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Superoxide radicals are not the main promoters of acceptor-side-induced photoinhibitory damage in spinach thylakoids

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Abstract

Superoxide anion radical formation was studied with isolated spinach thylakoid membranes and oxygen evolving Photosystem II sub-thylakoid preparations using the reaction between superoxide and Tiron (1,2-dihydroxybenzene-3,5-disulphonate) which results in the formation of stable, EPR detectable Tiron radicals.

We found that superoxide was produced by illuminated thylakoids but not by Photosystem II preparations. The amount of the radicals was about 70% greater under photoinhibitory conditions than under moderate light intensity. Superoxide production was inhibited by DCMU and enhanced 4–5 times by methyl viologen. These observations suggest that the superoxide in illuminated thylakoids is from the Mehler reaction occurring in Photosystem I, and its formation is not primarily due to electron transport modifications brought about by photoinhibition.

Artificial generation of superoxide from riboflavin accelerated slightly the photoinduced degradation of the Photosystem II reaction centre protein D1 but did not accelerate the loss of oxygen evolution supported by a Photosystem II electron acceptor. However, analysis of the protein breakdown products demonstrated that this added superoxide did not increase the amount of fragments brought about by photoinhibition but introduced an additional pathway of damage.

On the basis of the above observations we propose that superoxide radicals are not the main promoters of acceptor-side-induced photoinhibition of Photosystem II.

Abbreviations: DCBQ – 2,5-dichloro-*p*-benzoquinone; DCMU – 3- (3,4-dichlorophenyl)-1,1-dimethylurea; DMBQ – 2,5-dimethyl-*p*-benzoquinone; DMPO – 5,5-dimethyl-pyrrolin N-oxide; Hepes – N-(2-hydroxyethyl)-piperazine-N′-(2-ethanesulfonic acid); Mes – 2-(N-morpholino)-ethanesulfonic acid; methyl viologen – 1,1′-dimethyl-4,4′-bipyridinium dichloride; PS – Photosystem; SOD – Superoxide dismutase (EC 1.15.1.1); Tiron – 1,2-dihydroxybenzene-3,5-disulphonate; Tris – 2-amino-2-hydroxymethylpropane-1,3-diol

Introduction

High intensity illumination of photosynthetic systems inhibits their electron transport (Powles 1984). This damage, which is subsequently followed by the selective degradation of the D1 protein in the reaction centre of Photosystem (PS) II, is known as photoinhibition (recent reviews by Barber and Andersson 1992; Prasil et al. 1992; Aro et al. 1993).

The reaction centre of PS II is a thylakoid membrane spanning heterodimer of two proteins, D1 and D2, which either bind or contain the redox cofactors involved in the electron transport (Namba and Satoh 1987). The bound components are: the primary electron donor chlorophyll dimer (P_{680}), the primary acceptor pheophytin (Pheo), the first and second quinone electron acceptors (Q_A and Q_B). Electrons from the catalytic cleavage of water by a manganese containing cluster bound to the lumenal side of D1/D2 are deliv-

ered to P_{680} via Tyr_Z, a redox active tyrozine residue of D1. At the reducing side of PS II, the acceptors Pheo, Q_A and Q_B transfer the electrons from P_{680} to a mobile pool of plastoquinone molecules. These reduce redox active components of a cytochrome *b/f* containing protein complex which transports electrons towards PS I (for a review see Andersson and Styring 1991 and references therein).

In photoinhibition, not only the structural damage but also its promoter, the functional impairment is targeted to PS II. Disturbance of the electron transport can initiate photoinhibition either at the acceptor or at the donor side of PS II (reviewed by Aro et al. 1993). In samples with active water cleavage system, the dominant pathway of damage is believed to be acceptor-side-induced photoinhibition. This develops as an over-reduction of PS II quinone acceptors resulting in an unusual, double reduction and in the subsequent loss of Q_A (Van Mieghem et al. 1989; Styring et al. 1990; Vass et al. 1992) and, consequently, in the formation of triplet reaction centre chlorophyll (Vass and Styring 1992, 1993). It is generally accepted that the singlet oxygen forming reaction of oxygen with $^3P_{680}$ initiates photodamage and subsequent degradation of the D1 protein. Singlet oxygen production under photoinhibitory conditions has been confirmed in PS II reaction centre complexes (Macpherson et al. 1993; Telfer et al. 1994) and in oxygen evolving thylakoids (Hideg et al. 1994b).

Free radicals formed in PS II under light stress have also been suggested to be potential damaging species in photoinhibition (reviewed by Aro et al. 1993). Recently, we demonstrated that carbon centered (methyl- or hydroxymethyl-like) free radicals are produced in thylakoid preparations during acceptor-side-induced photoinhibition and showed how their production correlated to other steps of the damage. Preceding publications suggested the involvement of oxygen free radicals also. In an earlier interpretation, light induced inactivation of the PS II acceptor side was attributed to the formation of superoxide radicals from the oxidation of the secondary quinone (Q_B) by molecular oxygen (Kyle et al. 1984; Kyle 1987). Although the key role of high yield $^3P_{680}$ (and consequently $^1O_2$) production in the acceptor-side-induced damage has been revealed, there is still no consensus whether superoxide radicals also contribute to the process.

An indication for the role of superoxide in the acceptor-side-induced process is that oxygen radical scavengers reportedly retard the degradation of the D1 protein during photoinhibition. Sopory et al. (1990) showed that general free radical scavengers, such as uric acid or propyl-gallate, delayed the photoinhibitory damage in *Spirodella* leaves. In similar experiments, four other groups demonstrated the same protective effect of various SOD containing enzyme mixtures during the photoinhibition of higher plant (Barényi and Krause 1985; Richter et al. 1990; Mishra et al. 1993) and *Euglena* thylakoid membranes (Tschiersch and Ohmann 1993). They showed that SOD and a mixture of SOD and catalase decreased the photodamage by ca. 17–25% and 15–35%, respectively. On the other hand, recently Miyao (1994) showed that, although externally added SOD prevented the D1 protein degradation to some extent in PS II membrane preparations it was not protective in PS II core and in PS II reaction centre complexes.

Notably, there is no report on complete protection against photoinhibition by any of the antioxidant enzymes. The 20–30% less damage than usually observed after a certain exposure to high light in samples containing enzyme(s) as compared to ones which do not, can be interpreted both ways: as protection (because of the 20–30% retardation) or as the lack of protection (because 70–80% is damaged even in the presence of the enzyme).

In a previous report we demonstrated that superoxide radicals are not among the main free radical species detected in photoinhibited thylakoid membranes by EPR spectroscopy utilizing the spin trap DMPO (Hideg et al. 1994a). However, experiments with DMPO can only give a straightforward answer for the involvement of superoxide radicals if these are the main radical species produced (Hiramatsu and Kohno 1987; Buettner et al. 1990). A relatively small concentration of superoxide (manifesting as a small amount of DMPO-OOH as compared to other DMPO adducts) may not be noticed (Samuni et al. 1988) or may easily be detected as DMPO-OH (Finkelstein et al. 1979, 1980). The aim of the present study was to find direct experimental evidence whether superoxide radicals were produced in thylakoids under the conditions of acceptor-side-induced photoinhibition, thus, in this work we used Tiron, a superoxide specific spin trap (Greenstock and Miller 1975; Miller and Macdowall 1975). We also studied whether superoxide radicals could participate in the degradation of the D1 protein during photoinhibition by testing the effect of artificially generated superoxide radicals.

Materials and methods

Thylakoid membranes were isolated from market spinach (Takahashi and Asada 1982), suspended in a Hepes buffer (40 mM, pH 7.5) containing 0.4 M sucrose, 15 mM NaCl and 5 mM $MgCl_2$ and kept on ice in the dark until use.

PS II enriched (BBY-type) preparations were made according to a slightly modified version of the method described by Berthold et al. (1981), stored and studied in a buffer containing 50 mM Mes (pH 6.0), 0.4 M sucrose, 10 mM NaCl and 5 mM $MgCl_2$. PS II core complexes were prepared according to Ghanotakis et al. (1987) and kept in a buffer containing 20 mM Mes (pH 6.0), 0.4 M sucrose, 10 mM NaCl, 5 mM $CaCl_2$ and 2 mM dodecyl-maltoside. In order to achieve the conditions of acceptor-side-induced photoinhibition, core complex preparations were illuminated in the above buffer at pH 6.0, without any external electron acceptor.

Photoinhibition was performed by illumination (2500 or 5000 μmol m^{-2} s^{-1}) of the different samples diluted to 100 μg chlorophyll/ml with corresponding buffer. Illumination was provided by a KL-1500 (DMP, Switzerland) lamp focused onto a stirred temperature controlled glass cuvette at 22 °C with an optical fiber guide. In samples which were illuminated under non-photoinhibitory conditions photosynthetic electron transport was driven with 250 μmol m^{-2} s^{-1} intensity light using the same setup as in photoinhibition.

Steady-state oxygen evolution was measured with a Clark-type electrode (Hansatech, England) in a 1 ml reaction mixture containing thylakoids (50 μg chlorophyll) in the above Hepes buffer and 0.2 mM DMBQ or DCBQ as electron acceptor. In thylakoid membranes, the maximal rate of DMBQ supported oxygen evolution was 278 μM O_2 mg Chl^{-1} h^{-1}. In PS II preparations and PS II core complexes, 315 and 414 μM O_2 mg Chl^{-1} h^{-1} were measured, respectively, in the presence of DCBQ.

Degradation of the D1 protein was followed by immunoblotting as described by Barbato et al. (1991). Samples were dissolved immediately after treatment in 3% SDS, 6% glycerol, 40 mM Tris (pH 6.8) and 0.3 M dithiothreitol at room temperature and the polypeptides were separated by SDS-PAGE on a 12–17% linear acrylamide gradient gel containing 6M urea. The resolved proteins were electroblotted into nitrocellulose membranes and probed with two different anti-D1 polyclonal antisera. One of them (raised against the entire spinach D1 protein and kindly provided by Dr R. Barbato) was used in studies on the photoinduced loss of D1 protein in thylakoid preparations. The other antibody (raised against a synthetic polypeptide corresponding to the C-terminus of pea D1 protein, a kind gift from Dr P.J. Nixon) was used for immunodetecting the D1 C-terminal fragments in photoinhibited PS II core preparations. Densitometric analysis of the immunodecorated blots was carried out with an LKB laser ultrascan densitometer (LKB Bromma, 2222–010).

The superoxide inducing Mehler reaction of PS I was brought about in thylakoid preparations by illumination (20 min at moderate, 250 μmol m^{-2} s^{-1} intensity white light) in the presence of 1 mM methyl viologen.

Spin trapping EPR spectroscopy was performed in the presence of either 67 mM DMPO or 10 mM Tiron. Neither DMPO nor Tiron affected the photosynthetic activity of thylakoids at the applied concentrations (data not shown). Illumination of the spin traps only (i.e. in the absence of thylakoid preparations but under the same conditions as in the experiments in their presence) did not result in an EPR signal.

When comparing the trapping abilities of DMPO and Tiron in vitro, superoxide anion and hydroxyl radicals were produced from illuminated riboflavin (Grover and Piette 1981) and from Fenton's reaction (Halliwell 1978; Haber and Weiss 1984), respectively. In studies with superoxide, 0.02 mg riboflavin was dissolved in 1 ml distilled water and illuminated for 10 min at 2500 μmol m^{-2} s^{-1} in the presence of either DMPO or Tiron. Hydroxyl radicals were produced in the presence of either spin trap in the aqueous solution of 200 μM Fe^{2+} (from ammonium ferrous sulfate, $Fe(NH_4)_2(SO_4)_2$) upon the addition of 600 μM H_2O_2. The EPR spectra of the corresponding adducts were measured 10 s after initiating Fenton's reaction.

In studies on the effect of externally added superoxide, riboflavin was added to the samples before illumination. When applying 2500 or 5000 μmol m^{-2} s^{-1} illumination, the riboflavin concentration was 0.02 or 0.01 mg/ml, respectively. In the case of non-photoinhibitory illumination (250 μmol m^{-2} s^{-1}), the riboflavin concentration was higher, 0.2 mg/ml. In vitro spin trapping experiments show that equal amounts of superoxide radicals are produced under these three conditions.

EPR spectra were measured with a Bruker ECS-106 spectrometer. X-band spectra were recorded at room temperature with 9.45 GHz microwave frequen-

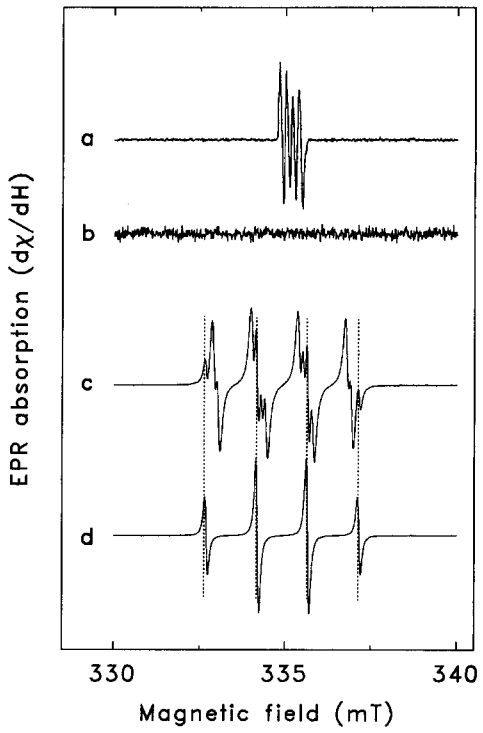

Fig. 1. EPR spectra of superoxide or hydroxyl radical adducts of Tiron or DMPO. Superoxide and hydroxyl radicals were produced from the photoreaction of riboflavin and from Fenton's reaction, respectively, as described in 'Materials and methods'. (a) Tiron + superoxide, (b) Tiron + hydroxyl radical, (c) DMPO + superoxide, (d) DMPO + hydroxyl radical. Dotted lines between the two lower traces mark DMPO-OH spectral lines. Amplification parameters are (a) 5×10^3, (b) 2×10^4, (c) 1.25×10^3 and (d) 8×10^2. Other EPR parameters are as described in 'Materials and methods'.

cy, 16 mW microwave power and 100 kHz modulation frequency, as described earlier (Hideg and Vass 1993). Free radical production under different conditions was compared on the basis of the area under the EPR absorption function (i.e. the double integral of the detected EPR signal).

Results

Detection of superoxide anions was based on the reaction of Tiron with superoxide which yields a stable EPR detectable Tiron (semiquinone) radical (Greenstock and Miller 1975; Miller and MacDowall 1975). Figure 1 shows spin trapping of free radicals from test tube reactions, i.e. without photosynthetic material. Figure 1a shows the characteristic EPR spectrum of the Tiron radical formed in a reaction between Tiron and superoxide radicals produced from illuminated riboflavin. Earlier studies on oxygen radical production in chlorophyll containing systems (Miller and MacDowall 1975) or in leaves undergoing senescence (McRae and Thompson 1983) already utilized Tiron for the identification of superoxide production. The second EPR spectrum (Fig. 1b) shows that, under our experimental conditions, contrary to earlier arguments (Van Ginkel and Raison 1980), hydroxyl radicals did not oxidize Tiron to the semiquinone radical form. Figure 1c illustrates that the application of another spin trap, DMPO, is not an unambiguous technique for detecting superoxide, since the reaction of DMPO and superoxide yields a mixture of DMPO adducts. The main product is the characteristic signal of the 12 line DMPO superoxide adduct (4 intense and 8 smaller lines, hyperfine splitting parameters: a_N = 14.3 G , a_H^β = 11.7 G and a_H^γ = 1.4 G) in accordance with the data of Harbour and Bolton (1975). Due to the decay of the DMPO-OOH adduct into DMPO-OH (Finkelstein et al. 1979) a 4 line spectrum ($a_N = a_H^\beta$ = 14.7 G, position marked by dotted lines in Fig. 1) also appears. The clear DMPO-OH signal is showed in Fig. 1d. In biological samples yielding superoxide the relative contribution of DMPO-OH and DMPO-OOH to the mixed EPR signal depends on the reactions involved, and thus superoxide anions can only be unambiguously detected with DMPO if they dominate the process (Hiramatsu and Kohno 1987; Buettner et al. 1990).

On the basis of the above results, we regarded the Tiron free radical as a selective indicator of superoxide production. Figure 2 shows that Tiron radicals are formed in illuminated thylakoid membranes (Fig. 2a), indicating the presence of superoxide anions. This superoxide production is about 70% enhanced if high intensity (photoinhibitory) illumination was applied (Fig. 2b). Previously, we found that these conditions of photoinhibition (2500 μmol m^{-2} s^{-1} intensity illumination for 20 min) result in a complete impairment of photosynthetic electron transport, as well as in the production of singlet oxygen (Hideg et al. 1994b) and carbon centered free radical (Hideg et al. 1994a). A comparison of the first and second EPR spectrum in Fig. 2 shows that, the same conditions did not enhance superoxide production extensively.

There was no marked superoxide production in thylakoids in the presence of DCMU, which blocks the electron transport between the first and second electron acceptors in PS II membrane preparations (Fig. 2c). Experiments with PS II membranes and PS II core complexes point out that superoxide production requires continuous electron flow through PS I: although these

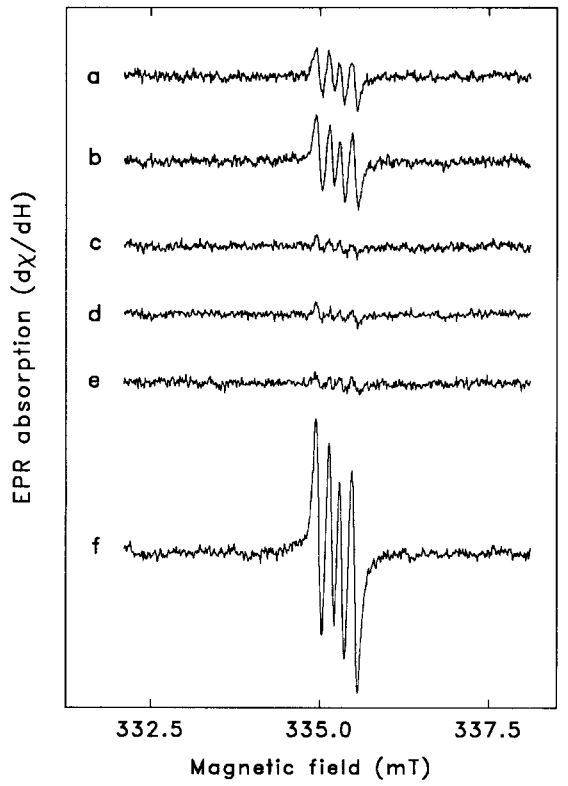

Fig. 2. EPR spectra of the Tiron radical formed in thylakoid membranes (a–c, f), PS II membranes (d) and PS II core complexes (e) in the presence of 10 mM Tiron. In experiments (c) and (f) thylakoid preparations contained 10 μM DCMU and 1 mM methyl viologen, respectively. Samples were illuminated with either 250 μmol m^{-2} s^{-1} (a, f) or 2500 μmol m^{-2} s^{-1} (b–e) intensity light for 20 min. EPR parameters are as described in 'Materials and methods'. Amplification was 2×10^4 for all spectra.

samples (containing PS II but not PS I) maintain electron transport they have no marked superoxide production (Fig. 2d and 2e).

It is well known that in thylakoids superoxide is produced by the univalent reduction of oxygen through the autooxidation of an electron acceptor in PS I under illumination (for review see Asada and Takahashi 1987). Since this process is enhanced by methyl viologen (Takahashi and Katoh 1984) we tested whether the superoxide production we observed was from this reaction. As shown in Fig. 2f, the amount of Tiron radicals (i.e. that of superoxide radicals) was markedly increased with the addition of methyl viologen to the thylakoids, even without photoinhibition. The experiments shown in Fig. 2 suggest that the superoxide production by thylakoid membranes under photoinhibition is not due to the effect of high light absorption by PS II but rather a consequence of electron flow through the acceptor side of PS I. Thus, it is not likely that photodamage in PS II is initiated by superoxide radicals. To examine this assumption more closely, we studied whether artificial, in situ generation of superoxide radicals in the sample increased the damage brought about by photoinhibition.

Illuminated riboflavin is a good, high flux source of superoxide radicals (Grover and Piette 1981). We compared functional (electron transport) and structural (D1 reaction centre protein) damage in the presence and in the absence of riboflavin. This pair of experiments was carried out under two different conditions: non-photoinhibitory (250 μmol m^{-2} s^{-1}) and photoinhibitory (2500 μmol m^{-2} s^{-1}). By adjusting the riboflavin concentration, the same amount of extra superoxide was produced under different light conditions. The superoxide concentration introduced to the samples by this procedure is about 15 times higher than the amount detected in thylakoids during photoinhibition (data not shown).

Figure 3A shows that high superoxide concentration did not change the time course of the high light induced impairment of PS II electron transport (open and closed circles, Fig. 3A). On the other hand, superoxide induced some loss of electron transport activity in samples illuminated with 250 μmol m^{-2} s^{-1} intensity light (closed squares, Fig. 3A), which does not cause significant decrease in the absence of artificial superoxide (open squares, Fig. 3A). Added superoxide also brings about an increase in the time course of D1 reaction centre protein degradation (Fig. 3B). While 2 hours of 250 μmol m^{-2} s^{-1} intensity illumination caused only 10% loss of D1 (open squares, Fig. 3B), the same treatment in the presence of superoxide resulted in 30% degradation of D1 (closed squares, Fig. 3B). This suggest that superoxide itself, without photoinhibitory illumination induces D1 damage to an extent which is about half of the protein loss caused by high intensity illumination only. On the other hand, added superoxide also accelerated D1 loss during photoinhibition (compare open and closed circles in Fig. 3B), although not as dramatically as its presence during non-photoinhibitory illumination.

We also examined whether the artificially generated superoxide influenced the fragmentation of D1 protein. Because the identification of fragments is difficult in thylakoids, PS II core complex preparations were illuminated with high intensity light in the presence or absence of riboflavin. As shown in Fig. 4, D1 protein fragmentation pattern was markedly changed by superoxide. While high intensity illumination itself

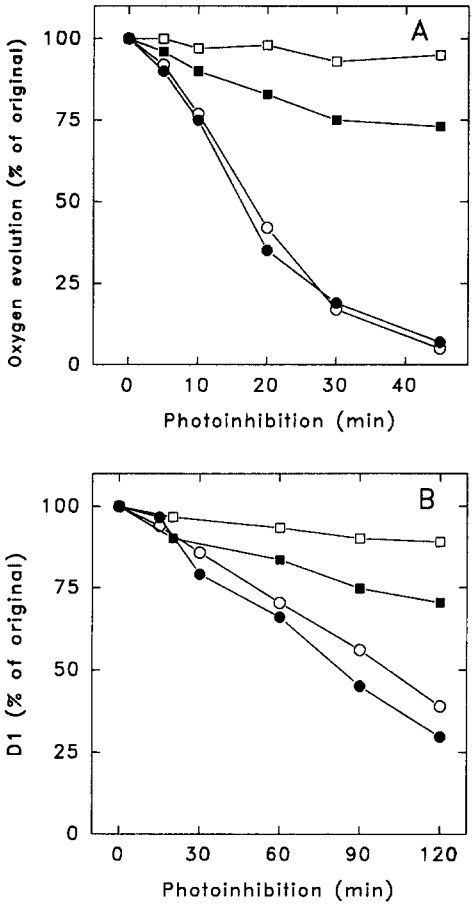

Fig. 3. Time course of oxygen evolution (A) and D1 protein (B) loss in thylakoid membranes during 250 μmol m^{-2} s^{-1} illumination (squares) or during photoinhibition achieved by 2500 μmol m^{-2} s^{-1} illumination (circles). Experiments were carried out in the presence (full symbols) or in the absence (open symbols) of superoxide radicals produced from the photoreaction of riboflavin.

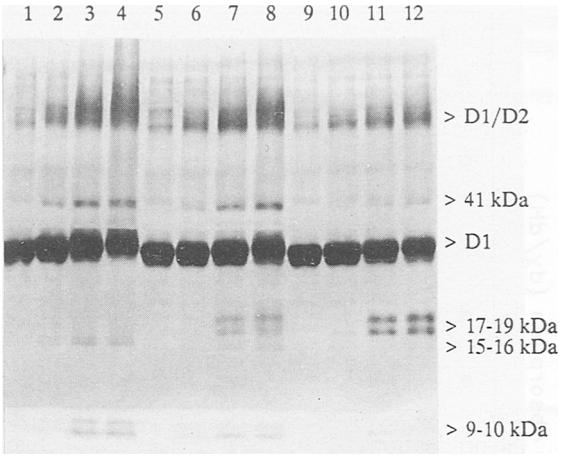

Fig. 4. Immunodetection of the photoinduced D1 protein C-terminal fragments in PS II core complexes illuminated with 5000 μmol m^{-2} s^{-1} intensity light without addition (lanes 1–4), in the presence of 0.01 mg/ml (lanes 5–8) or 0.2 mg/ml (lanes 9–12) riboflavin. Samples were kept in the dark for 60 min (lanes 1, 5 and 9), illuminated for 10 min (lanes 2, 6 and 10), 30 min (lanes 3, 7 and 11) and 60 min (4, 8 and 12) at room temperature, then solubilized in electrophoresis buffer immediately (see 'Materials and methods').

generated C-terminal products with apparent molecular weight of 15–16 and 9–10 kDa, the combination of the same light with high concentration of superoxide resulted in a decrease of these fragments and in the parallel appearance of two new fragments in the 17–19 kDa region. Also, the accumulation of two other indicators of photoinduced D1 damage, the D1/D2 heterodimer at around 65 kDa and the 41 kDa adduct, a cross linked product of D1 and the a subunit of cyt b_{559} (Barbato et al. 1992) is retarded by the presence of superoxide. Increasing the amount of riboflavin (and therefore that of superoxide) 20 times during photoinhibition made these changes even more pronounced (compare lanes 11,12 with lanes 7,8 of Fig. 4).

Discussion

Superoxide radicals are produced in photosynthetic organisms, mainly in PS I (Asada and Takahashi 1987) or, under special conditions, in PS II (Chen et al 1992; Ananyev et al. 1994). It was previously suggested that superoxide radicals promote the damage of photoinhibition both in the acceptor-side-induced (Kyle et al. 1984; Kyle 1987) and in the donor-side-induced process (Chen et al. 1992, 1995). The present study was focused on acceptor-side-induced photoinhibition, which many workers consider to be the main pathway in vivo (Aro et al. 1993).

Spin trapping EPR spectroscopy, i.e. detecting the signal from the stable product of the reaction between free radicals and spin traps (for review see Janzen 1971 and Evans 1979) has proved useful in earlier studies on photoinhibition, and experiments utilizing the spin trap DMPO demonstrated the production of hydroxyl and carbon centered free radicals (Hideg et al. 1994a). However, direct detection of superoxide with DMPO is not free of experimental limitations: although superoxide can be trapped by DMPO and the DMPO-OOH and DMPO-OH adducts are well distinguishable, DMPO-OOH may decompose into a false DMPO-OH adduct (Finkelstein et al. 1979). The use of Tiron as a spin trap for the identification of superoxide overcomes this uncertainty (Fig. 1).

Spin trapping EPR spectroscopy in the presence of Tiron demonstrated that superoxide radicals are produced in illuminated thylakoids and the production rate is increased under photoinhibitory conditions (Fig. 2). This superoxide production was inhibited by DCMU and was not present in PS II membranes or core complexes even with high intensity illumination. These observations show that superoxide production is not related to PS II. On the other hand, superoxide production by thylakoids is markedly (4–5 fold) enhanced by illumination in the presence of methyl viologen, a result suggesting that it originates from the Mehler-reaction in PS I (Asada and Takahashi 1987). Studying the production rate of Tiron radicals in thylakoids, Miller and coworkers demonstrated that the formation of superoxide is far slower than the oxidation of Tiron by superoxide (Greenstock and Miller 1975; Miller and MacDowall 1975), thus Tiron production directly monitors superoxide production by PS I. One may speculate that the superoxide which is photoproduced in PS I promotes the damage of PS II, but the fact that photoinhibition results in both electron transport and D1 protein loss not only in thylakoids (containing both PS II and PS I) but also in PS II preparations makes this possibility very unlikely.

We also studied whether the addition of riboflavin (i.e. parallel induction of superoxide production and photoinhibition) effected light induced damage. The rationale of this experiment was that if superoxide takes part in photoinhibition at any level, a large increase of its concentration should cause dramatic increase in damage. We found that in thylakoids the presence of superoxide did not affect the light induced impairment of DMBQ supported oxygen evolution by PS II under photoinhibitory conditions (2500 μmol m^{-2} s^{-1}) but it accelerated D1 protein degradation to some extent (from 50% D1 loss in 105 minutes to that in 80 min) (Fig. 3). The former result is in agreement with the results of spin trapping EPR spectroscopy suggesting that normally superoxide production is unrelated to PS II. The promotion of protein damage by superoxide can be interpreted in two ways: (i) superoxide radicals produced in PS I contribute significantly to photoinduced D1 degradation and thus an additional production of them from riboflavin accelerates the damage, or (ii) the superoxide radicals from PS I do not participate in acceptor-side-induced D1 degradation but the addition of external superoxide triggers a new pathway of D1 damage.

There are two experiments on photosynthetic electron transport activity supporting the latter assumption. First, additional superoxide, when combined with high intensity illumination, does not change the time course of the functional damage of DMBQ supported oxygen evolution, i.e. it does not influence the process responsible for initiating further, structural damage. Second, the addition of superoxide by itself, without high intensity illumination, can inactivate DMBQ supported oxygen evolution. The extent of this damage is similar to that of D1 protein loss (approx. 20–25% in 45 min, Fig. 3) suggesting that in the superoxide induced process, contrary to the sequence of events in photoinhibition, oxygen evolution is lost as a consequence of structural damage to the D1 protein PS II reaction centre.

Photoinhibition results in the selective degradation of the D1 reaction centre protein into specific fragments. The main fragments generated in acceptor-side-induced photoinhibition are two C-terminal fragments of 10 and 16 kDa and a 23 kDa N-terminal fragment (Barbato et al. 1991; Salter et al. 1992). In order to study the effect of superoxide on D1 protein degradation further, the C-terminal fragments produced in photoinhibition in the presence and in the absence of riboflavin were compared. Because the actual amount of fragments produced in the photoinhibition of thylakoids is very low (probably due to the rapid proteolytic digestion of the primary fragments or to their release from the membrane, Aro et al. 1993), we used oxygen evolving PS II core complexes in this experiment. The results clearly indicate that superoxide anions themselves, or products of their further reactions, attack a cleavage site which is different from the sites of photoinhibition induced fragmentation (Fig. 4). The apparent molecular weight of the superoxide induced fragments (17–19 kDa) suggest that this new, artificial cleavage site is within the lumen exposed loop between the C and CD helices, a site easily accessible for the externally generated superoxide. Our results infer that even though the presence of superoxide accelerates D1 protein degradation, this is due to an additional process and not to the enhancement of the reactions of acceptor-side-induced photoinhibition.

Previous reports on systems containing SOD are partly in favour (Barényi and Krause 1985; Sopory et al. 1990; Richter et al. 1990; Tschiersch and Ohmann 1993) and partly against (Miyao 1994) the importance of superoxide in photoinhibition. Unfortunately, it is difficult to judge the involvement of superoxide on the basis of the effect of addition of SOD, especially if it is used in combination with other enzymes. The protection achieved in such experiments may not be due to

SOD or may not be due to its enzyme activity. Similarly to results by Barényi and Krause (1985); Sopory et al. (1990); Richter et al. (1990) and Tschiersch and Ohmann (1993), we also found that the addition of SOD somewhat retarded the light induced impairment of oxygen evolution by thylakoid membranes. However, this delay of photodamage was characteristic to samples with more severe electron transport damage by photoinhibition than to the less affected ones: the 80% loss of DMBQ supported oxygen evolution caused by 30 min photoinhibition in the absence of SOD was reduced to 60% loss by the presence of 300 U/ml SOD, but the 40% loss brought about by 15 min photoinhibition was not affected by the presence of SOD (data not shown). Moreover, the same protection was achieved by adding heat inactivated SOD or BSA (data not shown), suggesting that protection by SOD does not necessarily indicate the involvement of superoxide radicals in photoinhibition. It should not be overlooked that SOD can itself be the target of active oxygen and effect the course of damage this way. For example, in vitro experiments proved that SOD reacts with singlet oxygen, and its quenching constant is about 10–12 times higher than that of histidine, which is among the most efficient, specific targets of singlet oxygen (Matheson et al. 1975). The inability of SOD to protect against photoinhibition in vivo was recently demonstrated by Herbert et al. (1992), who reported that although cyanobacteria mutants lacking of SOD activity were more sensitive to oxidative stress caused by methyl viologen or 100% oxygen atmosphere than wild types, but both strains were equally sensitive to photoinhibition of PS II by exposure to strong light.

In summary, we propose that although superoxide radicals are produced in illuminated thylakoids they do not take part in the series of damages occurring under the conditions of acceptor-side-induced photoinhibition of PS II. Nevertheless, the role of superoxide under more complex stress conditions should not be underestimated. When light stress is combined with other stress factors which may promote superoxide production (e.g. temperature stress, air pollution or herbicide effect), it is very likely that the reactions of these radicals are crucial.

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