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Dark production of reactive oxygen species in photosystem II membrane particles at elevated temperature: EPR spin-trapping study

Pavel Pospíšil *, Iva Šnyrychová, Jan Nauš

Laboratory of Biophysics, Department of Experimental Physics, Faculty of Science, Palacký University, tř. Svobody 26, 771 46 Olomouc, Czech Republic

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Abstract

In our study, EPR spin-trapping technique was employed to study dark production of two reactive oxygen species, hydroxyl radicals ($OH^{\bullet}$) and singlet oxygen ($^1O_2$), in spinach photosystem II (PSII) membrane particles exposed to elevated temperature (47 °C). Production of $OH^{\bullet}$, evaluated as EMPO-OH adduct EPR signal, was suppressed by the enzymatic removal of hydrogen peroxide and by the addition of iron chelator desferal, whereas externally added hydrogen peroxide enhanced $OH^{\bullet}$ production. These observations reveal that $OH^{\bullet}$ is presumably produced by metal-mediated reduction of hydrogen peroxide in a Fenton-type reaction. Increase in pH above physiological values significantly stimulated the formation of $OH^{\bullet}$, whereas the presence of chloride and calcium ions had the opposite effect. Based on our results it is proposed that the formation of $OH^{\bullet}$ is linked to the thermal disassembly of water-splitting manganese complex on PSII donor side. Singlet oxygen production, followed as the formation of nitroxyl radical TEMPO, was not affected by $OH^{\bullet}$ scavengers. This finding indicates that the production of these two species was independent and that the production of $^1O_2$ is not closely linked to PSII donor side.

Keywords: EMPO; EPR spin-trapping; Heat; Hydroxyl radical; Singlet oxygen; Photosystem II

1. Introduction

Molecular oxygen, even though essential for most of living organisms, serves as a precursor of reactive oxygen species (ROS) [1,2]. At low concentrations ROS play important role in cell signaling processes, but when formed in excess, they damage cellular macromolecules such as proteins, lipids and nucleic acids [3]. In thylakoid membrane, ROS are formed when absorption of light by chlorophylls exceeds the capacity for energy utilization by photosynthetic apparatus [4,5]. Besides this, evidence was given that ROS can be also produced under special conditions in the dark. Using EPR spin-trapping spectroscopy, Hideg and Vass [6] have demonstrated that singlet oxygen is produced in the dark in thylakoid membrane exposed to high temperature. However, the mechanism of ROS generation in the dark is still to the great extent a matter of debate.

The most thermally labile component of the thylakoid membrane is photosystem II (PSII) [7], namely its donor side, and the effect of heat treatment on Mn-complex has been extensively studied [8–10]. It has been shown that the heat inactivation of water-splitting complex involves the release of 18 kDa, 23 kDa and 33 kDa extrinsic proteins from the lumenal surface of thylakoid membrane [9,11], followed by stepwise release of Mn atoms [10]. It has been suggested that heat treatment affects chloride-binding site within Mn-complex [12], whereas EXAFS study showed that the early phase of stepwise disassembly of Mn-complex is coupled to the release of Ca^{2+} [10,11,13]. From these evidences it can be concluded that inorganic cofactors of water-splitting complex play important role in the process of heat inactivation of oxygen evolution. Thompson et al. [14] proposed that the heat-induced release of extrinsic proteins results in uncontrolled accessibility of water

Abbreviations: desferal, deferoxamine mesylate; DMPO, 5,5-Dimethyl-1-pyrroline *N*-oxide; EDTA, Ethylenediamine-*N,N,N′,N′*-tetraacetic acid; EMPO, 2-ethoxycarbonyl-2-methyl-3,4-dihydro-2*H*-pyrrole-1-oxide; EPR, electron paramagnetic resonance; PSII, photosystem II; ROS, reactive oxygen species; TEMP, 2,2,6,6-tetramethylpiperidine; TyrD, Tyr-161 of subunit D2 of PSII; TyrZ, Tyr-161 of subunit D1 of PSII

* Corresponding author. Tel.: +42 585634164; fax: +42 585225737.
E-mail address: pospip@prfnw.upol.cz (P. Pospíšil).

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to Mn-complex and subsequent divalent oxidation of water to hydrogen peroxide (H_2O_2). To our best knowledge, no direct evidence has been given on the generation of ROS linked to PSII donor side inactivation in the dark.

In this work we used EPR spin-trapping technique to study the dark production of $OH^{\bullet}$ in PSII membrane particles exposed to heat stress (47 °C). For the first time we have shown that heating of PSII membrane particles in the dark results in the production of $OH^{\bullet}$ and we suggest that this process is connected to heat-induced changes of PSII donor side. Further, we have shown that $OH^{\bullet}$ is unlikely related to the generation of $^1O_2$, which has also been observed in heated PSII membrane particles.

2. Materials and methods

2.1. Sample preparation

PSII membrane particles from spinach (*Spinacia oleracea*) were prepared using Triton X-100 purification according to the method of Berthold et al. [15] with modifications described in Ford and Evans [16]. PSII membrane particles were resuspended in medium containing 400 mM sucrose, 15 mM NaCl, 5 mM $MgCl_2$, and 40 mM Mes (pH 6.5) and stored at −80 °C.

2.2. EPR spin-trapping spectroscopy

EMPO (5-(ethoxycarbonyl)-5-methyl-1-pyrroline N-oxide, Alexis Biochemicals, Lausen, Switzerland) spin trap compound was used for the detection of $OH^{\bullet}$, whereas spin-trapping of $^1O_2$ was accomplished by TEMP (2,2,6,6-tetramethylpiperidine, Sigma-Aldrich). PSII membrane particles (500 μg Chl ml^{-1}) in 40 mM Mes (pH 6.5) were incubated in Eppendorf tubes immersed in water bath (47 °C) in the presence of 75 mM EMPO ($OH^{\bullet}$ trapping) or 50 mM TEMP ($^1O_2$ trapping). In the case of TEMP, 5% ethanol was present to avoid spin trap precipitation. Samples were heated at 47 °C in digitally controlled heater in complete darkness for time periods indicated in figures. After treatment, sample was transferred into a glass capillary tube (Blaubrand® intraMARK, Brand, Germany) and room temperature EPR spectra were recorded using EPR spectrometer MiniScope MS200 (Magnettech GmbH, Germany). Signal intensity was evaluated as a relative height of the central doublet (EMPO-OH) or the central peak (TEMPO) of EPR spectrum obtained as a first derivative of the EPR absorption signal. 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}.

2.3. 77K EPR spectroscopy

PSII membrane particles (1 mg Chl ml^{-1}) in 40 mM Mes (pH 6.5) were heated in Eppendorf tubes immersed in water bath (47 °C) under the same conditions as used for spin-trapping EPR measurements. After heating, the sample was transferred into EPR tube and $TyrD^{ox}$ EPR spectra were recorded at 77 K using EPR spectrometer MiniScope MS200 (Magnettech GmbH, Germany). EPR conditions were as follows: microwave power, 3 mW; modulation amplitude, 2.6 G; modulation frequency, 100 kHz; sweep width, 100 G; scan rate, 1.62 G s^{-1}.

3. Results

3.1. Dark production of ROS at elevated temperature

Dark production of ROS at elevated temperature in spinach PSII membrane particles was followed using EMPO and TEMP spin traps. EMPO is known to react with both $OH^{\bullet}$ and $O_2^{\bullet-}$, forming paramagnetic EMPO-OH and EMPO-OOH adducts, respectively [17,18]. TEMP is considered to react with $^1O_2$ to form nitroxyl radical TEMPO [19].

The EPR spectrum obtained after exposure of PSII membrane particles to 47 °C in the presence of EMPO can be solely ascribed to the EMPO-OH adduct (Fig. 1A). For comparison, EPR spectrum of the EMPO-OH adduct obtained using model Fenton system and EMPO-OOH spectrum obtained in xanthine/xanthine oxidase system are shown in Fig. 1A (two most upper traces). The finding that no EMPO-OOH adduct was detected may either indicate that no superoxide is produced or that the superoxide adduct is unstable at higher temperatures. No EMPO-OH EPR signal was observed after heating the buffered solution of pure spin trap, which indicates that PSII membrane particles are required for the appearance of this adduct (data not shown). Due to very high sensitivity of EMPO, the first detectable EMPO-OH adduct

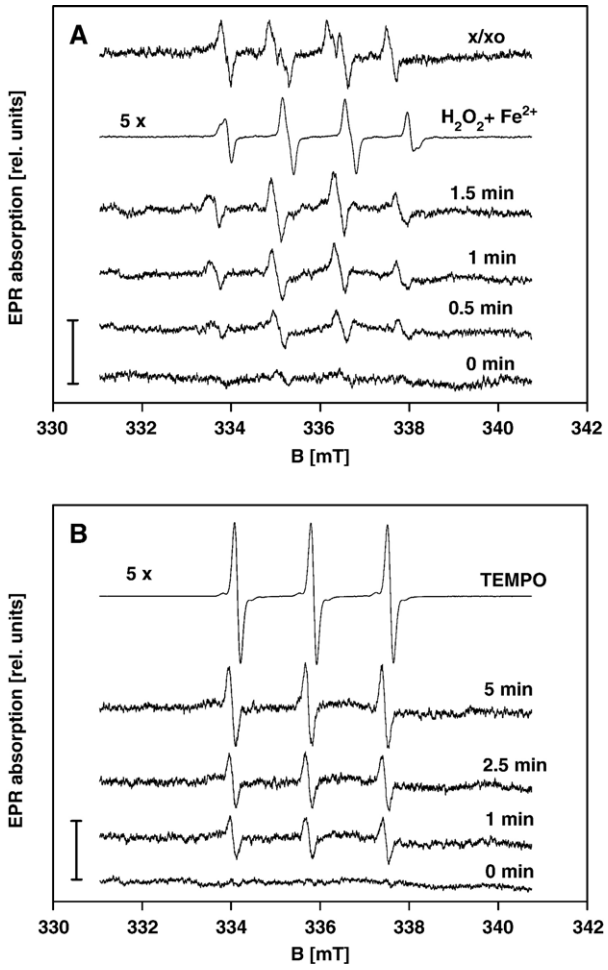

Fig. 1. Dark EMPO-OH adduct (A) and TEMPO (B) EPR spectra measured in PSII membrane particles exposed to 47 °C for the time indicated. EMPO-OH adduct EPR spectra were obtained in the presence of 75 mM EMPO, 500 μg of Chl ml^{-1} and 40 mM Mes (pH 6.5). Model EMPO-OH and EMPO-OOH adduct EPR spectra were obtained in $H_2O_2/FeSO_4$ and xanthine/xanthine oxidase systems, respectively, in the presence of 25 mM EMPO. TEMPO EPR spectra were measured in the presence of 50 mM TEMP, 5% ethanol, 500 μg of Chl ml^{-1} and 25 mM Mes (pH 6.5). Model EPR spectrum was obtained using 1 mM TEMPO. EPR spectra were plotted as the first derivative of the EPR absorption. Vertical bar represents 2000 relative units.

EPR signal was observed as fast as after first 15 s of heating (data not shown) and this gave us the possibility to detect $OH^{\bullet}$ in PSII membrane particles after a very short period of heat treatment (Fig. 1A). On the other hand, due to instability of EMPO-OH adduct, the corresponding EPR signal decreased during prolonged heating (data not shown).

Exposure of PSII membrane particles to 47 °C in the presence of TEMP spin trap results in the formation of TEMPO (Fig. 1B), whereas no TEMPO EPR signal was observed in the absence of PSII membrane particles (data not shown). No reduction of TEMPO radical occurred in the dark and therefore it was not necessary to perform any extraction and reoxidation that is unavoidable for experiments involving light [20]. Model spectrum obtained using pure TEMPO radical is shown in Fig. 1B (most upper trace).

3.2. Effect of $^1O_2$ and $OH^{\bullet}$ scavengers on EMPO-OH adduct

It is well known that DMPO spin-trapping experiments can be easily misinterpreted due to the fact that DMPO-OH adduct can be formed not only as a result of $OH^{\bullet}$ trapping, but also by different mechanisms. We have performed a set of experiments verifying that under our experimental conditions EMPO-OH adduct is formed by interaction of EMPO with $OH^{\bullet}$ and that it indeed reflects the production of $OH^{\bullet}$.

To exclude the possibility that EMPO-OH adduct is formed by the decomposition of EMPO-OOH, the effect of $OH^{\bullet}$ scavenger mannitol was tested. Our finding that heating of PSII membrane particles in the presence of mannitol completely prevented the formation of EMPO-OH adduct (Fig. 2) indicates that EMPO-OH is formed by direct trapping of $OH^{\bullet}$ and that the formation of EMPO-OH adduct by decomposition of EMPO-OOH adduct is unlikely.

To find out whether EMPO-OH adduct is formed by the interaction of spin trap with $^1O_2$, EMPO-OH adduct EPR spectra were measured in the presence of either $^1O_2$ scavenger sodium azide (NaN_3) or deuterium oxide (D_2O), which prolongs the half-lifetime of $^1O_2$ and enhances $^1O_2$-mediated effects. No pronounced effect of NaN_3 (suppression by 12±3%) and D_2O (suppression by 11±2%) on the formation of EMPO-OH adduct EPR signal has been observed (Fig. 2). These findings exclude the possibility that $OH^{\bullet}$ is formed as an artifact during the reaction between the spin trap and $^1O_2$, which has been suggested to occur in the presence of DMPO [21].

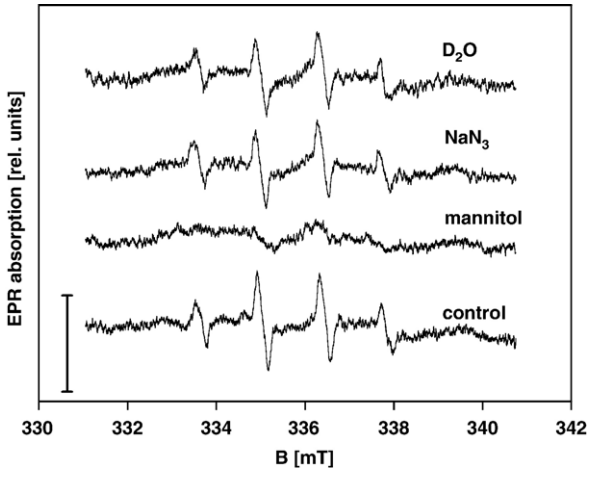

Fig. 2. Effect of $OH^{\bullet}$ and $^1O_2$ scavengers on EMPO-OH adduct EPR spectra measured in PSII membrane particles exposed to 47 °C for 1.5 min. 250 mM mannitol, 10 mM NaN_3 were added before heat treatment. 50% D_2O was used to replace H_2O in buffer medium. Other experimental conditions were as described in Fig. 1A. EPR spectra were plotted as the first derivative of the EPR absorption. Vertical bar represents 2000 relative units.

3.3. Effect of catalase, peroxide and chelators on $OH^{\bullet}$ generation

The most straightforward mechanism of $OH^{\bullet}$ production in biological systems involves the univalent reduction of H_2O_2 by reduced metals in a Fenton-type reaction [22]. To test whether this pathway is of importance for the formation of $OH^{\bullet}$ in PSII membrane particles at elevated temperature, effects of catalase and metal chelators were measured. Removal of H_2O_2 by catalase (5000 U ml^{-1}) completely suppressed $OH^{\cdot}$ production (Fig. 3). It has been demonstrated that catalase is thermally stable up to 50 °C; however, the activity of the enzyme is slightly lowered at high temperature [23]. This might provide explanation why partial $OH^{\bullet}$ production was observed at lower catalase concentration (2500 U ml^{-1}) (data not shown). In agreement with the effect of catalase, the addition of H_2O_2 to PSII membrane particles prior to heating doubled $OH^{\bullet}$ generation (increase by 107±7%) (Fig. 3). The presence of metal chelator EDTA lowered $OH^{\bullet}$ production (data not shown), however, removal of redox active iron by stronger chelator desferal completely suppressed EMPO-OH EPR signal (Fig. 3). Based on these results it is suggested that $OH^{\bullet}$ is likely formed via metal-catalyzed reduction of H_2O_2.

3.4. Effect of high pH, chloride and calcium on $OH^{\bullet}$ production

Evidence has been given that the light-induced formation of H_2O_2 on the donor side of PSII is promoted at high pH [24]. The authors proposed that at high pH one of the inorganic cofactors, namely chloride ion, is replaced by hydroxide ion and that this hydroxide ion can be oxidized to H_2O_2. Recently, it has been demonstrated that PSII membranes depleted of chloride are characterized by higher rates of light-induced $OH^{\bullet}$ production compared to active PSII membranes [25]. To reveal whether heat-induced dark production of $OH^{\bullet}$ is related to PSII donor side, pH dependence of EMPO-OH EPR signal and effect of inorganic cofactors of water-splitting complex on $OH^{\bullet}$ formation were studied (Fig. 4).

Increase in pH above physiological values induced significant enhancement in $OH^{\bullet}$ production (Fig. 4A). The fitting of pH dependence of EMPO-OH EPR signal intensity to a single proton titration curve gives apparent pK_a of 7.3. The excess of chloride or calcium ions in the medium during heat treatment suppressed EMPO-OH adduct EPR signal intensity by 32±4% and 41±6%, respectively (Fig. 4B). These observations indicate that $OH^{\bullet}$ is likely produced on PSII donor side.

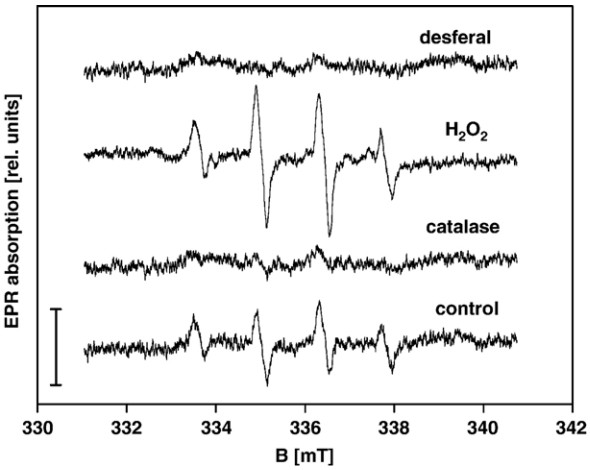

Fig. 3. Effect of catalase, H_2O_2 and desferal on EMPO-OH adduct EPR spectra measured in PSII membrane particles exposed to 47 °C for 1.5 min. 5000 U ml^{-1} catalase, 250 mM H_2O_2 and 1 mM desferal were added before heat treatment. Other experimental conditions were as described in Fig. 1A. EPR spectra were plotted as the first derivative of the EPR absorption. Vertical bar represents 2000 relative units.

The stability of PSII donor site at elevated temperatures was followed by the measurement of EPR signal corresponding to oxidized form of tyrosine-161 of D2 subunit of PSII, usually denoted as $TyrD^{ox}$. Exposure of PSII membrane particles to 47 °C results in a rapid loss of $TyrD^{ox}$ EPR signal (Fig. 4A, insert). Since TyrD is known to be in redox equilibrium with the water-oxidizing complex, the conversion of TyrD into its reduced form provides evidence of very fast heat-induced destabilization of the donor side of PSII.

3.5. Production of $^1O_2$ and its link to $OH^\bullet$ formation

The formation of $^1O_2$ in thylakoid membranes heated to high temperatures in the dark has already been described [6]. Even though the exact mechanism of $^1O_2$ production was not studied, the authors suggested that it might be related to the process of lipid peroxidation.

Lipid peroxidation can be induced by various factors and ROS usually act as initiators of this process. To clarify the role of $OH^\bullet$ in $^1O_2$ production at elevated temperature, the formation of TEMPO was followed in the presence of catalase and metal chelator. When catalase or desferal were added to PSII membrane particles before heating, no effect on $^1O_2$ production was observed (Fig. 5), whereas under the same conditions $OH^\bullet$ production was completely suppressed (Fig. 3). Similarly, $^1O_2$ production was unaffected when PSII membrane particles were heated in the presence of mannitol (Fig. 5). These observations indicate that $OH^\bullet$ produced at elevated temperature is unlikely coupled with $^1O_2$ production.

4. Discussion

4.1. Dark production of $OH^\bullet$ at elevated temperature

In this study, EPR spin-trapping spectroscopy was used to study dark production of ROS in PSII membrane particles at elevated temperature. Spin trap EMPO is sensitive to both $OH^\bullet$ and $O_2^{\bullet-}$ and the respective paramagnetic adducts are more stable in comparison to widely used trap DMPO [17,18]. So far, EMPO has been successfully employed for the detection of light-induced ROS in PSII membrane particles [26,27]. Even though EPR spin-trapping technique is the most direct and sensitive method for the detection of ROS in biological systems, the possibility of artifacts and misinterpretations has to be carried in mind [28]. Considering this fact, formation of EMPO-OH adduct does not necessarily reflect the production of $OH^\bullet$ in the investigated system and therefore scavenger effects were studied to verify the origin of EMPO-OH.

To exclude the possibility that EMPO-OH adduct is derived from the decomposition of EMPO-OOH adduct, which is known to occur in the case of DMPO, $OH^\bullet$ production was measured in the presence of $OH^\bullet$ scavenger mannitol (Fig. 2).

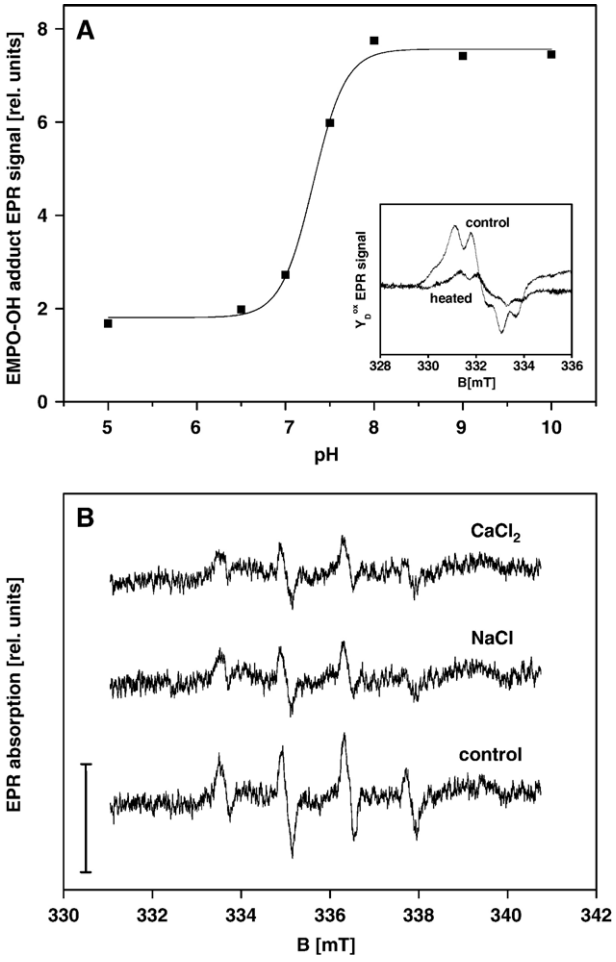

Fig. 4. (A) Effect of pH on EPR signal intensity of EMPO-OH adduct measured in PSII membrane particles exposed to 47 °C for 1.5 min. PSII membrane particles (500 μg Chl ml^{-1}) were heated in the presence of 75 mM EMPO, 40 mM acetate buffer (pH 5.0) or 40 mM Mes (pH 6.5) or 40 mM HEPES (pH 7.0–8.0) or CAPS (pH 9.0–10.0). Insert shows $TyrD^{ox}$ EPR signal measured in PSII membrane particles exposed to 47 °C for 0 and 1 min. (B) Effect of chloride and calcium on EMPO-OH adduct EPR spectra measured in PSII membrane particles (500 μg Chl ml^{-1}) exposed to 47 °C for 1.5 min. 100 mM NaCl and 50 mM $CaCl_2$ were added before heat treatment. Other experimental conditions were as described in Fig. 1A. EPR spectra were plotted as the first derivative of the EPR absorption. Vertical bar represents 2000 relative units.

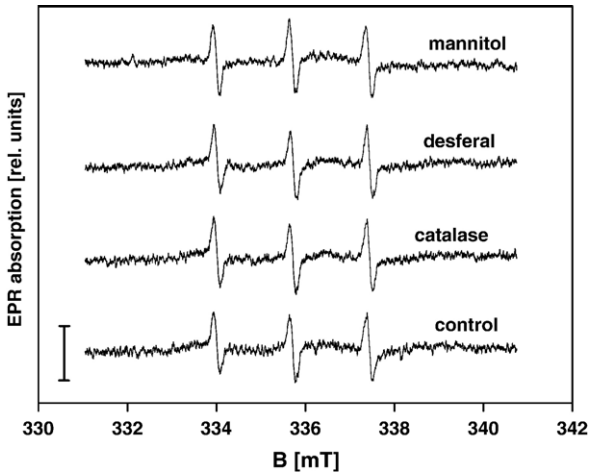

Fig. 5. TEMPO adduct EPR spectra measured in PSII membrane particles (500 μg Chl ml^{-1}) exposed to 47 °C for 5 min in the presence of 5000 U ml^{-1} catalase, 1 mM desferal or 250 mM mannitol. Other experimental conditions were as described in Fig. 1B. EPR spectra were plotted as the first derivative of the EPR absorption. Vertical bar represents 2000 relative units.

The fact that EMPO-OH adduct EPR signal was completely diminished by mannitol indicates that EMPO-OH is formed by the interaction of EMPO with $OH^{\bullet}$. Recently it has been demonstrated that the reaction of DMPO with $^1O_2$ can lead to artifactual formation of DMPO-OH [21]. To study whether the formation of EMPO-OH adduct is affected by $^1O_2$, EMPO-OH adduct EPR spectra were measured in the presence of NaN_3 and D_2O (Fig. 2). These two compounds are known to suppress and enhance $^1O_2$ mediated processes, respectively, but they lacked any significant effect on EMPO-OH adduct EPR signal. These results suggest that neither $O_2^{\bullet -}$ nor $^1O_2$ are involved in the formation of EMPO-OH adduct.

4.2. $OH^{\bullet}$ is formed by decomposition of H_2O_2 on PSII donor side

Based on the observation that catalase and desferal suppressed $OH^{\bullet}$ production (Fig. 3), it can be concluded that $OH^{\bullet}$ is formed by one-electron reduction of H_2O_2. It has been previously suggested that under heat treatment H_2O_2 is formed by partial oxidation of water on the donor side of PSII [14]. Release of extrinsic proteins was proposed to result in uncontrolled water accessibility to Mn-complex and oxidation of hydroxide ion to H_2O_2 [29]. Judging from the sigmoid shape of the pH dependence of EMPO-OH EPR signal intensity, it seems that the generation of $OH^{\bullet}$ is somehow affected by PSII cofactors exhibiting pK_a around 7.3. Interestingly, pK_a of two redox-active tyrosine residues on the donor side of PSII, denoted tyrosine Z and tyrosine D, ranges between 7 and 8 [30]. It is known that $TyrD^{ox}$ is reduced at high temperature, as demonstrated by the decrease in $TyrD^{ox}$ EPR signal (Fig. 4A, insert) [10,11,14]. It should be noted that the reduction of $TyrD^{ox}$, similarly to the appearance of EMPO-OH EPR signal, is very fast in heated PSII membrane particles ($TyrD^{ox}$ EPR signal decreased by 70% after 1 min at 47 °C). The reduction of $TyrD^{ox}$ is accompanied by its protonation [30] and therefore pK_a of TyrD in fact characterizes also the redox change between its oxidized and reduced form. It seems that the enhancement in $OH^{\bullet}$ production is correlated with the reduction of $TyrD^{ox}$, which in turn is generally considered as a sign of the destabilization of water-splitting complex. Based on these evidences we suggest that during heat treatment, $OH^{\bullet}$ is formed via univalent reduction of H_2O_2 in a process linked to the impairment of PSII donor side.

4.3. $OH^{\bullet}$ is not closely linked to $^1O_2$ production

Our observation that catalase, desferal and mannitol did not affect $^1O_2$ generation indicates that $OH^{\bullet}$ is not involved in the production of $^1O_2$. We suggest that the formation of $^1O_2$ is connected to some heat-stimulated oxidation processes, but detailed clarification of its origin is beyond the scope of this study. It has been previously suggested that $^1O_2$ formation can be related to the process of lipid peroxidation [6] and that it can be formed as its by-product via recombination of lipid radicals. In such case it would be likely that rather than $OH^{\bullet}$, another highly oxidizing species (such as ferryl, perferryl, ferrous–dioxygen–ferric complex) participate in the initiation of lipid peroxidation at higher temperature [31,32]. Hydroxyl radical is typical by its very short lifetime, diffusion controlled reaction rates and very high standard redox potential [2]. On the other hand, the extreme reactivity of $OH^{\bullet}$ restricts its harmful effects to targets in the close proximity of the site of its production. It is known that EMPO is rather hydrophilic, reacting with $OH^{\bullet}$ formed in polar phase [33]. In addition, scavengers like catalase and mannitol can hardly react with $OH^{\bullet}$ formed in lipid phase. Thus, the fact that the formation of $^1O_2$ was not affected by scavengers abolishing EMPO-OH EPR signal (Fig. 5) would not necessarily rule out the possible involvement of $OH^{\bullet}$ in a process leading to $^1O_2$ if it takes place in the interior of the thylakoid membrane.

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