Photoproduction and Detoxification of Hydroxyl radicals in Chloroplasts and Leaves and Relation to Photoinactivation of Photosystems I and II

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The light-dependent production of hydroxyl radicals (HO·) by thylakoids, chloroplasts and leaves of Spinacia oleracea was investigated using dimethylsulfoxide as HO· trapping agent. Maximum rates of HO· production by thylakoids as indicated by the formation of methane sulfonic acid were observed under aerobic conditions in the absence of added electron acceptors. They were higher than 2 μmol (mg Chl h)−1. Saturation of HO· production occurred at the low photon flux density of 100 μmol m−2 s−1. Trapping of HO· by dimethylsulfoxide suppressed, but did not eliminate light-dependent inactivation of PSI and PSII suggesting that HO· formation contributed to the photosensitivity of isolated thylakoids. DCMU inhibited HO· formation. Importantly, methylviologen decreased HO· formation in the absence, but stimulated it in the presence of Fe3+.

In intact chloroplasts, HO· formation became appreciable only after KCN had been added to inhibit effective O2 scavenging by ascorbate peroxidase. It was stimulated by ferricyanide, but not by ferricyanide which does not penetrate the chloroplast envelope. Infiltrated spinach leaves behaved similar in principle to intact chloroplasts in regard to HO· formation but HO· production was very slow if detectable at all by the formation of methylsulfonic acid indicating effective radical detoxification.

HO· formation is interpreted to be the result of a Fenton-type reaction which produces HO· in chloroplasts from H2O2 and reduced ferredoxin, when O2 is electron acceptor in the Mehler reaction and radical detoxification reactions are inhibited.

Key words: Chloroplasts — Electron transport — Haber-Weiss-reaction — Hydroxyl radicals — Photoinactivation — Radical formation — Thylakoids.

In photosynthesis, CO2 and water are substrates and carbohydrates and oxygen are products. However, intermediate reductants formed during electron transport to CO2 may react with oxygen giving rise to highly reactive and potentially damaging oxygen species. In the Mehler reaction, the univalent reduction of oxygen at the reducing side of PSI is known to lead to the superoxide anion radical O2− which, under the influence of superoxide dismutase, disproportionates rapidly to molecular oxygen and hydrogen peroxide. When not detoxified by ascorbate peroxidase (Foyer and Hall 1980, Asada 1994, Asada and Takahashi 1987, Elstner 1990), H2O2 can produce hydroxyl radicals (or species acting like HO·; Youngman and Elstner 1981, Elstner et al. 1978) in a reaction with reduced iron compounds (Fenton-type reaction, often referred to as Haber-Weiss-reaction). Recently, hydroxyl radicals were reported to be formed in the light at appreciable rates within PSII of the green alga Euglena gracilis (Tschiersch and Ohmann 1993). These radicals belong to the strongest oxidants known and may be involved in the photoinactivation of chloroplast electron transport. Whereas chloroplasts in leaves are normally reasonably well protected against photoinactivation, intact isolated chloroplasts are highly sensitive to bright illumination. Their electron transport chain may be largely inactivated during less than one hour exposure to sunlight conditions in the presence of oxygen owing to damage to both PSI and PSII. Under anaerobiosis, only PSII is rapidly damaged, whereas PSI is left unaffected (Heber et al. 1989). Recently, Sonoiie and Terashima (1994) and Sonoiie (1995) reported a selective inhibition of PSI in chilling sensitive Cucumis sativus and chilling insensitive spinach chloroplasts even at room temperature which might involve oxygen radicals.

We were interested in the question, at which rates and under which conditions hydroxyl radicals are produced by isolated chloroplasts and by chloroplasts in leaves and whether these radicals are involved in the photoinactivation of the chloroplast electron transport chain.

Materials and Methods

Intact chloroplasts were prepared from spinach leaves using a modification (Heber and Santarius 1970) of the isolation method of Jensen and Bassham (1966). The medium for the homogenization of the leaves contained 330 mM sorbitol, 30 mM KCl, 1 mM MgCl2, 1 mM MnCl2, 2 mM EDTA, 0.25 mM KH2PO4, 1.6 mM ascorbate, 3.9 mM cysteine and 50 mM MES (pH 6.1). The chloroplasts were finally suspended in reaction medium which contained 330 mM sorbitol, 30 mM KCl, 1 mM MgCl2, 1 mM MnCl2, 2 mM EDTA, 0.25 mM KH2PO4, and 50 mM HEPES (pH 7.6). The percentage of chloroplasts with intact envelopes was between 60 and 90% as measured by the ferricyanide method (Heber and San-

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Abbreviations: DCPIP, dichlorophenolindophenol; DMSO, dimethylsulfoxide; MSA, methane sulfonic acid; MV, methylviologen; PFD, photon flux density.

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Thylakoids were obtained by rupturing chloroplasts osmotically in an at least 10 fold larger volume of either water (thylakoids) or 2.5 mM HEPES/10 mM MgCl₂ (class D chloroplasts; Hall 1972). After brief exposure to hypotonic conditions, isoosmolarity was re-established by adding an equal volume of twice-concentrated reaction medium. Ferricyanide reduction of thylakoids was measured as oxygen evolution in a Clark type electrode (Bachofer, Reutlingen, Germany) in the presence of 0.6 μM nigericine under saturating red light (Schott RG 610, Calflex C, 2,000 μmol m⁻² s⁻¹). Reduction of oxygen in the presence of thylakoids, 2 mM ascorbate, 1 mM KCN and 0.6 μM nigericine was measured with the same equipment. Chl concentration was 30 μg ml⁻¹ for each experiment in the reaction medium mentioned above. Formation of hydroxyl radicals was measured under illumination in a stirred reaction vessel with 30 μg Chl ml⁻¹ at about 22°C in the presence of 0.7 M DMSO according to Babbs and Gale (1987) and Babbs et al. (1989). Photon flux densities were measured by a LI-185B quantum meter (Licor, Lincoln, Nebraska, U.S.A.). Modulated Chl fluorescence was measured with a PAM 101 Fluorometer (Walz, Effeltrich, Germany). The quantum yield of PSII was determined after light exposure of the chloroplasts from fluorescence data according to Schreiber et al. (1986) using the method of Genty et al. (1989). For photoinactivation experiments, thylakoids were illuminated with white light. Aliquots were removed at different times of light exposure and linear electron transport from water to MV was measured polarographically as oxygen uptake. The reaction medium for the assay of electron transport was the same as described above, but contained in addition 1 mM KCN, 4 mM NH₄Cl and 90 μM MV. Illumination for determining electron transport was provided by a Schott KL 1,500 lamp (Schott, Mainz, Germany) through a RG 610 cutoff filter (Schott) at a PFD of 1,000 μmol m⁻² s⁻¹. A very similar method was used for measuring electron transport from ascorbate to MV through PSI. In this case, the reaction medium contained 5 mM ascorbate, 0.2 mM DCPIP, 1 mM KCN, 0.2 mM MV, 1.33 μM nigericine and 5 μM DCMU.

To measure hydroxyl radical production in leaves, the method used for the chloroplasts was modified. Leaves were infiltrated in vacuo with 0.7 M DMSO (plus additions as shown in Table 2). They were then exposed to illumination on a thin layer of 0.7 M DMSO. After a cycle of 9 h in the light, 13 h in the dark and 2 more h in the light, leaves were removed, the surface dried and frozen in liquid nitrogen. The frozen leaf material was powdered, thawed and extracted with water. After removal of debris by centrifugation, the extract was acidified with HCl to pH 2.5 and shaken with a mixture of toluene and 2-butanol (3/1, v/v). The aqueous phase was passed through two Sep-Pak C₁₈ columns to remove apolar components. The part of the eluate containing MSA was used for the coupling reaction with Fast-Blue-BB-salt (Babbs and Gale 1987, Babbs et al. 1989, Tschiersch and Ohmann 1993). DMSO is membrane-compatible as shown by its ability to protect thylakoid membranes against inactivation by freezing (Heber and Ernst 1967). As a small amphiphilic molecule, it penetrates membranes rapidly and can be used to trap hydroxyl radicals even inside intact cells and organelles. To increase its trapping efficiency, it was used at the high concentration of 0.7 M. Fig. 1 shows the time course of the formation of hydroxyl radicals as indicated by the formation of MSA under illumination in the presence of osmotically shocked and intact chloroplasts. Osmotic shock liberates thylakoid membranes from chloroplasts and dilutes soluble chloroplast constituents. No electron acceptors were added to the preparations, but oxygen and some CO₂ were present owing to equilibration of the solutions with air. The rate of hydroxyl radical production was highest (about 2 to 4 μmol (mg Chl h)⁻¹) when thylakoids were obtained from chloroplasts by shocking them first in water and resuspending the membranes immediately afterwards in the same isotonic medium that was used for stabilizing the intact chloroplasts. When the chloroplasts were exposed to hypotonic shock in 2.5 mM HEPES and 10 mM MgCl₂, and then brought back to isotonic condi-

**Results and Discussion**

*Oxidation of dimethylsulfoxide by hydroxyl radicals in isolated thylakoids and intact chloroplasts—Hydroxyl radicals react rapidly with a large number of cellular components. To measure their formation, a highly competitive reaction must be introduced which yields a defined product that can be analyzed. DMSO reacts rapidly with hydroxyl radicals to form MSA which can be measured after a coupling reaction with Fast-Blue-BB-salt (Babbs and Gale 1987, Babbs et al. 1989, Tschiersch and Ohmann 1993). DMSO is membrane-compatible as shown by its ability to protect thylakoid membranes against inactivation by freezing (Heber and Ernst 1967). As a small amphiphilic molecule, it penetrates biomembranes rapidly and can be used to trap hydroxyl radicals even inside intact cells and organelles. To increase its trapping efficiency, it was used at the high concentration of 0.7 M. Fig. 1 shows the time course of the formation of hydroxyl radicals as indicated by the formation of MSA under illumination in the presence of osmotically shocked and intact chloroplasts. Osmotic shock liberates thylakoid membranes from chloroplasts and dilutes soluble chloroplast constituents. No electron acceptors were added to the preparations, but oxygen and some CO₂ were present owing to equilibration of the solutions with air. The rate of hydroxyl radical production was highest (about 2 to 4 μmol (mg Chl h)⁻¹) when thylakoids were obtained from chloroplasts by shocking them first in water and resuspending the membranes immediately afterwards in the same isotonic medium that was used for stabilizing the intact chloroplasts. When the chloroplasts were exposed to hypotonic shock in 2.5 mM HEPES and 10 mM MgCl₂, and then brought back to isotonic condi-
tions (class D chloroplasts), less hydroxyl radicals were formed, and a time lag in the formation of radicals suggested initial trapping of radicals by a competing reaction. In intact chloroplasts, the formation of hydroxyl radicals was negligible. However, when the chloroplasts were poisoned with 3 mM KCN which inactivates enzymes such as CuZn-superoxide dismutase and ascorbate peroxidase, hydroxyl radical formation was observed at an appreciable rate.

In Fig. 2, the light dependence of hydroxyl radical production by thylakoids (chloroplasts shocked in water and then resuspended; see Fig. 1) is shown. An appreciable rate of formation was already observed at very low PFD of 10 \( \mu \text{mol m}^{-2} \text{s}^{-1} \). The reaction was almost saturated at PFD = 100 \( \mu \text{mol m}^{-2} \text{s}^{-1} \), i.e. at less than 10\% of sun light. This is reminiscent of oxygen reduction in the Mehler reaction which is saturated by very low light intensities (Heber and French 1968). It leads, via \( \text{O}_2^- \), to the accumulation of \( \text{H}_2\text{O}_2 \) when ascorbate is absent. In thylakoid preparations, the ascorbate originally contained in the chloroplasts is highly diluted. Ascorbate peroxidase is inactivated by depletion of ascorbate or KCN (Amako et al. 1994, Asada 1992, 1994a, Miyake and Asada 1992).

**Participation of reduced ferredoxin in the light-dependent formation of hydroxyl radicals**—Univalent oxygen reduction and the subsequent formation of \( \text{H}_2\text{O}_2 \) can be increased far beyond the limits set by the Mehler reaction, when MV is added as an electron acceptor to thylakoids (Harbour and Bolton 1975, Elstner et al. 1978, Youngman and Dodge 1979, Härtil et al. 1992). However, Fig. 3 shows the unexpected result that MV, instead of increasing hydroxyl radical production, clearly decreased it in thylakoids. However, \( \text{HO}^- \) formation was stimulated when \( \text{Fe}_3\text{(SO}_4)_2 \) (0.5 mM) was added together with MV to illuminated chloroplasts, which reduce \( \text{Fe}^{3+} \) to \( \text{Fe}^{2+} \) (Table 1). In this case, the rate of hydroxyl radical production as indicated by MSA formation was about 5 \( \mu \text{mol (mg Chl h)}^{-1} \) even in intact chloroplasts. In the presence of ferrous ions \( \text{HO}^- \) is formed in a Fenton-type reaction (see also Youngman and Elstner 1981):

\[
\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{HO}^- + \text{OH}^- + \text{Fe}^{3+} \tag{1}
\]

The suppression of \( \text{HO}^- \) formation by MV in the presence of oxygen in Fig. 3 indicates that MV interferes with the reduction of ferredoxin. MV is a highly effective electron acceptor. In the absence, but not in the presence of MV, ferredoxin is photoreduced. Oxygen is reduced in the Mehler reaction to \( \text{O}_2^- \) after oxidized ferredoxin is no longer available. \( \text{O}_2^- \) produces \( \text{H}_2\text{O}_2 \). Reduced ferredoxin reacts with \( \text{H}_2\text{O}_2 \) according to equation (1) (\( k = 5.2 \times 10^3 \text{M}^{-1} \text{s}^{-1} \); Ho-

**Table 1** Formation of hydroxyl radicals by intact chloroplasts under illumination with PFD = 900 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)

<table>
<thead>
<tr>
<th>Addition</th>
<th>HO$^-$ formation, ( \mu \text{mol (mg Chl h)}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>0.5 mM Fe$_3$(SO$_4$)$_2$ + 3 mM KCN</td>
<td>3.2</td>
</tr>
<tr>
<td>0.5 mM Fe$_3$(SO$_4$)$_2$ + 90 ( \mu \text{M MV} )</td>
<td>5.0</td>
</tr>
</tbody>
</table>
Sein and Palmer 1983). However, in the presence of MV ferredoxin is bypassed and the iron-sulfur-centers of PSI are oxidized by MV. In the absence of reduced ferredoxin or other reduced iron species, Fenton-type HO' formation is slow in thylakoids even though increased levels of H$_2$O$_2$ are available.

This conclusion was confirmed by a set of experiments with washed thylakoids. Thylakoids were freed of soluble ferredoxin by washing with water and 100 mM NaCl. Illumination of the resuspended thylakoids resulted in oxygen uptake in the Mehler reaction, but HO' formation was decreased compared to unwashed thylakoids. However, addition of ferredoxin to the washed thylakoids produced HO' radicals in a concentration-dependent manner (Fig. 4). Exogenous H$_2$O$_2$ also stimulated HO' formation, but less so in the absence than in the presence of ferredoxin, and HO' formation declined with time during illumination, perhaps because of damage to the thylakoids by the added H$_2$O$_2$ (Fig. 4).

**Inhibition of hydroxyl radical formation by DCMU—**

HO' formation in thylakoids is inhibited not only by MV but also by DCMU (10 µM, Fig. 3). Härtel et al. (1992) observed complete inhibition of oxygen radical formation in EPR experiments after adding 100 µM DCMU to thylakoid suspensions in the presence of MV, whereas Tschiersch and Ohmann (1993) reported appreciable DCMU-insensitive HO' formation. We observed half-maximal inhibition of linear electron transport from water to ferricyanide at a DCMU concentration of 125 nM with nigericine as uncoupler. Uncoupled oxygen uptake in the Mehler reaction in the presence of 2 mM ascorbate and 1 mM KCN showed two components, one DCMU-sensitive and one DCMU-insensitive. The sensitive component revealed half-maximal inhibition below 300 nM DCMU, i.e. close to the DCMU concentration which also inhibited ferricyanide-dependent oxygen evolution. The DCMU-insensitive component was dependent on the presence of ascorbate. It was still observed after the thylakoids were heated for 5 min to 95°C. This oxygen reduction is thought to reflect a photosensitized oxidation of ascorbate. It may explain conflicting observations regarding the DCMU sensitivity of HO' formation. By preventing electron flow through PSI, DCMU inhibits both the accumulation of reduced ferredoxin and formation of H$_2$O$_2$ in the Mehler reaction, prerequisites of the occurrence of reaction (1). Still, some formation of HO' radicals at the level of PSI (Tschiersch and Ohmann 1993, Shiraishi et al. 1994) cannot be excluded, although it was not detectable in our experiments.

Intact chloroplasts supported significant HO' production only when KCN was present to inhibit effective H$_2$O$_2$ detoxification (Fig. 1). Fe$^{3+}$ in the form of Fe$_3$(SO$_4$)$_2$ also supported HO' formation whereas K$_3$[Fe(CN)$_6$] was ineffective (data not shown). The ferricyanide anion is unable to penetrate the chloroplast envelope (Heber and Santarius 1970). It is an effective electron acceptor only for broken chloroplasts. However, MV reduction in combination with Fe$^{3+}$ ions led to fast HO' formation. Apparently, Fe cations, in contrast to ferricyanide anions, penetrate the chloroplast envelope.

The data suggest that hydroxyl radicals are primarily formed in the absence of ferric ions by the interaction of reduced ferredoxin and H$_2$O$_2$ close to PSI. Usually it is assumed that in leaves damage to PSI is responsible for the sensitivity of the chloroplast electron transport chain to excessive illumination. However, in isolated intact chloroplast PSI is not much less damaged by excess light than PSI, when oxygen is present (Heber et al. 1989). Sonoiike (1995) and Sonoiike and Terashima (1994) also observed a fast light-dependent inactivation of PSI in chloroplasts of spinach and cucumber, which might be mediated by oxygen radicals.

**Participation of HO' in the photoinactivation of the chloroplast electron transport chain—**

HO', when formed, is a highly aggressive oxidant. Just because it will attack anything oxidizable, it is not clear whether the damage it causes is specific enough to play a significant role in the light-dependent inhibition of chloroplast electron transport. This question is particularly relevant in view of the observation that HO' formation was saturated at low light (Fig. 2) whereas the photoinactivation of the chloroplast electron transport chain is fast under high intensity illumination and insignificant at low light.

Fig. 5 shows an experiment, where thylakoids were ex-
posed to light in the presence of $O_2$, but in the absence of DMSO or MV. PFDs ranged from 10 to 2,000 $\mu$mol m$^{-2}$ s$^{-1}$. At different times of light exposure, aliquots were removed from the thylakoid suspension and PSI dependent electron transport from ascorbate to MV was measured in the presence of DCMU to inhibit water oxidation. Even though hydroxyl radical formation was largely light-saturated already close to PFD=100 $\mu$mol m$^{-2}$ s$^{-1}$ (Fig. 2), electron transport through PSI was inactivated faster at PFD=2,000 $\mu$mol m$^{-2}$ s$^{-1}$ than at PFD=100 $\mu$mol m$^{-2}$ s$^{-1}$ (Fig. 6). Still, DMSO which traps HO' by competing with thylakoid constituents for this highly reactive oxygen species decreased inactivation of PSI (Fig. 7). Even though hydroxyl radicals are unlikely to be solely responsible for damage to PSI, the protective effect of DMSO strongly suggests that HO' participated in the destruction of PSI reaction centers which is indicated by the inactivation of PSI-dependent electron transport (Fig. 5 and 6). Results very similar to those shown in Fig. 7 were obtained, when the quantum yield of electron transport through PSII was measured as the ratio of the flash-induced increase in modulated Chl fluorescence ($F_m'$–$F$) to the maximum fluorescence $F_m'$ after thylakoids had been exposed to strong illumination in the absence of MV (Fig. 8). The data show, that not only PSI, but also PSII is partially protected against photoinactivation by DMSO. Hydroxyl radicals have been considered to trigger D1-protein degradation in the case of donor-side induced photoinhibition of PSII, whereas $O_2$ is thought to be a candidate for marking the D1-protein for degradation in the case of acceptor-side induced photoinhibition (Hideg et al. 1994, Aro et al. 1993). Even though trapping of HO' by DMSO revealed appreciable HO' formation by illuminated thylakoids (Fig. 1–4) and electron transport was rapidly inactivated (Fig. 5–8), bleaching of Chl could not be demonstrated during ex-

![Fig. 5 Inactivation of PSI-dependent electron transport of thylakoids in isotonic suspension (without DMSO, with about 270 $\mu$M $O_2$; membranes obtained as in Fig. 2) at different PFDs. The thylakoids were preilluminated in the absence of artificial electron acceptors as shown (○ $<$10 $\mu$mol m$^{-2}$ s$^{-1}$; □ 100 $\mu$mol m$^{-2}$ s$^{-1}$; ▲ 500 $\mu$mol m$^{-2}$ s$^{-1}$; ● 2,000 $\mu$mol m$^{-2}$ s$^{-1}$). Aliquots were removed as indicated and electron transport from ascorbate to MV was measured as light-dependent $O_2$ uptake at PFD=1,000 $\mu$mol m$^{-2}$ s$^{-1}$. The reaction medium for the measurement of electron transport contained 5 mM ascorbate, 0.2 mM DCFIP, 0.2 mM MV, 1 mM KCN, 1.3 $\mu$M nigericin and 5 $\mu$M DCMU.

![Fig. 6 Inactivation of PSI-dependent electron transport of thylakoids in isotonic suspension in the absence of DMSO as a function of light intensity. Illumination time 10 min.

![Fig. 7 Effect of DMSO (0.7 M ●; ○ without DMSO) on the inactivation of PSI-dependent electron transport of thylakoids (obtained as in Fig. 2). Preillumination of the thylakoids with PFD=2,000 $\mu$mol m$^{-2}$ s$^{-1}$ in the absence of artificial electron acceptors. Measurement of electron transport as in Fig. 5.]
Fig. 8 Effect of DMSO on the photoinactivation of PSII of thylakoids (obtained by shocking intact chloroplasts in hypotonic HEPES/MgCl₂ solution) during illumination with a PFD of 900 \( \mu \text{mol m}^{-2} \text{s}^{-1} \). Oxygen concentration about 270 \( \mu \text{M} \). Modulated Chl fluorescence \( F \) was measured during illumination in the presence of a very weak measuring light. Maximum fluorescence \( F_{m} \) was obtained by excitation with saturating 1 s flashes. Relative quantum efficiency is expressed as \( \frac{F - F_{m}}{F_{m}} \) according to Gentzky et al. (1989). • with 0.7 M DMSO; ○ without DMSO.

posure of thylakoids to light for one hour.

Scavenging of HO' in intact chloroplasts and leaves—In intact chloroplasts appreciable HO' formation was observed only in the presence of cyanide which inhibited the radical scavenging system of superoxide dismutase and ascorbate peroxidase (Asada and Takahashi 1987). When spinach leaves were infiltrated with a solution containing 0.7 M DMSO very little MSA was formed in the light and a long period of illumination was required for reliable MSA measurements (Table 2). The leaf system was more difficult to analyze for MSA than the chloroplast system owing to the presence of solutes in leaves which interfered with the MSA determination (see Material and Methods section). When KCN was present in the infiltration medium in addition to DMSO, MSA formation was stimulated by a factor of 5 compared to a control without KCN addition. With ferrisulfate, a stimulation by a factor of 10 was observed. MV which produced extensive chlorophyll bleaching during illumination in the leaf experiment suppressed MSA formation as it did in thylakoid experiments. Less bleaching than in the MV experiment was observed in the presence of ferrisulfate and KCN and none in the control. In regard to MSA formation, the leaf data were similar in principle to data obtained with chloroplasts, although much longer illumination times were required to observe sufficient MSA accumulation for reliable measurements (Table 2). We interpret this as indicating highly effective radical detoxification in leaves. Still, damage by oxygen radicals may even occur in vivo in situations where radical detoxification cannot keep pace with radical production (Kim and Jung 1993, Moran et al. 1994, Sonoike and Terashima 1994).

Conclusions—Several observations made during this study are considered to bear on the problem of photoinactivation of chloroplast electron transport:

(1) Intact isolated chloroplasts and leaves are equipped with protective systems not lost during isolation which are capable either of preventing the formation of highly reactive and destructive hydroxyl radicals during illumination or of scavenging them as they are formed. Suppression of radical formation and radical scavenging are particularly effective in intact leaves.

(2) After inactivation of radical scavenging systems by KCN or diluting them by rupturing the chloroplast envelopes, hydroxyl radicals are generated by illuminated thylakoid membranes. They are involved in the photoinactivation of PSI and II of the chloroplast electron transport system.

(3) Hydroxyl radical formation is almost saturated at the low PFD of 100 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) suggesting HO' formation as a consequence of \( O_2 \) reduction in the Mehler reaction. Instead of stimulating HO' formation, MV reduction suppresses it, although MV reduction, like the much slower Mehler reduction, results in the formation of \( O_2^- \) and the subsequent disproportionation to \( H_2O_2 \). Since, in contrast to the Mehler reaction, MV reduction does not lead to the accumulation of reduced ferredoxin or reduced Fe-S-centers, light-dependent HO' formation by chloroplast thylakoids is proposed to be brought about by a Fenton-type reaction between reduced iron-species and \( H_2O_2 \). This emphasizes the importance for chloroplast function of the highly active ascorbate peroxidase system, which is efficient to scavenge \( H_2O_2 \), and of proper control of PSI, which regulates accumulation of reduced ferredoxin.

Table 2 Formation of hydroxyl radicals in spinach leaves as indicated by the accumulation of MSA after infiltrating leaves with 0.7 M DMSO plus additions as indicated

<table>
<thead>
<tr>
<th>Addition</th>
<th>MSA in leaves after 11 h illumination in the presence of DMSO</th>
<th>( \text{nmol (g FW)}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>MV, 90 ( \mu \text{M} )</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>KCN, 3 mM</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>( \text{Fe}_2(\text{SO}_4)_3 ), 0.5 mM</td>
<td>122</td>
<td></td>
</tr>
</tbody>
</table>

Illumination of the leaves for 11 h with a PFD of 500 \( \mu \text{mol m}^{-2} \text{s}^{-1} \). For comparison with the data of Table 1: spinach leaves with 1 g FW contain about 1.5 mg Chl.
Hydroxyl radicals in illuminated chloroplasts

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