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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 cynobacterium *Synechocystis* 6803 was studied using chemical trapping by histidine, which leads to O_2 uptake during illumination. The rate of O_2 uptake, measured by a standard Clark-type electrode, is enhanced in the presence of D_2O, which increases the lifetime of $^1O_2$, and suppressed by the $^1O_2$ quencher NaN_3. Due to the limited mobility of $^1O_2$ these data demonstrate that exogenous histidine reaches close vicinity of $^1O_2$ 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 O_2 uptake method we showed that $^1O_2$ production linearly increases with light intensity even above the saturation of photosynthesis. We also studied $^1O_2$ 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 $^1O_2$ production concomitant with decreased rate of photodamage relative to the WT, whereas both $^1O_2$ production and photodamage were enhanced in the D1-Gln130Leu mutant. The data are discussed in the framework of the model of photoinhibition in which ^{3}P680 mediated $^1O_2$ production plays a key role in PSII photodamage, and nonradiative charge recombination of the primary charge separated state provides a photoprotective pathway.

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 Photosytem 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).

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 ($^1\Delta_gO_2$, which will be abbreviated as $^1O_2$) is produced via interaction of ground state molecular oxygen ($^3\Sigma_gO_2$) with Chl triplets in PSII, and has been implicated in the process of photoinhibition as an important mediator of light induced damage. Production of $^1O_2$ 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

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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[21,22]. In thylakoid membrane particles EPR spin trapping was applied successfully for $^1O_2$ 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 $^1O_2$ 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 μmol quanta m^{-2} s^{-1}) for the induction of the SOSG fluorescence signal [32] together with $^1O_2$ 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 $^1O_2$ in intact *Synechocystis* 6803 cells, and demonstrate that $^1O_2$ production linearly depends on light intensity. We also show that the efficiency of $^1O_2$ 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 μmol quanta m^{-2} 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 μg Chl mL^{-1} concentration. Before starting high light treatment cells were left for 1 h under 40 μmol quanta m^{-2} 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 μmol quanta m^{-2} s^{-1} light in the presence of the protein synthesis inhibitor lincomycin (300 μg/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 μg 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 μg Chl mL^{-1} was used in each measurement, and three replicates were measured.

2.5. Histidine mediated oxygen uptake measurements

$^1O_2$ production in cell free BG-11 medium was initiated by illumination in the presence of 1 μM Rose Bengal (RB). $^1O_2$ was detected by measuring the rate of light induced oxygen uptake in the presence of 5 mM histidine. For the enhancement of $^1O_2$ production H_2O was replaced with D_2O in BG-11, while quenching of $^1O_2$ 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 radials (500 μM H_2O_2 + 200 μM $Fe(NH_4)_2(SO_4)_2$), 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}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 $^1O_2$ 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 $^1O_2$ [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 $^1O_2$-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 $^1O_2$ 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 $^1O_2$ 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 $^1O_2$ 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 $^1O_2$ trapping by organic ingredients of BG11.