A γ-subunit point mutation in Chlamydomonas reinhardtii chloroplast F1Fo-ATP synthase confers tolerance to reactive oxygen species

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\textbf{ABSTRACT}

The chloroplast F1Fo-ATP synthase (CF1Fo) drives ATP synthesis and the reverse reaction of ATP hydrolysis. The enzyme evolved in a cellular environment where electron transfer processes and molecular oxygen are abundant, and thiol modulation in the γ-subunit via thioredoxin is important for its ATPase activity regulation. Especially under high light, oxygen can be reduced and forms reactive oxygen species (ROS) which can oxidize CF1Fo among various other biomolecules. Mutation of the conserved ROS targets resulted in a tolerant enzyme, suggesting that ROS might play a regulatory role. The mutations had several side effects in vitro, including disruption of the ATPase redox regulation [F. Buchert et al., Biochim. Biophys. Acta, 1817 (2012) 2038–2048]. This would prevent disentanglement of thiold- and ROS-specific modes of regulation. Here, we used the F1 catalytic core in vitro to identify a point mutant with a functional ATPase redox regulation and increased H2O2 tolerance. In the next step, the mutation was introduced into Chlamydomonas reinhardtii CF1Fo, thereby allowing us to study the physiological role of ROS regulation of the enzyme in vivo. We demonstrated in high light experiments that CF1Fo ROS targets were involved in the significant inhibition of ATP synthesis rates. Molecular events upon modification of CF1Fo by ROS will be considered.

1. Introduction

In the course of evolution, the F-ATP synthase (F1F0) has been remarkably conserved in terms of structure and function [reviewed in 1]. The enzyme is embedded in energy-producing membranes of bacteria, mitochondria and chloroplasts. The chloroplasts F1F0 (CF1Fo) consists of two portions, the membrane-attached F1 with five subunits (αβγεδ), and the membrane-spanning F0 with four subunits (abb′c14). At the expense of a trans-thylakoid electrochemical proton gradient (Δμ\text{H}+), ATP is produced from inorganic phosphate and ADP at the catalytic sites, mainly located in β-subunits. Several mechanisms regulate the activity of CF1Fo, and are believed to be important for the optimization of photosynthetic activity. The first one is a regulation by the substrate itself, the Δμ\text{H}+. A Δμ\text{H}+ threshold level, Δμ\text{H}+\text{trigger}, is necessary to transition CF1Fo into an active state in isolated chloroplasts [2,3]. This transition has also been demonstrated in vivo [4,5] by using electrochromic shift (ECS) measurements [reviewed in 6]. Dissipation of Δμ\text{H}+ via CF1Fo, is achieved by protonation and deprotonation of a Glu residue in the c-subunit, eventually driving rotation of subunits γεδc14. The static β- and the rotating γ-subunit share temporary contacts during the catalytic cycle, probably guided by ionic interactions [7,8]. F1Fo also catalyzes the reverse reaction by generating a Δμ\text{H}+ upon ATP hydrolysis. Both isolated F1 and the catalytic core αβγδ perform ATP hydrolysis in vitro. Other mechanisms are involved in ATPase activity regulation and have been investigated especially in vitro. Tightly bound MgADP on the catalytic sites produces low ATPase rates, which is referred to as ADP inhibition [9]. Anions [10] and detergents like LDAO [11] can alleviate ADP inhibition by facilitating the release of MgADP from the catalytic sites. Besides the c-subunit with its intrinsic ATPase inhibitor function [12], CF1Fo has a unique feature located in the γ-subunit. A short segment harbors a disulfide that can be reduced by thioredoxin, thereby unmasking ATPase activity [13]. This mechanism is referred to as ATPase redox regulation. Insertion of the corresponding spinach γ-fragment

\textbf{Abbreviations:} F1F0, F1Fo-ATP synthase; CF1Fo, chloroplast F1Fo-ATP synthase; F1-redox, redox-sensitive F1 derived from the αβγδ complex of T. elongatus BP-1; γMLCA, set of mutations in the F1\textsubscript{\textgamma}− subunit γM24L/γC90A/γM280L/γM283L; ROS, reactive oxygen species; Trx, thioredoxin; LDAO, N-dimethyldodecylamine-N-oxide; PSI, photosystem I; PSII, photosystem II; Oc, inorganic phosphate; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; HA, hydroxylamine; ECS, electrochromic shift; Δμ\text{H}+, trans-thylakoid electrochemical proton gradient; ΔΨ, electric field component of the Δμ\text{H}+; Δμ\text{H}+\text{trans}, Δμ\text{H}+ established in dark-adapted samples via ATP hydrolysis by CF1Fo; ΔΔΨ, light-induced changes of Δμ\text{H}+ and ΔΨ, respectively, compared to values in the dark; Δμ\text{H}+\text{trigger}, a critical threshold Δμ\text{H}+ to be exceeded for triggering high CF1Fo activity

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transferred the CF\textsubscript{\gamma}\textsubscript{\alpha}-specific redox regulation feature to a cyanobacterial F\textsubscript{\gamma} enzyme \cite{14}. The engineered F\textsubscript{\gamma}, termed F\textsubscript{\gamma-redox} in this study, helped to identify a redox regulation interface between the \beta- and \gamma-subunit distant from the \gamma-dithiol \cite{15}. This subunit interface plays an important role in torque generation \cite{16}. It also makes CF\textsubscript{\gamma}\textsubscript{\alpha} susceptible to activity impairment upon exposure to reactive oxygen species (ROS) since it harbors a conserved set of sulfur-containing Met and Cys residues \cite{17}. The in vitro target study used a hybrid F\textsubscript{\gamma} system with spinach CF\textsubscript{\gamma} \gamma-subunit and \textit{R. rubrum} F\textsubscript{\gamma} \alpha\beta\beta heterotrimer. Removal of the redox-regulatory Cys pair did not change ROS effects, indicating that the \gamma-disulfide formation is not involved in ROS-mediated ATP\textsubscript{\gamma} activity loss. However, the ROS-resistant mutants had lost ATP\textsubscript{\gamma} redox regulation.

The main focus of this report is the ROS-mediated regulation of ATP synthesis rates under high light in vivo. ROS production during photosynthesis is inevitable as molecular oxygen can accept electrons via a multitude of processes \cite{18}. Therefore, various ROS detoxification mechanisms exist such as the Mehler reaction where, under high light, superoxide anion radicals are produced that are subsequently detoxified in the water-water cycle yielding H\textsubscript{2}O\textsubscript{2} as an intermediate to be further detoxified \cite{19,20}. ROS-mediated protein modifications usually involve oxidation of Met, Cys, and aromatic residues which, to a certain extent, can be reversible \cite{21}. Studying the specific regulation of CF\textsubscript{\gamma}\textsubscript{\alpha} activity by ROS in vivo requires the design of a resistant mutant that displays no side effects regarding the other modes of regulation, e.g., via \gamma-disulfide formation/cleavage. The functional redox-regulatory feature is necessary since the extent of activity impairment by singlet oxygen exposure depended on the \gamma-subunit redox state in spinach thylakoids \cite{22}.

In this study, a point mutation of a conserved \gamma-subunit Arg yielded an alternative ROS-resistant F\textsubscript{\gamma} enzyme in vitro that displayed normal ATP\textsubscript{\gamma} redox regulation. Thus, by eliminating the major drawback of previous in vitro mutants, this allowed transition to in vivo measurements. By using \textit{Chlamydomonas reinhardtii}, we conferred H\textsubscript{2}O\textsubscript{2} tolerance to an in vitro system and analyzed the consequences of this ROS tolerance upon high light treatment of the cells. We observed a high light-induced slowdown of the wild type enzyme which could play an important role in the \Delta\phi\textsubscript{m}-dependent regulation of photosynthesis that would be prevented in the mutant. This significant inhibition occurred independently of the \Delta\phi\textsubscript{m} regulation. On this basis, we propose a molecular scenario for ROS-modified CF\textsubscript{\gamma}\textsubscript{\alpha} and a preliminary outline on bioenergetic consequences.

2. Materials and methods

2.1. Materials

Aldrithiol-2, ATP, pyruvate kinase, lactate dehydrogenase, and phosphoenolpyruvate were purchased from Sigma. NADH was purchased from Roche Diagnostics. Other chemicals were of the highest grade commercially available. Inhibitors were added from 1000 \times stock solutions.
 fragment from spinach CF1 into a cyanobacterial γ-subunit [14]. The used plasmid constructs, the mutant F1-redoxγMLCA (γM24L/γC90A/ γM280L/γM283L), and the purification protocol were published previously [15]. In order to generate F1-redoxγR279Q, abutting forward (5′-ATGACGCGCATGAAACAGCCGACGCG) and reverse primers (5′-CTGTCG- CGCCAATTTCTGAGG) were used. As described previously [15], cleavage (50 mM DTT) and formation (0.5 mM Aldrithiol-2) of the γ-subunit disulfide was promoted prior to ATP hydrolysis measurements in a regenerating assay [24]. ATP hydrolysis rates in the presence of substrate-coordinating Mg2+ [25] are referred to as MgATPase activity. In some experiments the addition of DDAO [11,26] and sodium sulfite [10], respectively, served to favor the release of tightly bound MgADP, thus enhancing ATP hydrolysis rates. Where indicated, H2O2 pretreatment was carried out as described previously [17].

2.3. Nuclear transformation and growth of Chlamydomonas reinhardtii cultures

EST clones were ordered from www.kazusa.or.jp (Accession No. AV634065). The ATP CDS was cloned as a BamHI/EcoRI fragment into the pPEARL expression vector (GenBank: KU531882.1) by using forward (5′-CCGAGGATCCCGAGGCTGCATGCTGAGG) and reverse primers (5′-GTTCGAATTCCATGGCCGCTATGCTCGCC). The latter oligonucleotide was used in combination with a mutational primer (5′-CGAGCTGGCTGCCCAGATGAACGCCATG) to generate a megaprimer [27] that harbored γR277Q (C. reinhardtii numbering). A non-phototrophic mutant that did not accumulate ATP transcripts [28] served as a recipient strain cultured on TAP medium [29]. Transformants were obtained by electroporation [30] using 25 μCF and 1000 V cm−1. Expression of wild type and γR277Q ATPC transgene restored phototrophic growth. For this study, transformants were cultured at 50 μmol photons m−2 s−1 on agar-supplemented and liquid minimum medium [29]. Before physiological experiments, liquid cultures were bubbled with sterile air at 50 μmol photons m−2 s−1 constant light, and kept in the exponential phase (0.5–3 × 106 cells/mL) at 23 °C by dilutions every 1–2 days. Where indicated, early log-phase cultures were illuminated for 90 min at 800 μmol photons m−2 s−1 under agitation and air bubbling.

2.4. Spectroscopy

C. reinhardtii cultures were harvested and concentrated by centrifugation (3 min, 7500 rpm), and adjusted to 1 × 107 cells/mL in minimum medium supplemented with 10% (w/v) Ficoll to minimize baseline drifts by phototaxis and sedimentation. The samples were shaken vigorously in the dark for at least 15 min before transferring to the cuvette. In vivo photosynthetic parameters were measured at room temperature in a JTS-10 spectrophotometer (Biologic, France) using a similar setup as described previously [31]. Dark-adapted samples were illuminated by a single-turnover laser flash or a short saturating light pulse. The illumination generated an electric field ΔΨ which is completed in < 100 μs (“a” phase), and a slow increase phase in the ms range, which corresponds to the turnover of the cytochrome bψ (“b” phase). After the “b” phase, the ECS signals decay mainly due to ATP synthesis by H+ translocation via CF1Fo. In dark-adapted samples illuminated by a single-turnover flash, the ECS signal

![Figure 2](image-url)

**Fig. 2. C. reinhardtii** CF1Fo activity in the dark probed by flash-induced ECS signal decay and the impact of exogenously added H2O2. (A) Representative 500-ms ECS decay phases illustrate CF1Fo activity. The “b” phase in the 20 ms-range is highlighted in gray and explained in Section 3.2. Refer also to Fig. S2 for demonstration of the “b” phase upon on

**2.2. In vitro studies: Bacterial expression vectors, recombinant protein purification, ATPase activity measurements, and post-translational enzyme modifications**

**Escherichia coli** strains DH5α were used for cloning and BL21(DE3) unC702 [23] were used for expression of the αβγ complex of *T. elongatus* BP-1, respectively. The latter strain was a kind gift from Dr. C. S. Harwood (University of Iowa). For in vitro studies, a *T. elongatus* BP-1 F1_redox chimera enzyme was used that showed plant-specific redox modulation of ATP hydrolysis upon insertion of a Cys-containing
after completion of the "b" phase was used for calculation of CF$_2$F$_3$ ATP synthesis activity via the reciprocal of the ECS decay half-time. The "a" phase in the presence of the PSII inhibitors DCMU (10 μM) and HA (1 mM) was measured at the end of each experiment and used to calibrate ECS signals to one charge separation per PSI. In fluorescence analysis says the maximum quantum yield (Fv/Fm), the photochemical quantum yield of PSII, $\Phi_{\text{PSII}}$, and representative kinetics are shown in Fig. S1. (A) The maximum quantum efficiency of PSII Fv/Fm, is shown and was averaged at two actinic light intensities which were used to calculate (B) the photochemical quantum yield of PSII, $\Phi_{\text{PSII}}$, and (C) the PSII efficiency factor, $q_p$. Redox kinetics of P700 were calculated during 5 s illumination with (D) 150 and (E) 350 μM$^{-2}$s$^{-1}$ actinic red light, respectively. The quantum yield of PSI (YI), electron donor side limitation (YND) and acceptor side limitation (YNA) are indicated.

Table 1
Spectroscopic determination of photodamage upon high light treatment.

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<td>SD</td>
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Inhibitors were 10 μM DCMU + 1 mM HA.

3. Results and discussion

3.1. The F$_1$-redox System is an in vitro tool to identify alternative ROS-resistant mutants

The heterologously expressed F$_1$-redox enzyme was designed to study ATPase redox regulation, exclusively found in the chloroplast enzyme [14]. In F$_1$-redox, a cyanobacterial F$_1$ αβ$_3$γ complex, the regulatory Cys-containing fragment from spinach CF$_1$ γ-subunit was inserted. Here, the enzyme was used as a tool in a follow-up study that links ROS tolerance of F$_1$ ATPase activity to a set of mutations [17], referred to as γMLCA (γM24L/γC90A/γM280L/γM283L). The mutations showed additional effects by perturbing ATPase redox regulation, although the γ-disulfide formation and cleavage was not hampered [15]. Numerous second-site mutations in F$_1$-redoxγMLCA background failed to re-establish the regulatory feature, most likely because the primary ROS targets are found in a delicate βγ-subunit interface that is involved in ATPase redox regulation. In addition to the redox-regulatory disturbance, the γM24 substitution in the γMLCA mutant bears a potential risk to perturb the coupling of H$^+$ translocation from nucleotide catalysis in vivo, as shown for E. coli F$_1$F$_0$ [36]. Therefore, an alternative approach was carried out without mutating primary ROS targets. We found the F$_1$-redoxγR279Q mutant (Fig. 1A) showing wild type-like ATPase redox regulation (Fig. 1B) while displaying different responses to sul fate and LDAO, respectively. The redox-insensitive F$_1$-redoxγMLCA with modified ADP inhibition served as a reference [15]. The chemicals are known to alleviate ADP inhibition, as shown by the 1.8- and 7.1-fold ATPase activity stimulation in the wild type for sul fate and LDAO, respectively. Both approaches failed to enhance F$_1$-redoxγR279Q ATPase activity in a wild type-like manner. While sulfate was slightly inhibiting, LDAO enhanced the ATPase activity by a factor 2, a trend that was also observed in F$_1$-redoxγMLCA. The F$_1$-redoxγMLCA in our follow-up ROS response study can be compared with the previously reported homologous mutant consisting of recombinant spinach CF$_1$ γ-subunit and R. rubrum F$_1$ αβ$_3$γ heterohexamer [17]. Before focusing on the ROS effect of F$_1$-redox.
His composed two components, dark and Δ changes. Although in insets). For more details see Section 3.3. reciprocal half-time decay of ECS signals after strong pH buffering capacitance of the lumen. The ΔΔΨ, is in thermodynamic equilibrium with the [ATP]/([ADP]*[Pi]) ratio. Accordingly, (A) ΔΨ changes due to the small number of charge separations during the short pulse, and due to the strong pH buffering capacitance of the lumen. (B) ΔΨ changes were negligible due to the coin- ciding change of ADP inhibition properties in ROS-tolerant mutant F1 enzyme suggests that the ROS impact on the wild type modulates ADP inhibition, as suggested in the model in Fig. 1F. The ionic track, involving rotation-guiding interactions between negatively charged βDELESEED motif residues and γArg/Lys [8], was modified in F1, redox/R279Q. ROS-mediated oxidation of the γMet/Cys targets might influence [Glu/Asp interactions of the DELESEED motif with γArg279. Thus, a change in mechanistic properties could modulate ADP inhibition. For instance, when restricting relative movements of the γ-termini in T. elongatus BP-1 F1 αβγ by engineered disulfides, ADP inhibition was low and high ATPase activity was observed [24]. A similar, ROS-mediated restriction could result in elevated ATPase activity upon H2O2 treatment in F1, redox (Fig. 1B) and a modification of γ-terminal moves could have caused inhibition of the spinach enzyme [17]. However, understanding of the ROS impact on F1 is purely based on in vitro studies. Despite earlier studies on isolated thylakoids using photosensitizers [22], it is not known whether the ROS-mediated CF1Fo modification occurs in vivo as well. The mutated γArg described in F1, R277Q/R279Q is highly conserved. Since ATPase redox regulation was not disturbed in the mutant, site-directed mutagenesis was carried out in the photosynthetic model organism Chlamydomonas reinhardtii to analyze CF1Fo modulation by ROS in vivo.

3.2. Laser flash-induced electrochromic shift measurements of C. reinhardtii cells revealed enhanced H2O2 resistance in CF1Fo γR277Q

Based on the observations in Fig. 1, the transition to an in vivo model was made by generating Chlamydomonas reinhardtii CF1Fo γR277Q, referred to as γR277Q. The mutation did not interfere with phototrophic growth and transformants were cultured on minimum medium. Due to modified pigment absorbance spectra in the presence of an electric field, photosynthetic membranes are spectroscopic voltmeters [reviewed in 6]. Generated by light-driven electron transfer and H+ translocation processes, the electrochromic shift (ECS) of the pigments serves as a probe for the electric field component (ΔΨ) of the trans-thylakoid electrochemical proton gradient ΔΨfl. The flash-induced ECS decay kinetics is multi-phasic and has been used for decades to monitor ATP synthesis in a non-invasive manner [2,4,38]. It comprises three phases: a fast phase of increase corresponding to charge separations in photosystems ("a" phase), a second phase of increase corresponds to the electric activity of the cytochrome b6f complex which generates an additional electric field [6] ("b" phase), and a phase of ECS decay which is mainly due to ATP synthesis by H+ translocation via CF1Fo. ECS decay rates at the end of the "b" phase were used for ATP synthesis activity calculations, referred to as CF1Fo activity hereafter for simplicity (Methods, Section 2.4). It is important to note that the rate of H+ translocation via CF1Fo also modifies the kinetics and amplitude of the "b" phase because the two phases partly overlap. The slower the CF1Fo-mediated decay of the electric field, the more pronounced the electrogenic activity of the cytochrome b6f complex appears [6]. This was previously clearly revealed in a CF1Fo-lacking strain [4]. We noticed a faster flash-induced ECS decay in dark-adapted mutant samples (Fig. 2A), suggesting higher CF1Fo activity in the mutant. Accordingly, amplitude and duration of the "b" phase in the 20 ms-range after the flash (gray indication in Fig. 2A) were more pronounced in the wild type. We ruled out that higher ECS decay rates in the mutant could be independent from CF1Fo activity, e.g., caused by a partial H+ leak. The opposing particularities of the two F1 systems were observed in basal ATPase activities and ADP inhibition properties: Compared to the corresponding wild type, the F1, redoxγMLCA was highly active and sulfite had an inhibiting effect. Additionally, the wild type ATPase activity
H⁺ translocation via CFo, which depends on deprotonation of a Glu in the rotating c-subunits, is inhibited by CFo-binding venturicidin [39]. ECS decay in the presence of venturicidin, reflecting the CF1Fo-independent decay of the ΔΨ, was similar in wild type and mutant, and the amplitude and duration of the “b” phase were enhanced in the same manner (Fig. S2). This demonstrates that the faster ECS decay measured in the mutant was likely linked to higher CF1Fo activity.

To test whether the in vitro findings in F1-redoxγR279Q can be applied to C. reinhardtii γR277Q, H2O2 was added to the cells. This treatment slowed down flash-induced ECS decay significantly, in both wild type (Fig. 2B) and the mutant (Fig. 2C). Photochemistry was not impaired because charge separations by a single-turnover laser flash were not altered during the experiment. This was demonstrated in dark-adapted samples by the constant values of light-induced ΔΨ changes during the unresolved “a” phase (~1.8 charge separations/PSI).

Based on the approximate half-time of the ECS decay in the controls, the H2O2 impact after several minutes of treatment was about half as much in the mutant (Fig. 2D).

We concluded that, under the conditions tested, γR277Q rendered CF1Fo twice as tolerant to H2O2 in vivo. Clearly, 5 mM H2O2 was beyond physiological relevance and monitoring for longer periods was aggravated by optically drifting samples. As a next step, we wanted to use a more physiological ROS-generating treatment by testing whether high light could lead to a similar effect, promoting oxidative modifications of CF1Fo in a less artificial manner.

3.3. High light exposure slowed down wild type CF1Fo activity

Before analysis, the cultures were transferred from growth condition light to high light (800 μmol photons m⁻² s⁻¹) for 90 min, which promoted oxidative damage of the photosynthetic machinery. Chlamydomonas is routinely exposed to high light as a prerequisite for nonphotochemical fluorescence quenching measurements [40] and, as shown recently [41], expression of PSII repair cycle proteins is upregulated under high light stress, in conditions that were harsher than in our experiments. We chose a 90-min treatment and effects began to evolve at 30 min (Fig. S3). Based on parameter assessments of both photosystems, major damage occurred at PSII in all strains at similar extents. No significant differences were observed in loss of PSI and redox parameters of P700 during the applied photoinhibition period (Fig. 3, Table 1).

Photoinhibition resulted in a smaller number of charge separations...
upon a single-turnover laser flash (Table 1). When comparing the high light-induced loss of charge separations per PSI, both wild type and γR277Q were photoinhibited similarly (see also Fig. 4). However, the ECS kinetics after the high light treatment was clearly different between the two strains. In the wild type, the ECS decay phase was slower after high light and the “b” phase was significantly increased in amplitude (Fig. 4A). Such a change in the shape of the ECS cannot be explained by the decreased “a” phase. Indeed, the comparison of the ECS kinetics following a saturating flash or a non-saturating flash (yielding different “a” phase amplitudes) indicate that both treatments resulted in homothetic kinetics (Fig. S4). Instead, as described in the introduction, the two observations were both consequences of a slowdown of the CF1Fo activity. Interestingly, the ECS decay in the high light-exposed wild type re-activated after certain minutes in darkness (Fig. S5). However, this observation will be explored in more detail elsewhere.

The pronounced slowdown of the ECS decay beyond the “b” phase as in high light-treated wild type was not observed in the mutant (Fig. 4B). Such an observation strongly suggests that the physiological effect of ROS is the CF1Fo slowdown and that the γArg mutation appeared to increase ROS resistance in vivo, too. Slowing down H+ release from the lumen via CF1Fo could be important for generating a high ΔΨf, thus mediating efficient responses to high light. These could include the photostatic control, i.e., the slowdown of the cytochrome b6f [42] or the high energy quenching of the chlorophyll fluorescence, qE [43], which are both regulated by the luminal pH. However, at this stage, conclusions on CF1Fo activity drawn from flash-induced ECS decay kinetics should be drawn with care. Indeed, the rate of CF1Fo activity depends on other parameters including the regulation by its substrate, the ΔΨf (see Introduction). Since the ΔΨf across laser flash-energized membranes in dark-adapted cells was low, the ΔΨf regulation of CF1Fo, initially demonstrated by membrane energization with consequent short laser flashes [2], was not contributing to our observations. ATPase activity measurements in isolated chloroplasts showed that ΔΨf stimulated the rate-limiting product release [44], i.e., ΔΨf cancels ADP inhibition – the feature that was altered by the mutation in vivo. During the ATP synthesis mode, the main energy-consuming steps of ATP release and Pi binding [45] might be facilitated in laser flash-energized membranes harboring mutant CF1Fo. The γR277Q mutation as well as the actual ROS targets are found in a CF1Fo region that was structurally responding to ΔΨf [46]. In a physiological scenario under high light, ROS formation coincides with a high ΔΨf. Therefore, CF1Fo should be in its ΔΨf-activated state when it is supposedly slowed down by ROS. To test whether the activated enzyme is also slowed down by ROS, we modified the protocol and measured CF1Fo activity after generating a significant ΔΨf by short illumination.

3.4. After transitioning into the ΔΨf-activated CF1Fo conformation, the membrane potential decayed slower via the high light-treated wild type enzyme

A recently described method allowed us to monitor the generation of a membrane potential in dark-adapted samples by illuminating for several milliseconds with a saturating light pulse [31]. Before presenting the results, it is necessary to point out that in the dark, the ΔΨsfl is not null and a certain level, ΔΨsdark, is maintained via ATP hydrolysis in the dark [47]. As proposed by Junge [48] and summarized in Fig. 5, the ΔΨsdark is in equilibrium with the [ATP]/([ADP]×[Pi]) ratio. If the [ATP]/([ADP]×[Pi]) ratio is low, the ΔΨsdark will be low. On the contrary, if the ratio is high, the ΔΨsdark will be high, too. A critical threshold ΔΨsfl, ΔΨsflsaturate needs to be exceeded to promote high CF1Fo activity rates [2,48]. Regarding the method that we used here, three considerations are given:

1. It is assumed that in dark-adapted samples the pulse-generated change of ΔΨsfl (ΔΔΨsfl) mainly reflects a light-induced change of its electric component ΔΨ (ΔΔΨ), considering the relatively small number of charge separations during the ms-pulse in combination with the high buffering capacitance of the lumen [49]. The latter feature made changes in ΔpH negligible in first approximation.

2. A sufficiently large ΔΨsfl during the ms-pulse energized the membrane above the critical ΔΨsflsaturate, promoting high CF1Fo activity. The pulse duration was adjusted to reach a maximal ΔΨsfl (indicated as ΔΨsflmax in Fig. 6 insets).

3. Equal absolute ΔΨ values were obtained at the end of the pulse (measured as ΔΨsflmax), exclusively depending on the electric permeability of the membrane [31].

Since inhibition of the photosynthetic apparatus after high light treatment was similar in the samples (Fig. 3, Table 1) one could assume that it is valid to calibrate each sample internally by PSI, as for the flash-induced kinetics shown before (see Methods, Section 2.4).

Following the pulse, ECS signals decayed slower in the high light-treated wild type transformant lines (Fig. 6A–C). In addition, a very slow phase of variable amplitude with a lifetime > 20 s was observed.
After high light, the ΔΨ dissipation in the mutant was less compromised and the slow phase was not observed (Fig. 6D–F). In the following discussion, we advise to refer to Fig. 5 as well: In wild type and mutant controls (Fig. 6), the ΔΨ had similar amplitudes, reaching comparable ΔΨmax values. This implied that in the dark-adapted samples, given the thermodynamic equilibrium with the [ATP]/[ADP]?[Pi] ratio, the pre-existing ΔΨdark was comparable. Accordingly, the mutation did not cause a partial H+ leak via CF1Fo, which would otherwise lower both the [ATP]/[ADP]?[Pi] ratio and ΔΨdark. When comparing high light samples with controls, none of the treated mutants produced larger ΔΨ amplitudes – unlike the wild type. This points to the direction that in wild type #3, for instance, slightly lower plasticid ATP levels contributed to a smaller ΔΨdark. However, the high light effects on the ΔΨ amplitudes were variable in the wild type lines and conclusions should be drawn carefully. Following high light treatment, chloroplast ATP levels in dark-adapted samples depend on various processes such as ATP-consuming PSI repair [50], in addition to mitochondrial respiration rates [51]. We are aware that the calibration method could become ambiguous if the high light-induced abundance of field-indicating pigments was altered. Instead of the internal reference, high light-treated samples were calibrated to signals produced by one charge separation per PSI in the corresponding control, given equal cell concentrations (Fig. S6). However, the basic conclusions were the same.

Consider that equal absolute ΔΨ values were obtained at the end of the pulse, CF1Fo activities were compared. The initial CF1Fo-related decay phase could not be measured unbiased due to an overlap with cytochrome b6f activity after switching off the light, in analogy to the “b” phase described for flash-induced ECS decay above. Therefore, the first 12 ms of the decay were disregarded, the ΔΨ value at 12 ms darkness was set to 0, and the ΔΨ decay up to 100 ms is shown in Fig. 7. In line with findings preformed with laser flashes, the compromised wild type (Fig. 7A) and unvaried mutant (Fig. 7B) clearly demonstrate that CF1Fo inhibition by high light treatment was independent of the ΔΨr, revealing that ROS did impair both the performance of the ΔΨr-activated and the non-activated CF1Fo conformation. The relatively large ECS decay amplitudes during the initial phase confirmed that the ΔΨr-activated CF1Fo conformation was obtained in both enzymes (Fig. 7C).

4. Conclusions and outlook

ROS production is an inevitable process during photosynthesis. The capacity to detoxify the reactive intermediates depends on the physiological state of the cell and can be surpassed during the high light treatment like the one we used here, favoring oxidative damage of various cellular components. Our in vitro ECS synthase analysis, which started as an in vitro study and used independent Chlamydomonas transformants, presents evidence that the CF1Fo function is compromised under high light stress that favors ROS production. Functional impairment most likely resulted from oxidation events in the γ-subunit neck region, harboring previously identified ROS target residues [17]. This could result in mechanistic modifications during rotatory catalysis which were bypassed in the mutant. We propose that ROS could play a regulatory role by modifying CF1Fo. Given that the substrate of CF1Fo, the ΔΨr, is also the main regulator of the high light responses in the photosynthetic electron transfer chain, the potential of a ROS-modified CF1Fo in the feedback-regulated photosynthetic apparatus could be important, too. Indeed, both the high light response mechanisms at the PSI level and the photosynthetic control at the cytochrome b6f level are regulated by the ApH, the osmotic component of the ΔΨr. Two main questions remain to be answered and will be examined in future studies. First, the regulations at the level of PSII and cytochrome b6f depend on the H+ conductivity of CF1Fo. For example, under CO₂-limiting and fluctuating light conditions, highly conductive Arabidopsis CF1Fo with point mutations in the γ-subunit had an impact on the photosynthetic machinery [52,53]. These experimental conditions were, in part, described to be favorable for ROS production but modification of CF1Fo by ROS was not considered. It will be interesting to test whether the mutation of our study produces similar results. Second, it is tempting to propose that the temporary inhibition in wild type CF1Fo involves enzymatic repair mechanisms, such as the Met sulfoxide reductase system [reviewed in 54]. This repair mechanism would ensure transient control of PSI and cytochrome b6f by a ROS-modulated CF1Fo and will be examined elsewhere.

Transparency document

The http://dx.doi.org/10.1016/j.bbabio.2017.09.001 associated with this article can be found, in online version.

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Appendix A. Supplementary data

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References


