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REVIEWS

The Mechanisms and Role of Photosynthetic Hydrogen Production by Green Microalgae

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Abstract—The review clarifies the mechanisms of H_2 production by green microalgae and the physiological role of the [FeFe] hydrogenase in adaptation of these organisms to varying environmental conditions, primarily to anaerobiosis. The work is focused at interrelationships and mutual regulation between biological hydrogen production, photosynthetic electron transport, fermentation and oxygen uptake under anaerobiosis.

Keywords: Chlamydomonas reinhardtii, hydrogenase, hydrogen production, photosynthesis, regulation

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The family of hydrogenases comprises numerous enzymes catalyzing reversible oxidation of molecular hydrogen by various microorganisms, primarily prokaryotes: $2H^+ + 2e^- \rightleftarrows H_2$ (Happe and Kaminski, 2002). The functional role of this reaction is optimization of the intracellular redox balance under anaerobic conditions in accordance with the metabolic requirements and depending on H_2 presence in the gas phase (Vignais and Billoud, 2007). Among oxygenic phototrophic microorganisms, only cyanobacteria and members of some green algae genera (including *Chlamydomonas*, *Chlorella*, and *Scenedesmus*) possess active hydrogenases. Only in algae hydrogen production is closely coupled to photosynthetic reactions.

Green microalgae posses a hydrogenase with the FeFe-type bimetallic cofactor, which exhibits high efficiency in the direction of hydrogen synthesis. The structural genes of algal hydrogenase are usually represented by several paralogues, e.g., the *HYDA1* and *HYDA2* genes in *Chlamydomonas reinhardtii*, which encode two structurally similar enzymes with different contribution to hydrogen production (Forestier et al., 2003; Meuser et al., 2012). Maturation of a hydrogenase requires the product of the *HYDEF* and *HYDG* genes, which are responsible for synthesis and incorporation of the cofactor (so-called H-cluster) into the apoenzyme (Posewitz et al., 2005; Mulder et al., 2011). The *C. reinhardtii* hydrogenase is a monomeric globular protein with the H-cluster located inside and connected with the globule surface by the channels for proton exchange and oxygen inflow. Importantly, the *HYD* genes are located in the cell nucleus, while hydrogenase is active only in the chloroplasts, where it is functionally coupled to photosynthetic electron transport via the ferredoxin (Fd) PetF. While six Fd forms were found in *C. reinhardtii* cells, only PetF was capable of effective interaction with both hydrogenase and the photosystem (PS) 1 (Winkler et al., 2010; Sawyer and Winkler, 2017). The FeFe-hydrogenases are known to be highly sensitive to molecular oxygen and require anoxic conditions for their functioning. Molecular oxygen inhibits hydrogenase activity in green microalgae by suppressing the expression of the *HYD* genes and enzyme maturation, and by inhibiting the catalytic site (Ghirardi et al., 2007; Swanson et al., 2015).

The emergence and structural modification of algal hydrogenase in the course of evolution is of special interest, as well as assessment of the role of this enzyme for green microalgae. The enzyme structurally similar to clostridial FeFe-hydrogenase was probably the precursor of the modern algal hydrogenase. Its genes were likely transported from a nonphotosynthetic bacterium to an ancestor of the modern green algae via lateral transfer (Meyer et al., 2007). In the course of algae evolution, the mechanisms were developed to incorporate hydrogenase activity in the chloroplast, where biosynthesis and posttranslational modification of the enzyme occur (Sawyer et al., 2017). Importantly, apart from the main H-cluster, bacterial FeFe-hydrogenases also contain the domains with additional FeS-centers (F-clusters), which are responsible for electron exchange between the H-cluster and bacterial-type Fd. Recent discovery of an F-cluster-containing bacterial type hydrogenase

† Deceased.

in the green microalga *Chlorella variabilis* NC64A (*Trebouxiophyceae*, *Chlorophyta*) provided insights on the evolution of this enzyme in algae (Meuser et al., 2012). This finding suggests that the modern algal hydrogenases containing only the H-cluster evidently originated from a more ancient, bacterial-type enzyme, which was preserved by some evolutionarily old lineages of green algae. The main result of the structural modification of algal hydrogenase is ability to interact efficiently with PetF that is not the case for bacterial-type hydrogenases, including the enzyme from *Chlorella variabilis* NC64A (Engelbrecht et al., 2017). Localization of the active hydrogenase in the chloroplast and its close interaction with the photosynthetic electron transport chain (ETC) are the most interesting aspects of the evolution of algal hydrogenase. These features evidently indicate a specific role of hydrogenase in the regulation of electron transport in the chloroplast under anoxic conditions, which are required for maintenance of hydrogenase activity (Melis, 2007; Hemschemeier and Happe, 2011; Tsygankov, 2014).

The present review aims to clarify the mechanisms and the possible regulatory role of H_2 production in the chloroplasts of green microalgae using *C. reinhardtii*, a unicellular organism with well-studied metabolism, as a model alga. Special attention is paid to the light-induced hydrogen production under nutrient depriation. Deficiency of such macroelements as nitrogen, phosphorus, iron, magnesium, or sulfur is known to be among the major natural stressors and is important for phytoplankton activity, many of which inhabit the oligotrophic areas of the World Ocean (Moore, 2013). Mineral limitation is used in biotechnology to stimulate the biosynthesis of such valuable products as biofuels and carotenoids (astaxanthin) (Solovchenko and Chekanov, 2014; Solovchenko, 2015; Shurin et al., 2016) and is considered as the approache for hydrogen production via water photolysis in the microalgal chloroplast (Scoma and Toth, 2015).

METABOLIC FEATURES OF *Chlamydomonas reinhardtii*

The unicellular green microalga *C. reinhardtii* is a commonly accepted model for investigation of various physiological and metabolic processes, including chloroplast biogenesis, flagellar motility, taxes, photosynthesis, signal pathways, and regulatory mechanisms (Harris, 2001). This alga is considered as a model organism due to simplicity of its life cycle, relative ease of mutant construction, and high ability of acclimation to unfavorable environmental conditions. Its life cycle includes the vegetative and generative processes, with the diploid phase occurring only at the zygote stage, while all other stages, including zoospores, adult cells, and gametes, are haploid. The haploid state of *C. reinhardtii* cells is convenient for investigation of genetic mutations, since recessive mutations result in the mutant phenotype. The genetic apparatus of *C. reinhardtii* is presently well-studied, and comprehensive studies using proteomic, transcriptomic, and metabolomic techniques (Nguyen et al., 2008; Matthew et al., 2009; Chen et al., 2010; González-Ballester et al., 2010; Schmollinger et al., 2014; Juergens et al., 2015) made this alga attractive for investigation of such problems as the principles and mechanisms of phototroph acclimation to varying environmental conditions (Rolland et al., 2009; Toepel et al., 2011; Aucoin et al., 2016).

The genus *Chlamydomonas* comprises ~500 species, which mainly occur in soil and freshwater environments. Under natural conditions, their main type of metabolism is autotrophic, with carbon dioxide as the main carbon source. *Chlamydomonas* species include both obligate autotrophs and mixotrophs with mixed nutrition, when both CO_2 and organic substrates may be used as carbon sources. Most *C. reinhardtii* laboratory strains can grow heterotrophically with extracellular acetate as a carbon source. Unlike higher plants, chloroplasts of this alga retain their structural organization and functional activity in the cultures grown in the dark on the medium supplied with acetate. This feature makes it possible to cultivate strains with photosynthetic mutations lethal to the cells under illumination.

Under photoautotrophic growth conditions, *C. reinhardtii* cells, similar to higher plants, store light energy using oxygenic photosynthesis, the key anabolic process of carbon metabolism (Fig. 1). *C. reinhardtii* cells contain a single chloroplast, which is spread along the surface in order to increase the light-harvesting area. The thylakoid membrane inside the chloroplast forms grana stacks connected by lamellae. The space between the inner chloroplast membrane and the grana is called the stroma. The space inside the thylakoid discs is called the lumen. Light-dependent photosynthetic reactions occur in the thylakoid membranes, while the dark reactions of CO_2 assimilation (the Calvin cycle) occur in the stroma. The chloroplast contains a large pyrenoid, a specialized micro-compartment which is a part of the CO_2-concentrating mechanism and the carboxylation center (Wang et al., 2011).

Acetyl coenzyme A (acetyl-CoA) plays the central role in *Chlamydomonas* carbon metabolism. It is the key player in many biochemical processes, including the TCA and glyoxylate cycles, lipid biosynthesis, and fermentation pathways (Mus et al., 2007; Johnson and Alric, 2013) (Fig. 1). The major sources of acetyl-CoA in algal cells are pyruvate, the terminal product of glycolysis, and exogenous acetate (in the case of mixotrophic or heterotrophic growth). The glyoxylate cycle catalyzes acetyl-CoA conversion to succinate, which may be then used for carbohydrate synthesis in gluconeogenesis, the process which is in fact reverse to gly-

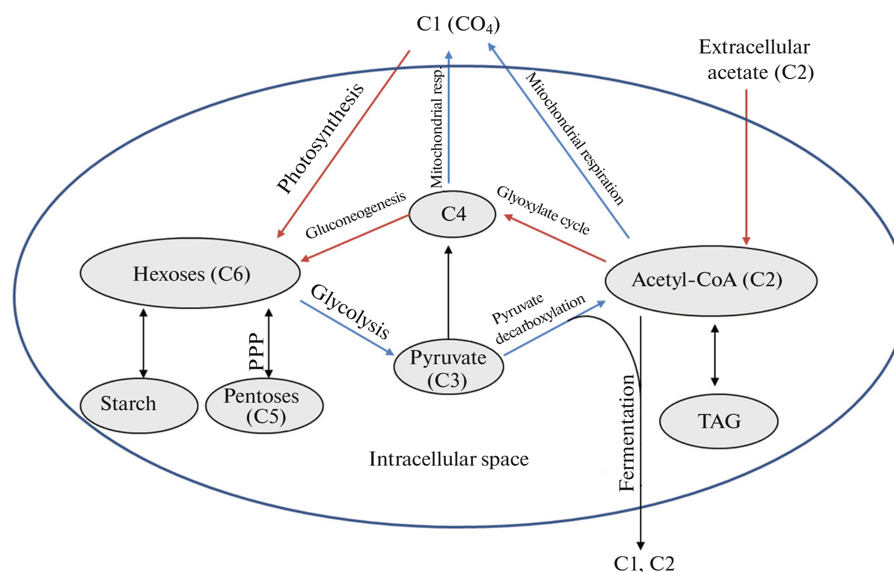

Fig. 1. The key pathways of carbon metabolism in the cell of *C. reinhardtii* (Antal, 2018). Designations: PPP, pentose phosphate pathway; TAG, triacylglycerols. Respiration includes the TCA cycle and the respiratory ETC. The numerical values indicate the number of carbon atoms per molecule.

colysis (Eastmond and Graham, 2001). Thus, in the presence of external acetate starch synthesis is carried out via gluconeogenesis either completely (heterotrophic growth) or partially (mixotrophic growth) (Ball et al., 1990).

The components responsible for glycolysis are located mainly in the chloroplast of *C. reinhardtii* cells, where they catalyze glucose oxidation to pyruvate, coupled to ATP and NADH generation (Fig. 1). Pyruvate, in turn, is converted to acetyl-CoA, which is metabolized aerobically via the TCA cycle. The pentose phosphate pathway resulting in pentose synthesis and NADPH formation, is an alternative to glycolysis pathway in *Chlamydomonas*. The metabolites of this pathway are precursors for nucleotide synthesis and may be involved in glycolysis reactions (Kruger and von Schaewen, 2003).

Ability to acclimate to oxygen deficit is a specific feature of *Chlamydomonas* metabolism. This ability is achieved through the activation of various fermentation pathways in anoxia aiming to breakdown of pyruvate to simpler organic compounds, H_2, and CO_2 in the absence of respiration (Mus et al., 2007; Atteia et al., 2013; Catalanotti et al., 2013; Yang et al., 2015).

REGULATION OF ELECTRON TRANSFER IN THE CHLOROPLAST

Hydrogen production by green algae in the light is coupled to photosynthetic reactions and is probably a mechanism regulating photosynthetic electron flow and related processes in anoxia. A number of thorough reviews on the organization and regulation of the light photosynthetic reactions in higher plants and green microalgae has been published in the recent decade (Eberhard et al., 2008; Rochaix, 2011; Alric, 2014; Tikhonov, 2015; Yamori and Shikanai, 2016; Govindjee et al., 2017). The major pathway of primary photosynthetic processes (PPP) is linear electron flow from water to $NADP^+$ and then to the Calvin cycle, which involves three pigment-protein complexes in the thylakoid membranes of the chloroplast: PS1, PS2, and the b_6/f cytochrome complex (Fig. 2). The photosystems are responsible for absorption of light energy, primarily by the light-harvesting antenna complexes (LHC), and conversion of absorbed energy to the energy of reduced and oxidized forms of electron carriers in the photosystem. The redox interactions between three types of pigment-protein complexes are provided by mobile electron carriers: plastoquinone (PQ), plastocyanin (Pc), and Fd.

The principles of linear photosynthetic electron transport in oxygenic photosynthetic organisms are described by the so-called Z-scheme, in which the sequence of electron transfer reactions is determined by the redox potential of the chain components (Govindjee et al., 2017). In PS2 electron transport starts by electron donation of the Chl *a* dimer (P680) in a singlet excited state to the transport chain at the acceptor side of the photosystem, in which the last is Q_B, a PQ molecule noncovalently bound to the Q_B site of PS2 (Fig. 2). At the donor side of PS2, the $P680^+$ cation radical oxidizes the manganese cluster of the oxygen-evolving PS2 complex. Sequential accumulation of four positive charges in the manganese cluster results in oxidation of two water molecules, formation of an oxygen molecule, and release of four protons into the luminal space.

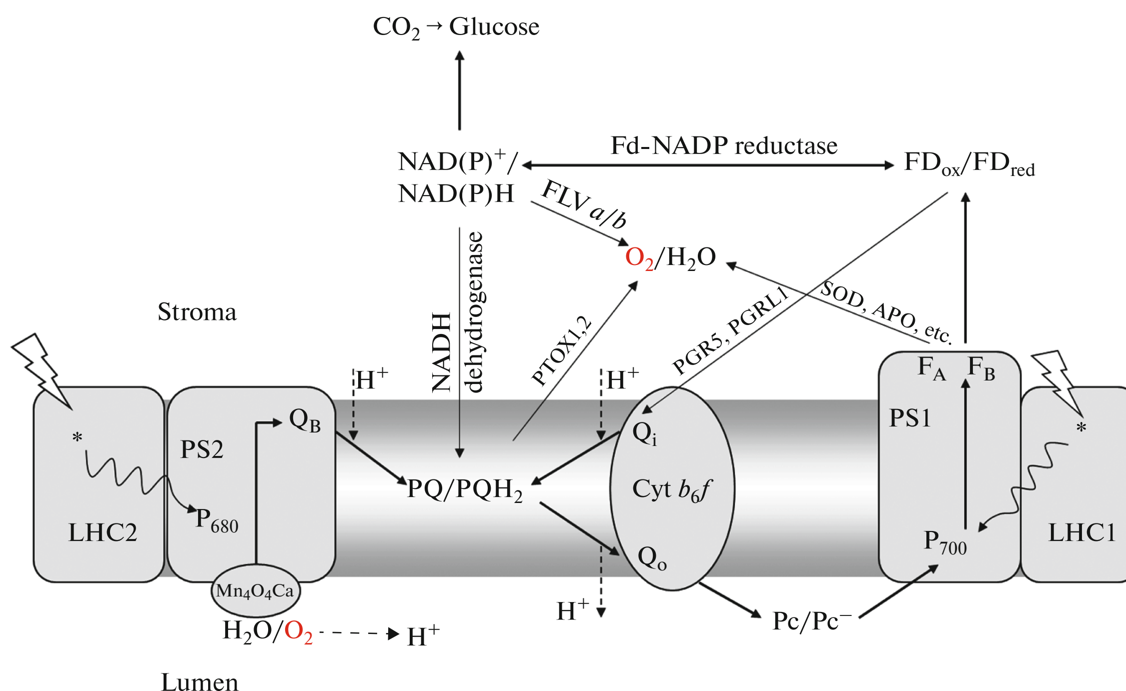

Fig. 2. Electron transport (broad straight arrows), alternative pathways for electron transport (thin straight arrows), and proton translocation (dashed lines) in a *C. reinhardtii* chloroplast under aerobic conditions.

The doubly reduced Q_B molecule is protonated from the stroma, loses affinity to the binding site in PS2, and moves into the PQ pool as a plastohydroquinone (PQH_2), while a new oxidized molecule occupies its place. PQH_2 diffuses inside the lipid bilayer and binds to the Qo-site of the cytochrome b_6f complex, where it is deprotonated and oxidized. The protons are released into the lumen, one electron is transferred to Pc via a chain of cofactors, and the second electron is transported to the PQ molecule bound to the Qi-site of the cyt b_6f complex. Reverse electron transport from the cyt b_6f complex to the PQ pool forms the so-called Q-cycle, which is used to increase the number of protons translocated from the stroma to the lumen, by the number of electrons transferred from PS2 to PS1.

In PS1 the primary electron donor, Chl *a* dimer (P700) in its excited singlet state, reduces the F_A and F_B FeS clusters via several intermediate electron transporters. The oxidized P700 form is in turn directly reduced by Pc. At the stromal side of the thylakoid membrane the Fd molecule interacts with PS1, oxidizing the F_B FeS cluster. The electrons are then transported to the two-electron acceptor $NADP^+$ by Fd-$NADP^+$ reductase (FNR) (Fig. 2).

The processes of water oxidation in PS2 and PQ oxidation/reduction in the cyt b_6f complex cause protonation of the lumen, which results in formation of the electrochemical potential in the membrane and therefore in generation of the proton-motive force, which is used for ADP phosphorylation to ATP by the chloroplast ATP synthase, a multiprotein complex responsible for phosphorylation.

Only several PPP components of *C. reinhardtii* exhibit differences from their counterparts in higher plants, including the LHC of the photosystems and involvement, apart from Pc, of cytochrome c_6 in electron transfer from the cyt b_6f complex to PS1 (Gorman and Levine, 1966; Drop et al., 2014).

Photosynthesis regulation in plants involves adaptive modifications at the levels of the organism, chloroplasts, thylakoid membranes, and individual photosynthetic components (Tikhonov, 1999; Niyogi, 2000; Peltier et al., 2010; Rochaix, 2011). According to their character and function, the major regulatory mechanisms in the chloroplast may be classified as antioxidant, photoprotective, and the ones controlling the electron and energy fluxes, as well as enzymatic activity. This classification is, however, somewhat arbitrary, since some regulatory molecules or systems may have several functions. Two major photosynthetic parameters are involved regulation of photosynthesis: the pH gradient across the thylakoid membrane and the redox state of electron carriers in the thylakoid membrane and the chloroplast stroma. The pH-dependent mechanisms include the energy-dependent component (qE) of nonphotochemical quenching (NPQ) and photosynthetic control of electron transport via the cyt b_6f complex. State transition, the thioredoxin system, and PQ-dependent redox regulation

of gene expression are associated with the redox status of the ETC (Tikhonov, 2013, 2015).

The regulatory mechanisms of the primary photosynthetic processes may be conventionally subdivided into the slow and rapid ones (Rubin and Krendeleva, 2003). Slow regulation involves modification of the chloroplast structure and of the number and properties of the photosynthetic components due to *de novo* protein synthesis. While this type of regulation is responsible for ability of the organism to acclimate to environmental changes by controlling gene expression and the quantity and activity of enzymes or metabolic pathways as a whole, these changes are not preserved at the genetic level. Structural rearrangement of the PS1 and PS2 antenna complexes is an important acclimation mechanism, in which changes in capacity for light absorption result from the changes in the composition and organization of the antenna subunits (Drozak and Romanowska, 2006; Eberhard et al., 2008).

The rapid regulatory mechanisms change the properties of PSA components within the range of minutes or seconds, which does not involve protein synthesis. These processes are primarily associated with post-translational modification of the proteins due to: (1) protonation/deprotonation of amino acids, depending on pH of the stroma and lumen; (2) reversible formation of disulfide bonds between the cysteine thiol groups, which involves thioredoxin; and (3) phosphorylation/dephosphorylation of amino acids by kinases/phosphatases. This type of regulation is especially important for light induction of photosynthesis and under conditions of intermittent light or excessive illumination. NPQ, state transition, and photosynthetic control and regulation of enzymatic activity involving the thioredoxin system are the most important rapid regulatory processes (Finazzi et al., 2002; Peers et al., 2009; Shinopoulos and Brudvig, 2012; Tikhonov, 2015).

PS2 photoprotection is achieved mainly by regulation of energy dissipation in LHC2 (Bukhov, 2004; Joliot and Finazzi, 2010; Tikhonov, 2013). NPQ changes are caused mainly by pH-dependent regulation of the xanthophyll cycle and by conformational transitions of specialized proteins (Johnson and Ruban, 2011; Fan et al., 2015). The main xanthophyll cycle in the chloroplasts of higher plants and green algae involves conversion of violaxanthin, a photosynthetic xanthophyll, to the photoprotective xanthophyll zeaxanthin (Horton, 2014). In higher plants, the PsbS protein is also required for efficient nonphotochemical quenching; its pH-dependent modifications affect the functional interactions between LHC2 and the PS2 core complex. In green algae, instead of the PsbS proteins, pigment-containing stress proteins of the LHCSR family (Light-Harvesting Complex Stress-Related) play an important part in NPQ (Bonente et al., 2011), such as the LHCSR3 protein (Finazzi et al., 2006; Tibiletti et al., 2016).

Phosphorylation of the LHC2 proteins is one of important mechanisms regulating light energy distribution between PS1 and PS2 (Tikkanen et al., 2011; Tikkanen and Aro, 2012). Reduction of the PQ pool in the light activates the LHC protein kinase due to interaction between PQH_2 and the Qo-site of the cyt b_6f complex (Vener et al., 1997; Zito et al., 1999). After phosphorylation the LHC2 antennae lose connection with the PS2 centers, become aggregated, or migrate to the stromal part of the thylakoids, where they interact with PS1. Thus, the membranes switch from state 1, in which the LHC2 are bound to PS2, to state 2, in which some LHC2 lose connection to PS2. Reverse transition from state 2 to state 1 is caused by activity of the chloroplast phosphatase (PPH1/TAP38), which catalyzes LHC2 dephosphorylation, so that the antenna complex regains its ability to interact with PS2. In higher plants the number of the LHC2 complexes involved in state transition is known not to exceed 20%, while in green microalgae this value may be as high as 80% (Allen, 1992; Delosme et al., 1996), which indicates the more important adaptive role of this process in microalgae. Unlike higher plants, state transition in microalgae plays a photoprotective role, e.g., in adaptation to oxygen and ATP limitation (Cardol et al., 2009).

Decreased CO_2 availability and other stress factors stimulate alternative pathways for electron transport in chloroplasts, which results from partial switching of the main (linear) photosynthetic electron flow to $NADP^+$ and further to CO_2 to electron flow to O_2 as the terminal electron acceptor or to cyclic mode (Kuvykin et al., 2008; Curien et al., 2016) (Fig. 2). Activation of alternative pathways may be considered as a fast redox-dependent regulatory mechanism, although this process depends also on expression of the relevant genes.

Cyclic electron transport around PS1 (CET) returns electrons from the stromal acceptors to the PQ pool along at least two pathways (Yamamoto et al., 2011; Tikhonov, 2015). The first pathway is involved in direct electron transfer from Fd to the PQ pool by the PGR5 and PGRL1 proteins (Hertle et al., 2013; Labs et al., 2016), while the key reaction of the second pathway is electron transfer from NAD(P)H to the PQ pool by NADH dehydrogenase (NDH) (Nishikawa et al., 2012, Yamori and Shikanai, 2016) (Fig. 2). PGRL1 is the essential component of the first pathway in *Chlamydomonas* cells, while the second pathway involves type 2 NDH; its activity does not depend on the lumen protonation (Jans et al., 2008; Desplats et al., 2009; Iwai et al., 2010). CET is the dominant process of electron transport at the initial stages of photosynthesis induction and under stress conditions, when linear electron transport is suppressed due to inactivation of the Calvin cycle or CO_2 or O_2 deficit.

Regulation of the ratio between ATP and NADPH and activation of pH-dependent NPQ and photosynthetic control are the major CET functions (Shikanai, 2016; Shikanai and Yamamoto 2017).

Pseudocyclic electron transport in the chloroplast involves formation of the superoxide radical (O_2^-) in PS1 in the light and the pathways for its safe reduction to water by the antioxidant enzyme system, which includes superoxide dismutase (SOD), ascorbate peroxidase (APO), and other enzymes (Asada, 1999, 2006) (Fig. 2). The pseudocyclic pathway is evidently a safe way for utilization of electrons from the photosynthetic ETC at low rates of CO_2 fixation. *Chlamydomonas* is characterized by considerable oxygen consumption by the chloroplast in the light (up to 80%), mainly due to the pseudocyclic transport (Liran et al., 2016). A characteristic feature of green microalgae is an additional bacterial-type pseudocyclic pathway, which carries out safe oxygen reduction to water in the chloroplast by the activity of FlvA and FlvB iron-containing flavoproteins using NADPH as a substrate (Peltier et al., 2010; Dang et al., 2014) (Fig. 2).

In higher plants, the plastid terminal oxidase (PTOX) is involved in carotenoid synthesis and chloroplast biogenesis (Okegawa et al., 2008). PTOX is traditionally considered to be located at the stromal side of the thylakoid membrane, not forming complexes with other membrane components, and participates in PQ pool oxidation (Bennoun, 1982; Nixon, 2000; Joët et al., 2002) (Fig. 2). Two terminal oxidases were found in *C. reinhardtii* chloroplasts (PTOX1 and PTOX2), which are not involved in carotenoid synthesis, but promote cell acclimation to reducing conditions (Houille-Vernes et al., 2011).

It should be noted that many of the rapid regulatory mechanisms described above may function efficiently not only under oxic conditions, but also under anoxic conditions in the light. Thus, state transition, CET, and NPQ facilitate acclimation of algal cells to conditions of high ETC reduction and lack of energy under oxygen limitation.

MECHANISMS AND ROLE OF HYDROGEN PRODUCTION IN GREEN MICROALGAE

Characteristics of Anaerobic Metabolism

The metabolism of oxygenic phototrophs is adapted to oxic conditions. Hypoxia is, however, relatively common in some soil and aquatic environments inhabited by microalgae and cyanobacteria, although it is usually temporary. Oxygen limitation may be caused by a number of environmental factors, including insufficient illumination, limited aeration, and high activity of heterotrophic microorganisms. Algal or cyanobacterial blooms, as well as formation of cell films and local aggregations of phytoplankton may also result in development of anoxic or microaerobic conditions even in daytime.

In nature, algae most usually switch to anaerobiosis during the night. Under these conditions the cells experience serious energy limitation due to simultaneous cessation of both photo- and oxidative phosphorylation. Thus, aerobic dark metabolism provides for efficient energy generation in the presence of storage carbohydrates, since complete oxidation of a glucose molecule to CO_2 and H_2O results in formation of over 30 ATP molecules. Under anoxic conditions, however, respiration is suppressed, and glucose is oxidized incompletely (to pyruvate) in the course of glycolysis, which does not require oxygen, but releases only 2 ATP molecules. Glycolysis is accompanied by NADH generation, which reoxidation is required to maintain the process. Moreover, pyruvate utilization in the TCA cycle is hindered by the absence of respiration. Therefore, utilization of glycolysis products is the central problem of anaerobic metabolism in algae.

Chlamydomonas are widespread in humid soils, where the cells often experience anoxic conditions and thus require the special pathways of bacterial-type anaerobic metabolism. The recently obtained genomic, transcriptomic, and biochemical data contributed significantly to the understanding of anaerobic metabolism of *C. reinhardtii* (Dubini et al., 2009; Terashima et al., 2010; Catalanotti et al., 2013; Subramanian et al., 2014). A number of fermentative pathways are used for anaerobic utilization of glycolysis products by decomposing pyruvate to simpler organic compounds, H_2, and CO_2, which are then released into the environment (Atteia et al., 2013, Catalanotti et al., 2013, Yang et al., 2015). Since fermentation reactions in *C. reinhardtii* are coupled to ATP synthesis and/or NADH oxidation, additional energy may be generated and excessive reducing equivalents may be utilized; thus, the balance between energy supply and the redox state of electron carriers is additionally regulated (Atteia et al., 2013, Catalanotti et al., 2013). In *C. reinhardtii* cells, the fermentation enzymes are involved in production of acetate, ethanol, formate, and CO_2 as the major end products, while glycerol, lactate, succinate, and H_2 are produced in minor amounts. The main fermentation reactions in *C. reinhardtii* cells are shown on Fig. 3. Two parallel reactions are responsible for pyruvate conversion to acetyl-CoA; they are catalyzed by pyruvate-formate lyase (reaction 2) and pyruvate-ferredoxin oxidoreductase (reaction 3), and result in formation of formate and CO_2 as side products. Reaction 3 is coupled to Fd reduction. In turn, acetyl-CoA may be sequentially reduced to ethanol by acetaldehyde dehydrogenase and alcohol dehydrogenase (reaction 4), using 2 NADH molecules as electron donors. Conversion of acetyl-CoA to acetate by phosphate-acetyltransferase and acetokinase is an alternative pathway of acetyl-CoA metabolism (reaction 5). In this process the energy of the acetyl-CoA macroergic bond is used for phosphorylation of one ADP molecule.

Hydrogen Production in the Dark and Light Conditions

Anaerobic hydrogen production by *C. reinhardtii* in the dark involves mainly sequential activity of pyruvate-Fd oxidoreductase and FeFe-hydrogenase and is therefore a part of the fermentation process. As was noted above, pyruvate-Fd oxidoreductase catalyzes pyruvate conversion to acetyl-CoA, which results in formation of one CO_2 molecule and reduction of two Fd molecules in the chloroplast; the latter may donate electrons to hydrogenase (Noth et al., 2013) (see reactions 3 and 6 on Fig. 3). An additional pathway for hydrogen production in the dark may be associated with the reaction of reversible electron transfer from Fd to $NADP^+$, catalyzed by FNR (Fig. 2). The equilibrium of this reaction is strongly shifted to $NADP^+$ reduction due to a significant difference between the redox potentials of plant-type Fd (–400...–450 mV) and NADP (~350 mV). However, when NADPH concentration in the chloroplast is considerably higher than that of reduced Fd (e.g., under anaerobic incubation in the dark), the probability of the reverse reaction (Fd reduction by NAD(P)H) increases.

The *C. reinhardii* mutant CC-5128 *hydEF-1* mt+ lacks hydrogenase activity due to a mutation in the *HYDEF* gene (Posewitz et al., 2005). This mutant and its parent strain were used for investigation of the physiological role of hydrogenase in dark anaerobic metabolism (Dubini et al., 2009). Comparison of the parameters of the cultures of the parent and mutant strains revealed lower CO_2 production (reaction 3 on Fig. 3) and higher succinate production by the cells of *HydEF.* The absence of hydrogenase activity did not result in impaired physiological state of the culture, probably due to metabolic compensation, when disruption of one fermentation pathway results in altering the balance of the remaining ones. Anaerobic hydrogen production in the dark is generally low and has no significant effect on adaptation to oxygen limitation.

Transition of the anaerobic culture from darkness to the light results in the abrupt increase of hydrogen production by several orders of magnitude; these changes are transitory, decaying within several minutes. This phenomenon was discovered for *Scenedesmus* cells over 70 years ago (Gaffron and Rubin, 1942) and was the first evidence of light-induced hydrogen production in eukaryotes. A drastic hydrogen release under these conditions results from rapid induction of photosynthetic electron transport and reduction of PetF and $NADP^+$ in the chloroplast (Cournac et al., 2002) prior to the activation of the Calvin cycle enzymes (Michelet et al., 2013). As known, activation of CO_2 fixation in the light depends on a number of factors, including stromal pH, thioredoxin redox state, ATP content, etc. (Werdan et al., 1975). Inactive state of the Calvin cycle at the initial stage of photosynthesis induction results in inhibition of photosynthetic electron transport, which in turn hinders the activation of the Calvin cycle. Under oxic conditions this problem is solved by partial redirection of electrons from the photosynthetic chain to molecular oxygen via alternative pathways, as was described above (Fig. 2). Under oxygen limitation, however, protons may act as an alternative electron acceptor in green microalgae. Thus, during transition of an algal culture from anoxic, dark conditions to light excessive electrons in the photosynthetic chain are utilized via hydrogen production. In this case, the role of hydrogenase is similar to that of pseudocyclic electron transport under oxic conditions. Moreover, hydrogen formation in the chloroplast results in alkalization of the stroma due to proton relocation from stroma to lumen. This is important for the activation of RuBisCO, the key enzyme of the Calvin cycle; its activity is low at neutral pH and increases at higher pH values (Mott and Berry, 1986). Hydrogen yield in the light decreases rapidly due to activation of the Calvin cycle and oxygen generation in PS2, which inhibits hydrogenase activity. Thus, the obvious physiological role of hydrogenase during transition from darkness to the light is electron utilization at the initial stage of photosynthesis induction. Indeed, investigation of photosynthesis induction in the *C. reinhardtii HydEF* mutant revealed that the presence of hydrogenase resulted in more rapid activation of Calvin cycle after anaerobic incubation in the dark (Ghysels et al., 2013). Importantly, the authors of this work revealed also the important role of state transition in photosynthesis induction in *C. reinhardtii*. Thus, anoxic conditions induce transition to state 2 in the cells of green microalgae, which is favorable for high CET activity (Cardol et al., 2009). In turn, CET supports phos-

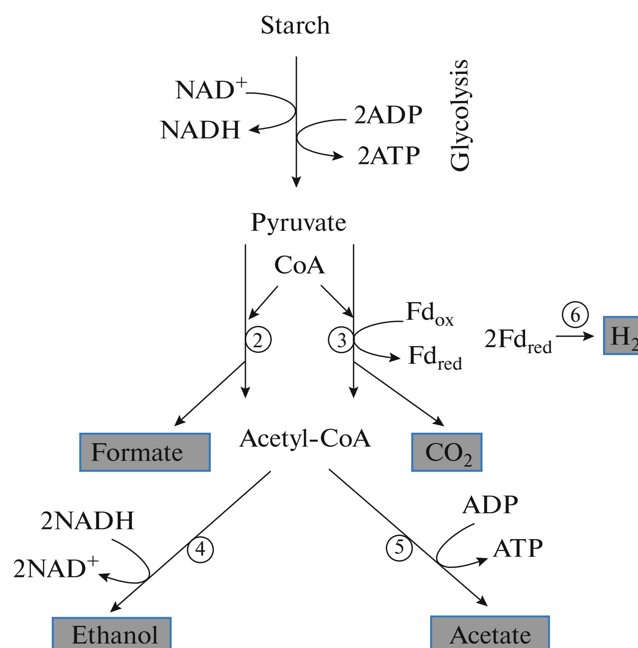

Fig. 3. The major fermentation reactions in *C. reinhardtii*.

phorylation, providing ATP for the activation of the Calvin cycle. While neither the ability to perform state transition, nor hydrogenase activity are obligatory required for the activation of CO_2 fixation, involvement of these processes, however, accelerates activation of photosynthesis considerably.

Intermittant illumination (pulse sequence) makes it possible to maintain high hydrogen production per pulse for prolonged periods. Thus, illumination of a *C. reinhardtii* culture with 1-s pulses of saturating light with longer dark intervals (9 s) was shown to result in intense photoproduction of hydrogen for several days (Kosourov et al., 2018). These results indicate the possible photoprotective role of hydrogenase as an electron acceptor in the chloroplast under intermittent light, similar to the role of pseudocyclic electron transport pathways under oxic conditions. This hypothesis, however, has not yet been confirmed experimentally.

Light-induced hydrogen production was previously shown to occur for a long time (several weeks) in *C. reinhardtii* cultures grown on acetate and illuminated with low-intensity light ~10 μmol quanta m^{-2} s^{-1}) (Scoma et al., 2014). At low light intensity, the rate of photosynthetic oxygen production is below the respiration level, since the compensatory point for photosynthesis by this alga corresponds to the values between 30 and 70 μmol photons m^{-2} s^{-1}, depending on the cultivation conditions. Insufficient illumination results in oxygen deficit in the cell and hydrogenase activation. Under such conditions the electrons for hydrogen synthesis arrive mainly via PS2-dependent pathway (Scoma et al., 2014). Oxygen produced by PS2 is efficiently consumed by respiration, and exogenous acetate acts as the main substrate for mitochondrial respiration. At low illumination the intracellular starch reserve is low, an carbohydrate catabolism has therefore little effect on hydrogen production. The rate of hydrogen evolution under such conditions is insignificant, and its possible physiological role has not been studied, although light limitation is widespread among phytoplankton habitats.

Another method to achieve long-term light-induced hydrogen production by *C. reinhardtii* cells was proposed by Nagy et al. (2018). It involves artificial substrate limitation of the Calvin cycle by CO_2 removal from the gas phase, absence of exogenous acetate and therefore autotrophic metabolism, and very high light intensity (3000 μmol photons m^{-2} s^{-1}). The absence of CO_2 in the gas phase causes photosynthetic electron transport to switch from CO_2 to alternative electron acceptor O_2, which results in low oxygen content in the cell. High light intensity, at which the photosynthetic chain, including the PQ pool, is almost completely transferred to a reduced state, also inhibits oxygen production in PS2. These conditions lead to a significant decrease of oxygen content in the cell and to hydrogenase activation. Light-induced hydrogen production according to this protocol is due to electron leakage from the photosynthetic chain under predominance of electron transport from PS2 to oxygen. While this method makes it possible to maintain a relatively high rate of hydrogen production for several days, assessment of the physiological role of the hydrogenase reaction under these conditions, which a priori do not exist in nature, is hardly appropriate.

Hydrogen Production at Nutrient Limitation

Deprivation of algal cells of the main mineral nutrients is the best-studied approach for maintaining long-term hydrogen production in light (Saroussi et al., 2017; Antal et al., 2018). Thus, sulfur limitation is widely used as the method for regulation of carbon metabolism in order to induce transition of the culture from aerobic to anaerobic conditions, followed by long-term hydrogen photoproduction. The mechanisms responsible for this phenomenon have been investigated since the early 2000s, mainly on *C. reinhardtii*. According to the previously proposed protocol for obtaining a sulfur-deprived culture (Melis et al., 2000), *C. reinhardtii* cells has to be transferred from complete Tris-acetate-phosphate medium into the modified sulfur-depleted medium where sulfate is replaced by chloride. The culture is then incubated under constant illumination in a closed-type photobioreactor (without access to atmospheric air). Under these conditions, the algae undergo sequentially the aerobic and anaerobic stages of deprivation. The first (aerobic) stage is approximately one day long and is characterized by rapid decrease in the rates of cell division and photosynthesis, as well as by active acetate uptake from the medium and accumulation of starch; its content in the chloroplast may increase 10- to 20-fold. Starch synthesis is promoted by transitory upregulation of genes involved in external acetate consumption and gluconeogenesis (Toepel et al., 2013). A switch of the culture to anaerobiosis is rather due to inactivation of photosynthetic reactions in starving cells. It should be noted that the Calvin cycle reactions are most sensitive to sulfur limitation, and their inhibition occurs more rapidly than that of the reactions of photosynthetic electron transport (Zhang et al., 2002). Preferable inactivation of the Calvin cycle results in the reduced state of photosynthetic ETC and activation of alternative pathways of electron flow to oxygen in the chloroplast, leading to the low oxygen content in the cell even at relatively high content of functional PS2. Unlike photosynthesis, the activity of components of the mitochondrial respiratory chain does not decline under sulfur limitation (Melis et al., 2000), and the respiration rate increases due to excess of available reducing equivalents (Antal et al., 2006). Inactivation of photosynthetic reactions at the high respiration rate is the main cause of transition of the sulfur-deprived culture to anaerobiosis.

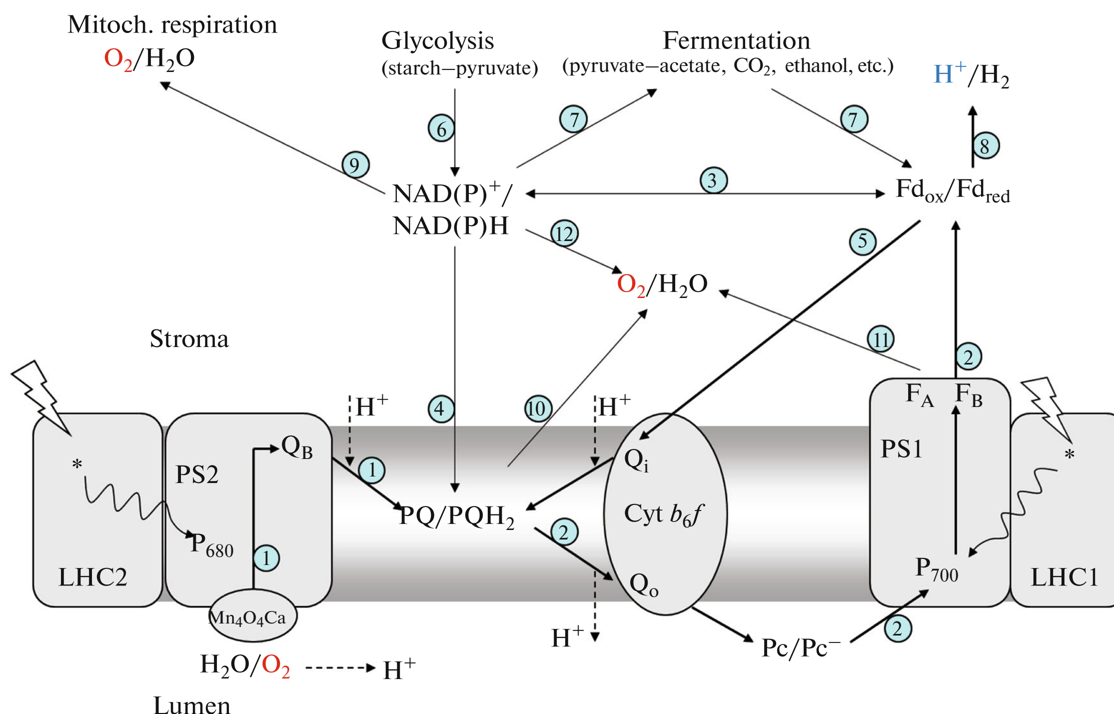

Fig. 4. Electron transport pathways in *C. reinhardtii* chloroplast during the anaerobic stage of nutrient deprivation. The dominant pathways are marked by thick straight arrows. The numbers indicate individual reactions or pathways of electron transfer.

After complete consumption of oxygen from the medium, anaerobic metabolic pathways are activated, including fermentation and hydrogen photoproduction (Fig. 4). Hydrogen production in *C. reinhardtii* cultures continues for several days, up to cell death, and is accompanied by gradual starch degradation and accumulation of the fermentation products, such as acetate, ethanol, formate, lactate, CO_2, etc. Importantly, hydrogen photoproduction is the result of synergetic interaction of two factors: sulfur and oxygen deficiency.

By the time of transition of the starving culture to anaerobiosis, the Calvin cycle is completely inhibited, and oxygen cannot be used as the terminal electron acceptor. Electron carriers of the photosynthetic and respiratory chains are therefore almost completely reduced. Indeed, when oxygen concentration in the medium reaches zero, PS2 is sharply inactivated due to reduction of the PQ pool and therefore the blockage of electron transport in PS2 (Antal et al., 2003). Overreduction of electron transport chains in the cell results in decreased rates of photo- and oxidative phosphorylation. Hydrogenase activation under these conditions promotes reoxidation of the photosynthetic ETC, partial reactivation of PS2, and increase in the oxygen production by the cell (Antal et al., 2003).

It should be noted that the starving cell performs water biophotolysis in the chloroplast in the course of electron transfer from PS2 to hydrogenase: $2H_2O \rightarrow 2H_2 + O_2$. Hydrogen is released from the cell into the medium, while oxygen is rapidly utilized in the processes of respiration and alternative electron transport in the chloroplast (pathways 9, 10, 11, and 12 on Fig. 4). Although light-induced hydrogen production is attributed mainly to the activity of PS2, which supplies ~80% electrons for hydrogen production (pathways 1, 2, and 8 on Fig. 4), the rate of this process is limited by the rate of intracellular utilization of photosynthetically produced oxygen. Thus, it was shown (Volgusheva et al., 2013) that high rate of oxygen utilization by starving cells results in elevated hydrogen evolution. Acetate assimilation from the medium is known to be suppressed during anoxic stage of sulfur deprivation (Tsygankov et al., 2002), and mitorespiration is therefore maintained mostly due to NADH generation in glycolysis which takes plays mainly in the chloroplast (pathways 6 and 9 on Fig. 4). Importantly, a redox equilibrium is maintained between the chloroplast and mitochondria in a plant cell due to the malate-oxaloacetate shuttle, and the reducer generated in the chloroplast stroma may be redistributed to the mitochondria. NADH in the chloroplast stroma also reduces the photosynthetic chain at the PQ pool level, providing a minor contribution to light-induced hydrogen production (pathways 6, 4, 2, and 8 on Fig. 4). Thus, the following interrelated processes are involved in the regulation of the redox and energy balance in the cell during the anaerobic stage of sulfur deprivation: light-induced production of hydrogen

and oxygen in the chloroplast, oxygen utilization, and starch degradation. Reactions of fermentation with NAD and Fd as redox partners also contribute to this balance.

A close relation between light-induced hydrogen production, state transition, CET, respiration, and energization of the thylakoid membranes was shown. For instance, CET inhibits hydrogen photoproduction, which indicates competition with hydrogenase for reduced Fd (pathways 5 and 8 on Fig. 4) (Antal et al., 2009).

The effect of deficiency of such elements as nitrogen, phosphorus, or magnesium on cellular metabolism is similar to that of sulfur limitation and is characterized by inactivation of photosynthesis, starch accumulation, and transition of the culture to anaerobiosis with subsequent long-term hydrogen production in the light (Batyrova et al., 2012; Philipps et al., 2012; Volgusheva et al., 2015). This fact indicates existence of common acclimation mechanisms to limitation of the major macroelements in green microalgae. Although the functional role of light-induced hydrogen production in algae acclimation to mineral starvation is insufficiently studied, the relevant research is presently under way. Thus, data were obtained in our laboratory on decreased viability of the *C. reinhardtii HydEF* mutant compared to the parent strain under sufur deprivation (in press). The physiological role of hydrogenase in starving cells may be due to the following factors. First, hydrogenase activation at the anaerobic stage of deprivation supports the photosynthetic electron transport and photophosphorylation. Second, hydrogenase-induced PS2 reactivation results in increased light-induced production of oxygen, which is utilized in the reactions of cell respiration and thus enhances the electron transport in the mitochondrial respiration chain and supports other aerobic metabolic pathways. Third, the PS2-independent pathway for hydrogen production supports glycolysis by utilization of its product—NADH. It is also important that hydrogen photoproduction is accompanied by deprotonation of the chloroplast stroma and likely by altering the balance between different fermentation pathways. In general, the ability of green microalgae to produce hydrogen in the light under nutrient limitation may present a mechanism for cell acclimation to highly reducing conditions by regulating oxygen evolution, phosphorylation, and glycolysis.

CONCLUSIONS

The physiological role of the hydrogenase reaction in green microalgae remains presently an insufficiently explored aspect of the hydrogen photoproduction phenomenon; the latter has been studied primarily due to the interest in development of green energy technologies. To understand this role, it is important to consider the fact that significant production of hydrogen is possible only in association with the photosynthetic electron transport. The algal hydrogenase retained high sensitivity to oxygen typical of the FeFe-hydrogenases, which indicates its involvement in the regulation of the photosynthetic electron transport and related processes under anoxic or microoxic conditions. Apart from oxygen limitation, efficient hydrogen production requires low activity of the Calvin cycle, the main consumer of reduced equivalents in the chloroplast in the light. In the chloroplast, the excess reducing power occurs at the initial stage of photosynthesis, at CO_2 limitation, and in the presence of various stress factors. The latter include deficiency of mineral nutrients, which is an important stress for phytoplankton. The physiological role of hydrogenase during photosynthesis induction in green algae has been reliably documented, whereas the effect of this enzyme on algae acclimation to nutrient limitation requires more thorough studies. In general, one can conclude that the algal hydrogenase plays the role similar to that of the Mehler reaction under aerobic conditions, optimizing the redox state of the electron transport chains and stromal pH; it also affects the processes of glycolysis and fermentation. Deeper understanding of the role of hydrogen photoproduction in green microalgae requires both laboratory studies and proves that this process occurs in natural environments. Breakthroughs in this direction are expected in near future due to development of highly sensitive hydrogen sensors, which make in situ measurement of low hydrogen concentrations possible.

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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