Production of reactive oxygen species in chloride- and calcium-depleted photosystem II and their involvement in photoinhibition

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Abstract

Mixed photosystem II (PSII) samples consisting of Cl⁻/Ca²⁺-depleted and active, or Ca²⁺-depleted and active PSII enriched membrane fragments, respectively, were investigated with respect to their susceptibility to light. In the presence of Cl⁻/Ca²⁺-depleted PSII, active centers were damaged more severely, most likely caused by a higher amount of reactive oxygen species formed in the nonfunctional centers. Cl⁻ depletion led to an increased H₂O₂ production, which seemed to be responsible for the stimulation of PSII activity loss. To distinguish between direct H₂O₂ formation by partial water oxidation and indirect H₂O₂ formation by oxygen reduction involving the prior formation of O₂⁻, the production of reactive oxygen species was followed by spin trapping EPR spectroscopy. All samples investigated, i.e. PSII with a functional water splitting complex, Ca²⁺- and Cl⁻/Ca²⁺-depleted PSII, produced upon illumination O₂⁻ radicals on the acceptor side, while Cl⁻-depleted PSII produced additionally OH⁻ radicals originating from H₂O₂ formed on the donor side of PSII.

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1. Introduction

The Mn cluster of photosystem II (PSII) catalyzes the oxidation of water to molecular oxygen in the light. The five intermediate catalytic oxidation states of the Mn cluster which are formed by sequential transitions are called S₀–S₄, with S₄ being the oxidation state in which oxygen is released (for review see Refs. [1–3]). Photosynthetic oxygen evolution is reversibly inhibited by depleting the water splitting complex of its obligatory cofactors chloride or calcium. Depletion of the Mn cluster of Cl⁻ leads to an inhibition of the transition from the S₂⁻ to the S₃-state in the main proportion of the reaction centers [4], while Ca²⁺ depletion evokes the formation of a modified S₃-state and an inhibition of the S₃→S₀ transition [5,6].

In addition to the normal water oxidizing process, misfunctioning can occur during the oxidation of water resulting in the formation of peroxide, especially when the cofactor Cl⁻ of the Mn cluster is missing [7] or when PSII is deprived of its extrinsic proteins [8], which normally shield the water splitting complex from the lumenal space. Alternatively, oxygen can be reduced on the acceptor side of PSII leading to the formation of superoxide [8–13]. Superoxide itself can be converted to H₂O₂, a reaction which is accelerated by SOD.

A PSII-associated catalase has been described previously [14,15] which may play an important role in the detoxification of the produced peroxide. An investigation of the activity of this catalase after Cl⁻-depletion treatments or removal of the extrinsic proteins has not been described in the literature.

Reactive oxygen species are involved in the light-induced damage of PSII (photoinhibition). In vivo, ¹O₂ seems to be the main species responsible for photoinhibition [16,17],...
however, it was shown in vitro using PSII with a nonfunctional water splitting system that other reactive oxygen species like hydroxyl and carbon-centered radicals are formed [18,19] and may cause photoinhibition. In addition, superoxide is produced in leaves during photoinhibitory illumination as has been shown recently [20]. It was also reported that photoinactivation of PSII activity and degradation of the D1 protein can be induced by $H_2O_2$ in the dark [21].

Cl$^-$ and Ca$^{2+}$-depleted PSII enriched membrane fragments are more susceptible to light than PSII enriched membrane fragments which possess an active water splitting complex. In a direct comparison we could show that Cl$^-$-depleted PSII is even more susceptible to photoinhibition than Ca$^{2+}$-depleted PSII [19,22]. It has been reported previously that $H_2O_2$ formed in Cl$^-$/Ca$^{2+}$-depleted membrane fragments at pH 4.2 in room light, which leads to the degradation of both $O_2$ and $O_2^-$ radicals. In the presence of transition metals, $H_2O_2$ can react to OH$^\cdot$ and OH$^-$, the so-called Fenton reaction. We used ethanol/POBN as a spin trapping system which reacts with OH$^\cdot$, but not with $O_2^-$, forming a relatively stable hydroxethyl adduct which can be detected by room temperature EPR [26]. In addition, we investigated the production of both $O_2^\cdot$ and OH$^\cdot$ by using DEPPO or EMPO as spin traps, which form characteristic, distinguishable, and relatively stable adducts with the OH$^\cdot$ and $O_2^\cdot$ radicals [27]. Furthermore, the activity of the PSII-associated catalase was measured after PSII was subjected to the Ca$^{2+}$- and Cl$^-$-depleted treatment.

2. Materials and methods

2.1. Preparation of PSII samples

PSII enriched membrane fragments (PSII particles) were prepared from spinach as described in Ref. [28] with modification as described in Ref. [29]. The activity of the samples was 400–500 μmol O$_2$ mg Chl$^{-1}$ h$^{-1}$. The activity of the samples was measured in a Clark-type O$_2$-electrode in a buffer containing 0.3 M sucrose, 10 mM NaCl, 25 mM MES pH 6.5 in the presence of 1 mM pPBQ as electron acceptor.

2.2. Cl$^-$ depletion

Cl$^-$ depletion of PSII particles was done by alkaline pH-treatment as described in Ref. [25]. PSII particles (50 μg Chl/ml) were incubated at pH 10 for 30–45 s in a buffer containing 0.4 M sucrose (ultrapure) and 2 mM CAPS before lowering the pH to 6.5. The remaining activity of the samples was about 20% compared to untreated samples. Upon re-addition of 20 mM NaCl, 70–80% of the activity prior to the treatment was restored.

2.3. Ca$^{2+}$ depletion

Ca$^{2+}$ depletion of PSII particles was done by incubating PSII particles (50 μg Chl/ml) for 3–5 min at pH 4.2 in room light in a buffer containing 300 mM sucrose, 20 mM KCl and 20 mM glycyglycin. The samples were then washed in a buffer containing 300 mM sucrose, 20 mM KCl and 20 mM MES (pH 6.5). The remaining activity of the samples was about 20% of the original activity. Upon re-addition of 50 mM CaCl$_2$, 80% of the activity of a control sample was obtained in these samples.

2.4. Mn depletion

Mn was released by incubating PSII particles (0.5 mg Chl/ml) in a buffer containing 0.5 M Tris (pH 8.4) for 10 min in the light on ice. The sample was washed twice with a buffer containing 300 mM sucrose, 10 mM NaCl, 50 mM MES (pH 6.5). The remaining Mn content of the samples was 0.65–0.3 Mn/PSII as determined by atomic absorption spectroscopy.

2.5. Photoinhibitory treatment

Photoinhibition was performed by illuminating the samples (30 μg Chl/ml) at 20 °C with white light, intensity of 1000 μmol quanta m$^{-2}$ s$^{-1}$ in a 25 mM MES pH 6.5 buffer which contained 300 mM sucrose. The O$_2$-evolution activity of the samples at a given time point was measured in the presence of 1 mM pPBQ as electron acceptor.
2.6. Photometric H$_2$O$_2$ measurements

H$_2$O$_2$ production of PSII samples was followed by thiobenzamide (1 mM) oxidation by lactoperoxidase (10 $\mu$g/ml) in a buffer which contained 300 mM sucrose and 25 mM MES (pH 6.5). The samples (5 $\mu$g Chl/ml) were illuminated in the presence of thiobenzamide for a given time with a light intensity of 1000 $\mu$mol quanta m$^{-2}$ s$^{-1}$. Thiobenzamide sulfoxide production was quantified by its absorbance at 370 nm ($\varepsilon = 2.92 \times 10^3$ M$^{-1}$ cm$^{-1}$). No inhibitory effect on O$_2$-evolving activity of the PSII samples was observed during up to 20-min preincubation of the samples with 1 mM thiobenzamide (data not shown).

2.7. SDS-PAGE and Western blotting

SDS gel-electrophoresis was carried out in 14% polyacrylamide gels. Western blotting was performed using a Multiphor II Novablot Unit (Pharmacia Biotech). For detection, the ECL system (Amersham) was used according to the manufacturer’s protocol.

2.8. EPR measurements

X-band room temperature EPR spectra were recorded with a Bruker 300 spectrometer at 9.69-GHz microwave frequency, 63-mW microwave power and 100-kHz modulation frequency, 2-G modulation amplitude. The samples (150 $\mu$g Chl/ml) were illuminated with a light intensity of 1000 $\mu$mol quanta m$^{-2}$ s$^{-1}$ for 15 min in the presence of the different spin traps (10 mM POBN and 170 mM ethanol (0.63% v/v) or 25 mM DEPMPO or EMPO, respectively) in a 25 mM MES pH 6.5 buffer. The central position of all spectra measured was at $g = 2.0$. OH$^*$ production by H$_2$O$_2$ in the presence of 500 $\mu$M Fe(II) (Fenton reaction) was used to calibrate the EPR signal. The size of the signal (or the integrated area) of the EPR signals correlates linearly with the concentration of the trapped radicals (data not shown).

2.9. Preparation of PSII-associated catalase

Catalase preparation was performed as described in Ref. [14] by incubating the samples (2 mg/ml Chl) at 30 $^circ$C for 90 min in a 25 mM MES pH 6.5 buffer. The samples were then centrifuged, the pellet and the supernatant were tested for catalase activity in a Clark-type O$_2$-electrode in the presence of 5 mM H$_2$O$_2$.

3. Results

3.1. Photoinhibition of heterogeneous PSII samples

Fig. 1 shows photoinhibition experiments which were performed with mixed samples consisting of either 50% “Cl$^-$-depleted” PSII particles or 50% Ca$^{2+}$-depleted PSII particles and, in both cases, 50% active particles, which were able to evolve oxygen. In these heterogeneous samples the light-induced loss of oxygen evolution of the active PSII particles (and the part of PSII centers with remaining water splitting activity after the depletion treatments) was measured while the particles with an inactivated water splitting complex were not detectable in this assay. As shown in Fig. 1, the loss of oxygen evolution of the active PSII particles was increased when “Cl$^-$-depleted” samples were present compared to the situation when Ca$^{2+}$-depleted samples were present. The loss of activity was almost the same in the presence of 50% Ca$^{2+}$-depleted samples as in 100% active samples. This indicates that Ca$^{2+}$-depleted centers did not affect the rate of photodamage in active centers. In contrast, “Cl$^-$-depleted” PSII produced more or different toxic species, most likely reactive oxygen species which were responsible for the observed increase in the damage of the active centers. The curve obtained with 50% “Cl$^-$-depleted” and 50% active samples seems to be biphasic. The faster loss in the activity during the first 10 min of the photoinhibitory treatment might be caused by an increased susceptibility to light of those centers which still showed oxygen-evolving activity after the depletion procedures. In addition to the data shown in Fig. 1, the protective effect of catalase and SOD on photoinhibition of active PSII mixed with nonfunctional PSII was studied (Fig. 2). In the case of 50% active and 50% “Cl$^-$-depleted” PSII, the active samples were protected by catalase and, to a low extent, also by SOD (Fig. 2A). In the case of 50% active and 50% Ca$^{2+}$-depleted PSII, no protection effect by catalase was observed (Fig. 2B) while, however, addition of SOD prevented photoinhibition to some extent.

Fig. 1. Photoinhibition of heterogeneous PSII samples. Samples consisting of 50% “Cl$^-$-depleted” and 50% active (squares), 50% Ca$^{2+}$-depleted and 50% active (triangles) and 100% active PSII enriched membrane fragments (open diamonds) were illuminated with white light ($I = 1000$ $\mu$mol quanta m$^{-2}$ s$^{-1}$) for a given time. The loss of the activity was measured by the rate of oxygen evolution in the presence of 0.5 mM pPBQ. The initial activity of the mixed samples was 220–250 $\mu$M O$_2$ mg Chl$^{-1}$ h$^{-1}$. The average value of five independent measurements is given; the error bars show the standard deviation.
3.2. H$_2$O$_2$ measurements

The protection effect by catalase led to the assumption that H$_2$O$_2$ is involved in the photoinhibition of active PSII centers stimulated by the presence of “Cl$^{-}$/C0”-depleted” PSII. Fig. 3 shows time-dependent H$_2$O$_2$ production in active, “Cl$^{-}$”, and Ca$^{2+}$-depleted PSII particles measured after illumination with white light. “Cl$^{-}$-depleted” PSII showed the highest level of H$_2$O$_2$ production. The amount of H$_2$O$_2$ generated by active PSII (measured in the presence of 10 mM CaCl$_2$ to exclude the possibility of cofactor depletion in some centers) was about 60% lower and Ca$^{2+}$-depleted PSII produced even less H$_2$O$_2$. When “Cl$^{-}$-depleted” samples were illuminated under anaerobic conditions, the amount of H$_2$O$_2$ produced was lowered by approximately 30% (data not shown), indicating that part of the hydrogen peroxide was formed via reduction of O$_2$ on the acceptor side of PSII. Reduction of O$_2$ on the acceptor side leads to the formation of superoxide, which then disproportionates spontaneously or in an SOD-catalyzed reaction to H$_2$O$_2$. The yield of H$_2$O$_2$ formation in “Cl$^{-}$-depleted” PSII was not pH-dependent between pH 6.5 and 7.5; however, in active PSII, it was stimulated at pH 7.5 indicating that Cl$^{-}$ got lost at the elevated pH value [25].

In the presence of silicomolybdate, an electron acceptor which did not disturb the photometric assay, H$_2$O$_2$ production was reduced in active PSII by 30–40%, while the yield stayed unchanged in “Cl$^{-}$-depleted” samples (data not shown). In active PSII, oxygen evolution was measured in the presence of silicomolybdate (110 $\mu$mol O$_2$ mg Chl$^{-1}$ h$^{-1}$), showing that silicomolybdate functioned as an electron acceptor. The addition of DCMU reduced the O$_2$-evolution activity of the samples by 10%.

No change in the yield of H$_2$O$_2$ production was observed upon variation of the sucrose concentration in the assay medium (data not shown). Upon addition of the heme catalase inhibitors azide and triazolamine, H$_2$O$_2$ production was stimulated by about 30% in active and Cl$^{-}$-depleted PSII particles (data not shown).

3.3. Polypeptide composition of “Cl$^{-}$”, Ca$^{2+}$-depleted and active PSII particles

To exclude the possibility that the cofactor depletion treatments at extreme pH values led to a loss of the extrinsic proteins and therefore to an increased H$_2$O$_2$ production and photoinhibition, the polypeptide composition of PSII particles was investigated by SDS-PAGE and immunoblots using antibodies raised against the 33-, 23- and 17-kDa extrinsic proteins. After “Cl$^{-}$” and Ca$^{2+}$ depletion, no change in the content of the extrinsic proteins was detected.
compared to the control (Fig. 4). In the case of “Cl” depletion” by high pH-sulfate treatment [30] and also in the case of Ca\(^{2+}\) depletion at pH values lower than 4.0 (data not shown), partial loss of the 23- and 17-kDa proteins was found.

3.4. Detection of superoxide and hydroxyl radicals by spin trapping

To further analyze the production of reactive oxygen species in the different PSII samples, hydroxyl radical (\(\text{OH}^\cdot\)) and superoxide anion radical (\(\text{O}_2^\cdot\)) formation was followed by spin trapping experiments. First, we used the spin trap assay ethanol/POBN in which only hydroxyl radical formation is detected. Ethanol/POBN does not react with superoxide. Fig. 5 shows the EPR signal of the ethanol/POBN adduct which reflects the formation of \(\text{OH}^\cdot\) radicals upon illumination. After illumination with 1000 μmol quanta m\(^{-2}\) s\(^{-1}\) for 15 min, “Cl”-depleted” samples produced the highest amount of \(\text{OH}^\cdot\) while Ca\(^{2+}\)-depleted and active PSII produced a lower amount. These results correlate with the H\(_2\)O\(_2\) measurements which also showed the highest production rate in “Cl”-depleted” PSII. The hydroxyl radicals detected in the spin trapping assay may originate from either hydrogen peroxide formed directly at the water splitting complex or from peroxide which originates from superoxide produced on the acceptor side of PSII. H\(_2\)O\(_2\) can react in the presence of transition metals to \(\text{OH}^\cdot\) and \(\text{OH}^\cdot/\text{O}_2^\cdot\), the so-called Fenton reaction. It might be also possible that \(\text{OH}^\cdot\) is directly formed by incomplete water oxidation at the level of the Mn cluster.

Fig. 6 shows the effect of re-addition of Cl\(^-\) on the hydroxyl production of “Cl”-depleted” PSII. On addition of Cl\(^-\), the signal size of the spin trap adduct, i.e. the \(\text{OH}^\cdot\)
production, was reduced to the level found for active PSII particles (compare Fig. 6A and B with Fig. 5). In “Cl−-depleted” samples the addition of Cl− restored the water splitting activity of the samples by approximately 80%. The presence of catalase totally prevented the formation of OH radicals (Fig. 6d), while in the presence of SOD a small signal was detectable (Fig. 6c). Addition of Fe(II), a catalyst of the Fenton reaction, increased the OH production (Fig. 6e). Furthermore, azide, an inhibitor of heme catalases, also increased the amount of OH produced (data not shown).

In the presence of DCMU, the size of the EPR signal decreased by about 40% (data not shown) and the size of the signal was the same like for Ca2+-depleted and active PSII. These results indicate that a significant amount of the OH radicals produced in “Cl−-depleted” PSII originates from H2O2, which is produced directly via a turnover of the Mn-cluster, and not via the reduction of O2 to superoxide. In Ca2+-depleted and active PSII, most of the hydroxyl radical adduct detected seems to originate from superoxide produced on the acceptor side.

To determine more directly whether the reactive oxygen species originate from the donor or the acceptor side of PSII, another spin trapping assay was applied. The spin trap DEPMPO forms specific and characteristic adducts with both OH− and O2− radicals and can therefore be used to distinguish between these two species [26]. This spin trap is much more sensitive and a shorter illumination time and a lower light intensity was sufficient to detect clear differences in the three samples used. This is a big advantage because we do not want to study photoinhibitory effects which may overrun the specific differences between “Cl−-depleted”, Ca2+-depleted and active PSII. The disadvantage is that the spectra of the DEPMPO adducts are much more complex than the spectrum of the POBN adduct. Fig. 7 shows typical EPR spectra of the DEPMPO adducts produced by illuminating active, “Cl−-depleted” and Ca2+-depleted PSII (spectra a, b and c). “Cl−-depleted” samples showed characteristics of both OH− and O2−-DEPMPO adducts, while Ca2+-depleted and active PSII showed mainly the spectrum of the O2− adduct. Upon addition of SOD the signal in active and Ca2+-depleted PSII was almost completely eliminated, while the “Cl−-depleted” PSII produced a clear OH− radical signal. The activity of the Cu,Zn-SOD used was not affected by the low amount of H2O2 formed (data not shown).

EMPO, another spin trap, which also forms stable and not interconvertible adducts with OH− and O2− radicals [27], was used to study further the formation of OH− and O2− in the presence of artificial electron donors, acceptors or inhibitors. EMPO spectra of the superoxide and hydroxyl radical adducts are simpler and easier to assign than the DEPMPO spectra, but the spin trap is less sensitive and the halftime of the superoxide adduct is shorter. Table 1 summarizes the oxygen radical production measured by the EMPO adducts. Without addition, active and Ca2+-depleted PSII produced O2− and to a very low extent OH−, while “Cl−-depleted” PSII produced both O2− and OH− radicals as shown in Fig. 7 by using DEPMPO. The addition of an electron acceptor (DCPIP) resulted in a complete loss of the EPR signal in active and Ca2+-depleted PSII, while in “Cl−-depleted” samples OH production was observed. In the presence of DCMU only O2− was produced in all samples. If azide was added, active PSII particles produced both types of oxygen radicals; in “Cl−-depleted” samples the amount of OH− radicals strongly increased. In Ca2+-depleted PSII, only a very small signal of the OH− adduct was detected in the presence of azide. Mn-depleted PSII showed O2− but no OH− production in the presence of electron donors. These results indicate that in active and Ca2+-depleted PSII, O2− was formed on the acceptor side while in Cl−-depleted PSII both O2− production on the acceptor side and H2O2/OH− formation on the donor side took place. The OH− radicals observed originate most likely from H2O2 produced at the donor side of the PSII. The lack of the cofactor Cl− of the water splitting complex seems to play an important role in the H2O2 production.

3.5. Activity of the PSII-associated catalase

As shown in Table 1, azide, an inhibitor of heme catalases, stimulated the production of OH− radicals in “Cl−-
depleted” PSII. A PSII-associated catalase has been identified earlier in spinach [14,15]. According to Sheptovitsky and Brudvig [14], the catalase can be released from PSII particles by a mild heat treatment. We analyzed the catalase activity from active, “Cl−/C0−” and Ca2+ -depleted PSII to show whether this catalase activity was modified by the different depletion methods and influenced the H2O2 production in the different PSII samples. Table 2 shows the catalase activity of the samples prior to the heat treatment, and of the pellet and the supernatant after the heat treatment. Treatment of the samples by a short incubation at pH 10, i.e. “Cl−/C0−-depleted” PSII, or an incubation at pH 4.2, i.e. Ca2+ -depleted PSII, did not lead to a significant inactivation of the catalase activity. After incubating the samples at 30 °C, part of the catalase was released, while the main catalase activity was still found in the PSII containing pellet. The heme catalase inhibitors triazolamine and azide inhibited the catalase activity completely (data not shown), indicating that all activity originated from a heme catalase and not from a pseudocatalase activity of the Mn cluster. In addition, the presence of a catalase in the supernatants after the heat treatment was determined by an activity stain using tetramethylbenzidine and peroxide in a nondenaturing polyacrylamide gel (data not shown).

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>Active PSII</th>
<th>“Cl−-depleted” PSII</th>
<th>Ca2+-depleted PSII</th>
<th>Mn-depleted PSII</th>
</tr>
</thead>
<tbody>
<tr>
<td>O2·− OH·</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>O2·− OH·</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>n.d.</td>
</tr>
<tr>
<td>30 μM DCMU</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
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<td>20 μM DCMU</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td>1 mM azide</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1 mM DPC</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
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OH· and O2·− radical production was measured by using EMPO as a spin trap. EPR spectra of the adducts were measured after 15-min illumination with 1000 μmol quanta m−2 s−1.

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>Initial activity</th>
<th>Pellet after heat treatment</th>
<th>Supernatant after heat treatment</th>
</tr>
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<tbody>
<tr>
<td>Active PSII particles</td>
<td>627 ± 107</td>
<td>451 ± 42</td>
<td>149 ± 13</td>
</tr>
<tr>
<td>“Cl−-depleted” PSII</td>
<td>551 ± 145</td>
<td>322 ± 34</td>
<td>75 ± 25</td>
</tr>
<tr>
<td>Ca2+-depleted PSII</td>
<td>644 ± 146</td>
<td>328 ± 76</td>
<td>51 ± 10</td>
</tr>
</tbody>
</table>

PSII enriched membrane fragments were incubated at 30 °C for 90 min. The catalase activity was measured in a Clark-type O2-electrode in the dark in the presence of 5 mM H2O2. The activity is given in μmol O2/mg Chl h; the average value of at least four independent measurements is given.
4. Discussion

A comparison of the rate of H$_2$O$_2$ production in active, “Cl$^{-}$” and Ca$^{2+}$ -depleted PSII shows that Cl$^{-}$ depletion stimulates this reaction in PSII. The higher amount of H$_2$O$_2$ produced correlates with the loss of PSII electron transport activity rate of active PSII in the presence of “Cl$^{-}$”-depleted” PSII. In contrast to superoxide formation (Table 1), H$_2$O$_2$ production by “Cl$^{-}$”-depleted” PSII was inhibited by DCMU, indicating that a turnover of the S-cycle of the Mn-cluster is necessary for this process. The presence of siliconolylate as electron acceptor in “Cl$^{-}$”-depleted” PSII did not stimulate the H$_2$O$_2$ formation, indicating that no efficient electron transport could be established by supplementing the system with an electron acceptor. The rate of H$_2$O$_2$ formation in “Cl$^{-}$”-depleted” PSII was very low (about 6 μmol H$_2$O$_2$/mg Chl*h). These results seem to be only slightly affected by the activity of the PSII-associated catalase, as can be seen by the stimulation of the H$_2$O$_2$ formation by about 30% in the presence of the heme catalase inhibitor azide or triazolamolase. As shown in Table 2, the catalase activity was almost unchanged after the Cl$^{-}$ depletion procedure, indicating that not a reduced catalase activity was responsible for the higher H$_2$O$_2$ production rate in “Cl$^{-}$”-depleted” PSII compared to active PSII.

In the literature there is a debate about whether H$_2$O$_2$ originates from an incomplete water oxidation or from oxygen reduction on the acceptor side [8,23,31 – 33]. In addition, the pseudocatalase activity of the water splitting complex [32,34] and the PSII-associated catalase [14,15] lead to further complications. Fine and Frasch [7] proposed that the oxidation of water to H$_2$O$_2$ occurs via a modified S-cycle (S$_1$ → S$_2$ → S$_0$), involving the modified S$_2$-state. Although the yield of H$_2$O$_2$ production is rather low and it is not a favored reaction, it might be interesting to study the modified S$_2$-state in “Cl$^{-}$”-depleted” PSII and the S-states involved in H$_2$O$_2$ production in detail, in order to understand the enzymatic mechanism of the water splitting process.

In addition to the production of H$_2$O$_2$, other reactive oxygen species can be produced in PSII and detected by spin trapping assays [13,16,18,19,35]. It has been reported that the formation of OH$^-$ radicals is characteristic for PSII with an inactivated water splitting complex [16,18,19]. As shown in Fig. 7 and Table 1, both superoxide and hydroxyl radicals were produced in the light. Superoxide is produced on the acceptor side in the absence of an artificial electron acceptor independently of the activity state of the water splitting complex, as shown by the spin trapping assays with DEPMPO and EMPO. In Ca$^{2+}$ -depleted and active PSII, hydroxyl radicals were also detected although the yield of H$_2$O$_2$ formation was lower than in “Cl$^{-}$”-depleted” samples. These OH$^-$ radicals seem to originate mainly from superoxide via its disproportionation to H$_2$O$_2$, because the formation of the spin adducts was almost completely suppressed in the presence of SOD (Fig. 7). In “Cl$^{-}$”-depleted” PSII, however, the signal of the hydroxyl radical adduct was detectable in the presence of SOD, indicating that it is, at least partially, produced from H$_2$O$_2$ originating from the donor side of PSII.

It was suggested that O$_2$ is reduced to O$_2^-$ via electron transfer from Pheo$^-$ or from QA$^-$ [11]. Ca$^{2+}$ depletion of PSII inhibits not only the water splitting complex but induces in addition a shift of the midpoint potential of the redox couple QA/QA$^-$ by about 150 mV towards a more positive potential [36]. Here we see no effect of the midpoint potential of QA in the yield of O$_2^-$ production; therefore, it might be possible that Pheo$^-$ or QA$^-$ is the electron donor to O$_2$. In the case that QA$^-$ was the electron donor to O$_2$, an effect of the different redox potentials of QA on the yield of O$_2$ production would have been expected.

Reactive oxygen species play an important role in light-induced loss of PSII activity and degradation of the D1 protein. Although the yield of H$_2$O$_2$ production in “Cl$^{-}$”-depleted” PSII is rather low, it is more susceptible to photoinhibitory illumination than Ca$^{2+}$ -depleted PSII [22]. As shown in Figs. 1 and 2, active PSII was more damaged by light when “Cl$^{-}$”-depleted” PSII was present in the assay than when Ca$^{2+}$ -depleted PSII was present. Hydrogen peroxide formation of the “Cl$^{-}$”-depleted” PSII seems to be responsible for this effect with OH$^-$ being the most reactive and damaging species and not H$_2$O$_2$ itself. One has to assume that H$_2$O$_2$ diffuses into the medium and reacts at PSII with a transition metal in a Fenton reaction to OH$^-$.

OH$^-$ formed directly in the “Cl$^{-}$”-depleted” PSII is too short-lived to induce damage in neighbouring centers. Bradley et al. [23] reported also an acceleration of photoinhibition in “Cl$^{-}$”-depleted” PSII due to H$_2$O$_2$ production. Activity loss and degradation of the D1 protein can be induced by incubating PSII membranes in darkness in the presence of H$_2$O$_2$ [21]. Miyao et al. [21] used PSII membranes which were depleted of metal ions and much higher H$_2$O$_2$ concentrations were needed to induce damage. Under the conditions used in the present study, metals were not removed and a low H$_2$O$_2$ concentration was sufficient to stimulate the light-induced activity loss in active PSII.

H$_2$O$_2$ produced by “Cl$^{-}$”-depleted” PSII can damage active PSII centers in the in vitro assays shown in Figs. 1 and 2. It has to be pointed out that this is an artificial situation which is interesting to study on its own. One has to keep in mind that it is not possible to draw conclusions from this in vitro assay for photoinhibition in a physiologically relevant system. There are no indications in the literature that the initial step of photoinhibition is associated with a loss of chloride, while Ca$^{2+}$ depletion might occur when the proton concentration in the lumen is low [33]. Instead of H$_2$O$_2$, superoxide formation may play a role in photoinhibition of PSII under more physiologically relevant conditions when the availability of electron acceptors is limited. It has been shown in vitro that Mn-depleted PSII can be protected against photoinhibition by SOD while catalase has no protective effect, indicating that superoxide is the damaging species in these samples [9,10]. As shown in Fig. 2, a small...
protection effect against photoinhibition was observed when SOD was added to active PSII mixed with “Cl 2 −” or Ca 2+ -depleted PSII, respectively. The higher susceptibility to light of Ca 2+ -depleted PSII compared to active PSII [19,22] was not caused by a higher yield of superoxide production (Fig. 7) but rather by the light-induced accumulation of highly oxidizing species like P 680 + or TyrZ at the donor side of PSII [37]. Superoxide production by PSII may contribute under certain physiological conditions to photoinhibition in vivo [20], although one has to keep in mind that PSI is a more important source for superoxide formation in the lack of final electron acceptors.

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