flow does not act as a sink for electrons, but rather each electron leaving the PSI acceptor side is redirected towards the PSI donor side. Actually, the main control of electron transport reactions during oxygenic photosynthesis (called photosynthetic control) is the down-regulation of cytochrome b_6f complex activity by the trans-thylakoidal proton gradient[52,53]. The stimulating effect of uncoupling agents on H_2 photoproduction was first reported by Bishop and Gaffron[7], and it was proposed to result from a limitation on electron transfer reactions (possibly at the level of the cytochrome b_6f complex) by the trans-thylakoidal proton gradient[54]. Furthermore, H_2 photoproduction was strongly enhanced in a mutant affected in PGRL1-mediated CEF[49]. The differential effect of the uncoupler, FCCP, on H_2 photoproduction measured in the wild-type strain and in the *pgrl1* mutant, also led Tolleter et al. to conclude that electron flow is down-regulated in the wild-type strain by the proton gradient component linked to the activity of CEF[49]. These results clearly show that CEF can limit H_2 production by triggering the photosynthetic control mechanism, that down-regulates the flow of electrons from PSII to the [FeFe]-hydrogenase.

8.3.3 Non-photochemical reduction of the plastoquinone pool

During the indirect pathway of H_2 production, stromal reducing equivalents generated by the degradation of starch are injected into the plastoquinone pool in a non-photochemical manner by Nda2, and in turn transferred to PSI and then to the [FeFe]-hydrogenase. Given the potential of indirect biophotolysis[30], and its low activity compared to the direct biophotolysis process, an important question to consider for further biotechnological improvements is to determine what is the limiting step of the indirect pathway. Indeed, the indirect pathway can potentially be limited by the supply of reducing equivalents from starch catabolism, by the activity of Nda2 or by downstream limitations, such as the photosynthetic control taking place at the level of the cytochrome b_6f complex. Contrasting conclusions have been discussed in the literature, recognizing that different limitations

may occur depending on the experimental conditions. By studying the effect of DCMU on H_2 photoproduction in the *pgrl1* mutant deficient in CEF, Tolleter et al. concluded that the indirect pathway, like the direct pathway, is limited by photosynthetic control[49]. However, Baltz et al.[55], studying the effect of Nda2 over-expression on H_2 production under different conditions of nutrient supply, concluded that the indirect pathway is limited by the non-photochemical reduction of plastoquinones. This occurs either by a limitation in the available stromal pool of electrons or by the activity of Nda2 itself, when the stromal pool of electrons is sufficient such as under nutrient-depleted conditions[55]. It was suggested that the long-term stimulation of H_2 photoproduction by the indirect pathway previously observed in the *pgrl1* mutant as compared to the WT in response to sulfur deprivation[49] may result from an indirect effect, such as the increased capacity of non-photochemical reduction of the PQ pool [55]. Under nutrient replete conditions the indirect pathway is limited by the supply of reducing equivalents from metabolism[55]. Therefore, again limitation of the indirect pathway may strongly vary depending on the experimental conditions.

8.4 Oxygen concentration in the vicinity of the [FeFe]-hydrogenase

Because of the extreme O_2-sensitivity of the [FeFe]-hydrogenase and the O_2-producing activity of PSII, sustained H_2 photoproduction by direct biophotolysis strongly depends on cellular processes capable of decreasing the O_2 partial pressure in the vicinity of the active site by consuming intracellular O_2[38]. Another biotechnological approach for addressing the O_2-sensitivity problem is discussed in Chapter 15.

8.4.1 Role of mitochondrial respiration

Mitochondrial respiration was recognized early as a major player for sustainable H_2 photoproduction in microalgae[21]. It was shown that by decreasing the activity of PSII below the level of respiration, sulfur deprivation allows for establishing anaerobiosis, thus triggering the induction of the [FeFe]-hydrogenase and promoting sustained H_2 photoproduction[21]. The use of respiratory inhibitors showed that both cytochrome oxidase and the alternative oxidase are involved during the process of H_2 production in sulfur-deprived cells[38]. Diminishing the photosynthesis/respiration ratio has been proposed as a way to improve H_2 photoproduction by the direct pathway[56]. By using inhibitors of mitochondrial respiration Antal et al.[57] observed an enhancement of H_2 photoproduction, and proposed that mitochondrial respiration functions as an electron sink, the inhibition of which promotes redirection of electrons to the [FeFe]-hydrogenase. However, the study of respiratory mutants led to contradictory conclusions. A *C. reinhardtii* mutant (*stm6*), affected in the regulation of respiratory complexes in response to light, showed increased starch accumulation and enhanced

H_2 photoproduction[51,58]. However, later studies of respiratory mutants affected at different levels of the mitochondrial respiratory chain showed decreased H_2 production and decreased starch accumulation in the mutants[59]. The dependency of starch accumulation on mitochondrial activity may explain the apparent discrepancy between the inhibitor and mutant studies. Indeed, the addition of respiratory inhibitors after the starch accumulation phase boosted H_2 photoproduction[57], while a lack of mitochondrial respiration during the initial phase of accumulation compromised starch accumulation, leading to a decreased H_2 photoproduction[59].

Although the contribution of mitochondrial respiration to H_2 photoproduction is widely recognized, the nature of the substrates fueling respiration has been a matter of debate. Indeed, either intracellular substrates (recently synthesized photosynthetic products or reserve compounds such as starch) or extracellular substrates (such as an external acetate supply) can serve as a source of reducing power for the mitochondrial respiratory chain. During the early phase of sulfur deprivation, mitochondrial respiration can be fueled by starch catabolism (provided sufficient starch accumulated within the cell) or by externally supplied acetate, thus resulting in the establishment of anoxia[4,39]. Acetate, however, is not required for H_2 photoproduction, which can occur under fully photoautotrophic conditions, provided PSII activity is decreased either by DCMU addition[4] or by lowering the light intensity in the photobioreactor[39]. Note that sustained H_2 production could also be observed in photoautotrophic, non-starved cells exposed to low light intensity[60]. Actually, acetate may only be needed to reach anoxia when PSII activity is high[39], or in the absence of starch reserves[40]. In the absence of an external acetate supply, the reducing power generated in the chloroplast, either by the photosynthetic electron transfer reactions or by starch catabolism, can be transferred to other cellular compartments, *via* metabolic shuttles such as the malate valve, and then used as a substrate by the mitochondrial respiratory chain[61]. Mitochondrial respiration may therefore serve to decrease the intracellular O_2 concentration and allow H_2 photoproduction. Note that if the entire flux of electrons generated by the photosynthetic chain were used to consume all of the O_2 produced at PSII, no electrons would be left for H_2 production. This points out the need for an additional source of electrons to maintain anoxia, which could be either intracellular (e.g., starch) or extracellular (an acetate supply).

8.4.2 Role of plastidial O_2 uptake processes

Two main electron transfer pathways leading to the reduction of O_2, the Mehler reaction and chlororespiration, have been described in chloroplasts[62]. These pathways may also participate in decreasing intracellular O_2 concentration, and may thus protect the hydrogenase from O_2 attack. Their efficiency may even be higher than mitochondrial respiration since O_2 photoreduction occurs

in chloroplasts, on the PSI acceptor side in the case of the Mehler reaction and between PSII and PSI in the case of chlororespiration (Figure 4). By using propyl-gallate, an inhibitor of the plastidial terminal oxidase, PTOX, Antal *et al*.[38] concluded that the contribution of chlororespiration is minor. On the other hand, high H_2 photoproduction has been described in a Rubisco-less mutant, which was ascribed to the existence of a high O_2 uptake rate through the Mehler reaction promoting anaerobiosis under conditions of sulfur deprivation[34]. More recently, based on the observation that H_2 production could be observed in *C. reinhardtii* cells even in the presence of O_2 in the culture medium, it was proposed that O_2 uptake pathways involved in chloroplast O_2 photoreduction might locally decrease the O_2 concentration in the vicinity of the hydrogenase, thus allowing significant H_2 production under these conditions[63]. Flavodiiron proteins (Flvs) are known to act in Mehler-like reactions in cyanobacteria[64,65], and genes encoding for such proteins are present in algae[62]. Based on the observation that the FlvA and FlvB transcripts are strongly expressed during the early phase of sulfur deprivation, it was proposed that these proteins might be involved in reaching anaerobiosis during sulfur deprivation[66]. This pathway, by creating micro-oxic niches at the vicinity of the [FeFe]-hydrogenase, may allow H_2 photoproduction in the presence of O_2 in the culture medium (Figure 4)[63].

Engineered protein fusion between the [FeFe]-hydrogenase and other proteins has been shown recently to decrease the O_2-sensitivity of the enzyme. A ferredoxin-hydrogenase fusion resulted in a lower O_2-sensitivity of the hydrogenase, and this was attributed either to a local decrease of O_2 thanks to its reduction into superoxide by the ferredoxin moiety or to a blockage in the access of O_2 to the active site [67]. A superoxide dismutase (SOD)-hydrogenase fusion was recently shown to enhance activity of the [FeFe]-hydrogenase, although the effect was not O_2 dependent[68]. Redirecting part of the photosynthetic electron flow to O_2-scavenging enzymes such as SOD or Flv, either freely in the chloroplast stroma or by fusion with the hydrogenase, may be a way in the future to optimize H_2 production in the direct biophotolysis pathway by creating micro-oxic niches in the vicinity of the [FeFe]-hydrogenase.

8.4.3 Effect of the PSII/PSI ratio

Producing H_2 by the indirect pathway relies on the ability to reversibly switch from oxygenic photosynthesis, in which both PSII and PSI are functioning, to anaerobic H_2 production, in which only PSI is active. On the other hand, producing H_2 by the direct pathway requires fine tuning of PSII activity in order to balance O_2-production and O_2-consuming mechanisms in order to maintain low O_2 concentration in the vicinity of the [FeFe]-hydrogenase. Both pathways, therefore, rely on the ability to regulate PSII/PSI stoichiometry, particularly the activity of PSII in a reversible manner.

This can be achieved by monitoring sulfur concentration in the culture[21], which differentially affects PSII and PSI activity. However, repeated exposure to nutrient starvation has deleterious effects on cell survival and H_2 photoproduction[69], thus encouraging researchers to develop better strategies to regulate PSII activity. The reversible control of PSII was demonstrated by placing Nac2, a nuclear gene involved in the stabilization of a PSII subunit transcript, under the control of an inducible promoter [70]. The potential of this strategy for H_2 production was demonstrated, opening the way for cyclic and sustainable H_2 production with an efficient electron partitioning between H_2 and cell growth[70]. Note, however, that only monitoring PSII activity might not be sufficient since another important effect of nutrient starvation is to induce the massive accumulation of starch, which is critical for sustained H_2 production.

During photosynthesis, the partitioning of excitation energy between PSII and PSI is controlled by a mechanism called state transition, which modulates the relative size of antennae attached to PSI and PSII. During transition from state 1 to state 2, LHCII antennae are phosphorylated by the STT7 kinase and migrate from PSII (state 1) to PSI (state 2)[71]. Anaerobiosis is known to induce transition to state 2[72] thus diminishing the PSII cross-section for light absorption. Although the effect of a state transition defect has not been reported so far on long-term H_2 photoproduction (i.e., during sulfur deficiency), this mechanism has been shown to involve the hydrogenase during the induction of photosynthesis under anaerobic conditions. In the *stt7* mutant blocked in state 1, induction of photosynthesis under anaerobiosis is faster than in the wild-type, indicating that state 2 cells must return to state 1 to efficiently resume photosynthetic O_2 production[73]. However, the induction of photosynthesis under anaerobiosis was severely compromised in a double mutant affected in both hydrogenase and state transition, thus showing that both mechanisms can independently promote the activation of photosynthesis[73].

From the study of a set of *Chlamydomonas* mutants affected in PSII, Torzillo et al. concluded that the high H_2 production rate observed in some mutant strains was at least partly due to the presence of reduced levels of chlorophyll[74], reduced antenna size being recognized as favoring biomass productivity of algae when grown in photobioreactors[75] (see Chapter 14).

8.5 Physiological function of hydrogen photoproduction

Some algal species harbor a [Fe-Fe] hydrogenase in their genome (Table 1), but many others like red algae do not (Figure 5). In chlorophytes, an active hydrogenase has been found in *C. reinhardtii*[76], *Scenedesmus obliquus*[2] or *Tetraselmis* [77]. Because molecular hydrogen has the ability to quickly diffuse outside algal cells, H_2 production represents a loss of energy for the cell. The presence of a [FeFe]-hydrogenase in all sequenced Chlorophyceae and Chlorodendrophyceae (Figure 5) indicates

that this enzyme may confer a selective advantage in some species. By following H_2 and O_2 exchange in algal cells placed under anaerobiosis, Kessler observed that H_2 production precedes O_2 production in algal species that exhibit hydrogenase activity, while algal species devoid of hydrogenase activity do not produce H_2[41]. It was proposed that H_2 photoproduction by effectively removing excess of reducing power in the photosynthetic electron transport chain would favor the start of photosynthesis[41]. Similar delay between H_2 production, and photosynthetic O_2 and carbon dioxide exchange were observed in the H_2-producing species, *C. reinhardtii*[54]. In line with this view, *C. reinhardtii* mutants affected in maturation factors, HydEF and HydG, and unable to synthesize active hydrogenases HydA1 and HydA2, showed a lag phase in the induction of photosynthesis under anaerobiosis in comparison to wild-type strains[73]. Recently, the study of a double mutant affected in PGRL1-mediated CEF and [FeFe]-hydrogenase maturation factor HydG showed that CEF or H_2 photoproduction are necessary and sufficient to start photosynthesis under anaerobiosis[78]. It was proposed that the [FeFe]-hydrogenase by acting as a sink for electrons under anaerobiosis, would contribute to the establishment of a proton gradient in concert with CEF, required for the production of ATP and the induction of the photosynthetic carbon reduction cycle[78].

The physiological function of the algal [FeFe]-hydrogenase may also be related to dark anaerobic metabolism[79,80]. Indeed, [FeFe]-hydrogenase may contribute to fermentative metabolism by allowing reduced ferredoxin to be re-oxidized without producing high levels of other fermentative products, such as formic acid or ethanol, which are toxic at high concentration[81]. A recent transcriptomic study performed on synchronized *C. reinhardtii* cells showed that the level of both hydrogenases transcripts is increased by more than a 100 fold when switching from light to dark periods under aerobiosis[82], indicating that the [FeFe]-hydrogenase may be expressed under such conditions. However, hydrogenase genes have been so far found as essentially transcribed under anaerobic conditions[80,83,84]. This suggests the existence of micro-oxic niches within algal cells that may generate a signal for hydrogenase transcription. Whether active hydrogenases are present in hypothetic micro-oxic niches, however remains to be determined.

8.6 Future directions

Since the initial discovery of Hans Gaffron[1], the field of H_2 photoproduction by microalgae has been very active. If the role of the [FeFe]-hydrogenase is central in this process, photosynthetic electron transfer reactions and metabolic pathways are critical to supply electrons to the enzyme, and may limit H_2 photoproduction.

At the metabolic level, given the importance of starch as a source of electrons for H_2 production by both direct and indirect biophotolysis, the understanding of starch catabolism and its

regulation appears critical. Indeed, if reactions of starch biosynthesis are relatively well described, our knowledge of starch catabolism is still very fragmentary.

At the level of electron transport reactions, future work should aim at better characterizing regulatory mechanisms that may limit the supply of electrons to the [FeFe]-hydrogenase (see also Chapter 10). Our knowledge of the competition occurring between the different acceptors downstream of PSI is still fragmentary (see Chapter 9), and understanding the dynamics of electrons partitioning is of great importance for redirecting flux towards hydrogenase and bypassing this competition. Since the [FeFe]-hydrogenase may act as a safety valve evacuating excess of reducing power generated by the photosynthetic electron transport chain, modifying regulatory mechanisms may result in a higher availability of electrons for the [FeFe]-hydrogenase. This was nicely illustrated by the demonstration that CEF impairment boosts H_2-production capacities and should be extended in the future to other regulatory mechanisms, such as state transition or non-photochemical quenching, to determine to what extent they may limit H_2 photoproduction.

An important point to consider in the future relates to the possible existence of intracellular micro-oxic niches allowing for the production of H_2 in the presence of low O_2 levels in the culture medium. It will be important to determine the molecular players and their regulations, and further optimize the partitioning of electrons originating from photosynthesis to the different sinks, including [FeFe]-hydrogenase and O_2-scavenging pathways. A promising strategy may be to artificially create micro-oxic environments in the vicinity of the hydrogenase by fusing O_2-reducing enzymes to the hydrogenase and channeling electrons to such a scavenging device.

Strong interest in H_2 photoproduction by microalgae has catalyzed research in different but related scientific fields for decades, and should keep driving for future research, particularly in the field of photosynthesis research.

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Species	Class	Number of [Fe-Fe] hydrogenase	Accession	Reference
Chlamydomonas reinhardtii	Chlorophyceae	2	XM_001694451.1 XM_001693324.1	Merchant et al, 2007
Volvox carteri f. nagariensis	Chlorophyceae	2	XM_002948441.1 XM_002948437.1	Prochnik et al, 2010
Monoraphidium neglectum	Chlorophyceae	2	XM_014051392.1 XM_014045807.1	Bogen et al, 2013
Gonium pectorale	Chlorophyceae	2	KXZ54929.1 KXZ55261.1	Hanschen et al, 2016
Scenedesmus obliquus	Chlorophyta	1	AF276706.1	Wunshiers et al, 2001
Chlamydomonas moewusii	Chlorophyta	1	AY578072.1	Kamp et al, 2008
Tetraselmis subcordiformis	Chlorodendrophyceae	1	JQ317304.1	D'Adamo et al, 2014
Tetraselmis sp. GSL018	Chlorodendrophyceae	2	KC820788.1 KC820787.1	D'Adamo et al, 2014
Tetraspora sp. CU2551	Chlorodendrophyceae	1	KT984857.1	Maneeruttanarungroj et al, 2010
Chlorella variabilis NC64A	Trebouxiophyceae	2	XM_005848550.1 XM_005847085.1	Blanc et al, 2010
Chlorella sp. DT	Trebouxiophyceae	1	GU354311.1	Chien et al, 2012
Nannochloropsis gaditana CCMP526	Eustigmatophyceae	1	XM_005854475.1	Radakovitz et al, 2012
Thalassiosira pseudonana CCMP1335	Mediophyceae	1	XM_002295124.1	Ambrust et al, 2004 Bowler et al, 2008
Nitzschia sp.	Bacillariophyceae	1	GBCF01034699.1	Cheng et al, 2014

Table 1. List of algae exhibiting a [Fe-Fe] hydrogenase in their genome. Both *Chlamydomonas reinhardtii* HydA1 and HydA2 hydrogenase proteic sequences (https://phytozome.jgi.doe.gov/pz/portal.html) have been used for protein blast against translated sequenced genomes, transcriptome shotgun assembly for *Nitzschia* or protein data base for *Gonium pectorale* (ncbi.gov tblastn). Putative proteins have been selected on the basis of having a similarity score higher than 200 hits with one of the two *C. reinhardtii* hydrogenases. Protein sequences were analysed and only the ones containing crucial residues for hydrogenase activity[85] were selected. Class of organisms was set as defined by algae base (http://www.algaebase.org/).

Legends of Figures

Figure 1. Electron transport reactions of oxygenic photosynthesis in microalgae. Two light reactions take place during oxygenic photosynthesis, one at PSII using water as an electron donor that results in the reduction of the plastoquinone pool (PQ/PQH_2), and the other at PSI using reduced plastocyanin (Pc) as an electron donor that results in the reduction of ferredoxin (Fd). In the "Z" scheme of photosynthesis, electrons are transferred from PSII to PSI through the cytochrome (Cyt) b_6/f complex and plastocyanin. Cyclic electron flow (CEF) around PSI, which involves two major components, (Proton Gradient 5 (PGR5) and Proton Gradient Like 1 (PGRL1), is linked to Fd for the generation of additional proton gradient (not shown on this scheme for simplification). The plastidial type II NADH dehydrogenase (Nda2) catalyzes the non-photochemical reduction of plastoquinones from the stromal NAD(P)H pool. The flavodiiron protein (Flv) catalyzes the reduction of oxygen to water using NADPH as an electron donor. The [FeFe]-hydrogenase (H_2ase) catalyzes the reduction of protons to molecular H_2 by using reduced ferredoxin as a substrate.

Figure 2. Indirect pathway of hydrogen photoproduction. With indirect pathway, reducing power produced by oxygenic photosynthesis is temporarily stored as reserve compounds (e.g., starch) during a preliminary aerobic phase. During a subsequent anaerobic phase (illustrated in the figure), PSII activity is inactivated (either by means of PSII inhibitors such as DCMU or decreased using sulfur deprivation). The reducing power generated by starch catabolism is injected into the inter-system electron transport chain by Nda2, resulting in the reduction of plastoquinones and subsequent production of H_2 by PSI. Abbreviations are described in the legend of Figure 1.

Figure 3. Direct pathway of hydrogen photoproduction. With the direct pathway, electrons produced by the "Z" scheme of photosynthesis (see Figure 1) are used for the production of H_2. Oxygen produced by PSII is scavenged by mitochondrial respiration, using either intracellular starch or an external acetate supply as the source of reducing power. Although its role has not been as yet established, flavodiiron (Flv) could participate in creating micro-oxic niches at the level of the [FeFe]-hydrogenase. The mitochondrial electron transport chain harbors complex I (NDH-1), the ubiquinone pool (UQ/UQH_2), the alternative oxidase (AOX), the cytochrome bc_1 complex (Cyt bc_1 complex), and the cytochrome aa_3 terminal oxidase (Cyt aa_3 oxidase). Other abbreviations are described in the legend of Figure 1.

Figure 4. Hypothetical scheme showing intracellular oxygen concentrations during hydrogen production by the direct pathway within a *Chlamydomonas* cell. PSII is mainly located in the grana lamellae, where it produces molecular O_2 during oxygenic photosynthesis. Two oxygen-scavenging systems are considered here. The first is related to mitochondrial respiration, which is generally considered as the major O_2-scavenging system associated with the direct pathway of H_2 photoproduction (see Figure 3). The second is purely hypothetical at this point and involves a possible role for flavodiiron proteins in scavenging O_2 in the chloroplast stroma. Differential intracellular locations of O_2 sources and sinks create local variations of O_2 concentration within the chloroplast that may allow the [FeFe]-hydrogenase to be active locally. Two hypotheses are considered for the role of Flv: (1) Flvs may specifically interacting with the [FeFe]-hydrogenase, providing an efficient local micro-oxic niche, or (2) Flvs are randomly distributed and only a few micro-oxic niches are created for the [FeFe]-hydrogenase.

Figure 5. Repartition of [Fe-Fe] hydrogenase on the phylogenetic tree of microalgae. Organisms shown in Table 1 were placed on the tree as well as sequenced organisms that do not exhibit a sequenced [Fe-Fe] hydrogenase[86-108]. Absence of a [Fe-Fe] hydrogenase was defined as the absence of any gene meeting the requirements used in Table 1. Presence or absence of a [Fe-Fe] hydrogenase is shown by the color of the organism name (respectively blue or red). The color of the tree branches represents green algae (green), red algae (red), glaucophytes (pale green) or chromista (dark blue).

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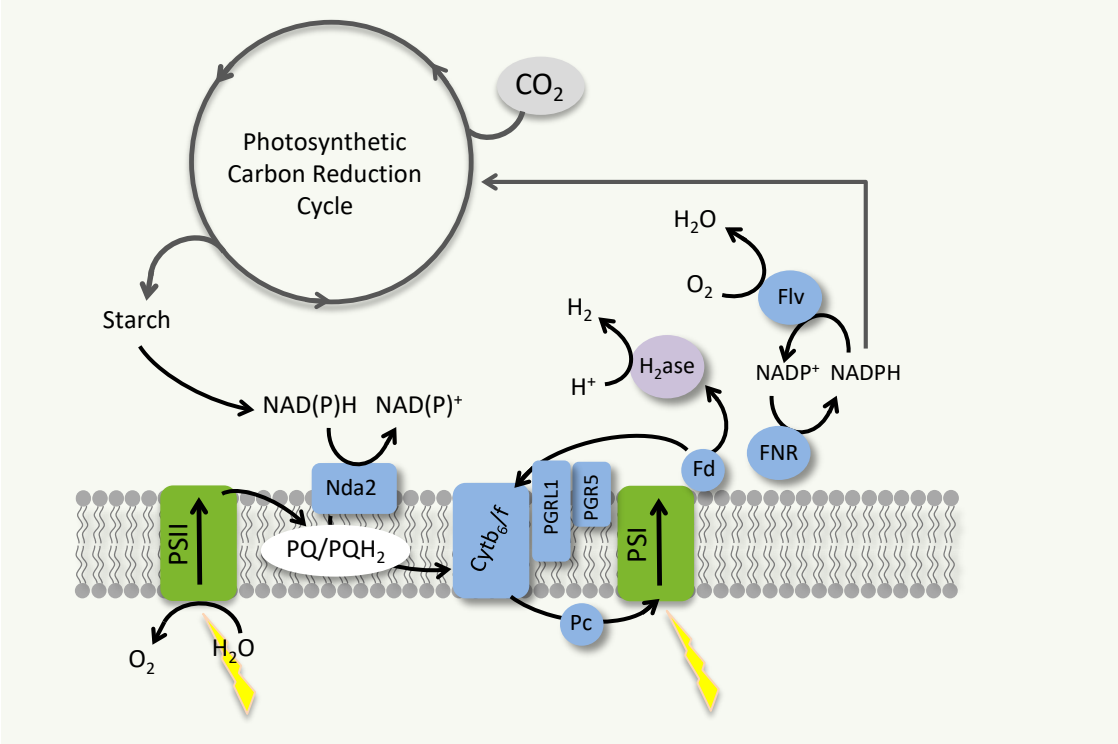

Figure 1. Electron transport reactions of oxygenic photosynthesis in microalgae. Two light reactions take place during oxygenic photosynthesis, one at PSII using water as an electron donor and resulting in the reduction of the plastoquinone pool (PQ/PQH_2), and the other at PSI using reduced plastocyanin (Pc) as an electron donor and resulting in the reduction of ferredoxin (Fd). In the "Z" scheme of photosynthesis, electrons are transferred from PSII to PSI through the cytochrome (Cyt) b_6/f complex and plastocyanin. Cyclic electron flow around PSI, which involves two major components, (PROTON GRADIENT 5 (PGR5) and PROTON GRADIENT LIKE 1 (PGRL1), allows generating additional proton gradient (not shown on this scheme for simplifaction). The plastidial type II NADH dehydrogenase (Nda2) catalyzes the non-photochemical reduction of plastoquinones from the stromal NAD(P)H pool. The flavodiiron protein (Flv) catalyzes the reduction of oxygen into water using NADPH as an electron donor. The [FeFe]-hydrogenase catalyzes the reduction of protons into molecular hydrogen by using reduced ferredoxin as a susbstrate.

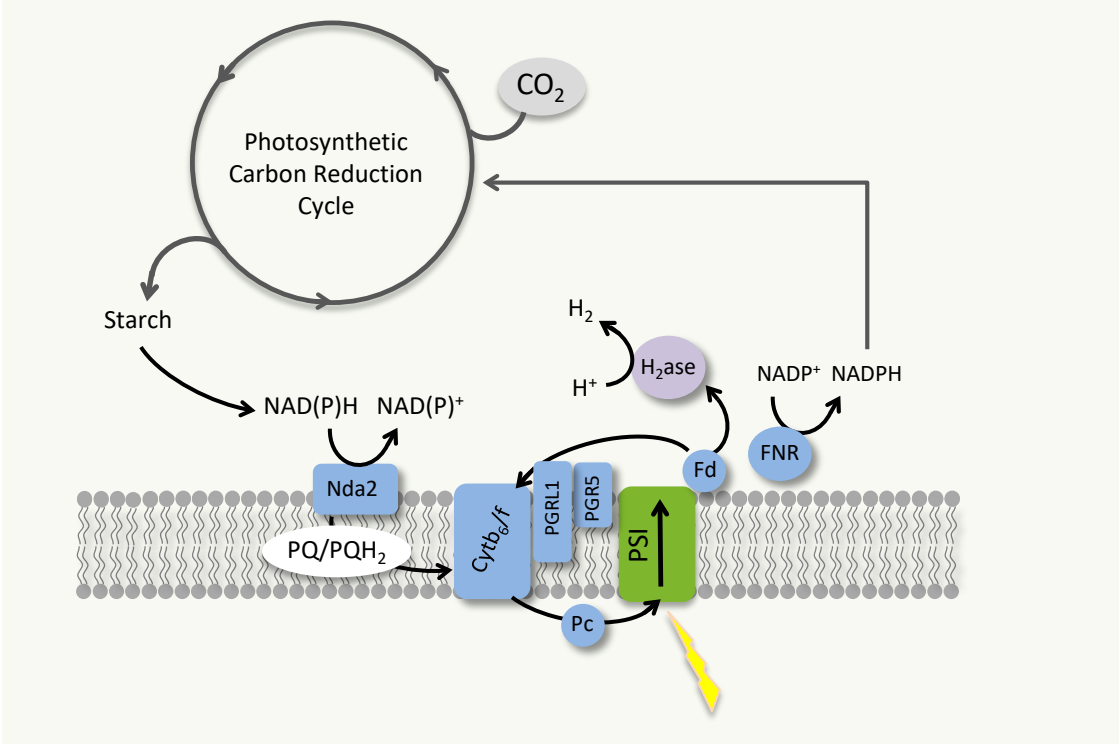

Figure 2. Indirect pathway of hydrogen photoproduction. During the indirect pathway, reducing power produced by oxygenic photosynthesis is temporarily stored into reserve compounds (starch) during a first aerobic phase. During a subsequent anaerobic phase, PSII activity is inactivated (either by means of PSII inhibitors such as DCMU or by using sulfur deprivation). The reducing power generated by starch catabolism is injected into the inter-system electron transport chain by Nda2, resulting in the reduction of plastoquinones and production of hydrogen by PSI. Abbreviations are described in the legend of Figure 1.

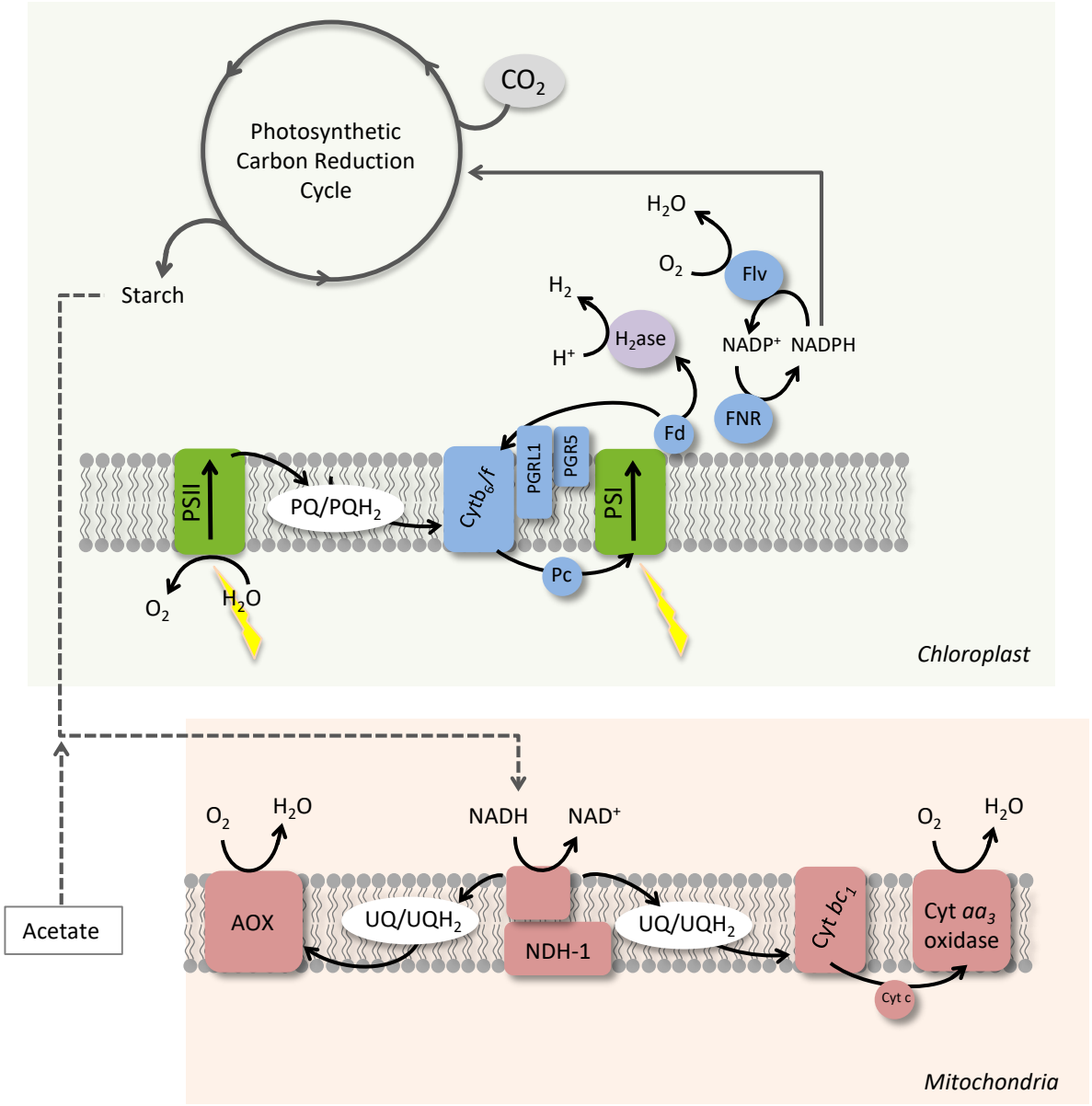

Figure 3. Direct pathway of hydrogen photoproduction. During the direct pathway, electrons produced by the "Z" scheme of photosynthesis (see Figure 1) are used for the production of hydrogen. Oxygen produced at PSII is scavenged by mitochondrial respiration, using either intracellular starch or external acetate supply as a source of reducing power. Although its role has not been yet established, flavodiiron (Flv) could participate to create micro-oxic niches at the level of the [FeFe]-hydrogenase. The mitochondrial electron transport chain harbors the complex I (NDH-1), the ubiquinone pool (UQ/UQH_2) the alternative oxidase (AOX), the cytochrome bc_1 complex and the cytochrome aa_3 terminal oxidase (Cyt aa_3 oxidase). Other abbreviations are described in the legend of Figure 1

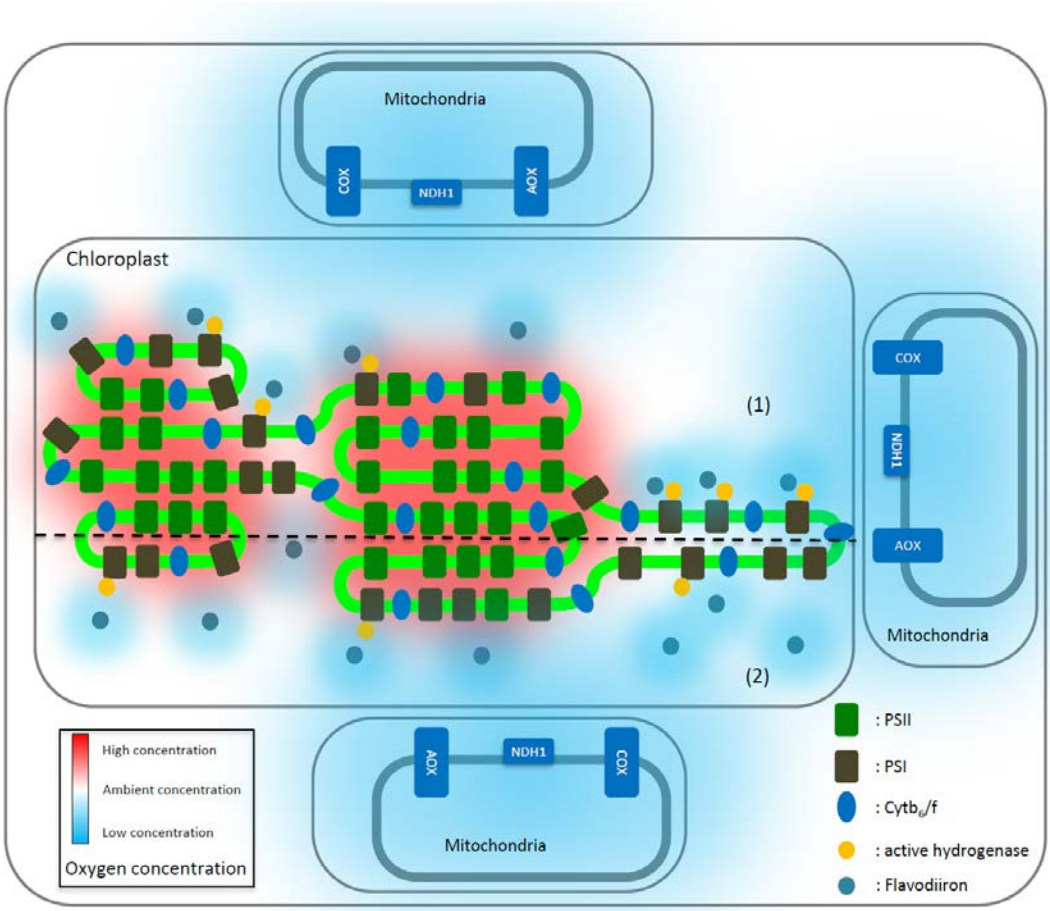

Figure 4. Hypothetical scheme showing intracellular oxygen concentrations during hydrogen production by the direct pathway within a *Chlamydomonas* cell. PSII is mainly located in grana lamellae, where it produces molecular oxygen during oxygenic photosynthesis. Two oxygen-scavenging systems are considered here. The first is related to mitochondrial respiration, which is generally considered as the major scavenging oxygen system during the direct pathway of hydrogen photoproduction (see Figure 3). The second is purely hypothetical and involves a possible role of flavodiiron proteins scavenging oxygen in the chloroplast stroma. Differential intracellular locations of oxygen sources and sink create local variations of oxygen concentration within the chloroplast that may allow the [FeFe]-hydrogenase to be active locally. Two hypotheses are considered for a role of Flv: (1) Flvs are specifically interacting with the [FeFe]-hydrogenase, providing an efficient local micro-oxic niche, (2) Flvs are randomly distributed and only a few micro-oxic niches are created for the [FeFe]-hydrogenase.

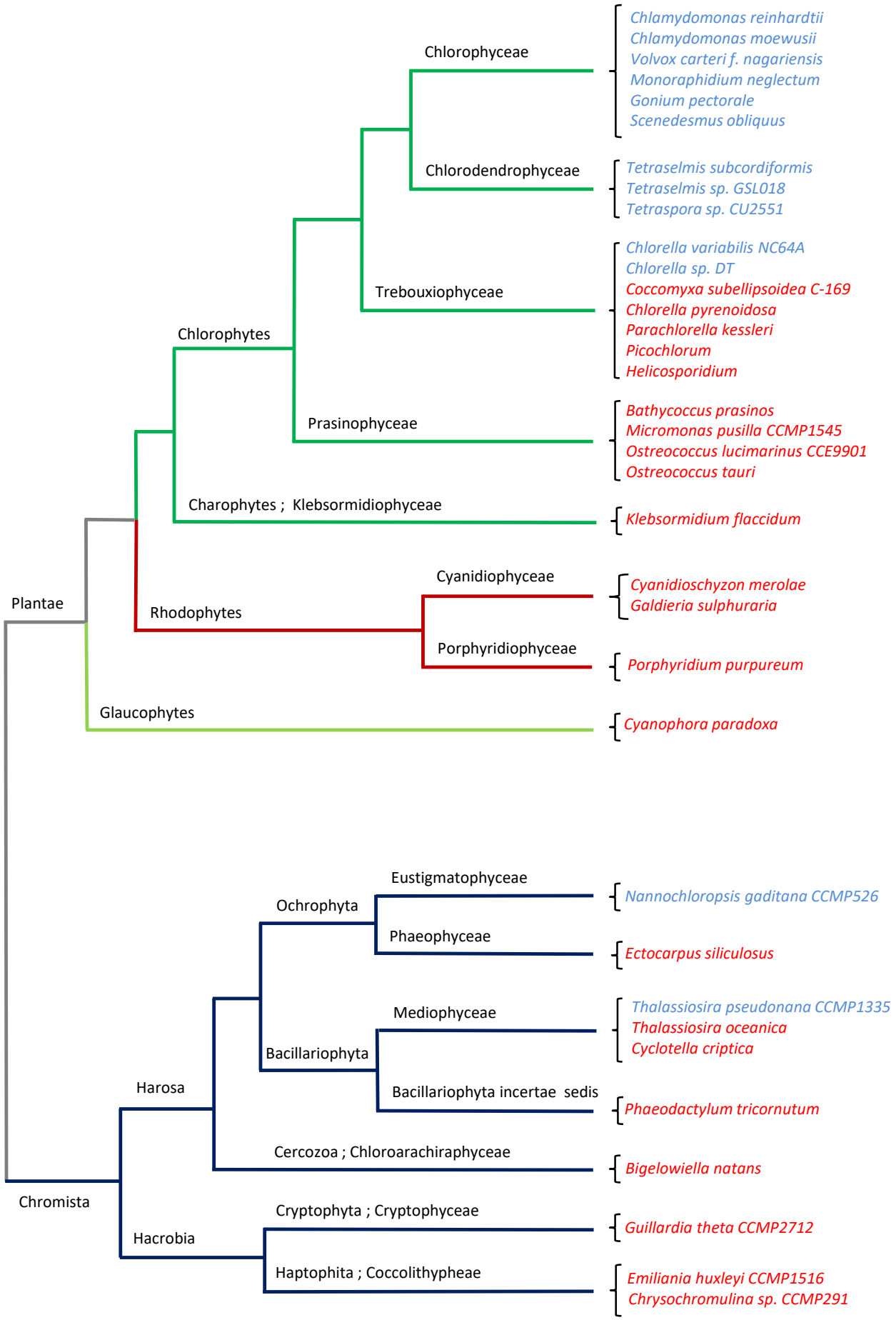
Chlorophyceae
Chlamydomonas reinhardtii
Chlamydomonas moewusii
Volvox carteri f. nagariensis
Monoraphidium neglectum
Gonium pectorale
Scenedesmus obliquus
Chlorodendrophyceae
Tetraselmis subcordiformis
Tetraselmis sp. GSL018
Tetraspora sp. CU2551
Trebouxiophyceae
Chlorella variabilis NC64A
Chlorella sp. DT
Coccomyxa subellipsoidea C-169
Chlorella pyrenoidosa
Parachlorella kessleri
Picochlorum
Helicosporidium
Chlorophytes
Prasinophyceae
Bathycoccus prasinos
Micromonas pusilla CCMP1545
Ostreococcus lucimarinus CCE9901
Ostreococcus tauri
Charophytes ; Klebsormidiophyceae
Klebsormidium flaccidum
Cyanidiophyceae
Cyanidioschyzon merolae
Galdieria sulphuraria
Plantae
Rhodophytes
Porphyridiophyceae
Porphyridium purpureum
Glaucophytes
Cyanophora paradoxa
Eustigmatophyceae
Nannochloropsis gaditana CCMP526
Ochrophyta
Phaeophyceae
Ectocarpus siliculosus
Mediophyceae
Thalassiosira pseudonana CCMP1335
Thalassiosira oceanica
Cyclotella criptica
Bacillariophyta
Harosa
Bacillariophyta incertae sedis
Phaeodactylum tricornutum
Cercozoa ; Chloroarachiraphyceae
Bigelowiella natans
Chromista
Cryptophyta ; Cryptophyceae
Guillardia theta CCMP2712
Hacrobia
Haptophita ; Coccolithypheae
Emiliania huxleyi CCMP1516
Chrysochromulina sp. CCMP291

Figure 5. Repartition of [Fe-Fe] hydrogenase on the phylogenetic tree of microalgae. Organisms shown in Table 1 were placed on the tree as well as sequenced organisms that do not exhibit a sequenced [Fe-Fe] hydrogenase[86-108]. Absence of a [Fe-Fe] hydrogenase was defined as the absence of any gene meeting the requirements used in Table 1. Presence or absence of a [Fe-Fe] hydrogenase is shown by the color of the organism name (respectively blue or red). The color of the tree branches represents green algae (green), red algae (red), glaucophytes (pale green) or chromista (dark blue).