



Characterization of singlet oxygen production and its involvement in photodamage of Photosystem II in the cyanobacterium *Synechocystis* PCC 6803 by histidine-mediated chemical trapping

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ABSTRACT

Singlet oxygen production in intact cells of the cyanobacterium *Synechocystis* 6803 was studied using chemical trapping by histidine, which leads to O₂ uptake during illumination. The rate of O₂ uptake, measured by a standard Clark-type electrode, is enhanced in the presence of D₂O, which increases the lifetime of ¹O₂, and suppressed by the ¹O₂ quencher NaN₃. Due to the limited mobility of ¹O₂ these data demonstrate that exogenous histidine reaches close vicinity of ¹O₂ production sites inside the cells. Flash induced chlorophyll fluorescence measurements showed that histidine does not inhibit Photosystem II activity up to 5 mM concentration. By applying the histidine-mediated O₂ uptake method we showed that ¹O₂ production linearly increases with light intensity even above the saturation of photosynthesis. We also studied ¹O₂ production in site directed mutants in which the Gln residue at the 130th position of the D1 reaction center subunit was changed to either Glu or Leu, which affect the efficiency of nonradiative charge recombination from the primary radical pair (Rappaport et al. 2002, *Biochemistry* 41: 8518–8527; Cser and Vass 2007, *BBA* 1767:233–243). We found that the D1-Gln130Glu mutant showed decreased ¹O₂ production concomitant with decreased rate of photodamage relative to the WT, whereas both ¹O₂ production and photodamage were enhanced in the D1-Gln130Leu mutant. The data are discussed in the framework of the model of photoinhibition in which ³P680 mediated ¹O₂ production plays a key role in PSII photodamage, and nonradiative charge recombination of the primary charge separated state provides a photoprotective pathway.

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1. Introduction

Photosynthesis is driven by light, which can be highly energetic and potentially dangerous substance that can damage the photosynthetic apparatus (for a historical overview see Ref. [1]). The light-induced decline of photosynthetic activity is broadly termed as photoinhibition, and this important phenomenon has been a topic of intense research in the last 30 years. The major site of photoinhibition is the Photosystem II (PSII) complex whose electron transport is inhibited and protein structure is damaged as a consequence of light exposure (see Ref. [2] for a review).

Abbreviations: Chl, chlorophyll; D1 and D2, reaction center protein subunits of Photosystem-II; DanePy, 3-[N-(b-diethylaminoethyl)-N-dansyl]aminomethyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrole; DCMU, 3-(3',4'-dichlorophenyl)-1,1-dimethylurea; NPQ, non-photochemical quenching; PQ and PQH₂, plastoquinone and plastoquinol, respectively; PSII, Photosystem-II; P680, reaction center Chl; OCP, orange carotenoid protein; ROS, reactive oxygen species; SOD, superoxide dismutase; SOSG, Fluorescence Sensor Green; TEMP, 2,2,6,6-tetramethylpiperidine; TEMPD-HCl, 2,2,6,6-tetramethyl-4-piperidone hydrochloride

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Although significant efforts have been devoted to clarify the mechanisms of photoinhibition of PSII no consensus has been reached yet and different models are considered to explain the detrimental effects of visible light on PSII (for recent reviews see [3–7]). These models include: (i) Modifications in the functioning of the Q_B [8] and Q_A acceptors [9–11] under conditions of excess excitation when the capacity of secondary metabolic processes is not sufficient to utilize the electrons produced in the primary photoreactions. (ii) Charge recombination processes that result in triplet Chl formation [12] and their involvement in subsequent singlet oxygen production in the PSII reaction center [9,11,13–16]. (iii) Visible light induced direct damage of catalytic Mn complex of water oxidation has also been suggested [17,18], and it is known from EPR studies that spin state changes in the Mn cluster can be induced by light at the far-red edge of the visible range [19].

Singlet oxygen (¹Δ_gO₂, which will be abbreviated as ¹O₂) is produced via interaction of ground state molecular oxygen (³Σ_gO₂) with Chl triplets in PSII, and has been implicated in the process of photoinhibition as an important mediator of light induced damage. Production of ¹O₂ has been demonstrated in isolated PSII reaction center complexes by histidine- or imidazole mediated chemical trapping [20], and also by direct 1270 nm luminescence measurements

[21,22]. In thylakoid membrane particles EPR spin trapping was applied successfully for $^1\text{O}_2$ detection by using either TEMP [15,23,24], or TEMPD-HCl [25], see also Ref. [26] for a recent summary of the method. The direct 1270 nm luminescence measurements could also be used in isolated PSII complexes [27]. In intact plant systems the fluorescent $^1\text{O}_2$ traps DanePy [28,29] and Fluorescence Sensor Green (SOSG) have been applied [30]. However, detection of singlet oxygen production in intact cyanobacterial cells presents a significantly larger challenge since the EPR and fluorescent spin traps do not penetrate inside the cells (E. Hideg, personal communication), and application of the direct 1270 nm luminescence method is hampered by the influence of background Chl fluorescence [31]. It is of note that there has been an attempt to use SOSG in intact *Synechocystis* cells [32]. However the requirement of long illumination under photonhibitory conditions (3 h at $1000 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$) for the induction of the SOSG fluorescence signal [32] together with $^1\text{O}_2$ production by illuminated SOSG itself, which induces an artifactual fluorescence increase in the absence of exogenous singlet oxygen source [33] limits the applicability of this method for quantitative detection of singlet oxygen in *Synechocystis*.

Here we describe in detail the applicability of histidine mediated chemical trapping for the detection of $^1\text{O}_2$ in intact *Synechocystis* 6803 cells, and demonstrate that $^1\text{O}_2$ production linearly depends on light intensity. We also show that the efficiency of $^1\text{O}_2$ production is modulated by amino acid replacements at the 130th position of the D1 protein, which modifies the redox potential of Phe and affects the efficiency of non-radiative charge recombination of the primary radical pair. The presented results provide support for the photoprotective role of non-radiative charge recombination processes in the PSII reaction center.

2. Materials and methods

2.1. Cell cultures

Synechocystis sp. PCC 6803 (which will be referred to as *Synechocystis*) cells were propagated in BG-11 growth medium in a rotary shaker at 30°C under a 3% CO_2 -enriched atmosphere. The intensity of white light during growth was $40 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$. Cells in the exponential growth phase (A_{580} of 0.8–1) were used. The D1-Gln130Leu and D1-Gln130Glu mutants were constructed in the *psbA3* gene of *Synechocystis* sp. PCC 6803 by Peter Nixon as described previously [34].

2.2. Light treatment

Cells were harvested by centrifugation at 8000 g for 5 min and resuspended in 100 mL fresh BG-11 medium at $5 \mu\text{g Chl mL}^{-1}$ concentration. Before starting high light treatment cells were left for 1 h under $40 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ light at continuous stirring followed by a measurement of the control value of oxygen evolution, which was used as zero time point for the high light treatment. For photoinhibitory treatment cells were illuminated with $500 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ light in the presence of the protein synthesis inhibitor lincomycin ($300 \mu\text{g/mL}$).

2.3. Variable fluorescence measurements

Flash-induced increase and the subsequent decay of chlorophyll fluorescence yield was measured by a double-modulation fluorometer (PSI Instruments, Brno) [35] in the 150 μs to 100 s time range as described earlier [36]. The sample concentration was $5 \mu\text{g Chl/mL}$. The same instrument was used for the measurement of variable fluorescence under continuous illumination by using the so called OJIP protocol.

2.4. Oxygen evolution measurements

Oxygen evolution for quantification of light induced loss of PSII activity was measured with a Hansatech DW2 O_2 electrode at saturating light intensity in the presence of 0.5 mM DMBQ, as an artificial electron acceptor. 2 mL of cells at $5 \mu\text{g Chl mL}^{-1}$ was used in each measurement, and three replicates were measured.

2.5. Histidine mediated oxygen uptake measurements

$^1\text{O}_2$ production in cell free BG-11 medium was initiated by illumination in the presence of 1 μM Rose Bengal (RB). $^1\text{O}_2$ was detected by measuring the rate of light induced oxygen uptake in the presence of 5 mM histidine. For the enhancement of $^1\text{O}_2$ production H_2O was replaced with D_2O in BG-11, while quenching of $^1\text{O}_2$ was achieved by the addition of 10 mM NaN_3 . The effect of further reactive oxygen species on His-mediated oxygen uptake was probed by oxygen measurements in BG-11 medium containing 5 mM His in the presence of 500 μM H_2O_2 , or artificially generated hydroxyl radicals (500 μM H_2O_2 + 200 μM $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$), or artificially generated superoxide (100 μM xanthine + 0.025 unit/mL xanthine oxidase [37,38]).

Singlet oxygen production in intact cells was detected by measuring the rate of light induced oxygen uptake in the presence of 5 mM His as described earlier for isolated PSII reaction center complexes [20]. *Synechocystis* cells were centrifuged and resuspended in fresh BG-11 medium before O_2 uptake measurements, which were performed by using a Hansatech DW2 O_2 electrode in the absence of artificial electron acceptors.

2.6. Simulation of PSII electron transport

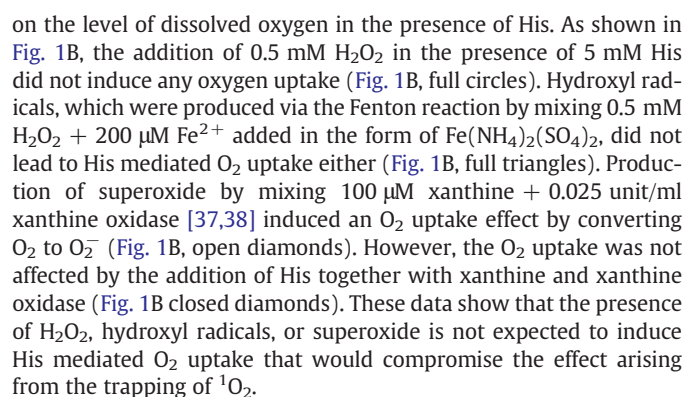
Light induced accumulation of Q_A^- as well as $^3\text{P680}$ was simulated on the basis of a coupled differential equation system describing 18 different states of PSII based on the electron transport network shown in Scheme 1 (see below). For the sake of simplicity some rarely populated states are omitted from Scheme 1 although they were considered for the calculations. The equations were solved numerically by using MATLAB with the rate constants shown in Table I of the Supplementary material.

3. Results and discussion

In order to establish a method that can be applied for detection of $^1\text{O}_2$ production in intact *Synechocystis* cells we used a chemical trapping method in which a good singlet oxygen acceptor, such as histidine (or imidazole) reacts with $^1\text{O}_2$ [39]. This reaction occurs via the addition of the singlet oxygen molecule to the imidazole ring to yield short-lived peroxide species and leads to incorporation of oxygen into oxidized endproducts (see Refs. [40,41]). This process removes dissolved O_2 from the cell suspension in a $^1\text{O}_2$ -concentration dependent way, which effect can be easily quantified by oxygen uptake measurements by using a standard oxygen electrode.

3.1. His-mediated O_2 uptake reflects $^1\text{O}_2$ production in cell free culture medium

As shown in Fig. 1A the O_2 level was not affected by illumination in the BG11 medium in the presence of 5 mM His alone (closed circles). When BG11 was supplemented with Rose Bengal (RB), which is a strong sensitizer of light induced $^1\text{O}_2$ production, switching on the light induced a rapid O_2 uptake in the presence of His, which is due to the removal of dissolved O_2 by His-mediated $^1\text{O}_2$ trapping (Fig. 1A, down triangles). It is of note that a small light induced decrease of the O_2 level was also observed when RB was added without His (up triangles). This effect is most likely the consequence of $^1\text{O}_2$ trapping by organic ingredients of BG11.



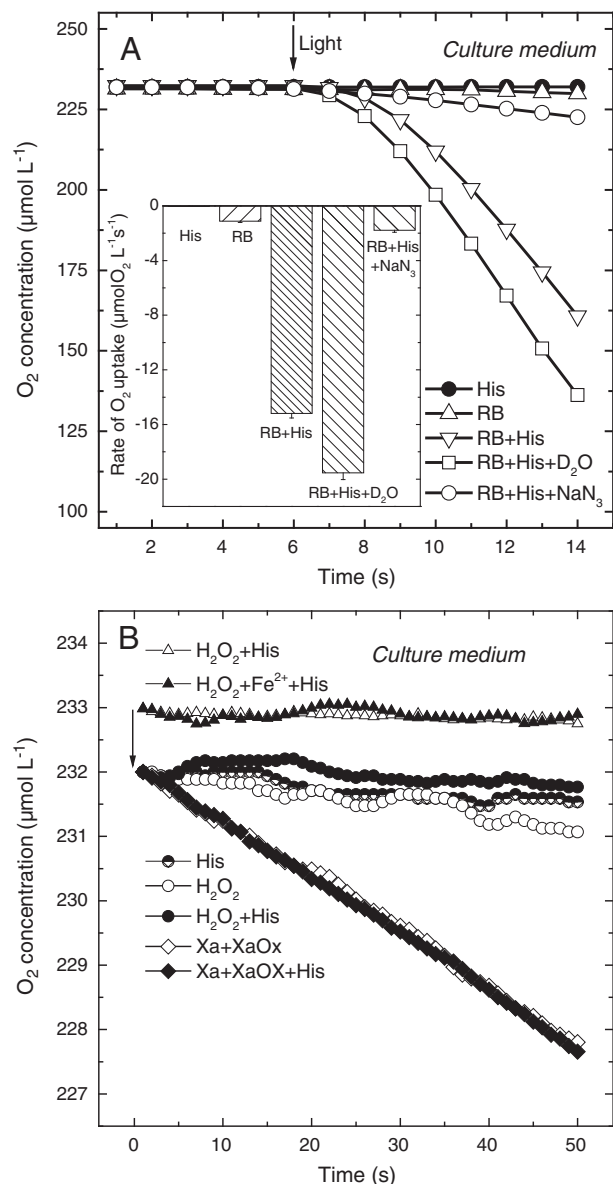


Fig. 1. His-dependent oxygen uptake in BG11 culture medium. A, Concentration changes of dissolved oxygen were measured by a Clark type oxygen electrode in cell free BG11 culture medium in the presence of 5 mM His (closed circles), 1 μ M Rose Bengal (RB) (up triangles), 5 mM His + 1 μ M RB (down triangles), 5 mM His + 1 μ M RB in D₂O containing BG11 (squares), 5 mM His + 1 μ M RB + 10 mM NaN₃ (open circles). The light intensity was 2300 μ mol quanta m⁻² s⁻¹. The inset shows rates of oxygen uptake calculated from traces like those shown in the presence of His, RB, RB + His, RB + His + D₂O, and RB + His + NaN₃. The data represent mean values from 3 independent measurements with the indicated errors. B, Oxygen uptake measurements were performed in the dark in the presence of 500 μ M H₂O₂ (open circles), 5 mM His (half-closed circles), 500 μ M H₂O₂ + 5 mM His (closed circles), 100 μ M xanthine + 0.25 unit/mL xanthine oxidase (open diamonds), 100 μ M xanthine + 0.25 unit/mL xanthine oxidase (open diamonds) + 5 mM His (closed diamonds). In a separate experiment the effect of 500 μ M H₂O₂ + 200 μ M Fe²⁺ (added in the form of Fe(NH₄)₂(SO₄)₂) + 5 mM His was also checked (closed triangles). This curve is shown together with its 500 μ M H₂O₂ + 5 mM His control (open triangles). For the sake of clarity the H₂O₂ + His, and H₂O₂ + His + Fe²⁺ curves are shown after shifting along O₂ concentration axis by 1 μ mol O₂ L⁻¹. The actual position of these curves is shown by the arrow.

3.2. His-mediated O₂ uptake reflects ¹O₂ production in intact *Synechocystis* cells

An important prerequisite of ¹O₂ trapping in intact cells is that the trap molecules should reach cell compartments where ¹O₂ is produced. Due to their short lifetime the mobility of ¹O₂ molecules

is very limited (ca. 200 nm in water and 400 nm in membranes [45]), which prevents their migration from the cells to the external medium. Therefore, the observation that His induces light dependent O₂ uptake in the suspension of *Synechocystis* cells (see below in Fig. 3), while it has no effect in cell free BG11 medium (Fig. 1A) shows that exogenous His penetrates inside the cells reaching close vicinity of the site of ¹O₂ production.

A further requirement for the applied trap is that it should not inhibit PSII electron transport activity, which could influence O₂ production and therefore lead to a misleading calculation of singlet oxygen production. Such an effect would represent an artifact in the O₂ uptake measurements in *Synechocystis* cells, which produce O₂ themselves during illumination. The effect of exogenously added His on PSII was assessed by flash induced variable Chl transients, which reflect the functioning of electron transport both at the donor and acceptor side of PSII. Illumination of *Synechocystis* cells with a single turnover saturating flash reduces the Q_A acceptor, which leads to increased fluorescence yield (Fig. 2A). Subsequent reoxidation of Q_A⁻ in the dark results in the relaxation of fluorescence yield exhibiting three main decay phases. The two faster decay components reflect the reoxidation of Q_A⁻ by PQ molecules which are already bound to the Q_B site at the time of the flash ($\tau \sim 500$ –600 μ s) or bind from the PQ pool after the flash ($\tau \sim 5$ –10 ms), respectively [46]. Whereas, the slow phase of the decay ($\tau \sim 10$ –15 s) arises from back reaction of the S₂ state of the water-oxidizing complex with Q_A⁻, which is populated via the equilibrium between Q_A⁻Q_B and Q_AQ_B⁻ ([47,48]). In the presence of DCMU, which blocks the Q_B binding site the fluorescence relaxation occurs via recombination of Q_A⁻ with oxidized PSII donors components and the process is dominated by the S₂Q_A⁻ recombination [48].

It is clear from Fig. 2A, that neither the amplitude nor the kinetics of the fluorescence signal is affected at 5 mM His concentration, in the absence or presence of DCMU. As a consequence His does not induce modification of PSII electron transport at the level of the Q_A and Q_B quinone electron acceptors, or the water oxidizing complex. However, at higher concentrations (above 10 mM) His addition induces a small decrease in the amplitude of flash induced Chl fluorescence relaxation traces (not shown), which shows that exogenously added His can reach the immediate vicinity of the PSII complex, or its antenna. The so called OJIP variable Chl fluorescence transient, which reflects the efficiency of electron transfer from the water oxidizing complex to the acceptor side of PSII, was also measured. Again, this variable fluorescence transient was not affected by 5 mM His either in the absence or presence of DCMU (Fig. 1B). Based on these data we can conclude that up to 5 mM concentration His does not interfere with PSII activity and potentially suitable for ¹O₂ trapping in intact *Synechocystis* cells.

The ¹O₂ quencher NaN₃ is known to affect photosynthetic activity and expected to decrease the rate of oxygen evolution (see [49]), which would interfere with the O₂ uptake assay if it is applied in cells with functional oxygen evolving activity. Therefore, NaN₃ was applied in the presence of DCMU, which blocks the Q_B site and eliminates O₂ evolution. As shown in Fig. 2B addition of 10 mM NaN₃ induced a decrease of variable fluorescence in the presence of DCMU showing a partial inhibition of PSII activity. However, this effect is not expected to interfere significantly with the His mediated O₂ uptake when performed in the presence of DCMU (see below).

We have also tested the effect of imidazole, which was used earlier as a chemical trapping agent for ¹O₂ detection in isolated PSII reaction center complexes [20]. However, in contrast to histidine 5 mM imidazole induced a significant inhibition of PSII activity as revealed by the decreased amplitude and retarded relaxation kinetics of the flash induced Chl fluorescence traces, as well as by the decreased induction of the OJIP fluorescence transient (not shown). The mechanism of this inhibitory effect of PSII activity by imidazole is not clear at present, and we restricted our studies to using His as chemical ¹O₂ trap.

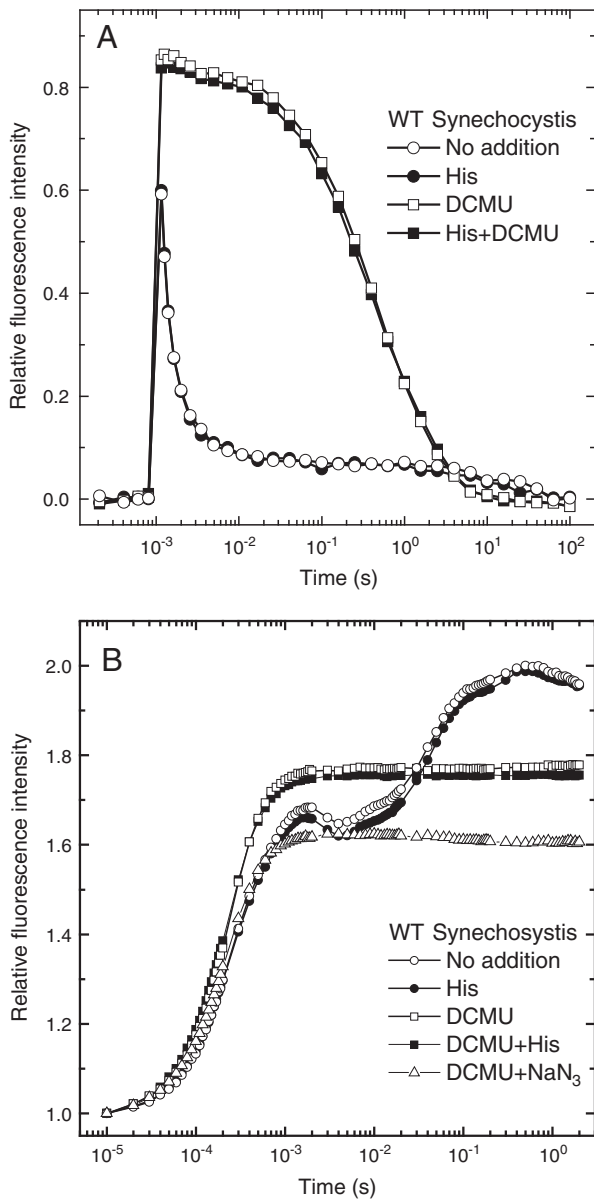


Fig. 2. Effect of histidine addition on variable fluorescence characteristics of *Synechocystis* cells. A, Flash induced Chl fluorescence traces in the absence (open symbols) and presence of 5 mM His (closed symbols). The curves, which were measured either without electron transport inhibitor (circles), or in the presence of 10 μ M DCMU (squares) are shown after shifting them to the same initial F_0 fluorescence level. B, Variable Chl fluorescence traces in the absence (open symbols) and presence of 5 mM His (closed symbols). The traces were measured either without electron transport inhibitor (circles), or in the presence of 10 μ M DCMU (squares and triangles). The effect of 10 mM NaN_3 is shown in the presence of DCMU (triangles). The curves are presented after shifting them to same initial F_0 fluorescence level.

When the effect of 5 mM His was checked on the O_2 evolving activity of *Synechocystis* cell cultures no modification of the rate of dark respiration was observed, in contrast the apparent rate of light induced oxygen evolution was decreased (Fig. 3A, open and closed squares). Since inhibition of PSII electron transport does not occur at this His concentration (see above, Fig. 2), the apparent decrease of the O_2 evolution rate indicates that His induces an O_2 removal process from the cell suspension. This process is in competition with light induced oxygen evolution and can be related to His-mediated $^1\text{O}_2$ trapping. This hypothesis was verified by using D_2O in the suspension medium instead of H_2O , which extends the lifetime of $^1\text{O}_2$ [43,44] and therefore enhances the probability of $^1\text{O}_2$ trapping by His (Fig. 3A, open and closed circles). Although D_2O in itself decreased the rate

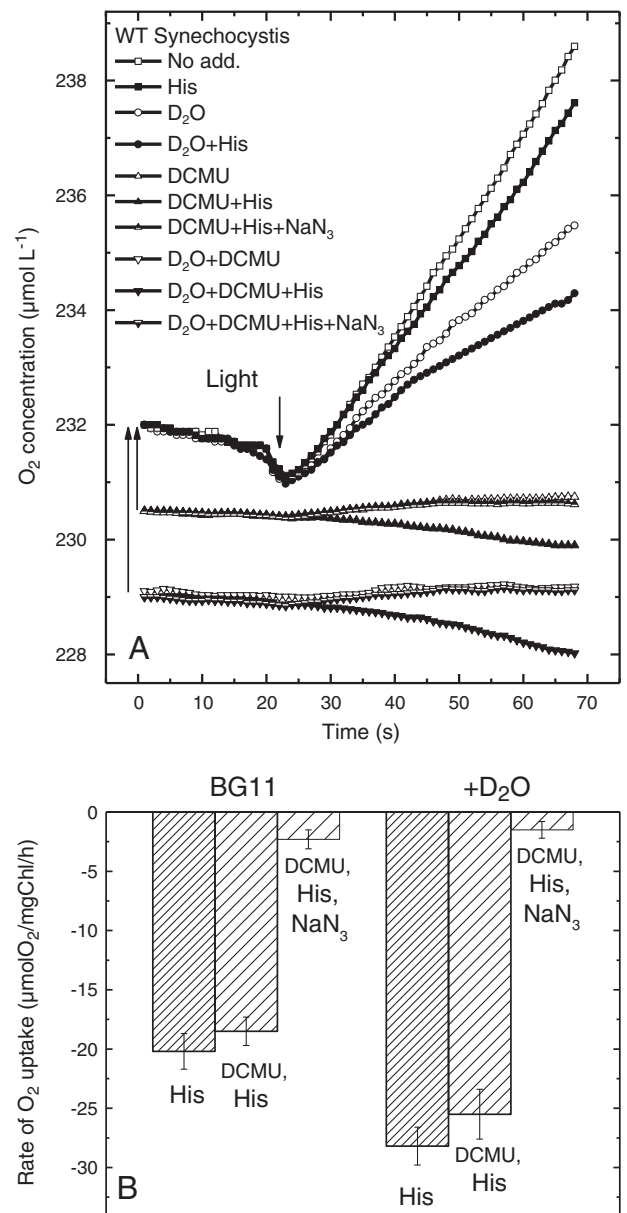


Fig. 3. Effect of D_2O and sodium azide on His-mediated O_2 uptake in *Synechocystis*. A, Light induced changes in the concentration of dissolved oxygen were followed in *Synechocystis* cell suspensions containing H_2O (squares and up triangles) or D_2O (circles and down triangles) in the absence (open symbols), and in the presence of 5 mM His (closed, and half closed symbols). The measurements were also performed in the presence of 10 μ M DCMU (triangles) where the effect of 10 mM NaN_3 in the presence of 5 mM His was also tested (half closed symbols). Light intensity during the oxygen measurements was $2300 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$. For the sake of clarity some curves are shown after shifting along O_2 concentration axis. The actual position of these curves is shown by the arrow. B, Rate of His-mediated O_2 uptake in H_2O and D_2O containing cell suspensions. The data are mean values from 3 measurements performed at $2300 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ light intensity with the indicated errors.

of O_2 evolution the extent of O_2 uptake by His addition was clearly increased relative to that obtained in the H_2O containing medium (Fig. 3B), which supports the idea that His-mediated O_2 uptake reflects $^1\text{O}_2$ trapping.

Further support for the idea that His-mediated O_2 uptake arises from $^1\text{O}_2$ trapping is provided by the observation of real O_2 uptake in the presence of DCMU, which blocks O_2 evolution by occupying the Q_B binding site. When only DCMU was added to the *Synechocystis* cells light induced increase in the level of dissolved oxygen was almost completely eliminated (Fig. 3A, open up triangles). The apparent small residual O_2 evolution could arise from the non-complete inhibition of

PSII activity by the applied 10 μM DCMU. However, this effect must be very small since the flash induced fluorescence relaxation curves (Fig. 2A) and steady state variable fluorescence transients (Fig. 2B) show a large extent inhibition of electron transport at the acceptor side of PSII. Another reason for the apparent residual O_2 evolution in the presence of DCMU could be a light induced decrease in the rate of respiration, as was shown earlier for another cyanobacterium *Trichodesmium* spp. by membrane inlet mass spectrometry, which effect was assigned to partial diversion of electrons that would end up at the terminal oxidase(s) in the dark towards NADP^+ in the light via PSI [50]. The rate of respiration in the presence of DCMU is apparently smaller than in the absence of this inhibitor. This effect most likely arises from the usage of different cell culture batches for the measurements, which were performed in the absence and presence DCMU. We did not observe DCMU affect on the respiration rate when the measurements were performed in the same cell culture with and without DCMU (not shown). The previous membrane inlet mass spectrometry data did not show DCMU effect on the respiration rate either [50]. Illumination in the presence of DCMU + His an absolute O_2 uptake was observed (Fig. 2A, closed up triangles), which effect was enhanced in the presence of D_2O (Fig. 2A, closed down triangles) in agreement with the increased lifetime of $^1\text{O}_2$ in D_2O . These observations confirm that His-mediated O_2 uptake in intact *Synechocystis* cells in the absence of DCMU cannot be caused by a small inhibition of O_2 evolution by His addition, which may cause such a small extent of change in the variable fluorescence characteristics in Fig. 2 that is masked by experimental error.

Based on these findings DCMU inhibition of O_2 evolution could be a standard part of the His-mediated O_2 uptake measurement protocol. However, electron transport inhibitors, which act at the Q_B site modify the redox potential of Q_A and influence charge recombination pathways in PSII and modify $^1\text{O}_2$ production efficiency [51,52]. Such an effect may interfere with the modifications of charge recombination characteristics induced by the studied mutations (see below). Therefore, we prefer to perform the O_2 uptake measurements without DCMU unless other treatments, such as NaN_3 , induce partial inhibition of O_2 evolution which should be masked by complete inhibition of O_2 evolving activity.

Further support for $^1\text{O}_2$ trapping by His comes from the suppression of O_2 uptake in the presence of the $^1\text{O}_2$ quencher NaN_3 . Since NaN_3 inhibits O_2 evolution [49] its $^1\text{O}_2$ quenching effect was tested in the presence of DCMU where the artifact that would arise from the decreased of O_2 evolution rate could be avoided. Addition of NaN_3 almost completely reversed O_2 uptake either in H_2O , or D_2O containing cell suspensions (Fig. 3A, half closed up and down triangles, respectively).

It has to be noted that NaN_3 causes a partial inhibition of PSII activity even in the presence of DCMU (Fig. 2B). However, this inhibitory effect is not expected to eliminate $^1\text{O}_2$ production, which can be observed even in isolated PSII reaction center complexes that lack completely O_2 evolving activity [20]. It is also of note that NaN_3 is not only a $^1\text{O}_2$ quencher, but also an inhibitor of SOD [53] and catalase [49]. Therefore, NaN_3 addition could lead to accumulation of O_2^- and/or H_2O_2 in the cells besides quenching $^1\text{O}_2$. However, it is highly unlikely that this effect could cause an artifact that would be responsible for the observed elimination of His-mediated O_2 uptake in the presence of NaN_3 as observed in Fig. 3 since our data show that neither O_2^- , nor H_2O_2 interacts with His (Fig. 1B) that would modify the level of dissolved O_2 . Therefore, the NaN_3 induced elimination of His-mediated O_2 uptake in the presence of DCMU provides strong support for the idea that the O_2 uptake effect is indeed due to $^1\text{O}_2$ trapping by His.

Based on the above presented key observations, which show that illumination in the presence of His induces O_2 uptake both in the absence and presence of DCMU, that is enhanced by D_2O and suppressed by NaN_3 we can conclude that His at 5 mM concentration

is a suitable chemical trap for $^1\text{O}_2$ detection in intact *Synechocystis* cells.

3.3. $^1\text{O}_2$ production is linearly dependent on light intensity

The production of singlet oxygen in photosynthetic systems involves the formation of triplet Chl states in the PSII reaction center, or in the light harvesting antenna (see Refs. [4,54,55]). This is followed by the interaction of Chl triplets with molecular O_2 , which also has triplet configuration in its ground state, and leads to the formation of highly reactive $^1\text{O}_2$. Since the amount of O_2 is usually high in comparison with the amount of Chl triplets the yield of $^1\text{O}_2$ production is limited by the availability of ^3Chl . On the other hand, the amount of Chl triplets is expected to be linearly dependent on light intensity not only in the light harvesting antenna, but also in the case of the PSII reaction center Chl P680 (see discussion below). Therefore, the yield of $^1\text{O}_2$ production is expected to be linearly increasing with increased light intensity. This expectation has been supported earlier in isolated PSII membranes by using EPR spin trapping [56]. The hypothesis that light intensity dependence of $^1\text{O}_2$ production can be followed by His-mediated O_2 uptake was tested in an *in vitro* system of BG11 medium and Rose Bengal as $^1\text{O}_2$ sensitizer, which indeed showed linear light intensity response in the 34–2300 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ intensity range (not shown).

In photosynthetic systems most of the $^1\text{O}_2$ production is expected to arise from the triplet state of the P680 reaction center Chl assembly. $^3\text{P680}$ is produced via charge recombination from the triplet charge separated state ($^3[\text{P680}^{++}\text{Phe}^{--}]$) whose amount is determined by the competition of spin conversion and forward electron transport from the singlet charge separated state ($^1[\text{P680}^{++}\text{Phe}^{--}]$), as well as of reverse electron flow from the $\text{P680}^{++}\text{PheQ}_\text{A}^-$ state to the singlet and triplet charge separated states (Scheme 1).

In low light, when secondary electron transport toward CO_2 fixation and other light-independent processes (such as the water–water cycle and chlororespiration) are able to utilize the electrons which are liberated from water by the primary charge separation event, Q_A is mainly oxidized, and PSII is in the so called open state. Under these conditions charge stabilization via forward electron transport from Phe^- to Q_A can compete efficiently with spin conversion, therefore reverse electron flow from $\text{P680}^{++}\text{PheQ}_\text{A}^-$ to $^3[\text{P680}^{++}\text{Phe}^{--}]\text{Q}_\text{A}$ is the main source of $^3\text{P680}$ formation in low light (Scheme 1A.). With the increase of light intensity the electron transport chain will be gradually reduced, which stabilizes Q_A^- and converts PSII into the so called closed state. Under these conditions the newly formed primary radical pair can be stabilized only via electron donation to P680^{++} from secondary PSII donors (Scheme 1B). This process has a low rate (ca. $4 \times 10^6 - 2 \times 10^7 \text{ s}^{-1}$ depending on the oxidation state of the water-oxidizing complex [57]) relative to that of spin conversion (ca. 3×10^8 [58]). If the rate constant of primary charge separation and the stability of $^3\text{P680}$ would remain the same in the closed PSII centers as in the open centers the above processes would drastically enhance the yield of the $^3\text{P680}$ forming pathway as can be observed in isolated PSII reaction center complexes, which lack Q_A , or have doubly reduced Q_A that is neutralized by protonation [59]. Based on picosecond fluorescence and absorption change measurements the rate of charge separation and the yield of primary radical formation are expected to decrease by a factor of 2–3 in the presence of fully reduced Q_A [60]. Parallel EPR and flash absorption spectroscopy measurements also showed an ≈ 2 -fold decrease in the yield of primary radical pair formation [59]. In addition a significant, ca. 100-fold, acceleration of the decay of $^3\text{P680}$ was observed in the presence of Q_A^- in cryogenic temperatures [59] from which an accelerated decay of $^3\text{P680}$ can also be predicted at room temperature in closed PSII centers. Therefore, the decreased yield of primary charge pair formation can partly compensate the increase, which is expected to occur in the yield of $^3\text{P680}$ due PSII closure. In addition, the accelerated decay of $^3\text{P680}$ decreases the probability that $^3\text{P680}$ can interact with O_2 . These effects together partly compensate

the potential for the increase in the yield of $^1\text{O}_2$ in closed PSII centers, which however is expected to be still higher than in open PSII. Since the rate of primary charge separation is light intensity dependent the steady state level of $^3\text{P680}$ should increase with increasing light intensities in closed PSII centers, even if the yield of $^3[\text{P680}^+\cdot\text{Phe}^-]$ formation is low and $^3\text{P680}$ decay is accelerated in the presence of Q_A^- leading to light intensity dependent increase of $^1\text{O}_2$.

A computer simulation on the basis of Scheme 1, using the rate constants shown in Table I of the Supplementary material indeed shows that the concentration of $^3\text{P680}$ increases linearly in a wide intensity range even after Q_A is fully reduced (Fig. 4A). This prediction

could also be verified experimentally when $^1\text{O}_2$ production was measured in a suspension of intact *Synechocystis* cells and yielded linear light intensity dependence in the 30–2300 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ range (Fig. 4B).

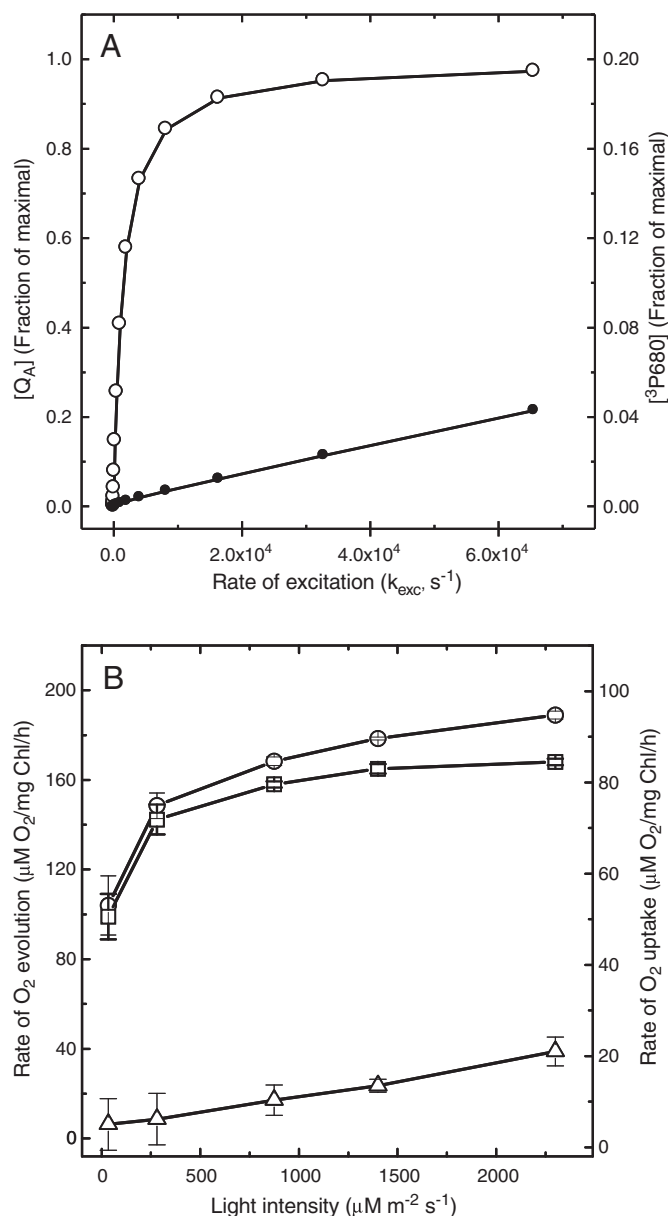
This result is highly important from the point of view of the light intensity dependence of photodamage. Experimental data demonstrate that the initial rate of photodamage linearly increases with light intensity even above the saturation of photosynthetic electron transport [61]. It has been argued that this finding can be explained only by assuming that photodamage of PSII is initiated by direct light induced inactivation of the Mn cluster of the water oxidizing complex [17,18]. The data, which were obtained earlier in isolated PSII membranes [56], and those presented here in intact *Synechocystis* cells demonstrate that the charge recombination mechanism of photodamage, in which $^3\text{P680}$ mediated $^1\text{O}_2$ production is a key event, can also explain the linear light intensity dependence of photoinhibition in a wide light intensity range, which extends well above the saturation level photosynthesis.

It has been reported recently that in higher plants ΔpH dependent dissipation of absorbed light energy via the non-photochemical quenching (NPQ) mechanism decreases $^1\text{O}_2$ formation and partially protects against photodamage [62]. In cyanobacteria NPQ does not depend on ΔpH , but occurs via light induced conformational change of the so called orange carotenoid protein (OCP) [63]. Since OCP-dependent NPQ is induced when light intensity increases one could expect a retardation of $^1\text{O}_2$ production at the onset of light energy dissipation via NPQ. Our data did not indicate such a process in the investigated light intensity range, which might be due to the saturation of the OCP-dependent quenching effect at high light intensities, or to some other effect whose background is not clear at present. This interesting question could be investigated in future studies by using *Synechocystis* mutants in which the level and activity of OCP-dependent NPQ can be controlled.

The rate of O_2 uptake at 2300 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ is ca. 20 $\mu\text{mol O}_2/\text{mg Chl/h}$ when measured in a *Synechocystis* cell suspension at 5 $\mu\text{g Chl/mL}$, corresponding to 5.6 $\mu\text{M Chl}$ concentration (Fig. 4B). In absolute terms this corresponds to 0.055 $\mu\text{mol O}_2/\text{L/s}$ rate of changing in the level of dissolved O_2 in the suspension. Considering that in cyanobacteria PSII and PSI contains ca. 35 and 96 Chl molecules based on their crystal structures [64,65], respectively, and also that the ratio of PSI:PSII is ≈ 3 [66] the 5.6 μM total Chl corresponds to ≈ 17 nM PSII concentration. In comparison, 1 μM RB in cell free BG11 induces ca. 16 $\mu\text{mol O}_2/\text{L/s}$ uptake rate at 2300 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ light intensity due to $^1\text{O}_2$ trapping by His (Fig. 1B). Although the $^1\text{O}_2$ producing efficiency of RB and Chl cannot be exactly compared due to their different absorption characteristics, from the above data we can conclude that under illumination with white light the overall $^1\text{O}_2$ producing efficiency of Chls in intact *Synechocystis* cells is ca. 0.06% that of RB when equal number of sensitizing molecules are considered. However, if we consider whole PSII units as $^1\text{O}_2$ sensitizers and assume also that contribution of antenna Chls in PSI to the overall $^1\text{O}_2$ production is negligible in comparison to PSII, which is supported by EPR spin trapping measurements [67], we obtain that one PSII complex can produce $^1\text{O}_2$ with ca. 20% efficiency of one RB molecule when identical white light illumination is used. Since the main source of $^1\text{O}_2$ production in PSII is expected to be $^3\text{P680}$ this represents very efficient $^1\text{O}_2$ formation in the heart of PSII.

3.4. $^1\text{O}_2$ production and photodamage are correlated, and modulated by the efficiency of nonradiative charge recombination in the PSII reaction center

It has been shown earlier that the amino acid residue at the 130th position of the D1 PSII reaction center subunit influences the redox potential of the primary electron acceptor Phe by modifying H-bonding interactions [68,69]. Higher plants have a Glu residue at this position,



whereas numerous cyanobacteria have multiple D1 protein forms, which have either D1-130Glu or D1-130Gln. The Glu residue occurs in the so called high light D1 forms, which are expressed under high light exposure, whereas Gln occurs in the so called low light D1 forms, which are expressed under low light exposure of the cells (see [70]). Studies with site directed mutants of *Synechocystis* 6803, which have the same D1 form with D1-Gln130 under both low and high light conditions have demonstrated that the replacement of Gln with Glu increases the midpoint redox potential of Phe by 33 mV, whereas the D1-Gln130Leu mutation decreases the $E_m(\text{Phe}/\text{Phe}^-)$ by 74 mV [68]. Biophysical characterization of these mutants have revealed that the D1-Gln130Glu accelerates charge recombination of the S_2QA^- state, whereas the D1-Gln130Leu slows it down [71,72]. These effects have been assigned to the modulation of the rate of nonradiative recombination pathway from the primary charge separated state $\text{P680}^+\text{Phe}^-$ [71,72]. It has also been suggested earlier that non-radiative charge recombination within PSII [73], and in particular from the singlet state of the primary radical pair $^1[\text{P680}^+\text{Phe}^-]$ acts as an important photoprotective pathway (indicated by k_{rd} in Scheme 1A), which competes with $^3\text{P680}$ formation and the consequent $^1\text{O}_2$ production [4,16,72]. Although this hypothesis is well supported by various lines of experimental evidence, so far only preliminary data were reported about the comparison of $^1\text{O}_2$ production and photodamage in intact cyanobacterial cells [74].

Here we applied the His-mediated O_2 uptake assay of $^1\text{O}_2$ detection in the D1-Gln130Glu and D1-Gln130Leu mutants in which the non-radiative recombination pathway is enhanced and retarded, respectively, in parallel with the measurement of PSII photodamage. As shown in Fig. 5A the rate of His-mediated oxygen uptake is smaller in the D1-Gln130Glu mutant, and higher in the D1-Gln130Leu mutant as compared to the WT. In order to confirm that the different rates of oxygen uptake in the mutants reflect different rates of $^1\text{O}_2$ production the measurements were performed in the presence of D_2O , which increases the lifetime of $^1\text{O}_2$. As expected D_2O increased the rate of O_2 uptake in both mutants and the WT (Fig. 5A) supporting its origin from singlet oxygen trapping.

This idea was further confirmed by the effect of NaN_3 , which is a specific singlet oxygen quencher. Since NaN_3 partially inhibits oxygen evolution the O_2 uptake assay was performed after blocking oxygen evolution by DCMU. Addition of NaN_3 to DCMU treated cells drastically decreased the His-mediated O_2 uptake, which supports further the correlation of $^1\text{O}_2$ production with O_2 uptake in the presence of His. The residual rate of O_2 uptake in the presence of NaN_3 may arise from the inability of NaN_3 to react with all $^1\text{O}_2$, which is accessible to His, or could reflect His oxidation by other ROS forms than $^1\text{O}_2$. The extent of His-dependent O_2 uptake, which can be quenched by NaN_3 is significantly higher in the D1-Gln130Leu than in the D1-Gln130Glu mutant, whereas the WT rate shows an intermediate level (Fig. 5A). These data confirm that the D1-Gln130Leu mutant produces $^1\text{O}_2$ at a significantly higher rate than the D1-Gln130Glu mutant, while the WT shows an intermediate rate.

Fig. 5A also shows that the rate of O_2 uptake due to His-mediated $^1\text{O}_2$ trapping is smaller in the presence of DCMU than without addition. It has been shown earlier that binding of electron transport inhibitors to the Q_B site shifts the redox potential of Q_A [51,75]. In case of DCMU $E_m(\text{Q}_\text{A}/\text{Q}_\text{A}^-)$ is shifted by +50 mV [51], which increases the free energy gap between $\text{P680}^+\text{Phe}^-$ and $\text{P680}^+\text{Q}_\text{A}^-$. As a consequence reverse electron transfer from the $\text{P680}^+\text{Q}_\text{A}^-$ charge separated state to $^3[\text{P680}^+\text{Phe}^-]$ is thermodynamically disfavored and its rate decreases at the expense of direct recombination (k_{rd} in Scheme 1A), which decreases the probability of $^3\text{P680}$ and $^1\text{O}_2$ formation (see Refs. [51,52]). This prediction has been validated earlier by EPR detection of $^1\text{O}_2$ in isolated PSII membranes [24,56], and supported further by our data in intact cells.

When susceptibility to photoinhibitory damage of PSII electron transport was measured in the presence of the protein synthesis inhibitor lincomycin, which prevents protein synthesis dependent repair

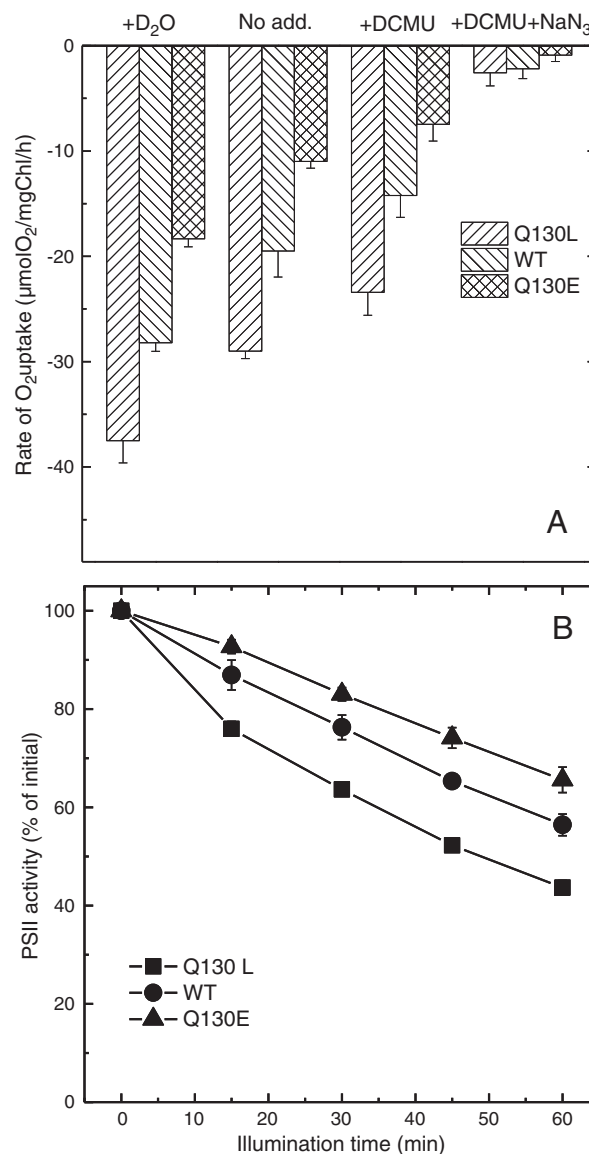


Fig. 5. The effect of D1-Gln130Glu and D1-Gln130Leu mutations on singlet oxygen production and photodamage in *Synechocystis* cells. A, $^1\text{O}_2$ production was assessed by measuring the rate by His-mediated oxygen uptake in WT, D1-Gln130Glu and D1-Gln130Leu *Synechocystis* strains. The measurements were performed using H_2O or D_2O in the BG-11 medium without further addition, or in the presence of 10 μM DCMU with or without 10 mM NaN_3 . B, Light induced damage of PSII activity was assessed by measuring the rate of O_2 evolution in the presence of 0.5 mM DMBQ as artificial electron acceptor during exposure of WT (circles), D1-Gln130Glu (triangles) and D1-Gln130Leu (squares) to illumination with 500 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ light intensity. The data represent mean values from three independent measurements using different cell cultures, with the indicated error.

of PSII, the D1-Gln130Glu cells were less inhibited than the WT (Fig. 5B). In contrast, the D1-Gln130Leu cells were damaged to a larger extent than either the D1-Gln130Glu, or the WT cells. This shows that the D1-Gln130Glu amino acid replacement provides protection against photodamage, while the D1-Gln130Leu replacement enhances it. It is very important to note that the extent of photodamage is correlated with the rate of $^1\text{O}_2$ production. Since the replacement of D1-Gln130 with Glu accelerates the efficiency of nonradiative charge recombination from the $^1[\text{P680}^+\text{Phe}^-]$ charge separated state, while the Leu replacement slows it down [72] these data provide further support for the earlier proposed [16,73] protective role of nonradiative charge recombination against $^1\text{O}_2$ mediated photodamage.

3.5. Concluding remarks

Here we report a method for detection of $^1\text{O}_2$ production in intact *Synechocystis* cells by using histidine as a chemical $^1\text{O}_2$ trap. This method requires only a standard oxygen electrode and can be easily applied in a wide range of organisms, provided that the exogenously added His penetrates through the cell wall. Therefore, the measurement of His-mediated O_2 uptake can remove the serious bottleneck of $^1\text{O}_2$ detection in intact aquatic photosynthetic organisms, which is imposed by the lack of EPR, or fluorescence $^1\text{O}_2$ sensors that can readily penetrate the cell wall of cyanobacteria or algae. By applying this method we show that singlet oxygen production is linearly dependent on light intensity even above the saturation of photosynthesis. This finding provides further evidence that the experimentally observed linear light intensity dependence of the rate of photoinhibition [61] can be explained by $^1\text{O}_2$ -mediated photodamage of PSII in agreement with earlier results obtained in isolated PSII membranes [56].

Our data also show that in the D1-Gln130Glu mutant, in which nonradiative charge recombination of the primary radical pair state is enhanced $^1\text{O}_2$ production is decreased, which effect is accompanied by decreased photodamage. In contrast, increased $^1\text{O}_2$ production concomitant with enhanced photodamage is observed in the D1-Gln130Leu mutant, in which nonradiative charge recombination of the primary radical pair state is enhanced. These data support the mechanism of photoinhibition in which $^1\text{O}_2$ production via interaction with $^3\text{P680}$ is a key step of PSII photodamage. The presented data provide support also for the hypothesis that nonradiative charge recombination of the singlet primary radical pair state provides photoprotection via competition with $^3\text{P680}$ and subsequent $^1\text{O}_2$ formation.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbabi.2013.02.016>.

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