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Singlet oxygen inhibits ATPase and proton translocation activity of the thylakoid ATP synthase CF1CFo

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ABSTRACT

Singlet oxygen ($^1O_2$) produced in plants during photosynthesis has a strong damaging effect not only on both photosystems but also on the whole photosynthetic machinery. This is also applicable for the adenosine triphosphate (ATP) synthase. Here we describe the impact of $^1O_2$ generated by the photosensitizer Rose Bengal on the ATP hydrolysis and ATP-driven proton translocation activity of CF1CFo. Both activities were reduced dramatically within 1 min of exposure. Interestingly, it is shown that oxidized thylakoid ATP synthase is more susceptible to $^1O_2$ than CF1CFo in its reduced state, a new insight on the mechanism of $^1O_2$ interaction with the γ subunit.

1. Introduction

Singlet oxygen ($^1O_2$) is a natural byproduct of photosynthesis, mainly formed at photosystem II even under low-light conditions [1–3]. However, following sudden environmental changes, the level of $^1O_2$ rises rapidly and $^1O_2$ is formed also at other locations, where triplet chlorophyll (Chl) molecules appear, mainly as a result of stress and/or high light conditions. Together with the observations that in contrast to previous estimations $^1O_2$ has a much longer half life in vivo [4] and could be detected even in the cytosol after being generated at photosystem II [5], it is likely that $^1O_2$ might also have an impact on proteins apart of the photosynthetic machinery. An initial hint was given by the observation that $^1O_2$ in the Arabidopsis *flu* mutant, which is able to produce $^1O_2$ in situ, has within minutes a structural impact on the γ subunit of the adenosine triphosphate (ATP) synthase [6].

These results prompted us to analyze the impact of $^1O_2$ on the CF1CFo complex in more detail. Using Rose Bengal (RB) as a photosensitizer for $^1O_2$ generation [7], we could show that under experimental conditions where thylakoid membrane integrity was not affected, almost instantaneously ATP hydrolysis and ATP-driven proton translocation dropped dramatically. Besides other energy-dependent factors that prevent ATP hydrolysis in the dark [8–11], the redox state of a disulfide bridge between the two regulatory cysteines of the γ subunit plays an important role on enzyme activity [12–14]. Taking this into account, we could show that ATPase activity and proton translocation capacity were differentially affected depending on the redox state of CF1CFo.

2. Materials and methods

2.1. Thylakoid membrane preparation

Preparation procedure was basically performed as previously described [15]. Fresh leaves of market spinach (*Spinacia oleracea*) were rinsed with water and larger midribs were removed. Isolation was performed at 4 °C. 10 g of leaf material was ground for 5 s in a pre-cooled Waring Blender using 50 mL of homogenization buffer (400 mM sucrose, 20 mM Tricine/NaOH pH 8.0, 10 mM NaCl). The homogenate was passed through four layers of Micracloth and centrifuged for 10 min at 5000×*g*. The resulting pellet was resuspended in a small volume of homogenization buffer to determine Chl content [16]. Afterwards thylakoids were diluted either to 1 mg Chl mL^{-1} or to 4 mg Chl mL^{-1}.

2.2. Thiol activation

Thylakoids equivalent to 100 μg Chl mL^{-1} were illuminated for 1 min (900 W m^{-2}/ca. 4.2 mmol m^{-2} s^{-1} photosynthetically active radiation (PAR); Philips 7158; Philips, Hamburg, Germany) at 25 °C in the presence of 50 mM Tricine/NaOH pH 8.0, 50 mM NaCl,

Abbreviations: ATP, adenosine triphosphate; 9-AA, 9-aminoacridine; ACMA, 9-amino-6-chloro-2-methoxyacridine; Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DTT, dithiothreitol; PAR, photosynthetically active radiation; RB, Rose Bengal; $^1O_2$, singlet oxygen

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5 mM $MgCl_2$, 25 μM pyocyanine and 10 mM dithiothreitol (DTT) to reduce regulatory γ-disulfide. Those thylakoids will be referred to as "activated thylakoids", although higher Mg^{2+}-ATPase activities could be obtained after 3 min of illumination [15]. However, 1 min of illumination in the presence of 10 mM DTT resulted in a roughly sixfold increase of Mg^{2+}-ATPase compared to non-illuminated samples. This is sufficient to obtain ATPase activities high enough to allow unambiguous detection of differences between control and RB treated thylakoids. Samples illuminated for 3 min showed comparable ATP hydrolysis rates as previously reported [15] (not shown).

2.3. Reduced/oxidized thylakoids and trypsin-activation

Alternatively, in some experiments reduced or oxidized thylakoids were obtained by incubating membrane preparations (1 mg Chl mL^{-1}) for 30 min at room temperature with 10 mM DTT to obtain reduced and 100 μM $CuCl_2$ to obtain oxidized thylakoids, respectively [17]. In some cases these oxidized or reduced thylakoids were used for trypsin treatment as previously described [18] to elicit higher Mg^{2+}-ATPase activities compared to their non-trypsinized counterparts, since CF1 containing the γ subunit cleaved by trypsin is not longer inhibited by the ε subunit [19,20]. Assay conditions during illumination were identical as for "activated thylakoids" except the presence of trypsin and absence of 10 mM DTT. Thylakoids equal to 100 μg Chl mL^{-1} were incubated for 1 min with 5 μg mL^{-1} of freshly prepared trypsin in the light (900 W m^{-2}; 25 °C). After illumination, sixfold excess (by weight) of trypsin inhibitor was added.

2.4. Singlet oxygen generation by Rose Bengal

One minute illumination (900 W m^{-2}; 25 °C) was performed in the presence of different concentrations of RB as a potential photosensitizer for $^1O_2$ formation [7]. Thylakoids were assayed immediately after illumination. For control experiments activated thylakoids were kept for 1 min with 10 μM RB in the dark.

2.5. Simultaneous measurement of electron transfer and membrane energization

Simultaneous assay was performed as described elsewhere [21]. Prior to assay, thylakoids (100 μg Chl mL^{-1}) were illuminated for 1 min (900 W m^{-2}; 25 °C) in a mixture containing 50 mM Tricine/NaOH pH 8.0, 50 mM NaCl and RB at different concentrations. For fluorescence measurements a reaction mixture (2 mL) containing 50 mM Tricine/NaOH pH 8.0, 50 mM NaCl, 0.2 mM ferricyanide, 2 μM 9-aminoacridine (9-AA) and thylakoids equal to 10 μg Chl was used. Measurements were performed using a FluoroMax-4 (HORIBA Jobin Yvon, NJ, USA). A neutral density filter (T = 10%) and a filter with T_{max} at 400 nm were placed in front of the excitation beam (399 nm, slit 0.5 nm) and emission beam (430 nm, slit 8 nm), respectively. Electron transfer was stimulated by illuminating the sample with actinic red light (140 μmol m^{-2} s^{-1} PAR) for 4 min while 9-AA fluorescence and ferricyanide reduction was monitored (see Supplementary Fig. S1). In some experiments, membranes were uncoupled with 2 μM gramicidin D and 4 mM NH_4Cl. ΔpH values were calculated as described elsewhere [22] assuming a ratio of external volume to internal thylakoid volume of 2500 to 1 in a reaction mixture with thylakoids equal to 20 μg of Chl mL^{-1} [23].

2.6. Measurement of proton translocation

Proton translocation of activated thylakoids was estimated by 9-amino-6-chloro-2-methoxyacridine (ACMA) fluorescence measurements performed as described elsewhere [18]. A neutral density filter (T = 10%) was placed in front of the excitation beam (410 nm, slit 0.75 nm). Emission was recorded at 475 nm (slit 2.5 nm). Thylakoids equal to 10 μg Chl were assayed in a mixture containing 50 mM Tricine/NaOH pH 8.0, 5 mM NaCl, 1.5 mM $MgCl_2$ and 2 μM ACMA. Quenching was initiated by adding 3 mM ATP. After reaching steady state fluorescence, 3 mM NH_4Cl was added to dissipate the proton gradient. In some experiments 5 mM sulfite was present. Results which represent ATP-dependent portion of the quenching are reported as $\Delta F/F$ (see Supplementary Fig. S2).

2.7. ATP hydrolysis measurements

Sulfite stimulated Mg^{2+}-ATP hydrolysis was monitored by measuring the release of inorganic phosphate photometrically [24]. Activated/trypsinized thylakoids equal to 6 μg Chl or reduced/oxidized thylakoids equal to 20 μg Chl were incubated for 5 min at 37 °C in a medium containing 50 mM Tricine/NaOH pH 8.0, 5 mM NaCl, 1.5 mM $MgCl_2$ and 3 mM ATP. Sulfite was present at 20 mM (activated/trypsinized) or 40 mM (reduced/oxidized). Reaction was stopped by adding trichloroacetic acid. Simultaneously thylakoids were added to a medium already containing trichloroacetic acid to obtain a zero value. Colorimetric measurements were performed at 740 nm (UV mini-1240; Shimadzu, Duisburg, Germany). One measurement represents the difference between the inorganic phosphate of a sample incubated for 5 min and its zero value.

3. Results

3.1. Influence of singlet oxygen on membrane integrity

$^1O_2$ has a strong potential to interact with various molecules, particularly lipids [25–27]. It is therefore essential to ensure thylakoid membrane integrity after and during $^1O_2$ exposure before evaluating any proton translocation activity of CF1CFo. According to Evron and McCarty [21] and Schuldiner et al. [22] we determined electron transfer rates and calculated ΔpH of thylakoids illuminated in the presence of different RB concentrations (Fig. 1). Upon 1 min of incubation with up to 10 μM RB only a cer-

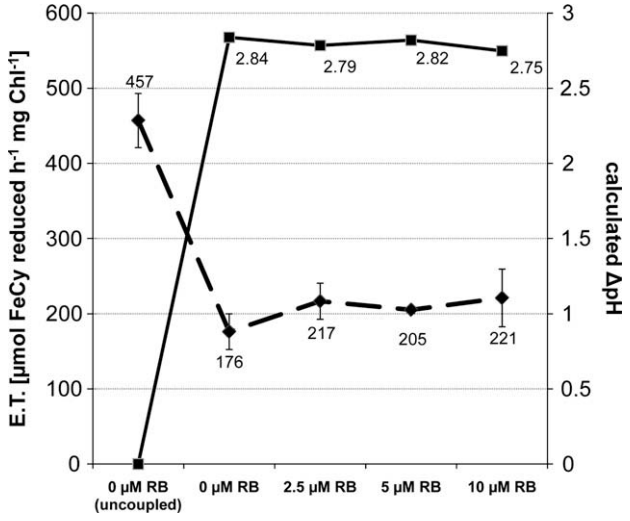

Fig. 1. Measured electron transfer (E.T.) rates and calculated ΔpH of thylakoids exposed to singlet oxygen. Assay conditions as described in Section 2. For E.T. and ΔpH assignment of RB treated thylakoids see *Supplementary* Fig. S1. Dashed line: E.T. rates of thylakoids equal to 10 μg Chl mL^{-1} illuminated with different RB concentrations or uncoupled with 2 μM gramicidin D and 4 mM NH_4Cl prior to assay. E.T. rates are expressed in μmol ferricyanide reduced h^{-1} mg Chl^{-1}; Solid line: calculated ΔpH of assayed thylakoids. n = 3.

tain increase of electron transfer rate could be observed (dashed line). Since electron transfer under continuous illumination correlates with proton efflux through thylakoid membranes [28,29], uncoupled membranes have roughly two and a half fold electron transfer rates compared to native membranes that were not exposed to $^1O_2$. A reversed, but expected situation occurred by analyzing the thylakoid membrane energization (solid line) at increasing RB concentrations (see methods for details). The extent of the generated ΔpH was slightly reduced at increasing concentrations of RB in strong contrast to uncoupled membranes, which showed no light induced 9-AA quenching. Together with the data obtained by analyzing the alkalization of medium pH during illumination of thylakoids in the presence of different concentrations of RB (see Supplementary Fig. S3), we deduce from our findings that up to a concentration of 10 μM, the effects of RB are caused by damage to the ATP synthase rather than to the thylakoid membranes. Samples that were treated with up to 50 μM RB (not shown) resembled 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) treated samples. Therefore, RB concentration applied in our experiments did not exceed 10 μM.

3.2. Effect of singlet oxygen on Mg^{2+}-ATP-dependent proton translocation of activated thylakoids

Based on the findings of Mahler et al. [6] we were interested to find out, if the structural impact of $^1O_2$ on the γ subunit of CF1CFo correlates with an altered proton translocation activity. As outlined in Fig. 2A, within 1 min of exposure to $^1O_2$ the ability of CF1CFo to generate an ATP-driven proton gradient drops dramatically at increasing concentrations of RB. Control samples kept in the dark showed comparable Δ*F*/*F* values similar to activated thylakoids without RB treatment. Measurement artifacts due to fluorescence interference of ACMA and RB can therefore be ruled out. Addition of sulfite to overcome the inhibition of Mg^{2+}-ATPase due to tightly bound Mg^{2+}-ADP molecules [30,31] has no effect on the rate of inactivation by $^1O_2$ (Fig. 2B). Sulfite increases the Δ*F*/*F* of trypsin treated membranes [18] that show an increased trypsin-mediated proton leakage [23].

3.3. Effect of singlet oxygen on sulfite stimulated Mg^{2+}-ATP hydrolysis of activated thylakoids

In a second approach we measured CF1CFo mediated hydrolysis of Mg^{2+}-ATP. As expected from the previous experiment also a gradual decrease of inorganic phosphate release of activated thylakoids exposed to increasing amounts of $^1O_2$ could be detected (Fig. 3). Interestingly, the effect of $^1O_2$ on phosphate release was less pronounced than its effect on ATP-driven proton translocation. Ten micromolars RB resulted only in a 50% reduction, while ATP-driven proton translocation was reduced to less than 25% under the same conditions (Fig. 2A). Again, sulfite was added to overcome ADP inhibition, thus obtaining values free from $^1O_2$-mediated uncoupling artifacts to ensure that the observed decline of Pi release is caused exclusively by $^1O_2$. Sulfite stimulated Mg^{2+}-ATP hydrolysis was also assayed in the presence of 2 μM gramicidin D and 4 mM NH_4Cl (not shown). As expected, due to the presence of sulfite, uncoupling had little effect on ATP hydrolysis of previously reduced thylakoids [9,32,33].

3.4. Effect of γ-cysteine redox state on the impact of singlet oxygen

Mahler et al. [6] showed that $^1O_2$ specifically interacts with the γ subunit of CF1CFo. Furthermore, it is known that the γ subunit plays a dominant role in proton gating [34] and that structural changes of the γ and ε subunit occur depending on a transmembrane potential and the γ redox state [35–40]. Fig. 4A shows ATP

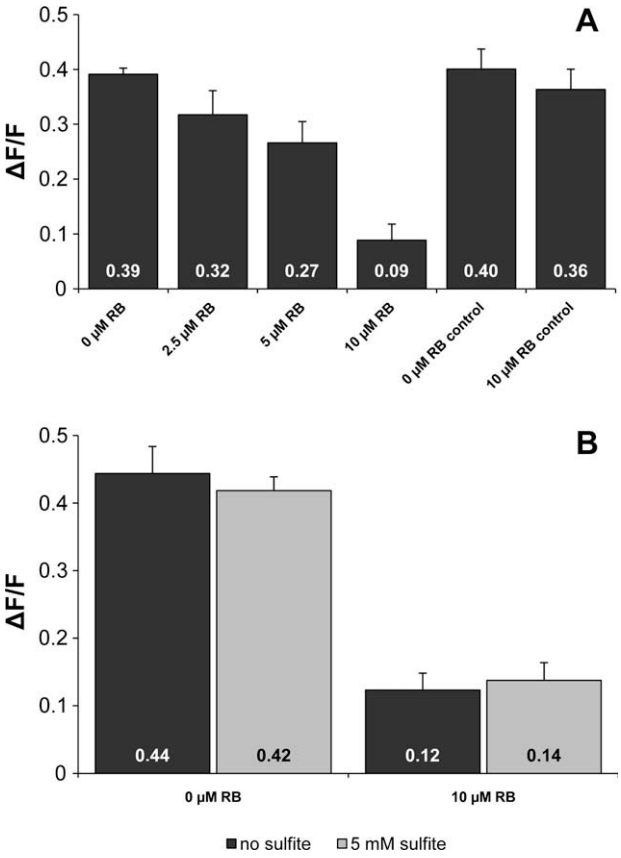

Fig. 2. ATP-driven ACMA quenching of activated thylakoids exposed to singlet oxygen. (A) Δ*F*/*F* values after exposure of thylakoids to $^1O_2$. Activation and assay conditions as described in Section 2. Values are obtained from raw data presented in *Supplementary* Fig. S2. In control experiments, activated thylakoids were kept 1 min in darkness in the absence or presence of RB. (B) Effect of 5 mM sulfite on Δ*F*/*F* of $^1O_2$-exposed thylakoids. Conditions in (B) are similar as in (A) unless indicated. $n = 3$.

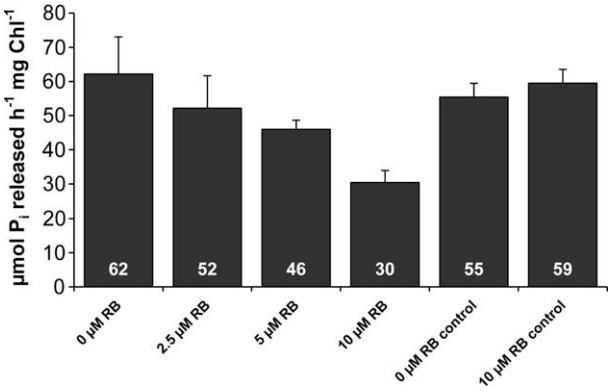

Fig. 3. Mg^{2+}-ATPase activity of activated thylakoids exposed to singlet oxygen. Activated thylakoids, equivalent to 6 μg Chl were assayed in the presence of 20 mM sulfite as described in Section 2. In control experiments, activated thylakoids were kept 1 min in darkness in the absence or presence of RB. The units of ATPase activity are expressed as μmol P_i released h^{-1} mg Chl^{-1}. Means of three independent measurements are shown.

hydrolysis of reduced and oxidized thylakoids exposed to $^1O_2$. Interestingly, ATPase activity of oxidized thylakoids is nearly three times more affected by $^1O_2$ compared to the ATPase of reduced thylakoids. To exclude any effect solely due to DTT in this context, in parallel reduced thylakoids were washed prior assaying $^1O_2$

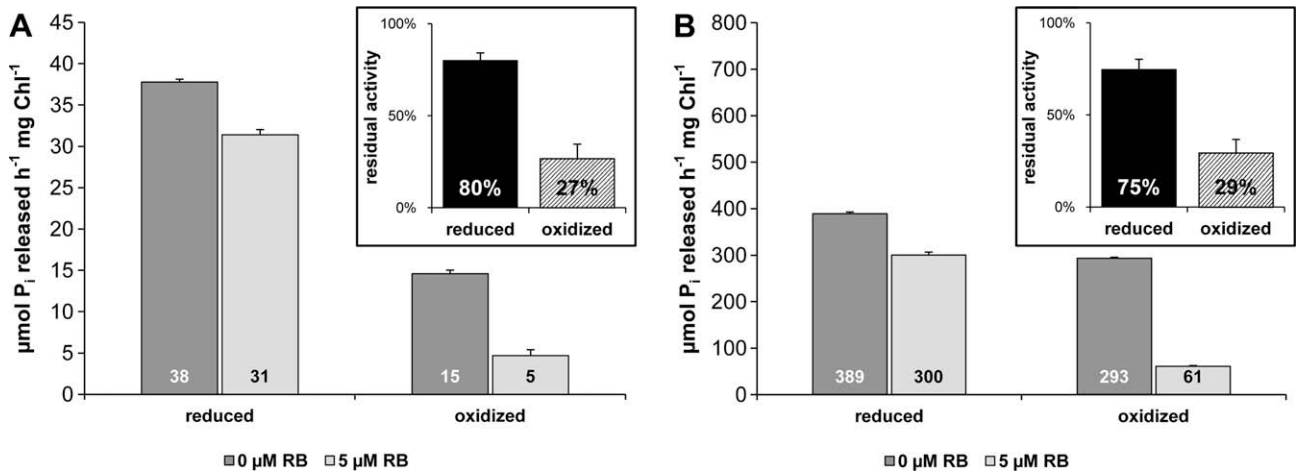

Fig. 4. Mg^{2+}-ATPase activity of reduced and oxidized thylakoids exposed to singlet oxygen. Reduced and oxidized thylakoids were prepared by incubating membrane preparations as described in Section 2. ATPase activity is expressed as µmol P_i released h^{-1} mg Chl^{-1} (means ± S.E. of three measurements). (A) Non-trypsinized preparations were illuminated with RB and assayed immediately in the presence of 40 mM sulfite. (B) Trypsin activated thylakoids were obtained as described in Section 2 prior to $^1O_2$ generation and assayed in the presence of 20 mM sulfite. The framed diagrams represent the residual relative ATPase activity of oxidized and reduced thylakoids after illumination with 5 µM RB (means ± S.E. of three preparations).

interaction. Thus, both experiments resulted in similar residual ATPase activities (not shown). The observed enhanced negative effect of $^1O_2$ on the Mg^{2+}-ATPase functionality of oxidized CF1CFo may indicate $^1O_2$ impact depends on structural properties of the γ subunit that is altered by its redox state. ATPase activity of CF1CFo is reported to be dependent on the interaction between γ and the inhibitory ε subunit [14] consolidated by the fact that oxidized CF1 has a 20-fold higher affinity to the inhibitory ε subunit resulting in a much lower catalytic activity compared to its reduced counterpart [19,41]. Thus, the degree of $^1O_2$-induced decay in activity would be dependent on an already existing interplay between those two subunits determined by the redox state of CF1CFo.

A different possibility to explain these results might be that $^1O_2$ provokes an altered configuration between γ and ε subunit. Therefore reduced and oxidized thylakoids were treated with trypsin prior incubation with $^1O_2$. Trypsin treatment of thylakoids minimizes the inhibitory effect of the ε subunit on ATP hydrolysis by cleaving the γ subunit at specific sites, thus loosening the interaction between both subunits and enabling enhanced ATPase activity, regardless if both γ-cysteines are oxidized or reduced [19,20,42]. Therefore, trypsin treatment might overcome the enhanced decay in activity as observed under oxidizing conditions. Intriguingly (Fig. 4B), Mg^{2+}-ATPase activity of oxidized trypsin treated thylakoids is again more susceptible to $^1O_2$ as the reduced counterpart. Residual activities after exposure to $^1O_2$ resemble each other, regardless if trypsin was applied or not. The same effect was observed when ATP-dependent proton translocation of trypsin treated thylakoids was assayed (Fig. 5). However, in this case trypsin treatment is essential, since untreated oxidized thylakoids have very marginal initial $\Delta F/F$ values [18]. After trypsin treatment, $\Delta F/F$ values of oxidized thylakoids exposed to $^1O_2$ generated by 5 µM RB dropped twice as much as of reduced thylakoids.

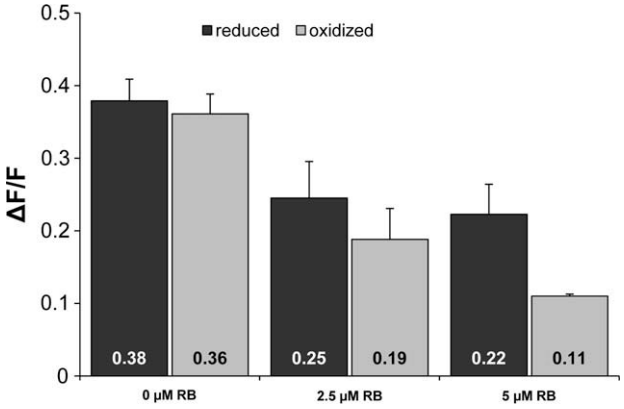

Fig. 5. ATP-driven ACMA quenching of reduced and oxidized trypsin treated thylakoids exposed to singlet oxygen. Decline of $\Delta F/F$ is shown after exposure of thylakoids to $^1O_2$. Trypsin activation and assay conditions as described in Section 2 in the presence of 5 mM sulfite. $n = 3$.

4. Discussion

Because of its half life of several microseconds [4], damage driven by $^1O_2$ should consequently not only occur in close vicinity of its origin around photosystem II, but also within the entire thylakoid membrane system and beyond [5]. Apparent target molecules for $^1O_2$ beside photosystem II itself are lipids and/or proteins of the thylakoid system. If at all, specific effects should be detectable almost instantaneously due to observed gene activation by $^1O_2$ within 30 min [43]. It was challenging that $^1O_2$ has within minutes a massive impact on the chloroplast ATP synthase in vivo [6] and as described here, in vitro. Firstly, it could be shown that the selected concentrations of RB to generate $^1O_2$ mostly preserved membrane integrity. $^1O_2$-dependent loss of ATP-driven proton translocation caused by excessive lipid peroxidation can therefore be ruled out. Secondly, ATPase and proton translocation activity are simultaneously affected. The most interesting finding of this work is that the magnitude of $^1O_2$ impact on both, ATPase and proton translocation activity is dependent on the redox state of the regulatory γ-cysteines. The widely accepted model of ATPase activity regulation proposes conformational changes within γ subunit [35–37], the ε subunit [38,39] and among ε and γ subunit [40]. A recently modeled structure of the regulatory γ subunit segment reveals a compact closed conformation of the inactive/oxidized state compared to the reduced state [44]. In our earlier work [6] a specific tryptic fragment between Phe176 and Lys195 of the γ subunit was altered upon exposure to $^1O_2$. This fragment contains the only γ-histidine (His187) and two lysines in close proximity to His187 at least in spinach and Arabidopsis. $^1O_2$ preferentially interacts with tyrosine, tryptophane, methionine, cysteine and histidine

[45,46], whereas histidine has the highest rate constant [26]. Furthermore, it is known that histidine can form $^1O_2$-mediated crosslinks with cysteines and lysines [47–52]. Interestingly, the histidine containing fragment is directly neighboring the redox regulatory fragment between Gly196 and Glu242 [17]. In the compact oxidized conformation the redox regulatory region, with Cys199 and Cys205 forming a disulfide bridge will be in closer spatial vicinity to His187. It might therefore be tempting to speculate that $^1O_2$ causes the formation of an intra-subunit crosslink preferentially in the oxidized state of the γ subunit. This crosslink should at least stabilize the compact γ subunit conformation to a certain extent since it is proposed that dithiol-modulated domain movement within the γ subunit is an essential step in the catalytic cycle [37]. The loss of catalytic activity might also be caused by formation of crosslinks between γ and ε subunits upon exposure to $^1O_2$, since crosslinking of both subunits performed in *Escherichia coli* F1 resulted in a loss of ATPase activity and ATP-driven proton translocation [53]. Thus, due to the tight interaction between γ and ε under oxidized conditions [54], an inter-molecular crosslinking might be possible. It is proposed that the C-terminal arm of the ε subunit, which is responsible for ATPase inhibition [55,56], wraps around the regulatory and central domain of the γ subunit, thereby stabilizing the closed compact conformation which keeps the enzyme inactive [44]. The last 45 C-terminal amino acids of spinach ε subunit contain three lysines as potential $^1O_2$-mediated crosslink partners for γHis187 [57]. However, trypsin treatment, which loosens the contact at specific positions between ε and γ subunit and therefore at least in theory should impede crosslink formation, had no effect on the magnitude of the $^1O_2$ effects on CF1 in the oxidized state. This would not support an inter-subunit crosslink hypothesis, although spatial range of the affected sites might not be changed by trypsin cleavage at all.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.11.040.

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