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Peroxynitrite inhibits electron transport on the acceptor side of higher plant photosystem II

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Abstract

Peroxynitrite is a strong oxidant that has been proposed to form in chloroplasts. The interaction between peroxynitrite and photosystem II (PSII) has been investigated to determine whether this oxidant could be a hazard for PSII. Peroxynitrite is shown to inhibit oxygen evolution in PSII membranes in a dose-dependent manner. Analyses by PAM fluorimetry and EPR spectroscopy have demonstrated that the inhibition target of peroxynitrite is on the PSII acceptor side. In the presence of the herbicide DCMU, the chlorophyll (Chl) *a* fluorescence induction curve is inhibited by peroxynitrite, but the slow phase of the Chl *a* fluorescence decay does not change. EPR studies demonstrate that the Signal II_{slow} and Signal II_{fast} of peroxynitrite-treated Tris-washed PSII membranes are induced at room temperature, implying that the redox active tyrosines Y_Z and Y_D of PSII are not significantly nitrated. A featureless EPR signal with a *g* value of approximately 2.0043 ± 0.0003 and a line width of 10 ± 1 G is induced under continuous illumination in the presence of peroxynitrite. This new EPR signal corresponds with the semireduced plastoquinone Q_A in the absence of magnetic interaction with the non-heme Fe^{2+}. We conclude that peroxynitrite impairs PSII electron transport in the Q_AFe^{2+} niche.
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Peroxynitrite is a strong oxidant and nitrating intermediate that can be rapidly produced by the nearly diffusion-limited reaction between nitric oxide ($^{\cdot}NO$) and superoxide radical ($O_2^{\cdot -}$) [1]. The two reactants, $^{\cdot}NO$ and $O_2^{\cdot -}$, are, respectively, reactive nitrogen and oxygen species that can be (enzymatically) formed in living organisms. $^{\cdot}NO$ and $O_2^{\cdot -}$ are produced at high and unregulated levels under several stress conditions and therefore formation of peroxynitrite is a non-enzymatic reaction that can occur in vivo [2]. Peroxynitrite and its homolytic decomposition products ($^{\cdot}OH$ and $^{\cdot}NO_2$) participate in many direct or radical dependent redox reactions, where key protein residues, cofactors, lipids and nucleotides are selectively oxidized and/or nitrated [3,4]. In vivo formation of peroxynitrite is well established in mammals, but less well recognized in plant tissues. However, some recent lines of evidence indicate that peroxynitrite is produced in plants [5–7]. In higher plants, $^{\cdot}NO$ is mainly produced by two major mechanisms; the NO_2^- pathway catalyzed by nitrate reductase present in the cytosol and the arginine pathway catalyzed by the (inducible) nitric oxide synthase

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(NOS)[1] present in mitochondria [8], chloroplasts [9], and peroxisomes [10,11] of plant cells. $O_2^{\bullet-}$ is produced by xanthine oxidase in the matrix of peroxisomes or by a NAD(P)H-dependent electron transport in peroxisomal membranes [12,13] as well as by the partial reduction of oxygen in chloroplasts and mitochondria [14]. Considering the hydrophobic and diffusible nature of $^{\bullet}NO$, peroxynitrite is proposed to be predominantly formed close to the site of $O_2^{\bullet-}$ generation [4,15]. Recently, Jasid et al. [16] have shown that there are at least two coexisting pathways for $^{\bullet}NO$ formation (i.e. NOS and NO_2^- pathways) in chloroplasts and that peroxynitrite formation is feasible inside this organelle.

The finding by Jasid et al. [16] that peroxynitrite oxidizes chloroplastic proteins and lipids, and intriguingly diminishes both the oxygen evolution and the fluorescence yield of photosystem II (PSII) has motivated us to conduct this study. PSII is well known to be a very sensitive thylakoid complex, which promptly shows inactivation under several types of stress conditions [17]. PSII has a number of transition metal cofactors and redox active residues in order to catalyze the light-driven oxidation of water [18,19]. On the PSII donor side, water molecules are oxidized in a bioinorganic cluster consisting of four redox active Mn ions and other non-redox active ions (Ca^{2+}, Cl^- and HCO_3^-). Between the Mn cluster and the primary electron donor chlorophyll (Chl) P680, there exists a redox active tyrosine residue named Z (Y_Z), which belongs to the PsbA (or D1) protein of the PSII reaction center and participates as a redox intermediate [20]. A dark living tyrosine radical (Y_D) is located in the symmetrical PsbD (or D2) protein of the PSII reaction center [21]; however, Y_D does not participate in the primary electron transfer reactions leading to water oxidation. On the PSII acceptor side, a non-heme Fe^{2+} is coordinated with a HCO_3^- ion and four histidines, two from each of the D1 and D2 proteins. The non-heme Fe^{2+} facilitates the one-electron transport between the plastoquinones Q_A and Q_B [22]. In addition, PSII has cytochrome *b*559, a heme protein closely associated with the reaction center [23]. Mn and Fe ions are well known to interact with peroxynitrite and to catalyze its homolysis, while tyrosine radicals can undergo oxidation or nitration in the presence of peroxynitrite [24]. The aim of this study is to determine the specific inhibition site of peroxynitrite in PSII. By means of polarographic oxymetry, pulse–amplitude–modulation (PAM) fluorimetry and electron paramagnetic resonance (EPR) spectroscopy, we conclude that the inhibition site of peroxynitrite is in the Q_AFe^{2+} niche of the PSII acceptor side.

Materials and methods

Preparation of Photosystem II and Tris-washed photosystem II membranes

PSII membranes from spinach were purified using the method described by Berthold et al. [25] with slight modifications to remove starch and adventitious metal ions [26]. PSII membranes with a final concentration of about 4–5 mg of Chl/mL were stored in 15 mM NaCl, 5 mM $MgCl_2$, 400 mM sucrose, 50 mM morpholine-ethane-sulfonic acid (MES) NaOH; pH 6.0 (storage buffer) at −80 °C until use. Tris-washed (TW) PSII membranes (lacking the PSII extrinsic polypeptides and the Mn cluster) were prepared by washing PSII membranes for 30 min with 0.8 M Tris–HCl; pH 8.4 [27]. Traces of Tris–HCl buffer were removed by washing the TW PSII membranes with 15 mM NaCl, 5 mM $MgCl_2$, 20 mM MES–NaOH; pH 6.0. The TW PSII were centrifuged, suspended in the storage buffer and stored at −80 °C until use.

Peroxynitrite synthesis

Peroxynitrite was synthesized by mixing $NaNO_2$ and acidified hydrogen peroxide as described in [28]. Aliquots of peroxynitrite were quickly frozen until use. Peroxynitrite solutions were treated with solid MnO_2 on ice for 2–3 h to remove completely excess hydrogen peroxide before use. Peroxynitrite stock concentrations were determined spectrophotometrically at 302 nm in 1 M NaOH ($\varepsilon = 1670\ M^{-1}\ cm^{-1}$). Stock solutions contained about 150–210 mM peroxynitrite, and less than 30% remaining nitrite (NO_2^-).

Western blot analysis

PSII membranes were subjected to 17% (w/v) SDS–polyacrylamide gel electrophoresis and transferred overnight to nitrocellulose membranes. Nitrocellulose membranes were stained with Ponceau S solution (Applichem, Germany) for 1 min to visualize PSII protein transfer and destained with water soon afterwards. The membranes were blocked with 5% (w/v) bovine serum albumin (BSA), 0.6% (w/v) Tween 20 in 150 mM NaCl, 50 mM Tris–HCl; pH 7.4 (TBS) (blocking buffer). For detection of 3-nitrotyrosine in PSII, membranes were incubated (1 h at 25 °C) with a polyclonal anti-nitrotyrosine antibody produced in our lab [29] (1/1000 dilution) in blocking buffer. After extensive wash in 0.6% (w/v) Tween 20 TBS, the immunocomplexed membranes were probed (1 h at 25 °C) with horseradish-peroxidase-linked secondary antibody (1/10000 dilution) in 0.3% (w/v) Tween 20, 0.1% (w/v) BSA TBS. Probed membranes were washed with 0.3% (w/v) Tween 20 TBS and immunoreactive proteins were visualized by luminol-enhanced chemiluminescence (Bio-Rad, Hercules, CA, USA).

Oxygen evolution

PSII oxygen evolution was measured polarographically using a Chlorolab 2 system (Hansatech Instruments, Norfolk England). PSII membranes at a concentration of 20 μg of Chl/mL were dispersed in 10 mM NaCl, 5 mM $CaCl_2$, 100 mM MES–OH; pH 6.0 (assay buffer) followed by peroxynitrite addition to ensure their interaction before peroxynitrite decomposition. The lifetime (τ) of peroxynitrite is pH-dependent and sharply decreases below pH 7.0 ($\tau \sim$ ms–s) [30,31]. After dark incubation for 1 min, 0.4 mM phenyl-*p*-benzoquinone (PPBQ) was added as electron acceptor before switching on the LED light.

[1] *Abbreviations used:* BSA, bovine serum albumin; Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dymethylurea; EPR, electron paramagnetic resonance; MES, morpholine-ethane-sulfonic acid; NaDT, sodium dithionite; NOS, nitric oxide synthase; PAM, pulse-amplitude-modulation; PPBQ, phenyl-*p*-benzoquinone; PSII, Photosystem II; P680, primary electron donor chlorophyll of photosystem II consisting of two chlorophyll *a* molecules denoted P_{D1} and P_{D2}; Q_A and Q_B, primary and secondary plastoquinone acceptors in photosystem II; TBS, 150 mM NaCl, 50 mM Tris–HCl, pH 7.4; Y_D, a tyrosine electron donor on the D2 polypeptide of photosystem II that gives rise to the EPR Signal II_{slow} when oxidized; Y_Z, *a tyrosine electron donor on the D1 polypeptide of photosystem II that in turn oxidizes the Mn cluster; TW, Tris-washed;* τ, lifetime.

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