Evidence on the Formation of Singlet Oxygen in the Donor Side Photoinhibition of Photosystem II: EPR Spin-Trapping Study

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Abstract

When photosystem II (PSII) is exposed to excess light, singlet oxygen (1O2) formed by the interaction of molecular oxygen with triplet chlorophyll. Triplet chlorophyll is formed by the charge recombination of triplet radical pair [P680+/Pheo−] in the acceptor-side photoinhibition of PSII. Here, we provide evidence on the formation of 1O2 in the donor side photoinhibition of PSII. Light-induced 1O2 production in Tris-treated PSII membranes was studied by electron paramagnetic resonance (EPR) spin-trapping spectroscopy, as monitored by TEMPONE EPR signal. Light-induced formation of carbon-centered radicals (R) was observed by POBN-R adduct EPR signal. Increased oxidation of organic molecules at high pH enhanced the formation of TEMPONE and POBN-R adduct EPR signals in Tris-treated PSII membranes. Interestingly, the scavenging of R by propyl gallate significantly suppressed 1O2. Based on our results, it is concluded that 1O2 formation correlates with R formation on the donor side of PSII due to oxidation of organic molecules (lipids and proteins) by long-lived P680+/TyrZ. It is proposed here that the Russell mechanism for the recombination of two peroxyl radicals formed by the interaction of R with molecular oxygen is a plausible mechanism for 1O2 formation in the donor side photoinhibition of PSII.

Introduction

Photosystem II (PSII) is a membrane pigment-protein complex located in the thylakoid membrane of oxygenic photosynthetic organisms (higher plant, algae and cyanobacteria). It is a homodimeric multisubunit complex, which is composed of proteins associated with various cofactors. Recent crystal structures of PSII from Thermosynechococcus elongatus and Thermosynechococcus vulcanus show that it is composed of 20 protein subunits, 35 chlorophylls, 12 carotenoids and 25 integral lipids per monomer [1–3]. It is involved in the conversion of light energy into chemical energy by water oxidation and plastoquinone reduction [4–7]. Light-driven water oxidation catalyzed by water-splitting manganese complex occurs via a step-wise release of four electrons and protons [8–12].

When higher plant, algae and cyanobacteria are exposed to high-light intensity illumination, PSII activity is inhibited in a process called photoinhibition [13–18]. Photo-inactivation of PSII is considered to be caused by damage to the D1 protein, one of the two proteins which formed a heterodimer with the D2 protein [19–22]. It is widely accepted that D1 damage is caused by two distinct mechanisms of photoinhibition i.e. the so called acceptor and donor side mechanism [19,23–24]. In the acceptor side photoinhibition, over-reduction of the primary electron acceptor QA leads to its release from the binding site in the D2 protein [25–27]. In donor side photoinhibition, the formation of long-lived highly oxidizing molecules P680*/TyrZ leads to the oxidation of the organic components such as proteins and lipids [18–19,21].

It has been reported that different types of reactive oxygen species (ROS) are formed in both the acceptor and the donor side photoinhibition [28–30]. In the acceptor-side photoinhibition, singlet oxygen (1O2) is considered the main ROS responsible for PSII damage. The primary charge separation results in the formation of a primary radical pair [P680+/Pheo−] which leads to the formation of a secondary radical pair [P680+/Qa−] by charge stabilization process. Under the complete or partial reduction of PQ pool, the reverse electron transport from QA− to Pheo− forms [P680+/Pheo−], which subsequently either recombines to the ground state P680 or converts to the triplet radical pair [P680+/Pheo−] by change in the spin orientation [18,27,30–31]. Singlet oxygen is generated by the interaction of molecular oxygen and triplet chlorophyll formed by the charge recombination of the triplet radical pair [P680*/Pheo−] [18,28–30]. Singlet oxygen formation was shown by electron paramagnetic resonance (EPR) spin-trapping in the thylakoid membranes [32–33], PSII membranes [34], by chemical trapping [35] and phosphorescence at 1270 nm in PSII reaction center [36]. Apart from the radical pair recombination mechanism in the PSII reaction center, the formation of 1O2 occurs in the PSII antenna complex by intersystem crossing from the singlet excited chlorophyll via triplet-singlet energy transfer from the triplet chlorophyll.
It has been proposed that \(^1\text{O}_2\) can be generated from either weakly coupled or energetically uncoupled triplet chlorophylls in the isolated light harvesting complex II (LHCII) [37–38]. Singlet oxygen formation in the isolated light harvesting complex II (LHCII) was shown by EPR spin trapping technique [39–40]. The authors concluded that \(^1\text{O}_2\) production in LHCII occurs as in the Type II photosensitization process. In this process, the triplet chlorophyll transfers its excitation energy to triplet molecular oxygen, while \(^1\text{O}_2\) is formed. In addition to \(^1\text{O}_2\) formation in the acceptor side photoinhibition, formation of the superoxide anion radical (\(\text{O}^2^-\)) and the hydroxyl radical (\(\text{HO}^\cdot\)) has been demonstrated in PSII membranes [41–42].

In the donor side photoinhibition, the oxidation of proteins and lipids by highly oxidizing molecules \(\text{P}680^*/\text{TyrZ}\) results in the formation of carbon-centered radical (\(R^*\)) [32]. Hydroxyl radical on PSII electron donor side was proposed to be formed by an unspecified reaction due to the photo-damage of thylakoid membrane by oxidizing reaction [32] and by the reduction of \(\text{H}_2\text{O}_2\) formed on the PSII electron donor side [43]. In our best knowledge, there is no evidence on the formation of \(^1\text{O}_2\) on the donor side photoinhibition of PSII. It has been shown that the photosensitized decomposition of molecular oxygen was increased six folds after the removal of water-splitting manganese complex from the PSII membranes [44]. Recently, lipid and protein hydroperoxides have been detected in Tris-treated PSII membranes [45]. It has been proposed that the loss of electron transport from water-splitting manganese complex to PSII reaction center leads to the formation of organic molecules by \(\text{P}680^*/\text{TyrZ}\) and consequently to the formation of organic \(R^*\) [44–47]. Apart from the above mentioned mechanism, manganese hypothesis has been recently assumed as another model in donor side photoinhibition [16,48]. In this manganese hypothesis, the excitation of manganese by UV or visible light inhibits the electron transfer from water-splitting manganese complex to \(\text{P}680^*\). Inactivation of water-splitting manganese complex occurs via the release of manganese and subsequently stabilizes the \(\text{P}680^*\) for a longer period which leads to the photoinhibition of PSII.

In spite of the above mentioned in vitro mechanisms, a unifying model has been given to explain the photosensitization of PSII under in vivo conditions [49–50]. It has been proposed that \(\text{P}680^+\) has the capability for photosensitization of PSII under steady state photosynthesis. The different ways of charge recombination have been regulated to the formation of primary radical pair \:^-\(\text{P}680^+\text{Pheo}^-\\), which leads to the photoinactivation of PSII under steady state photosynthesis in vivo.

In this present study, the evidence for the formation of \(^1\text{O}_2\) in donor side photoinhibition is provided by using EPR spin-trapping spectroscopy. Light-induced \(^1\text{O}_2\) formation in Tris-treated PSII membranes was detected with hydrophilic spin trap compound TMPS. It is proposed here that the generation of \(^1\text{O}_2\) in the donor side photoinhibition of PSII occurs by the recombination of peroxy radicals via the Russell mechanism.

## Results

Singlet oxygen formation in Tris-treated PSII membranes

In this study, light-induced formation of \(^1\text{O}_2\) in Tris-treated PSII membranes was measured by EPR spin-trapping technique. When spin-trapping was accomplished by utilizing the oxidation of lipophilic diaminogentic 2,2,6,6-tetramethylpiperidine (TEP) by \(^1\text{O}_2\) which yields paramagnetic 2,2,6,6-tetramethylpiperidine-1-oxyl (TEPNO), no TEMPO EPR signal was detected (data not shown). The absence of TEMPO EPR signal was due to the oxidation of TEMPO by highly oxidizing species in Tris-treated PSII membranes. It is well known that under highly oxidizing conditions TEMPO is easily oxidized to o xoanmonium salt [51]. To prevent the oxidation of paramagnetic TEMPO by highly oxidizing species formed on the PSII electron donor side, the spin-trapping was accomplished by utilizing the oxidation of hydrophilic diaminogentic 2,2,6,6-tetramethylpiperidine (TMPS) by \(^1\text{O}_2\) which yields paramagnetic 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPONE) (Fig. 1). Due to the fact that TMPS is a hydrophilic nitroxide spin trap, the nitroxyl radical TEMPO monitors predominantly the formation of \(^1\text{O}_2\) in the polar phase. The addition of TMPS to Tris-treated PSII membranes in the dark resulted in the appearance of negligible TEMPONE EPR signal. The negligible TEMPONE EPR signal observed in non-illuminated Tris-treated PSII membranes was due to impurity of the spin trap. The exposure of Tris-treated PSII membranes to continuous white light resulted in the generation of TEMPONE EPR signal (Fig. 1A). Figure 1B shows that TEMPONE EPR signal increases gradually with illumination period. These observations indicate that illumination of Tris-treated PSII membranes results in \(^1\text{O}_2\) formation.

### Carbon-centered radical formation in Tris-treated PSII membranes

In order to detect the formation of \(R^*\) in Tris-treated PSII membranes, we used EPR spin-trapping technique using POBN (4-pyridyl-1-oxide-\(N\)-tert-butylnitrone) as the spin-trap compound. In the dark, no detectable POBN-R adduct EPR signal was observed in Tris-treated PSII membranes. The exposure of Tris-treated PSII membranes to continuous white light resulted in the formation of POBN-R adduct EPR signal (Fig. 1B). In this study, the evidence for the formation of \(^1\text{O}_2\) in donor side photoinhibition was provided by using EPR spin-trapping spectroscopy. Light-induced \(^1\text{O}_2\) formation in Tris-treated PSII membranes was detected with hydrophilic spin trap compound TMPS. It is proposed here that the generation of \(^1\text{O}_2\) in the donor side photoinhibition of PSII occurs by the recombination of peroxy radicals via the Russell mechanism.
generation of POBN-R adduct EPR signal (Fig. 2A). Figure 2B shows that the POBN-R adduct EPR signal increases with illumination of Tris-treated PSII membranes. These observations indicate that illumination of Tris-treated PSII membranes results in R₇N formation.

Effect of pH on ¹O₂ and R₇ formation in Tris-treated PSII membranes

To study the correlation between the light-induced ¹O₂ and R₇ formation in donor side photoinhibition, the effect of pH on the formation of ¹O₂ and R₇ was measured in Tris-treated PSII membranes. The pH increase caused a significant enhancement in TEMPO (Fig. 3) and POBN-R adduct (Fig. 4) EPR signals. These observations indicate that the formation of ¹O₂ correlates with the formation of R₇ in the donor side photoinhibition. The enhancement of TEMPO and POBN-R adduct EPR signals at high pH reveals that the oxidation of organic molecules is involved in ¹O₂ formation in Tris-treated PSII membranes.

Effect of propyl gallate on ¹O₂ and R₇ production in Tris-treated PSII membranes

In order to confirm the correlation between light-induced ¹O₂ and R₇ formation in the donor side photoinhibition, the effect of free radical scavenger propyl gallate on TEMPO and EMPO-R adduct EPR signals was studied in Tris-treated PSII membranes. Figure 5A shows that the addition of propyl gallate to Tris-treated PSII membranes prior to illumination significantly suppressed the TEMPO EPR signal. In order to study the formation of R₇ in the presence of propyl gallate dissolved in ethanol, EMPO (5-(ethoxycarbonyl)-5-methyl-1-pyrroline N-oxide) spin trap was used instead of a POBN spin trap. As in the presence of ethanol, POBN reacts with a-hydroxyethyl radical (CH(CH₃)HO⁻) formed by HO⁻ with ethanol, the detection of R₇ by POBN in presence of ethanol is unfeasible. In the dark, no EMPO-R adduct EPR signal was observed in Tris-treated PSII membranes, whereas the exposure of Tris-treated PSII membranes to white light resulted in the generation of EMPO-R adduct EPR signal (Fig. 5B). The addition of propyl gallate to Tris-treated PSII membranes suppressed the EMPO-R adduct EPR signal in Tris-treated PSII membranes (Fig. 5B). These observations reveal that the scavenging of R₇ by the addition of exogenous propyl gallate results in the suppression of ¹O₂ in Tris-treated PSII membranes.

Discussion

It is well known that ¹O₂ is one of the most dangerous ROS in PSII, known to play a crucial role in the protein degradation of PSII under photoinhibitory conditions. The light-induced degradation of D1 protein occurs on both the electron acceptor and the electron donor side of PSII [19,23,52]. It is well established that ¹O₂ causes the damage of PSII in the acceptor site photoinhibition [18,28,30,53], whereas the donor side photoinhibition occurs by highly oxidizing long lived molecules P680⁺/TyrZ⁺ [18~19,21,23].

Using EPR spin-trapping spectroscopy, we demonstrated that the exposure of Tris-treated PSII membranes in the presence of
of organic molecules is involved in the production of 1O2 in Tris-treated PSII membranes. Observations that the addition of propyl gallate to Tris-treated PSII membranes suppressed the formation of 1O2 (Fig. 5A) and R
gallate to Tris-treated PSII membranes under light illumination for 30 min. The intensity of EPR signal was evaluated as the relative height of central peak of the first derivative of the EPR absorption spectrum. The data represent the mean value (±SD) of at least three experiments.

Protein-mediated lipid and protein oxidation forms R’ known to form a peroxyl radical (ROO•) in the presence of molecular oxygen [57–58]. Singlet oxygen is produced via the decomposition of linear tetraoxide intermediate which is formed by the combination of two ROO• [58–59]. Recently, it has been shown that 1O2 is formed by the enzymatic (cytochrome c and lactoperoxidase) decomposition of polyunsaturated lipid peroxide via the Russell mechanism [60]. Furthermore, it has been reported that the yield 1O2 is 10−10 times higher via decomposition of tetraoxide compared to the triplet excited peroxide via the Russell mechanism [60]. Furthermore, it has been reported that the yield 1O2 is 10−10 times higher via decomposition of tetraoxide compared to the triplet excited peroxide via the Russell mechanism [60].

Similarly, we propose here that the light-induced oxidation of lipids and proteins by long lived highly oxidizing molecules generates predominately 1O2 via Russell mechanism [61–62]. Similarly, we propose here that the light-induced oxidation of lipids and proteins by long lived highly oxidizing molecules generates predominately 1O2 via Russell mechanism [61–62].

It has been reported that the increase in photoconsumption of molecular oxygen at high pH is due to increased oxidation of organic molecules in Tris-treated PSII membranes [46–47]. In agreement with this, we showed here that the formation of 1O2 and R’ is enhanced in Tris-treated PSII membranes at high pH (Figs. 3 and 4). Similarly, we suggest that the increased oxidation of organic molecules at high pH enhances the formation of 1O2 via the Russell mechanism. Observations that the addition of propyl gallate to Tris-treated PSII membranes suppressed the formation of 1O2 (Fig. 5A) and R’ (Fig. 5B) reveals that the oxidation of organic molecules is involved in the production of 1O2 in Tris-treated PSII membranes.

The formation of 1O2 via the Russell mechanism was reported in chemical systems [55–56]. Radical-mediated lipid and protein oxidation forms R’ known to form a peroxyl radical (ROO•) in the presence of molecular oxygen [57–58]. Singlet oxygen is produced via the decomposition of linear tetraoxide intermediate which is
P680 and TyrZ formed by oxidation of lipids and proteins react with molecular oxygen distanced at 2.2 Å to the histidine residues D1-H198 and D2-H197, respectively [3]. The accessory chlorophylls Chl D1 and Chl D2 are hydrogen-bonded between the chlorine ring V and damage of PSII [64].

From the PSII reaction center in order to prevent the oxidative molecules provide an environment to keep molecular oxygen away photoinhibition [65]. Recently, it has been proposed that the lipid candidate for the initiation of lipid oxidation. Lipid molecules may provide structural flexibility around the PSII reaction center, thus providing the assembly and repair of PSII in the donor side photoinhibition [65]. Recently, it has been proposed that the lipid molecules provide an environment to keep molecular oxygen away from the PSII reaction center in order to prevent the oxidative damage of PSII [64].

In the PSII reaction center, chlorophyll dimer (P D1 and P D2) is distanced at 2.2 Å to the histidine residues D1-H190 and D2-H197, respectively [3]. The accessory chlorophylls Chl D1 and Chl D2 are hydrogen-bonded between the chlorine ring V and water molecule with a distance of 2.0 and 2.1 Å, respectively [3]. It has been reported that the midpoint redox potential of P680+/P680 and TyrZ/TyrZ redox couple ranges from 1.2 to 1.4 V and 1.1 to 1.2 V, respectively [5,7,10,31,66-67]. Due to the highest midpoint redox potential of P680+/P680, yields paramagnetic 2, 6-tetramethyl-4-piperidone-1-oxyl (TEMPONE) EPR signal. To eliminate impurity TEMPED EPR signal TEMPED was purified twice by vacuum distillation. Tris-treated PSII membranes (200 μg Chl ml⁻¹) were illuminated in the presence of 50 mM TMPD and 40 mM MES-NaOH (pH 6.5) at 20°C. Illumination was performed with a continuous white light (1000 μmol photons m⁻² s⁻¹) using a halogen lamp with a light guide (Schott KL 1500, Schott AG, Mainz, Germany). After illumination, the sample was centrifuged at 5000 g for 3 min to separate TEMPONE from Tris-treated PSII membranes. It has been reported recently that the separation of two phases prevents the reduction of TEMPONE by a non-specific reducing component in thylakoid and PSII membranes [34,71]. In this study, separation of the two phases was done to prevent the oxidation of TEMPONE by a non-specific oxidizing component in Tris-treated PSII membranes. After centrifugation, the upper phase was immediately transferred into the glass capillary tube (Blaubrand® intraMARK, Brand, Germany) and kept in liquid nitrogen until use. Prior to data collection, the capillary tube was taken away from the liquid nitrogen and EPR spin-trapping spectra were collected at room temperature.

Carbon-centered radicals were detected by either POBN (4-pyridyl-1-oxide-N-tert-butyl nitronite) (Sigma) or EMPO (5-(ethoxycarbonyl)-5-methyl-1-pyrroline N-oxide) (Alexis Biochemicals, Lausen, Switzerland) [72–73]. In the POBN detection system, Tris-treated PSII membranes (200 μg Chl ml⁻¹) were illuminated in the presence of 50 mM POBN and 40 mM MES-NaOH (pH 6.5). In the EMPO detection system, Tris-treated PSII membranes were first illuminated in the absence of spin trap for 30 min, whereas after illumination the sample was mixed with EMPO spin trap and further illuminated for 2 min. Illumination was performed with a continuous white light using a halogen lamp
with a light guide (KL 1500 electron, Schott, Germany). After illumination the sample was transferred into the glass capillary tube (Blaubrand® intraMARK, Brand, Germany) and EPR spectra were immediately recorded at room temperature. Spectra were recorded using EPR spectrometer MiniScope MS200 (Magnettech GmbH, Berlin, Germany). Signal intensity was evaluated as the height of the central peak of EPR spectrum. EPR conditions were as follows: microwave power, 10 mW; modulation amplitude, 1 G; modulation frequency, 100 kHz; sweep width, 100 G; scan rate, 1.62 G s⁻¹.

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Author Contributions
Conceived and designed the experiments: PP DKY. Performed the experiments: DKY. Analyzed the data: DKY. Contributed reagents/materials/analysis tools: PP. Wrote the paper: PP DKY.

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