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Role of chloride ion in hydroxyl radical production in photosystem II under heat stress: Electron paramagnetic resonance spin-trapping study

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Abstract Hydroxyl radical ($HO^{\bullet}$) production in photosystem II (PSII) was studied by electron paramagnetic resonance (EPR) spin-trapping technique. It is demonstrated here that the exposure of PSII membranes to heat stress (40 °C) results in $HO^{\bullet}$ formation, as monitored by the formation of EMPO-OH adduct EPR signal. The presence of different exogenous halides significantly suppressed the EMPO-OH adduct EPR signal in PSII membranes under heat stress. The addition of exogenous acetate and blocker of chloride channel suppressed the EMPO-OH adduct EPR signal, whereas the blocker of calcium channel did not affect the EMPO-OH adduct EPR signal. Heat-induced hydrogen peroxide (H_2O_2) production was studied by amplex red fluorescent assay. The presence of exogenous halides, acetate and chloride blocker showed the suppression of H_2O_2 production in PSII membranes under heat stress. Based on our results, it is proposed that the formation of $HO^{\bullet}$ under heat stress is linked to uncontrolled accessibility of water to the water-splitting manganese complex caused by the release of chloride ion on the electron donor side of PSII. Uncontrolled water accessibility to the water-splitting manganese complex causes the formation of H_2O_2 due to improper water oxidation, which leads to the formation of $HO^{\bullet}$ via the Fenton reaction under heat stress.

Keywords Photosystem II · Electron paramagnetic resonance · Hydroxyl radical · Spin trap · Heat stress · Chloride ion · Hydrogen peroxide · Amplex red

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Abbreviations

PSII	photosystem II
MES	4-morpholineethanesulfonic acid
EMPO	(5-(ethoxycorbonyl)-5-methyl-1-pyrroline N-oxide)
ROS	reactive oxygen species
EPR	electron paramagnetic resonance
DIDS	diisothiocyano-stilbene- 2,2 –disulfonic acid
$HO^{\bullet}$	hydroxyl radical
Amplex red	(10-acetyl-3,7-dihydroxyphenoxazine)
HRP	Horseradish peroxidase

Introduction

Photosystem II (PSII) is a pigment-protein complex embedded in the thylakoid membrane of oxygenic photosynthetic organisms (cyanobacteria, algae and higher plants). Photosystem II is an only unique natural enzyme, which is able to split water molecules to molecular oxygen. Recent crystal structures of PSII from cyanobacteria *Thermosynechococcus elongatus* and *Thermosynechococcus vulcanus* show that it is composed of about 20 protein subunits and number of cofactors, with a total molecular weight of 350 kDa (Ferreira et al. 2004; Guskov et al. 2009; Umena et al. 2011). The oxidation of two water molecules is catalyzed by the water-splitting manganese complex via consecutive stepwise removal of electrons, protons and release of molecular oxygen (Dau and Haumann 2008; Brudvig 2008; Renger 2011). The water-splitting manganese complex contains four manganese, one calcium and five oxygen atoms in the Mn_4CaO_5 cluster. Two chloride ions were identified in the vicinity of the Mn_4CaO_5 cluster surrounded by water molecules and a hydrophilic residue (Umena et al. 2011;

Kawakami et al. 2011). The extrinsic proteins (PsbO, PsbP, PsbQ) and cofactors (calcium and chloride ions) are associated with the water-splitting manganese complex to maintain its water-splitting activity (Roose et al. 2007; Enami et al. 2008; Gorkom and Yocum 2005; Popelková and Yocum 2007; Yocum 2008).

Among the cellular processes, photosynthetic activity of plants is one of the most heat-sensitive processes. Photosystem II is more thermolabile compared to photosystem I (PSI) in the thylakoid membrane (Weis and Berry 1988). The heat stress leads to the inactivation of electron transport on both the electron acceptor and the electron donor side of PSII (Allakhverdiev et al. 1996, 2008; Bukhov and Mohanty 1999; Carpentier 1999; Jajoo et al. 2001). It has been recently demonstrated that inactivation of PSII electron transport on both the electron acceptor and the electron donor side of PSII results in the formation of singlet oxygen ($^1O_2$) and hydroxyl radical ($HO^\bullet$), respectively (Pospíšil et al. 2007; Yamashita et al. 2008). It has been proposed that the D1 protein is damaged under heat stress conditions. The heat-induced suppression of CO_2 fixation leads to the inhibition of protein synthesis and the repair of PSII by the action of the reactive oxygen species (ROS) (Takahashi and Murata 2006; Murata et al. 2007).

On the electron acceptor side of PSII, heat-induced shift in the midpoint redox potential of redox couple Q_A/Q_A^- results in the inhibition of electron transfer from Q_A to Q_B (Pospíšil and Tyystjärvi 1999). It has been reported that the cleavage of the D1 protein occurs in the plastoquinone-binding pocket at the electron acceptor side of PSII (Yoshioka et al. 2006; Komayama et al. 2007; Yamashita et al. 2008). The suppression of cleavage and aggregation of D1 protein under anaerobic conditions provides the evidence for the involvement of molecular oxygen in the deleterious effect on the D1 protein in PSII (Yamashita et al. 2008). It has been suggested that $^1O_2$ is formed in the plastoquinone-binding pocket by the recombination of peroxyl radicals formed within the lipid peroxidation. Singlet oxygen caused the D1 protein degradation via the interaction with the DE loop of the protein in a similar manner as in the acceptor side photoinhibition of PSII (Yamashita et al. 2008; Yamamoto et al. 2008).

On the electron donor side of PSII, the previous study reported the deactivation of the water-splitting manganese complex under heat-stress (Nash et al. 1985). It has been demonstrated that the release of PsbO, PsbP, PsbQ proteins (Enami et al. 1994; Barra et al. 2005) and chloride (Coleman et al. 1988) posses heat inactivation of photosynthetic activity in PSII. Furthermore, the stepwise displacement of manganese is associated with the release of calcium from the water-splitting manganese complex under heat stress conditions (Pospíšil et al. 2003; Barra et al. 2005, 2006). The precise mechanism of heat inactivation of PSII is not explained completely till now.

It was previously proposed that the heat-induced release of PsbO, PsbP and PsbQ proteins leads to improper water accessibility to the water-splitting manganese complex and consequently to the formation of hydrogen peroxide (H_2O_2) (Thompson et al. 1989). Recently, we have showed that the $HO^\bullet$ production is suppressed by the addition of exogenous calcium and chloride ions (Pospíšil et al. 2007). The observation that $HO^\bullet$ is not formed in Tris-treated PSII membranes reveals that $HO^\bullet$ is produced on the electron donor side of PSII (Yamashita et al. 2008). Furthermore, the complete suppression of $HO^\bullet$ production occurs in the presence of exogenous catalase and metal chelator desferal. Based on these observations, it was proposed that $HO^\bullet$ formation is linked to the thermal disassembly of the water-splitting complex via the metal-catalyzed Fenton reaction (one electron reduction of H_2O_2 to $HO^\bullet$) on the electron donor side of PSII.

In the present study, we utilized the electron paramagnetic resonance (EPR) spin trapping technique to monitor the $HO^\bullet$ formation in PSII membranes under heat stress conditions. We have shown that the chloride ion is important to prevent the $HO^\bullet$ formation under heat stress. It is proposed here that the formation of $HO^\bullet$ is due to the uncontrolled accessibility of water to the water-splitting manganese complex caused by the release of chloride ion on the electron donor side of PSII.

Materials and methods

Photosystem II membranes preparation

Photosystem II membranes were isolated from fresh spinach leaves purchased from a local market according to the previously described method of Berthold et al. (1981) with the modifications described in Ford and Evans (1983). Photosystem II membranes were suspended in a buffer solution containing 400 mM sucrose, 10 mM NaCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$ and 50 mM MES-NaOH (pH 6.5) and stored at −80 °C. Prior to the experiment, the PSII membranes were slowly thawed at 4 °C. All the procedures of PSII membranes preparation were carried out under dim green light. Chlorophyll concentration was determined with 80 % acetone extract using a Unicam UV-550 spectrophotometer (Thermo Spectronic, Cambridge, U.K.).

Heat treatment of PSII membranes

Photosystem II membranes were subjected to heat treatment in a plastic Eppendorf tubes for the time period indicated in the figures. The tightly sealed Eppendorf tubes were

immersed in a water bath with water circulation maintained by a digitally controlled heater (Cole Parmer, Illinois, U.S.A.) in complete darkness. After the heat treatment, the sample was immediately subjected for further measurement.

EPR spin-trapping spectroscopy

EPR spin-trapping spectroscopy was used to measure $HO^{\bullet}$ production in PSII membranes. Spin-trapping was accomplished by spin-trap compound EMPO (5-(ethoxycarbonyl)-5-methyl-1-pyrroline N-oxide) (Alexis Biochemicals, Lausen, Switzerland) (Zhang et al. 2000). Photosystem II membranes (500 μg/ml chl) in 40 mM MES-NaOH (pH 6.5) and the presence of 75 mM EMPO were heated in Eppendorf tubes immersed in a water bath. Following heat treatment, PS II membranes were put into a glass capillary tube (Blaubrand® intraMARK, Brand, Wertheim, Germany) and EPR spectra were recorded using an EPR spectrometer MiniScope MS200 (Magnettech GmbH, Berlin, Germany). Signal intensity was evaluated from the relative height of the central doublet peak of the first derivative of the EPR absorption spectra. EPR conditions were as follows: microwave power (10 milliwatts), modulation amplitude (1 G), modulation frequency (100 kHz), sweep width (100 G) and scan rate (1.62 Gs^{-1}).

Detection of hydrogen peroxide

Hydrogen peroxide production was detected in PSII membranes using amplex red fluorescent assay. The amplex red (10-acetyl-3,7-dihydroxyphenoxazine) reacts with H_2O_2 in the presence of horseradish peroxidase to form the fluorescence compound resorufin (Zhou et al. 1997). After heating at 40 °C, the PSII membranes were immediately mixed with the horseradish peroxidase (10 unit/ml) and amplex red (10 μM). Fluorescence emission spectra were measured using a spectrofluorimeter F-4500 (Hitachi, Tokyo, Japan). For fluoresecence emission spectra, the resorufin was excited at 518 nm and fluorescence was recorded in the spectral range of 550–610 nm. When amplex red and HRP were mixed in buffer in the dark, a background fluorescence signal was observed, probably originated by autooxidation of amplex red. As amplex red is sensitive to light, heat and air providing an unspecific background signal by autooxidation of the probe, all the measurements were performed rapidly at room temperature in the complete darkness.

Results

Hydrogen peroxide production in PSII membranes

Heat-induced formation of H_2O_2 in PSII membranes was measured by a spectrofluorimetry technique. The reaction of amplex red with H_2O_2 catalyzed by horseradish peroxidase formed a fluorescence compound resorufin. Exposure of PSII membranes with amplex red to heat stress (40 °C) resulted in the generation of a resorufin fluorescence emission spectra (Fig. 1). The resorufin fluorescence signal increases with the increase of incubation time at 40 °C. The background fluorescence signal was observed at room temperature probably due to the autooxidation of amplex red (Fig. 1). These observations indicate that the exposure of PSII membranes to heat results in the H_2O_2 formation.

Effect of halides on H_2O_2 production in PSII membranes

To study the role of chloride ion in the formation of H_2O_2 in PSII membranes under heat stress, we used different exogenous halides such as NaCl, NaBr and NaI. The addition of exogenous halides to the solution mixture significantly suppressed the resorufin fluorescence signal in PSII membranes (Fig. 2a and b). The suppression of resorufin fluorescence signal by halides indicates that the chloride ion prevents the production of H_2O_2.

Effect of channel blocker and acetate on H_2O_2 production in PSII membranes

Evidence has been given that the chloride and calcium channel blockers bind to the electron donor side of PSII at the binding site of chloride or calcium respectively (Carpentier and Nakatani 1985). We detected the formation of H_2O_2 in the presence of the chloride channel blocker DIDS and Na acetate. The addition of exogenous DIDS (diisothiocyano-stilbene-2,2–disulfonic acid) to the solution mixture suppressed the resorufin fluorescence signal under heat stress (Fig. 3a and b). The exogenous acetate binds to the water-

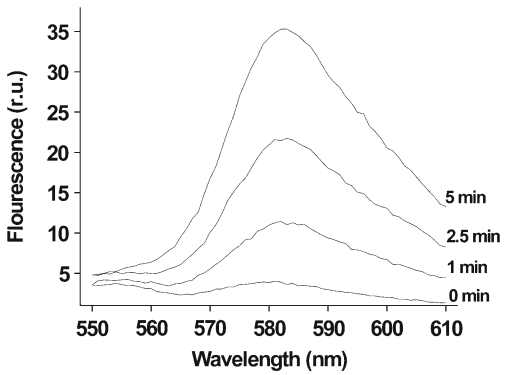

Fig. 1 Heat-induced resorufin fluorescence emission spectra measured in PSII membranes. After heating at 40 °C for the time period indicated in the figure, the PSII membranes (50 μg Chl ml^{-1}) were immediately mixed with the horseradish peroxidase (10 unit/ml) and amplex red (10 μM) in 50 mM sodium phosphate buffer (pH 7.4). To measure the fluorescence emission spectra, the resorufin was excited at 518 nm and fluorescence was recorded in the spectral range of 550–610 nm

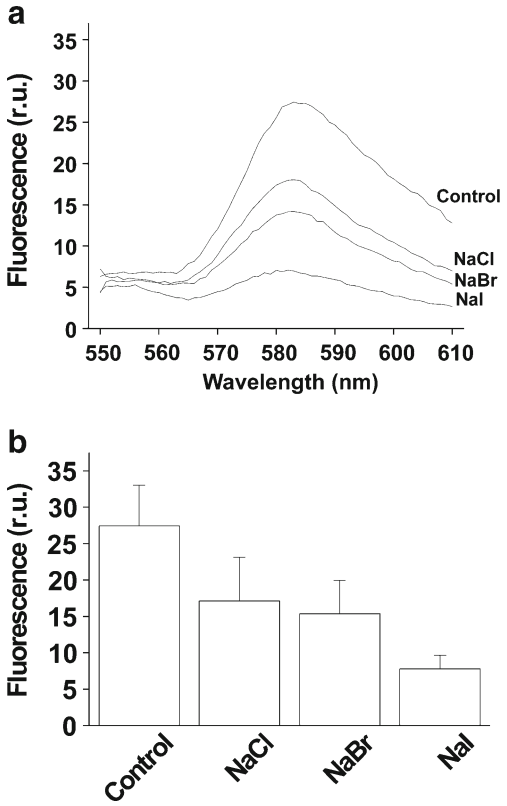

Fig. 2 Effect of halides on heat-induced resorufin fluorescence emission spectra measured in PSII membranes. **a** Photosystem II membranes were exposed to heat (40 °C) for 5 min in the presence of 100 mM NaCl, 100 mM NaBr and 100 mM NaI. **b** The intensity of resorufin fluorescence signal in the presence of different halides. The intensity of resorufin fluorescence signal is evaluated as the height of signal at 584 nm. The data represent the mean value (±SD) of at least three experiments. Other experimental conditions are the same as described in Fig. 1

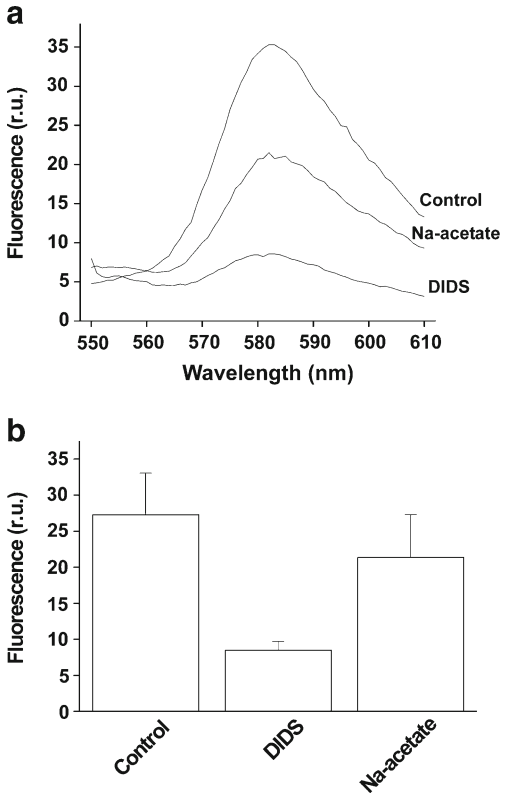

Fig. 3 Effect of channel blocker and acetate on heat-induced resorufin fluorescence emission spectra measured in PSII membranes. **a** Photosystem II membranes were exposed to heat (40 °C) for 5 min in the presence of 10 mM DIDS, 100 mM Na-acetate. **b** The intensity of resorufin fluorescence signal in the presence of DIDS and Na-acetate. The intensity of resorufin fluorescence signal is evaluated as the height of signal at 584 nm. The data represent the mean value (±SD) of at least three experiments. Other experimental conditions are the same as described in Fig. 1

splitting manganese complex in competition with chloride ion on the electron donor side of PSII. Heat-induced resorufin fluorescence signal was partially suppressed in the presence of exogenous Na-acetate in PSII membranes (Fig. 3). However, the calcium ion blocker nifedipine enhanced and the Ca-acetate completely suppressed resorufin the fluorescence signal probably due to unspecified reactions with the amplex red (data not shown). These observed data show that the chloride ion inhibits the H_2O_2 formation in PSII membranes under heat stress.

Hydroxyl radical production in PSII membranes

Heat-induced formation of $HO^{\bullet}$ in PSII membranes was measured by a EPR spin-trapping technique. Spin-trapping was accomplished by the reaction of EMPO (5-(ethoxycarbonyl)-5-methyl-1-pyrroline N-oxide) with $HO^{\bullet}$ forming an EMPO-OH adduct. The addition of EMPO to PSII membranes at room temperature did not result in the appearance of EMPO-OH adduct EPR signal. However, the exposure of PSII membranes to heat stress (40 °C) resulted in the generation of EMPO-OH adduct EPR signal (Fig. 4). The EMPO-OH adduct EPR signal increased with the increase

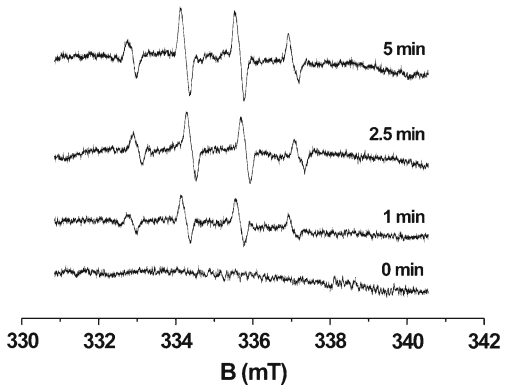

Fig. 4 Heat-induced EMPO-OH adduct EPR signal measured in PSII membranes. Photosystem II membranes (500 μg Chl ml^{-1}) were exposed to heat (40 °C) in the presence of 75 mM EMPO and 40 mM MES-NaOH (pH 6.5) for the time period as indicated in the figure

of incubation time at 40 °C. There was no EMPO-OH adduct EPR signal observed after heating of the pure spin-trap compound in the buffer solution (data not shown). These observations indicate that the exposure of PSII membranes to heat stress results in formation of $HO^{\bullet}$.

Effect of halides on $HO^{\bullet}$ production in PSII membranes

As we have reported recently, the heat-induced $HO^{\bullet}$ production is related to the electron donor side of PSII (Pospíšil et al. 2007; Yamashita et al. 2008). To study the role of chloride ion in the formation of $HO^{\bullet}$ in PSII membranes under heat stress, we used different exogenous halides such as NaCl, NaBr and NaI. The addition of exogenous halides to the solution mixture significantly suppressed the EMPO-OH adduct EPR signal in PSII membranes (Fig. 5). The different halides suppressed the $HO^{\bullet}$ production approximately to the similar extent in heat-treated PSII membranes. The suppression of the EMPO-OH adduct EPR signal by the halides indicates that the chloride ion prevents the production of $HO^{\bullet}$.

Effect of channel blockers and acetate on $HO^{\bullet}$ production in PSII membranes

We used DIDS and nifedipine as blockers of chloride and calcium ion, respectively. In the presence of the chloride ion blocker DIDS, the production of $HO^{\bullet}$ was significantly suppressed under heat stress (Fig. 6a). Interestingly, the calcium ion blocker nifedipine did not affect heat-induced $HO^{\bullet}$ production in PSII membranes (Fig. 6a). The EMPO-OH adduct EPR signal was partially suppressed in the presence of exogenous acetate (Fig. 6b). These data show that the chloride ion plays a crucial role in the heat-induced $HO^{\bullet}$ production in PSII membranes.

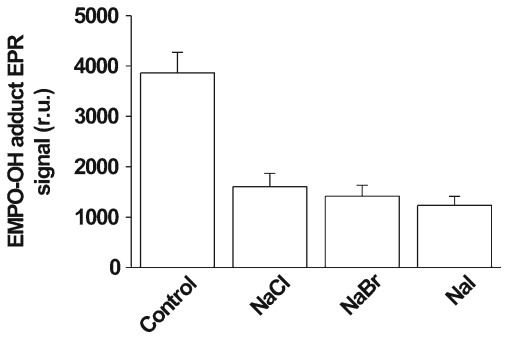

Fig. 5 Effect of halides on the heat-induced EMPO-OH adduct EPR signal measured in PSII membranes. Photosystem II membranes were exposed to heat (40 °C) for 5 min in the presence of 100 mM NaCl, 100 mM NaBr and 100 mM NaI. The intensity of the EPR signal was evaluated as the relative height of the central doublet peak of the first derivatives of the EPR absorption spectrum. The data represent the mean value (±SD) of at least three experiments. Other experimental conditions are the same as described in Fig. 4

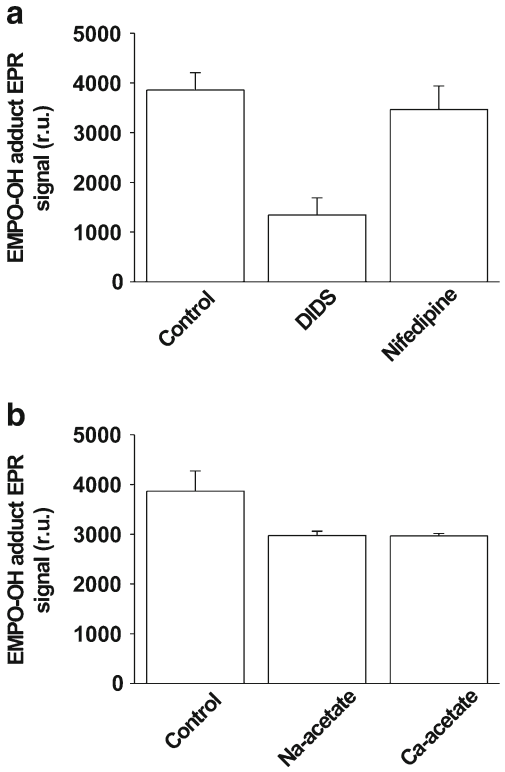

Fig. 6 Effect of channel blockers and acetate on the heat-induced EMPO-OH adduct EPR signal measured in PSII membranes. **a** Photosystem II membranes were exposed to heat (40 °C) for 5 min in the presence of 10 mM DIDS and 10 mM nifedipine. **b** Photosystem II membranes were exposed to heat (40 °C) for 5 min in the presence of 100 mM Na-acetate and 100 mM Ca-acetate. The data represent the mean value (±SD) of at least three experiments. Other experimental conditions are the same as described in Fig. 4

Discussion

Photosystem II is one of the most vulnerable complexes in thylakoids to be damaged under heat stress conditions. The primary effect of heat stress is the inactivation of electron transport on the electron donor side of PSII caused by inhibition of the water-splitting manganese complex. It is well known, that the different stress conditions (heat stress, high light intensity etc.) are responsible for the formation of ROS in thylakoid membrane (Bukhov and Mohanty 1999; Asada 2006; Krieger-Liszkay 2005; Krieger-Liszkay et al. 2008; Pospíšil 2009, 2012). In agreement with our previous study, we showed here by an EPR spin-trapping technique that $HO^{\bullet}$ is produced in spinach PSII membranes incubated at elevated temperature (Fig. 4) (Pospíšil et al. 2007; Yamashita et al. 2008). Previously, it was suggested that the water oxidation occurs at the S_4 state to form molecular oxygen, even though it cannot strictly rule out the incomplete water oxidation at lower S states of the water-splitting manganese complex (Rutherford 1989). It has been proposed that the formation of H_2O_2 could occur by impairment of the water-splitting manganese complex under heat stress conditions

(Thompson et al. 1989). Here, we have shown the heat-induced H_2O_2 formation in PSII membranes by using an amplex red fluorescent assay (Fig. 1).

It has been proposed that the water-splitting manganese complex is heat susceptible in PSII membranes. Chloride ion is a cofactor associated with the water-splitting manganese complex and important for the oxidation of water into molecular oxygen. The release of chloride ion leads to the inhibition of the water-splitting manganese complex and it is reversible in the presence of excess chloride ion (Rivalta et al. 2011; Kawakami et al. 2009; Popelková and Yocum 2007; Yocum 2008). Even though, the functional significance of chloride ion in PSII under heat stress remains unclear. Here, we showed that the presence of excess halides during heat treatment significantly suppressed the H_2O_2 formation (Fig. 2). In a similar way, these halides also showed suppression of heat-induced $HO^{\bullet}$ production by PSII (Fig. 5). It reveals the involvement of chloride ion in the formation of H_2O_2 and $HO^{\bullet}$ on the electron donor side of PSII under heat stress. Furthermore, the binding of acetate in the competition with chloride to the water-splitting manganese complex has been reported in PSII (Kühne et al. 1999). It has been shown that the blockers of chloride channel DIDS bind to the electron donor side of PSII and inhibit the oxygen evolution (Carpentier and Nakatani 1985). Our results showed that the presence of exogenous Na-acetate and chloride channel blockers suppressed the formation of H_2O_2 (Fig. 3a and b) and $HO^{\bullet}$ (Fig. 6) in PSII membranes exposed to heat stress

Recent crystal structure of PSII from cyanobacteria *Thermosynechococcus elongatus* and *Thermosynechococcus vulcanus* reported the presence of chloride ion in the vicinity of the water-splitting manganese complex (Guskov et al. 2009; Umena et al. 2011). It has been recently demonstrated the water molecule is located between the manganese and chloride ions (Guskov et al. 2009, 2010). It has been suggested that different channels within the protein matrix proceed the delivery of substrate water to manganese cluster along with removal of proton and molecular oxygen (Ishikita et al. 2006; Murray and Barber 2007; Ho and Styring 2008; Ho 2008; Gabdulkhakov et al. 2009). Chloride ion has been proposed to block the channels filled with water molecules and thus restrict the leakage of water molecules out of the channels towards the water-splitting-manganese complex (Guskov et al. 2009; Gabdulkhakov et al. 2009). It has been previously suggested that H_2O_2 is formed due to the incomplete water oxidation on the electron donor side of PSII under heat stress (Thompson et al. 1989). Wydrzynski et al. (1996) has given the water accessibility hypothesis to the formation of H_2O_2 in the structurally perturbed PSII under various treatments. According to this hypothesis, the accessibility of water to the water-splitting manganese complex is controlled by the surrounding proteins. Disturbance of the integrity of the protein matrix leads to an uncontrolled water access to the water-splitting manganese complex resulting in the incomplete water oxidation into H_2O_2 (Wydrzynski et al. 1996). In this study, it is proposed that the release of chloride ion from its binding site causes the uncontrolled accessibility of water toward the water-splitting manganese complex and leads to the formation of H_2O_2 under heat stress conditions.

The X-ray structure of PSII from *Thermosynechococcus vulcanus* at a resolution of 1.9 Å showed that the Mn_4CaO_5 cluster resembled a distorted chair, with the seat base formed by 3 Mn, 1 Ca, and 4 oxygen atoms and the back of chair formed by Mn (4) and 1 oxygen atom. The manganese Mn (4) atom of the water-splitting manganese complex is associated with two water molecules (W1 and W2) (Umena et al. 2011; Kawakami et al. 2011). Here, it is proposed that the chloride ion is required to avoid the formation of H_2O_2 and maintain the bonding of proteins to the water-splitting manganese complex for proper water oxidation. Under heat stress conditions, the enhancement of the accessibility of excess water to the water-splitting manganese complex due to the release of chloride ion leads to the two-electron oxidation of water (probably W1 and W2) into H_2O_2. According to previously reported data (Pospíšil et al. 2007; Yamashita et al. 2008), H_2O_2 reacts with transition metals to form $HO^{\bullet}$ in PSII under heat stress.

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