# Effect of Mn cluster on the formation of superoxide radicals in photoinhibition of photosystem Ⅱ

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**Abstract** **To further realize the action of superoxide radicals ($O_2^{-}$) in photoinhibition of photosystem Ⅱ (PSⅡ), we employed 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) as a spin trap, associated with EPR spectroscopy, to study the effect of illumination time on $O_2^{-\bullet}$ formation during high light photoinhibition in PSⅡ membranes and Mn-depleted PSⅡ membranes. Results indicated that the removal of Mn cluster from PSⅡ membranes has a strong influence on the dynamics of superoxide formation. The relative mechanism was also discussed. These novel findings may further promote the studies of the structure and function of PSⅡ and the mechanism of photoinhibition.**

**Keywords: Mn cluster, photosystem Ⅱ, photoinhibition, superoxide radicals, EPR.**

In green plants, light energy harvested by antenna chlorophyll-protein complexes was transferred to the chlorophyll of PSⅡ reaction center (RC) to initiate photochemical reaction in PSⅡ and result in the plastoquinone reduction and the water oxidation to molecular oxygen. But prolonged illumination with high intensities leads to the functional impairment of PSⅡ electron transport and the structural damage of the D1 protein in PSⅡ RC. This is described as the so-called photoinhibiton[1,2].

In recent years, great progress has been made in the research on the molecular mechanism of photoinhibition, but there still exist many problems to be resolved. Generally, photoinhibition is considered to proceed by two different molecular mechanisms: One is the so-called acceptor-side-induced photoinhibition, it is mainly due to the over-reduction of $Q_A$, which results in the charge recombination to form the triplet states of P680, and subsequently to generate singlet oxygen, which promotes the D1 degradation[3]. The other is the donor-side-induced photoinhibition, it is mainly caused by the light-induced accumulation of highly oxidizing species ($P680^+$ and/or the secondary electron donor in D1 protein $TyrZ^+$), which promote the D1 degradation[4]. Using various free radical scavengers, many scholars[5,6] demonstrated that active oxygen species are involved in the process of photoinhibition. However, the role of $O_2^{-\bullet}$ is still controversial[7—13].

In this work, we used 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) as the spin trap, associated with EPR spectroscopy, to carry out an comparative study on $O_2^{-\bullet}$ formation during photoinhibition of control Spinach PSⅡ membranes and Mn-depleted PSⅡ membranes. Mn cluster is composed of four differnent valent Mn atoms. It can act as a catalyst with the help of other cofactors to split $H_2O$ into $O_2$, and afford electron to P680[14]. $NH_2OH$ treatment can remove Mn while producing little effect on extrinsic polypeptides[15]. When the electron transport on the donor-side is blocked, illumination of Mn-depleted PSⅡ membranes can result in the donor-side-induced photoinhibition, which is quite different from the acceptor-side-induced photoinhibition when normal PSⅡ particles are as experimental materials. The possible mechanism was also discussed in this manuscript.

## 1 Materials and methods

Oxygen-evolving PSⅡ membranes were prepared from market Spinach leaves according to ref. [16] with minor modification. Freshly isolated PSⅡ membranes were suspended in SMN solution (0.4 mol/L sucrose, 5 mmol/L $MgCl_2$ and 10 mmol/L NaCl, 50 mmol/L MES-NaOH, pH 6.5) and stored at 77 K until use. Chlorophyll (Chl) content of the samples was determined according to Arnon[17]. The oxygen-evolution activity of PSⅡ membranes was about 280 μmol $O_2$ (mg of Chl)$^{-1}$ • $h^{-1}$.

Mn-depleted PSⅡ membranes were prepared from PSⅡ membranes as described in ref. [18] with slight modifications as follows. 2 mL PSⅡ membranes (1 mg Chl/mL) were diluted with an equal volume of 10 mmol/L $NH_2OH$, and kept at 4℃ in darkness for 30 min. Then the membranes were collected after centrifugation at 40000×*g* for 20 min and washed once with SMN (~5 mL). The resultant pellet of PSⅡ particles was suspended in SCN solution (0.4 mol/L sucrose, 10 mmol/L $CaCl_2$ and 180 mmol/L NaCl, 50 mmol/L Mes-NaOH, pH 6.5). The Mn-depleted PSⅡ membranes (5 mg Chl/mL) were frozen in liquid nitrogen and kept at 77 K until use. The oxygen-evolution activity was about 15 μmol $O_2$ (mg of Chl)$^{-1}$ • $h^{-1}$ (only about 5% of that in control PSⅡ membranes).

Photosynthetic oxygen evolution was measured with a Clark-type oxygen electrode in SMN solution at 25℃. 0.8 mmol/L 2,6-dichloro-*p*-benzoquinone (DCBQ) was used as the artificial electron acceptor.

Illumination was performed directly in the EPR cavity using a 500-W Xenon lamp. Light wavelength less than 400 nm was cut off by a long-pass filter. The light intensity on the surface of the cavity was $4.8\times10^2$ J • $m^{-2}$ • $s^{-1}$. A water filter was used for protection from far-red irradiation. Photoinhibition was performed in 10 mmol/L sodium phosphate (pH 6.5) containing 100 mmol/L sucrose, 5 mmol/L $MgCl_2$ and 50 mmol/L NaCl.

X-band EPR spectra were recorded at room temperature on a Varian E-112 spectrometer at 9.17 GHz. PS Ⅱ samples and DMPO were quantitatively injected into a flat quartz cell. Typical spectrometer parameters for spin trapping experiments were set as follows: microwave power, 50 mW; modulation amplitude, $1.25\times10^{-5}$ T; time constant, 0.125 s; receiver gain, $5\times10^4$.

The EPR spectrum of Mn was directly recorded in cavity after mixing PS Ⅱ samples with 0.5 mol/L HCl. EPR parameters were kept as the following: microwave power, 30 mW; modulation amplitude, $1\times10^{-3}$ T; time constant, 0.25 s; receiver gain, $2\times10^4$.

## 2 Results

(ⅰ) Manganese (Mn) content of PS Ⅱ and Mn-depleted PS Ⅱ membranes. In order to compare the contents of Mn in control PS Ⅱ membranes and Mn-depleted PS Ⅱ membranes, both membranes in the presence of 0.5 mol/L HCl were quantitatively injected into a flat quartz cell. The EPR spectra of $Mn^{2+}$ are shown in fig. 1. The characteristic EPR spectra of $Mn^{2+}$ (curve 1) were well consistent with literature report[19]. Curve 2 in fig. 1 shows that a great part of Mn was released after $NH_2OH$ (5 mmol/L) treatment of PS Ⅱ membranes. According to relative amplitude of the third-line in EPR spectrum, Mn content in the $NH_2OH$-treated PS Ⅱ samples was calculated to be ~20% of control PS Ⅱ samples. This indicated that the Mn-depleted PS Ⅱ membranes were suitable for the further study.

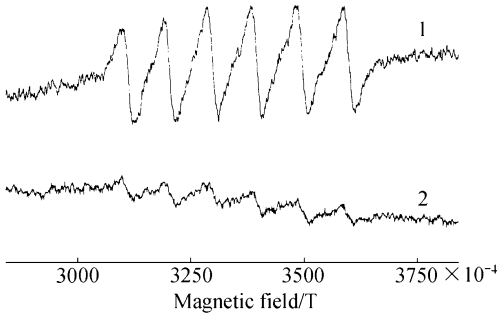


Fig. 1. EPR spectra of aqueous $Mn^{2+}$ released from: (1) PS Ⅱ membranes (0.5 mg/mL) in the presence of 0.5 mol/L HCl; (2) $NH_2OH$-treated PS Ⅱ membranes, other conditions as (1). For EPR parameters, see "Materials and methods".

(ⅱ) Relationship of acceptor-side- and donor-side-induced photoinhibition of PS Ⅱ with superoxide radicals. At present, DMPO is the most extensively used spin trap for $O_2^{\cdot-}$. It can react with $O_2^{\cdot-}$ to form DMPO-OOH adducts, showing the characteristic EPR signal. In this work, we also used this spin trap to study the formation of $O_2^{\cdot-}$ in photoinhibition process of PS Ⅱ.

Acceptor-side-induced photoinhibition was often achieved when the PS Ⅱ functional donor side was intact. However, donor-side-induced photoinhibition was commonly studied with impairment of oxidizing side of PS Ⅱ. In the absence of external electron acceptors, no EPR signal appeared when PS Ⅱ membranes (150 μg Chl/mL) were mixed with DMPO (50 mmol/L) in darkness (curve 1 in fig. 2). But after 1 min illumination, the EPR spectrum was immediately detected (as shown in curve 2 of fig. 2). The spectrum was well consistent with the standard DMPO-OOH signal ($\alpha^N = 14.2\times10^{-4}$ T, $\alpha_\beta^H = 11.2\times10^{-4}$ T and $\alpha_\gamma^H = 1.3\times10^{-4}$ T)[20].

Mn-depleted PS Ⅱ membranes were used as a model system for study of donor-side-induced photoinhibition. When the samples were mixed with DMPO, no EPR signals were detected in darkness (spectrum not shown). But upon illumination, the EPR signals of DMPO-OOH adducts also appeared (curve 3 in fig. 2). It is suggested that superoxide radicals should have been involved in both acceptor-side- and donor-side-induced photoinhibition of PS Ⅱ. After 1 min illumination (fig. 2), the relative signal intensity of DMPO-OOH adduct (also calculated from the ralative amplitude of the third line) in donor-side-induced photoinhibition was stronger than that in acceptor-side-induced photoinhibition.

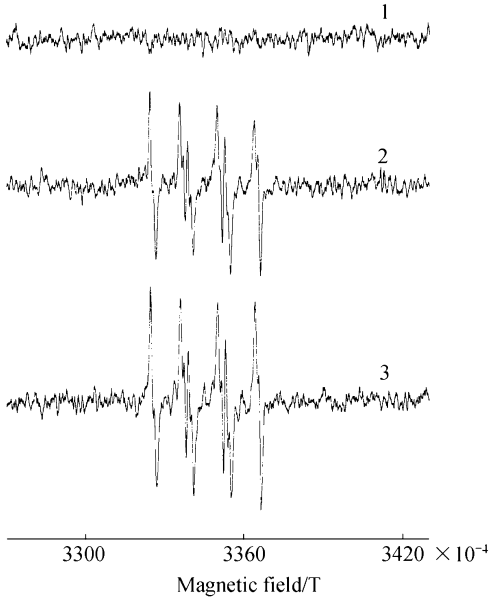


Fig. 2. EPR spectra for PS Ⅱ samples with different treatments in the presence of DMPO (50 mmol/L) at room temperature: (1) control, (2) after 1-min illumination, (3) $NH_2OH$-treated after 1-min illumination. Chl concentrations were 150 μg Chl/mL. For EPR parameters, see Section 1 "Materials and methods".

(iii) Effect of illumination time on EPR intensities of DMPO-OOH adducts. To further understand the action mechanism of $O_2^{-\bullet}$ in acceptor-side- and donor-side-induced photoinhibition of PS Ⅱ, the effect of illumination time on EPR intensities of DEPMPO-OOH adducts was studied with PS Ⅱ membranes and Mn-depleted PS Ⅱ membranes (as shown in fig. 3). It can be seen clearly that

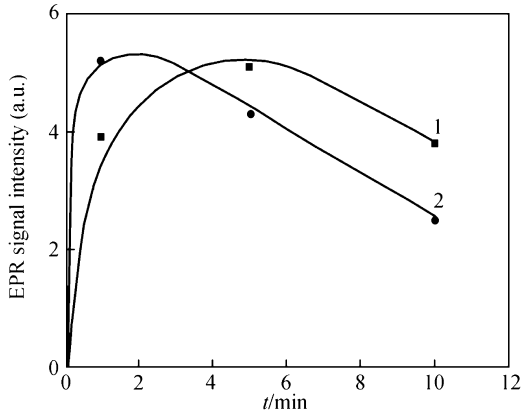


Fig. 3. Effect of illumination time on EPR signal intensities of DEPMPO-OOH adducts in different PS Ⅱ membranes. 1, Control; 2, $NH_2OH$-treated. Other experimental conditions were the same as in fig. 1.

the maximal EPR signal intensities of DMPO-OOH adducts of Mn-depleted PS Ⅱ membranes (for about 1-min illumination) appeared earlier than those of PS Ⅱ membranes (for about 5-min illumination).

## 3 Discussion

Considering the above results, it is proposed that the production of superoxide radicals could be initiated by both acceptor-side- and donor-side-induced photoinhibition of PS Ⅱ (fig. 2), which is in contrast to the results of Hedig et al.[9,10]. They used DMPO as the spin trap to study the radicals generated in photoinhibition of PS Ⅱ, but they did not detect $O_2^{-\bullet}$ successfully. One possible reason causing the measurement trouble might be that the illumination of samples was not carried out in the EPR cavity. The life time of DMPO-OOH adducts is very short (only ~1 min in neutral aqueous solutions). If the measurement of EPR spectra and illumination were not performed simutaneously, the DMPO-OOH adducts would probably decompose into a faulse DMPO-OH adduct. Therefore, the detected hydroxyl and alkoxyl radicals might be the products of the reaction of $O_2^{-\bullet}$ with substrates.

Comparing fig. 3 with fig. 1, we can conclude that the removal of Mn cluster from PS Ⅱ membranes exerts a strong influence on the dynamics of superoxide formation. The possible reasons may include the following three aspects: First, the intrinsic superoxide dismutase (SOD) was lost after the Mn cluster was released from PS Ⅱ membranes[21], since SOD can inhibit the formation of $O_2^{-\bullet}$; Second, there was probably no external electron donor to maintain the PS Ⅱ electron transfer after the removal of Mn by $NH_2OH$ washing. Our additional work demonstrated that superoxide radicals were generated probably from the reaction of $Q_B^-$ and $O_2$ (to be published). The termination of temporary electron transport may greatly influence the superoxide formation. And last, the large amount of $O_2^{-\bullet}$ formed rapidly after illumination of Mn-depleted PS Ⅱ membranes with high intensity can lead to the fast oxygen exhaustion, which in turn results in the appearance of the maximal EPR signal of DMPO-OOH. In fact, we obtained the similar results when using 5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide (DEPMPO) as spin trap. However, the life-time of DEPMPO-OOH (~14 min) is longer than that of DMPO-OOH (~1 min), so the maximal EPR signals of DEPMPO-OOH appeared relatively late than that of DMPO-OOH (to be published).

In conclusion, superoxide radicals are shown to be involved in both acceptor-side- and donor-side-induced photoinhibition of PS Ⅱ. Removal of Mn cluster from PS Ⅱ membranes has a strong influence on the dynamics of superoxide formation. These novel findings may promote the studies of the structure and function of PS Ⅱ and the mechanism of photoinhibition.

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