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Singlet oxygen production in herbicide-treated photosystem II

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Abstract **Photo-generated reactive oxygen species in herbicide-treated photosystem II were investigated by spin-trapping. While the production of $^{\bullet}OH$ and $O_2^{-\bullet}$ was herbicide-independent, $^1O_2$ with a phenolic was twice that with a urea herbicide. This correlates with the reported influence of these herbicides on the redox properties of the semiquinone $Q_A^{-\bullet}$ and fits with the hypothesis that $^1O_2$ is produced by charge recombination reactions that are stimulated by herbicide binding and modulated by the nature of the herbicide. When phenolic herbicides are bound, charge recombination at the level of $P^{+\bullet}Pheo^{-\bullet}$ is thermodynamically favoured forming a chlorophyll triplet and hence $^1O_2$. With urea herbicides this pathway is less favourable.**

Key words: Photosystem II; Oxidative stress; Herbicide; Charge recombination; Singlet oxygen

1. Introduction

When plants are exposed to higher light intensities than can be used in photosynthesis, photooxidative stress occurs. The major target of light-induced damage (photoinhibition) is photosystem II (PSII) (for review see [1,2]). Depending on experimental conditions, different mechanisms of photoinactivation seem to exist and can be investigated separately in vitro (e.g. [3–9]). It has been known for many years that charge recombination in PSII can result in formation of a chlorophyll triplet state (^{3}Chl) [10,11] and this chlorophyll triplet can react with $^3O_2$ to form $^1O_2$ [12–15]. Charge recombination can occur in PSII under several conditions that are physiologically relevant [16,17]. There is an increasing body of evidence that $^1O_2$ is the species responsible for photoinhibition in vivo. Hideg and co-workers [18] measured the formation of $^1O_2$ in vivo in chloroplasts of leaves of *Arabidopsis thaliana*. Trebst and co-workers [19] provided evidence that $^1O_2$ is the important damaging species during photoinhibition of *Chlamydomonas reinhardtii* cells. A special case is when herbicides are bound to PSII. Under these conditions electron transfer can take place only as far as $Q_A^{-\bullet}$ and charge recombination is the main outcome of light-induced charge separation. It was pointed out recently that the stimulated charge recombination occurring in herbicide-treated PSII could be an important factor in the herbicidal action mediated by $^1O_2$ [20]. This suggestion was based on a number of experimental observations and theoretical considerations:

1. Different herbicide classes have different influences on the photoinhibition of PSII [6,8,9,21–23]. Phenolic herbicides increase photodamage [6,21,22], while with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and other urea- and triazine-type herbicides photodamage is less marked [6,8,21,22] although all of these herbicides bind to the Q_B-binding site and inhibit forward electron transport.
2. Different classes of herbicides influence the charge recombination measured by differential changes in the thermoluminescence emission temperature. Phenolic herbicides decrease the emission temperature while urea- and triazine-type herbicides increase it [24,25].
3. Herbicide binding influences the midpoint potential (E_m) of the $Q_A/Q_A^{-\bullet}$ redox couple in PSII with phenolic herbicides lowering the E_m by 45 mV, while DCMU raises the potential by 50 mV [25].
4. Based on the situation in the purple bacterial reaction centre [26–29], it was suggested that two competing charge recombination pathways exists in PSII: one in which back-reactions occur leading to formation of $P^{+\bullet}Pheo^{-\bullet}$ which recombines to give ^{3}Chl and another where recombination takes place from $P^{+\bullet}Q_A^{-\bullet}$ without triplet formation. Which of these routes is favoured depends on the relative energy levels of the radical pairs.

Correlating these factors, it was suggested that herbicides modulate the recombination pathway within PSII [20,25] and thus the degree of $^1O_2$-mediated photodamage. Lowering of the redox potential of Q_A by binding of phenolics should increase the chances of repopulating the $P^{+\bullet}Pheo^{-\bullet}$ from $P^{+\bullet}Q_A^{-\bullet}$ by decreasing the energy gap between these two radical pairs. The $P^{+\bullet}Pheo^{-\bullet}$ radical pair can decay via the formation of the chlorophyll triplet state and hence the potential formation of $^1O_2$. Raising the redox potential of Q_A by DCMU binding increases the energy gap between the two radical pairs and thus should favour charge recombination via a direct route without the formation of $^1O_2$. A corollary of this suggestion was that $^1O_2$ generated by charge recombination could be important in herbicidal activity as mentioned above.

According to this idea it is predicted then that $^1O_2$ will be generated in herbicide-treated PSII and, importantly, that the

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Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EMPO, 2-ethoxycarbonyl-2-methyl-3,4-dihydro-2*H*-pyrrole-1-oxide; PSII, photosystem II; Q_A, primary quinone electron acceptor in PSII; Q_B, secondary quinone electron acceptor in PSII; Pheo, pheophytin – primary electron acceptor; PPBQ, phenyl-*p*-benzoquinone; SOD, superoxide dismutase; TEMP, 2,2,6,6-tetramethylpiperidine; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oyxl

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yield will be greater when phenolic herbicides are used compared to other classes of herbicide. It is known that photoinhibitory damage of PSII under various conditions can result in the formation not only of $^1O_2$ but also of other reactive oxygen species. In the present work we use spin trapping electron paramagnetic resonance (EPR) to monitor $^1O_2$, superoxide and hydroxyl radicals during photodamage in the presence of herbicides.

2. Materials and methods

PSII-enriched membrane fragments from spinach were isolated as in [30] with the modifications as described in [31]. The activity of the PSII preparations was approximately 600 μmol O_2 mg Chl^{-1} h^{-1} measured with a Clark-type electrode in the presence of 1 mM phenyl-*p*-benzoquinone (PPBQ) as electron acceptor in a buffer containing 0.4 mM sucrose, 10 mM NaCl, 50 mM 2-(*N*-morpholino)ethanesulphonic acid pH 6.5 and 20 μg Chl/ml.

2.1. Spin trapping of $^1O_2$ by TEMP

Spin-trapping assays were performed in the same buffer with a concentration of 100 μg Chl/ml, 10 mM 2,2,6,6-tetramethylpiperidine (TEMP) and 30 mM methanol (ultra-pure) final concentration. The herbicides 100 μM bromoxynil or 20 μM DCMU were dissolved in methanol. The concentration of methanol did not exceed 7% (v/v). Samples were illuminated for 15 min with different intensities of red light in a water bath at 20°C. Light was filtered by Calflex C and RG 620. The EPR signals of the samples were measured before and after exposure to light with a Bruker ESP 300 spectrometer. X-band spectra were recorded at room temperature with 9.7 GHz microwave frequency, modulation frequency 100 kHz, modulation amplitude 2 G. Because of the impurity of the spin trap TEMP purchased from different producers, purification was performed by a standard vacuum distillation at 15 mbar in the presence of analytic traces of zinc powder to provide a reductive atmosphere. The second of the four fractions of the distillation at 42°C showed the best properties with respect to stability and purity. At 43.5°C the majority of the sample was distilled. To estimate the concentration of $^1O_2$ produced and trapped by TEMP, a calibration curve was measured plotting the EPR signal size against the concentration of ultra-pure 2,2,6,6-tetramethylpiperidine-1-oyxl (TEMPO) (Sigma-Aldrich) under the same conditions.

2.2. Spin trapping of $^{\bullet}OH$ and $O_2^{-\bullet}$ by EMPO

Detection of $O_2^{-\bullet}$ and $^{\bullet}OH$ was carried out with 25 mM 2-ethoxycarbonyl-2-methyl-3,4-dihydro-2*H*-pyrrole-1-oxide (EMPO) as spin trap [32] under the same conditions as described above. The EPR signal of the pure $^{\bullet}OH$ adduct was obtained by adding 100 μM H_2O_2 and 100 μM Fe(II)SO_4 (Fenton reaction) to the same buffer in the presence of 25 mM EMPO. The EPR signal of the pure $O_2^{-\bullet}$ adduct was obtained by adding KO_2 crown ether reagent to the same buffer in the presence of 25 mM EMPO (data not shown). To distinguish between the $^{\bullet}OH$ and the $O_2^{-\bullet}$ adduct formed during the illumination of PSII-enriched membrane fragments, 2500 U/ml of catalase from bovine liver (EC 1.11.1.6) or 375 U/ml of superoxide dismutase (SOD) from bovine erythrocytes (EC 1.15.1.1) was added.

3. Results

Fig. 1 shows the characteristic EPR signal of the nitroxyl radical TEMPO, which is formed by the reaction of TEMP with $^1O_2$. $^1O_2$ was produced by PSII-enriched membrane fragments exposed for 15 min to high light intensity illumination (1800 μmol quanta m^{-2} s^{-1}). When bromoxynil was added prior to the illumination (Fig. 1c), the signal was approximately double the size of that obtained in the presence of DCMU at the given light intensity (Fig. 1b). A very small signal observable prior to illumination of the sample is due to the impurity of the spin trap even after the purification protocol used (Fig. 1a). The concentration of the phenolic herbicide used was low enough to block specifically the electron transfer on the electron acceptor side of PSII without any measurable influence of the electron donor side effect that is characteristic of phenolic herbicides at higher concentrations [33–35]. This was monitored by recording the thermoluminescence in the presence of the herbicides. With the concentration used the thermoluminescence curves showed only a displacement of the $S_2Q_A^{-\bullet}$ recombination band to a lower temperature without any quenching of this band (compared to that with DCMU) that is characteristic of the electron donor side effect of phenolic herbicides (data not shown, see also [25]).

The dependence of the amount of trapped $^1O_2$ on the light intensity is shown in Fig. 2. At low light intensities (up to 200 μmol quanta m^{-2} s^{-1}) no obvious difference was seen between the observable TEMPO formation in the presence of DCMU or bromoxynil while at higher light intensities approximately twice the amount of $^1O_2$ is trapped in the presence of bromoxynil compared with DCMU.

The light intensities and illumination times were chosen taking into account the trapping sensitivity and the fact that large amounts of reactive oxygen species destroy the integrity of the chlorophyll-binding proteins. The release of chlorophylls causes an oxygen uptake [36], which is thought to reflect the generation of reactive oxygen species. The destruction of the integrity of PSII is expected to induce a cascade in the production of reactive oxygen species. Therefore we used relatively short illumination times (15 min) and light intensity not higher than 1800 μmol quanta m^{-2} s^{-1}. These conditions provided clearly distinguishable slopes for the increase of $^1O_2$ production versus light intensity (Fig. 2).

The EPR signal size could be correlated to the TEMPO concentration so that a concentration of trapped $^1O_2$ could be estimated. Illumination with an intensity of 1800 μmol quanta m^{-2} s^{-1} resulted in rates of approximately 5.5 and 2.7 μmol mg Chl^{-1} h^{-} in the presence of bromoxynil and DCMU, respectively. At the same intensity of red light the

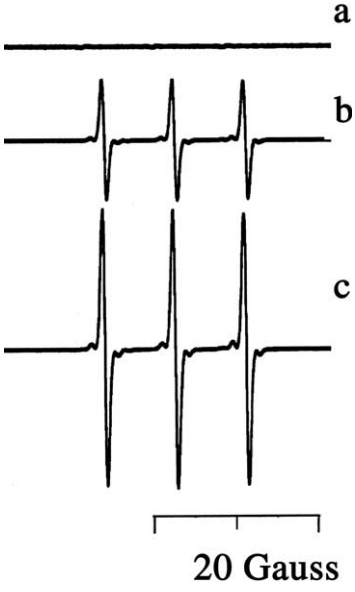

Fig. 1. Singlet oxygen trapping by TEMP. Typical EPR spectra of TEMPO as adduct of the reaction of TEMP with $^1O_2$ are shown. PSII-enriched membrane fragments were illuminated for 15 min with red light (1800 μmol quanta m^{-2} s^{-1}) in the presence of 10 mM TEMP. a: Non-illuminated sample in the presence of 100 μM bromoxynil. b: Samples illuminated in the presence of 20 μM DCMU. c: Samples illuminated in the presence of 100 μM bromoxynil.

PSII particles showed an oxygen evolution activity of 460 μmol O_2 mg Chl^{-1} h^- measured in the presence of 0.5 mM PPBQ as the electron acceptor.

To show that the different herbicides specifically affect the probability of singlet oxygen formation and not generally the production of reactive oxygen species by PSII, we measured the production of superoxide anion ($O_2^{-\bullet}$) and hydroxyl ($^\bullet$OH) radicals by spin trapping. It has been shown previously that $O_2^{-\bullet}$ and $^\bullet$OH radicals are mainly formed during illumination of PSII with an inactive water splitting complex [15,39]. However, in PSII preparations with an active donor side, carbon-centred radicals were found when DMPO was used as spin trap [15,39]. Fig. 3 shows that both the $^\bullet$OH and, to a lesser extent, the $O_2^{-\bullet}$ adduct of EMPO were detected in PSII membranes, which were subjected to photoinhibition ($I = 1800$ μmol quanta m^{-2} s^{-1}) for 15 min in the presence of DCMU (Fig. 3, curve a) and bromoxynil (Fig. 3, curve b). In this assay the amount of trapped radicals was the same in the presence of bromoxynil or DCMU. The same amount of trapped radicals was also seen in the absence of herbicides (data not shown). When catalase was added to the assay prior to illumination, no signals were detected (Fig. 3, curve c). Addition of SOD eliminated the signal of the superoxide adduct, however, a small signal of the hydroxyl adduct was still detected (Fig. 3, curve d).

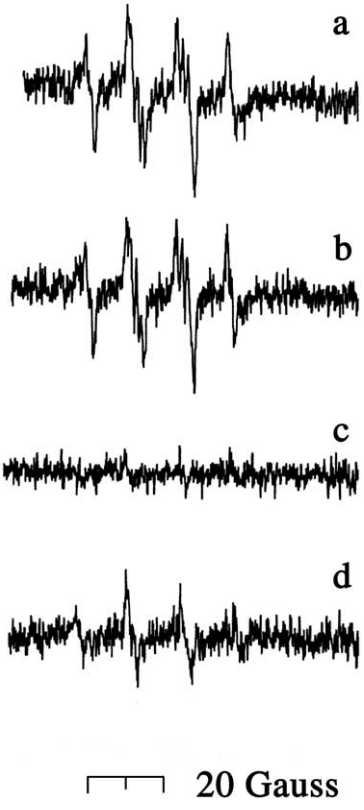

Fig. 3. Trapping of $OH^\bullet$ and $O_2^{-\bullet}$ radicals in the presence of the herbicides. PSII-enriched membrane fragments were illuminated for 15 min with red light (1800 μmol quanta m^{-2} s^{-1}) in the presence of 25 mM EMPO and (a) 20 μM DCMU or (b) 100 μM bromoxynil, (c) same as b with addition of catalase (2500 U/ml), (d) same as b with addition of SOD (375 U/ml) prior to illumination.

4. Discussion

A comparison of the amount of trapped $^1O_2$ in the presence of different herbicides showed that the yield of $^1O_2$ formation was affected by the type of herbicide. In the presence of the phenolic herbicide bromoxynil about twice as much $^1O_2$ was trapped compared to that in the presence of DCMU (Figs. 1 and 2).

The production of other reactive oxygen species, $^\bullet$OH and $O_2^{-\bullet}$ radicals, was not influenced by the different herbicides (Fig. 3). It was suggested that O_2 is reduced to $O_2^{-\bullet}$ via electron transfer from $Pheo^{-\bullet}$ or from $Q_A^{-\bullet}$ [40]. If $Q_A^{-\bullet}$ were the electron donor to O_2, an effect of the different redox potentials of Q_A on the yield of $O_2^{-\bullet}$ production would have been expected. Here we see no effect of herbicides on the yield of $O_2^{-\bullet}$ production; this seems to be more consistent with $Pheo^{-\bullet}$ being the main electron donor to O_2.

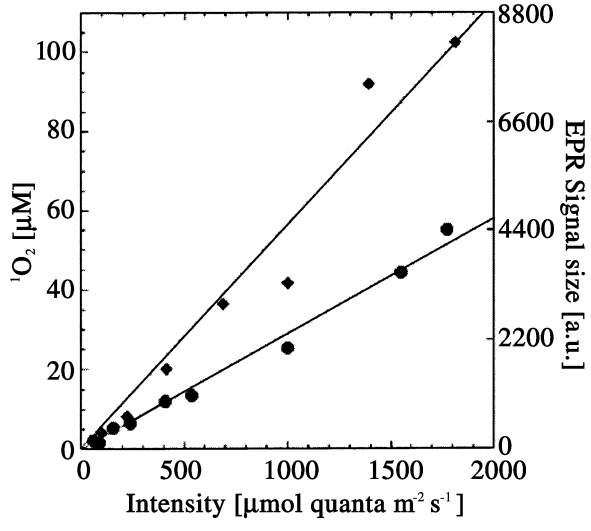

Fig. 2. Light dependence of $^1O_2$ trapping by TEMP in the presence of DCMU or bromoxynil. PSII-enriched membranes were illuminated for 15 min with red light in the presence of 10 mM TEMP and 20 μM DCMU (circles) or 100 μM bromoxynil (diamonds).

It is difficult to estimate the concentration of $^1O_2$ produced from the spin trapping experiments, however, we have obtained an approximate experimental value. The yield of $^1O_2$ formation is about 1/100 compared with the rate of oxygen evolution in the presence of a good artificial electron acceptor. The comparison, however, can only be made when we compare how many turnovers have occurred under the different experimental conditions. In the active system the rate-limiting step is assumed to be electron transfer from the reaction centre to the electron acceptor (PPBQ) and this is in the millisecond range. In the herbicide-treated system charge recombination is by far the dominant outcome of charge separation but the rate-limiting step is the lifetime of the $S_2Q_A^{-\bullet}$ state (1.5 s at 20°C [16,37]). Thus the number of charge separations (and hence recombinations in the herbicide-containing samples) that could produce singlet oxygen in the reaction centre is probably several hundred times less than the number of turnovers of the oxygen evolving enzyme.

In addition, when we take into account the following factors, (a) the less than stoichiometric yield of formation of ^3Chl upon recombination, (b) the less than stoichiometric yield of singlet oxygen from the chlorophyll triplet, (c) the effect of quenching of singlet oxygen by carotenoids and by other species in the sample, and (d) the trap efficiency, then we con-

clude that the estimated yield is bigger than would have been expected on a per turnover basis. This may be explained by a 'chain reaction' effect by which chlorophylls in damaged chlorophyll-bearing proteins become decoupled from their protective carotenoids and thus become potential sensitisers for $^1O_2$ production.

Overall the results clearly show that the type of herbicide bound to the Q_B-binding site significantly influences the yield of $^1O_2$ production. These data support the hypothesis that light-induced charge pairs in herbicide-inhibited PSII decay by a charge recombination route involving the formation of the primary radical pair ($P^{+\bullet}Pheo^{-\bullet}$) and of a chlorophyll triplet state which reacts with O_2 to form $^1O_2$ [20]. In the presence of the phenolic herbicide (bromoxynil) the midpoint potential of Q_A is lowered by about 50 mV as compared to the potential in the absence of herbicides [25] and therefore the charge recombination pathway via the primary radical pair is favoured. In the presence of DCMU, the midpoint potential of Q_A is about 100 mV more positive than in the presence of bromoxynil and the yield of $^1O_2$ formation is reduced by 50%. The safer direct recombination route is favoured which does not lead to the formation of the primary radical pair and thus formation of triplet chlorophyll and $^1O_2$.

We note that the modulation of the midpoint potential of Q_A should also result in a shorter lifetime for the $S_2Q_A^{-\bullet}$ state in bromoxynil-treated compared to DCMU-treated PSII. The predicted shorter lifetime of the $S_2Q_A^{-\bullet}$ state in the presence of bromoxynil should result in an increased number of the charge separation and recombination events. This lifetime effect, which is in fact just a different manifestation of the same charge recombination reactions that control $^1O_2$ production, is expected to amplify the difference in the yield of $^1O_2$ seen in the presence of the two different classes of herbicide.

The existence of two competing recombination pathways in PSII was suggested to rationalise the variations in the midpoint of Q_A seen upon photoactivation (light-dependent assembly of the Mn cluster) of PSII [38] and was later used to explain herbicide effects as described here [20,25]. Recently, other lines of evidence have also invoked the existence of the two competing pathways. Mutations either on the electron donor side [41] or on the electron acceptor side [42] that affect the energy gap between $P^{+\bullet}Q_A^{-\bullet}$ and $P^{+\bullet}Pheo^{-\bullet}$ do influence the recombination pathways (direct recombination versus indirect recombination via the primary radical pair). These reports and the present work lend credence to the hypothesis that PSII herbicides act by $^1O_2$-generating charge recombination reactions [20].

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