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Biochimica et Biophysica Acta 1186 (1994) 143–152

BBA Biochimica et Biophysica Acta

Singlet oxygen and free radical production during acceptor- and donor-side-induced photoinhibition. Studies with spin trapping EPR spectroscopy

Éva Hideg *, Cornelia Spetea, Imre Vass

Institute of Plant Biology, Biological Research Center, Hungarian Academy of Sciences, P.O. Box 521, H-6701 Szeged, Hungary

Received 1 February 1994

Abstract

High-intensity illumination of thylakoids results in the well-characterized impairment of Photosystem II electron transport (photoinhibition), followed by the degradation of the D1 reaction centre protein. The time-course and features of photodamage are different in fully functional thylakoid membranes, when photoinhibition is invoked by impairment of Photosystem II acceptor side electron transport, and in thylakoids which are unable to oxidize water, when the damage is a consequence of inactivation of Photosystem II donor side (reviewed by Aro, E.-M., Virgin, I. and Andersson, B. (1993) Biochim. Biophys. Acta 1134, 113–134). In the present study we followed the production of singlet oxygen and free radicals during both types of photoinhibition by EPR spectroscopy. Singlet oxygen was detected by following the formation of 2,2,6,6-tetramethylpiperidine-1-oxyl, a stable nitroxide radical yielded in the reaction of singlet oxygen with the sterically hindered amine 2,2,6,6-tetramethylpiperidine. Free radicals were detected as spin adducts of the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide, and identified on the basis of hyperfine splitting constants of the EPR spectra. We found the following. (i) Singlet oxygen, a non-radical form of active oxygen, was detectable only in samples undergoing acceptor-side-induced photodamage. (ii) The acceptor-side-induced process was accompanied by the oxygen dependent production of carbon centred (alkyl or hydroxyalkyl) radicals, probably from the reaction of singlet oxygen with histidine residues. (iii) Donor-side-induced photoinhibition was dominated by hydroxyl radicals, which were produced in anaerobic samples, too. The production rate of these radicals, as well as D1 protein degradation, was dependent on the possibility of electron donation from manganese ions to Photosystem II. The marked distinction between the active oxygen forms produced in acceptor- and donor-side-induced photoinhibition are in agreement with earlier reports on the different mechanism of these processes.

Key words: Thylakoid membrane; Photosystem II; Light stress; Oxidative damage; Active oxygen; Free radical

1. Introduction

Light, the driving force of life for photosynthetic organisms, also may promote their damage. High-intensity illumination of plants, their thylakoid membranes or sub-thylakoid fragments causes a complex set of stress reactions, known as photoinhibition [1]. While the time-course of the damage or the chance for a subsequent repair depends on the type of preparation as well as on the experimental conditions, the main target of photoinhibition is the reaction centre of Photosystem (PS) II in all preparations [1–4]. In higher plants, photoinhibition involves the functional impairment of photosynthesis in PS II electron transport followed by the structural damage of one of the PS II reaction centre proteins, D1, as reviewed in Refs. [3] and [4].

The reaction centre of PS II is a heterodimer of the D1 and D2 proteins in close association with cytochrome *b*-559 [5]. The D1 and D2 proteins bind or contain the molecules necessary for the primary processes of photosynthesis. Light energy is absorbed in or

Abbreviations: DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DMPO-OH, hydroxyl radical adduct of DMPO; DMPO-R, carbon-centred radical adduct of DMPO; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid; MDA, malondialdehyde; PS, Photosystem; TEMP, 2,2,6,6-tetramethylpiperidine; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl.

* Corresponding author. Fax: +36 62 434434.

SSDI 0005-2728(94)00039-8

transferred to the reaction centre chlorophyll (P680), which then conveys one electron to the primary electron acceptor, pheophytin (Pheo). After this primary separation, the charge pair on $P680^+$ $Pheo^-$ is stabilized in a series of redox reactions involving the first (Q_A) and second (Q_B) quinone acceptors on the acceptor side and a tyrozine residue of D1 (Tyr_Z) on the donor side. The lumenal side of the D1/D2 heterodimer provides the binding site for the catalytic Mn cluster of water oxidation (for review see Ref. [6]).

Depending on the physiological state of the sample, photoinhibition can be invoked by the photodamage of either the acceptor or the donor side of PS II ([2,4,7] and references therein). If photosynthetically active thylakoid membranes are illuminated with high-intensity light, photoinhibition is promoted by reactions occurring at the inhibited acceptor side. It was concluded that a light-induced over-reduction of the acceptor side results in an unusual, double reduction of Q_A [8–10]. This state, which is not present during normal photosynthesis, is inactive in mediating the photosynthetic electron transfer but promotes the formation of triplet chlorophyll ($^3P680$) in the reaction centre with a high yield [10–13]. Triplet chlorophyll is known to react with the triplet ground state of oxygen, producing the highly reactive singlet oxygen, as reviewed in Refs. [14] and [15]. Therefore, singlet oxygen was suggested to play an important role in damaging the D1 protein [10,16–18]. The presence of singlet oxygen during photoinhibition was further demonstrated by detecting its infrared chemiluminescence in isolated PS II reaction centre complexes [17] and its EPR active spin adduct in thylakoids [19].

There is a consensus that the damage of the D1 protein is a consequence of the light induced impairment of the PS II electron transport. D1 degradation is generally described in two main steps: First, the protein is triggered (i.e., marked for degradation in a yet unidentified way) by a photophysical process, which is followed by the light independent, probably proteolytic, degradation of the triggered protein [4,20–22]. The fragmentation of the triggered protein is well characterized. However, the details of the triggering step itself are not well understood. Recently, we found that in spinach thylakoids, during the time-course of acceptor-side-induced photoinhibition, the production rate of singlet oxygen was maximal when the degradation of D1 began and suggested to regard this as an evidence for the participation of singlet oxygen in marking D1 for degradation [19].

Singlet oxygen may directly attack important amino acids, such as histidines, of the D1 protein or may induce a conformational change of the reaction centre complex by damaging its chlorophyll or carotenoid components and thus expose the D1 protein to the proteolytic activity [21]. Singlet oxygen, which is a powerful active oxygen form but not a free radical, may also induce the formation of oxygen free radicals [14,15] whose involvement in photoinhibition has also been suggested on the basis of experiments in which externally added radical scavengers or protective enzymes partially prevented photodamage [22–24]. One aim of the present study is to use spin trapping EPR spectroscopy for searching direct evidence whether free radicals are formed in thylakoids during acceptor-side photoinhibition.

An other pathway, different from the above described mechanism, is the photoinhibition invoked by the photodamage in PS II donor side. This occurs if the electron transport from the water splitting enzyme to $P680^+$ is significantly slower than the rate of electron withdrawal from the reaction centre. In this case the damage is attributed to the generation of abnormally long lived states of cation radicals $P680^+$ and Tyr_Z^+ [25–30] which could attack chlorophylls, carotenoids or amino acids [2,28,30]. The generation of superoxide radicals has also been proposed [31]. Donor-side-induced photoinhibition has been extensively studied in vitro, in isolated reaction centre complexes or in PS II preparations in which the water oxidizing enzyme has been deactivated before photoinhibition [25–32], but it is also considered as a significant contributor to the photoinhibition of intact systems in vivo [4]. Contrary to the photodamage induced by acceptor side inactivation, donor-side-induced photoinhibition can partly proceed under anaerobic conditions [32,33] or under low light intensity [25]. Regarding the above differences, the second aim of the present study was to compare the active oxygen forms (singlet oxygen and radicals) produced in the thylakoid membrane during acceptor-side-induced and donor-side-induced photoinhibition.

Here, we report data on the detection of singlet oxygen and free radicals via their reactions with a sterically hindered secondary amine (TEMP) and a spin trap (DMPO), respectively, yielding long lived nitroxide radicals detectable by EPR spectroscopy. This sensitive technique enabled us to determine the kinetics of nitroxide production at various stages of photoinhibition in untreated and Tris-washed spinach thylakoids (model systems for acceptor- and donor-side-induced photoinhibition, respectively) and discuss the possible role of the trapped species in the photodamage.

2. Materials and methods

Thylakoid membranes were isolated from market spinach as described by Takahashi and Asada [34] and suspended in a Hepes buffer (40 mM, pH 7.5) containing 0.4 M sucrose, 15 mM NaCl and 5 mM $MgCl_2$.

Tris-washing, which removes Mn and the water soluble subunits of the water splitting enzyme [35] was performed as described in Ref. [36]. Tris-treated thylakoids were pelleted by centrifugation (5000 × *g*, 10 min), re-suspended under dim light in the above Hepes buffer containing 20 mM $NaHCO_3$ and stored on ice in the dark until use. As a result of this treatment, the oxygen evolving ability of the samples was decreased by more than 85%. When indicated, 20 mM Na-EDTA or 5 mM $MnCl_2$ was added to the Tris-washed thylakoids before photoinhibition. This extra manganese was removed from the $MnCl_2$ treated samples before EPR measurements by adding 20 mM Na-EDTA.

Untreated or Tris-treated samples, diluted with the

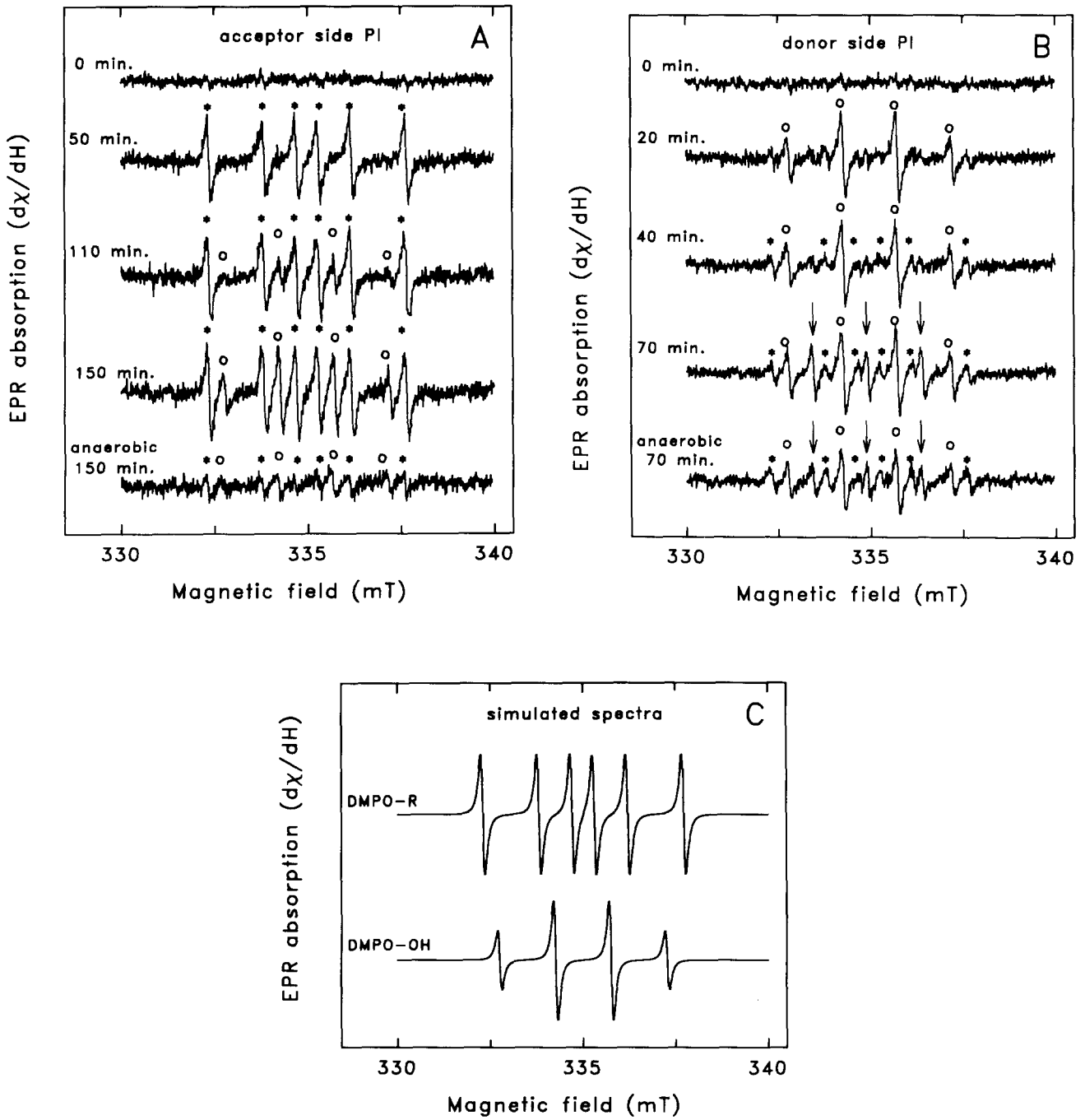

Fig. 1. EPR detection of free radicals trapped by DMPO in untreated (A) and Tris-washed (B) spinach thylakoids exposed to photoinhibition for the time indicated in the figures. Asterisks mark EPR spectra of the carbon centred radical adduct of DMPO (hyperfine splitting constants $a_N = 1.47$ mT and $a_H^\beta = 2.26$ mT), circles indicate spectra of DMPO-OH ($a_N = a_H^\beta = 1.47$ mT). Traces labeled as anaerobic represent samples exposed to photoinhibition while bubbled with argon. Downward arrows in (B) mark the triplet EPR spectrum from damaged DMPO. (C): EPR spectra simulated on the basis of the hyperfine splitting constants estimated from the spectra in Figs. A and B.

Hepes buffer to 100 μg chlorophyll/ml, were exposed to high-intensity (1000 $\mu E/m^2$ per s) illumination from a Tungsten lamp while being stirred in a temperature-controlled glass cuvette at 22°C.

Degradation of the D1 protein was followed by immunoblotting as described by Barbato et al. [37]. SDS-PAGE was performed on a 12–17% linear acrylamide gradient gel containing 6 M urea. The resolved proteins were electroblotted and identified by using an antibody raised against the D1 protein (kindly provided by Dr. R. Barbato). Densitometric analysis of the immunodecorated blots was performed using a Bio-Rad densitometer (Bio-Rad 1650) in reflectance mode, attached to a Hewlett Packard integrator (HP 33941).

Singlet oxygen was determined according to the method of Lion et al. [38,39], in samples containing 10 mM TEMP, by measuring the EPR absorption of the stable nitroxide radical (TEMPO) which is produced from the reaction between $^1O_2$ and TEMP. In order to avoid artifacts due to the production of *N*-hydroxylamine from the nitroxide, samples were extracted into ethylacetate and aired in the presence of catalytic amount of PbO_2 before EPR spectroscopy, as described earlier [19]. Spin trapping EPR spectroscopy was performed in the presence of 67 mM DMPO (5,5-dimethyl-1-pyrroline *N*-oxide) [40,41]. Neither TEMP nor DMPO 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 thylakoids but under the same conditions as in the experiments with thylakoids) did not result in an EPR signal.

The interaction of $^1O_2$ with histidine was studied in a reaction mixture containing 1 mM Toluidine blue, 100 mM histidine and 50 mM DMPO.

Free radical production during anaerobic photoinhibition was studied in argon atmosphere. Samples were bubbled with argon for 10 min under dim light before starting photoinhibition and the gas flow was maintained above the sample during the whole course of the experiment. The argon treatment, which removed more than 95% of the oxygen from the sample (as measured with an oxygen electrode, data not shown), was applied instead the glucose – glucose-oxidase – catalase system, because these enzymes may modify the course of nitroxide formation.

The rate of the nitroxide formation, both the spin adduct of DMPO and TEMPO, was measured in short intervals. In order to achieve this, untreated samples were pre-illuminated with high-intensity light, then TEMP or DMPO was added to the illuminated suspension and photoinhibition was continued for 15–20 min. Samples were collected regularly during this period of time and the rate of nitroxide production was calculated from the increase of EPR absorption (double integral of the detected EPR signal). This way, the decay of the nitroxide radicals into EPR-silent forms, which occurs in the hours time-scale, did not distort the data.

EPR spectra were measured with a Brucker ECS-106 spectrometer. X-band spectra were recorded at room temperature with 9.45 GHz microwave frequency, 16 mW microwave power and 100 kHz modulation frequency, as described earlier [42]. In order to insure comparative EPR quantitation, all spectra of spin traps in thylakoid membranes were measured under identical experimental conditions regarding both the samples (volume, concentration, uniform sample holders and the same central position in the cavity) and the EPR parameters (constant gain and modulation). Data collection and all calculations were performed utilizing the spectrometer's ECS-106 data acquisition software.

The general structural destruction of the photosynthetic membrane was estimated by measuring the production of malondialdehyde (MDA) from 1 ml alliquotes of samples photoinhibited for the time intervals shown in the figures. MDA was detected on the basis of its reaction with tiobarbituric acid yielding a pink chromogen, which was measured spectrophotometrically as $\Delta A_{532\ nm}$, according to Ref. [43]. The assay was calibrated using samples containing known concentrations of 1,1,3,3-tetraethoxypropane and untreated thylakoids.

3. Results

Extending our earlier study in which singlet oxygen was trapped by a sterically hindered amine [19] we have performed spin trapping EPR spectroscopy in photoinhibited thylakoids with DMPO. The spin trap itself is diamagnetic (has no EPR signal), but upon reacting with a free radical its radical adduct is paramagnetic (well detectable with EPR). The shape of the EPR spectrum, determined by the hyperfine splitting constants, is characteristic of the radical trapped by DMPO [40,41,44].

Fig. 1A and B show EPR spectra of nitroxide radicals produced in reactions between the free radicals formed during acceptor-side- and donor-side-induced photoinhibition in thylakoids, respectively, and the spin trap DMPO. There was no detectable radical formation in untreated samples. (This also demonstrates the purity of the spin trap, i.e., that the applied DMPO was not contaminated or damaged during handling and storage, the major source of artifacts in spin trapping experiments [41].)

As shown in Fig. 1A, there is free radical production during acceptor-side-induced photoinhibition. The free radical dominant after 50 min and DMPO yield a nitroxide radical which gives a six line EPR spectrum (marked with asterisks in Fig. 1). The hyperfine split-

ting constants obtained from this spectrum ($a_N = 1.47$ mT, $a_H^\beta = 2.26$ mT) are characteristic to a carbon centred, probably alkyl or hydroxyalkyl, adduct of DMPO (DMPO-R) [44]. The EPR spectrum calculated with these hyperfine splitting constants is shown as the upper trace of Fig. 1C. In thylakoids photoinhibited for more than 50 min, an other type of nitroxide radical (marked with circles in Fig. 1A) appears besides DMPO-R. Its hyperfine splitting constants ($a_N = a_H^\beta =$ 1.47 mT, see simulated spectrum in Fig. 1C) are the same as the ones reported for DMPO-OH [41,44], demonstrating that a small amount of hydroxyl radicals are produced in the latter phase of photoinhibition. Free radical production is markedly smaller in samples undergoing anaerobic photoinhibition (compare the last two traces in Fig. 1A). This indicates that the radicals trapped during the acceptor-side-induced process, both oxygen and carbon centred ones, are produced in a reaction requiring oxygen.

Free radicals are also produced during the donor-side-induced photoinhibition occurring in Tris-washed thylakoids. However, contrary to the acceptor-side-induced reactions, the hydroxyl radicals dominate this process (Fig. 1B). More prolonged photoinhibition results in the appearance of other free radicals, too. The EPR spectra in Fig. 1B illustrate that besides DMPO-OH (circles), some DMPO-R (asterisks) is also present. The last spectrum in Fig. 1B shows that, contrary to the acceptor-side-induced process, these radicals are produced at a high yield even in anaerobic samples.

The three-line EPR spectrum (marked with arrows in Fig. 1B) is probably from DMPO molecules damaged by some product of donor-side-induced photoinhibition. One possibility is that a strong oxidizing agent replaces the β-hydrogen on a spin adduct of DMPO. However, the direct oxidation of DMPO, similar to the reaction described by Floyd and Soong in the study of free radical production in hematin [45], can not be excluded, either. In both cases, the hyperfine splitting in the oxidized DMPO product is provided by the nitrogen atom only, which results in a three line EPR spectrum, independently from the nature of the trapped radical(s).

In a previous work we have demonstrated $^1O_2$ formation during acceptor-side-induced photoinhibition. It is very important to understand how $^1O_2$ and free radical formation are related to each other and to protein and membrane damage. To this end we measured the rate of active oxygen production during photoinhibition and compared these to the time-course of photodegradation of the D1 protein and the membrane structure. The production rate of free radicals and singlet oxygen was measured in several, relatively short time intervals, as the production rate of DMPO spin adducts and TEMPO, respectively. The precursors of these nitroxides (DMPO and TEMP, respectively)

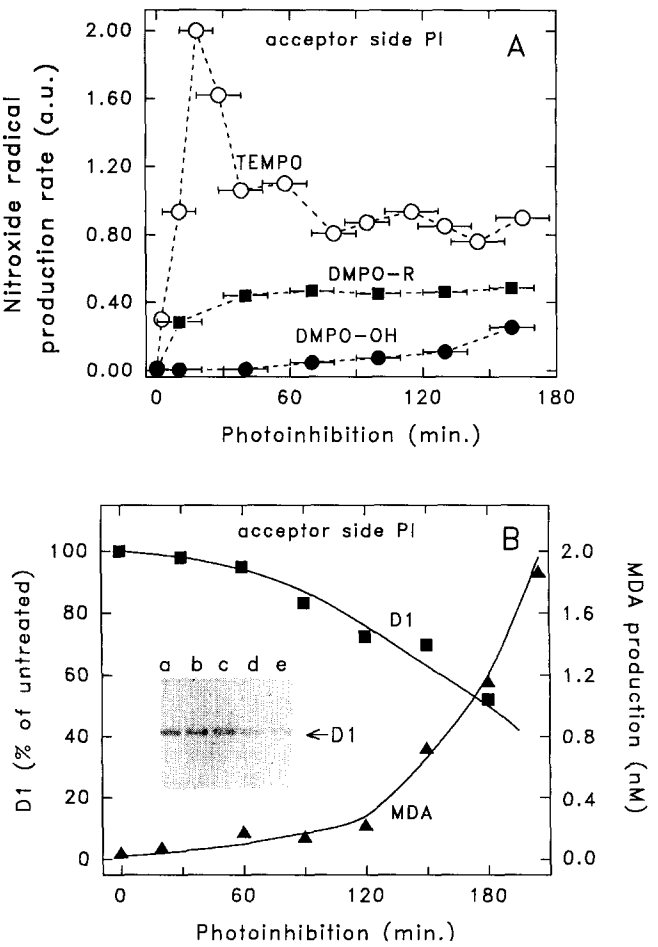

Fig. 2. (A) Production rates of TEMPO (○), DMPO-OH (●) and a carbon centred free radical adduct of DMPO (■, DMPO-R) in spinach thylakoids exposed to photoinhibition. Horizontal bars mark the time interval in which the trapping rate represented by the corresponding symbol was determined. (B) The kinetics of D1 protein degradation (■) and MDA production (▲) during photoinhibition. The insets show immunoblots serving as the basis of D1 degradation data. For the immunoblot thylakoids were kept in the dark for 180 min (a), photoinhibited for 30 (b), 90 (c), 120 (d) or 180 (e) min.

were only added to the sample at the beginning of these intervals (see Materials and Methods for details). Horizontal bars in Figs. 2A and 4A correspond to these intervals in which the production rate represented by the matching symbol is valid. Besides the loss of D1 protein, photodegradation was also characterized by the production of malondialdehyde (MDA), an indicator of lipid peroxidation and other breakdown products of general membrane destruction [43,46].

During acceptor-side-induced photoinhibition active oxygen formation is dominated by $^1O_2$ (Fig. 2A). The production rate of carbon centred radicals is increasing during the first 40–50 min, although not as fast as the rate of $^1O_2$ production (observed as TEMPO). After this initial rise, DMPO-R is produced at an approximately constant rate, indicating that the carbon cen-

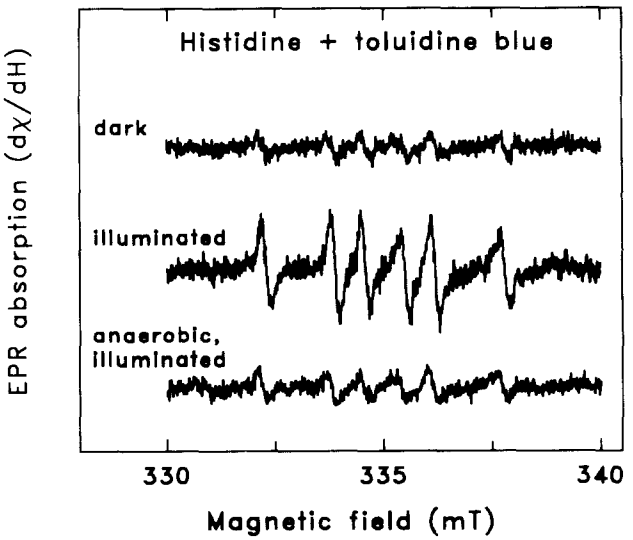

Fig. 3. EPR spectra of the radical adduct trapped by DMPO in the reaction mixture containing Toluidine blue and histidine in the dark (upper trace) and during illumination in the presence of oxygen (middle trace) or under anaerobic conditions (lowest trace).

tred radicals are continuously yielded and trapped (Fig. 2A). D1 protein degradation starts later than the formation of $^1O_2$ and DMPO-R. However, the time range of intense D1 protein degradation agrees with the range of constant $^1O_2$ and DMPO-R production, while the late appearance of DMPO-OH corresponds to the more general membrane damage featuring MDA production after 90–100 min (compare Figs. 2A and 2B).

In order the examine the possibility of a connection between $^1O_2$ and carbon centred radical production during acceptor-side-induced photoinhibition we studied the free radicals generated from the interaction of $^1O_2$ and histidine. This amino acid was chosen because histidine reportedly quenches $^1O_2$ with a high rate [47] and in PS II histidines provide the binding site for the reaction centre chlorophyll [48,49], the proposed site of $^1O_2$ production [10,16,19]. In this experiment $^1O_2$ was generated photodinamically, from the interaction of a triplet dye (illuminated Toluidine blue) and oxygen [50] in the presence of the amino acid. The free radicals yielded from this reaction were trapped with DMPO. As it is shown in Fig. 3, the interaction of $^1O_2$ and histidine resulted in the production of the same DMPO-R radical adducts as the ones detected in thylakoids during acceptor-side-induced photoinhibition. The appearance of the characteristic six line EPR spectrum required both illumination and the presence of oxygen (Fig. 3), indicating that the amino acid reacted with the $^1O_2$ molecules which were produced from oxygen in the photodynamic process. Similar EPR spectra were observed when repeating the above experiment with imidazole, but not with tyrosine (data not shown).

Singlet oxygen and free radical production, D1 protein and membrane damage are shown during the progress of donor-side-induced photoinhibition in Fig. 4. A striking distinction between acceptor and donor-side-induced photoinhibition is the almost complete absence of singlet oxygen production during the latter process. Singlet oxygen is produced at a high rate from the beginning of acceptor-side-induced photoinhibition, but we have failed to observe TEMPO production in Tris-washed thylakoids exposed to high-intensity illumination (compare hollow circles in Figs. 2A and 4A). The other, marked difference is that contrary to acceptor-side photoinhibition, the dominant free radical species of donor-side-induced photodamage are hydroxyl radicals, produced at a high rate from the beginning of the process (full circles in Fig. 4A). The production rate of DMPO-OH was increasing rapidly, practically from the beginning of illumination. It reached a constant level after about 30–40 min and

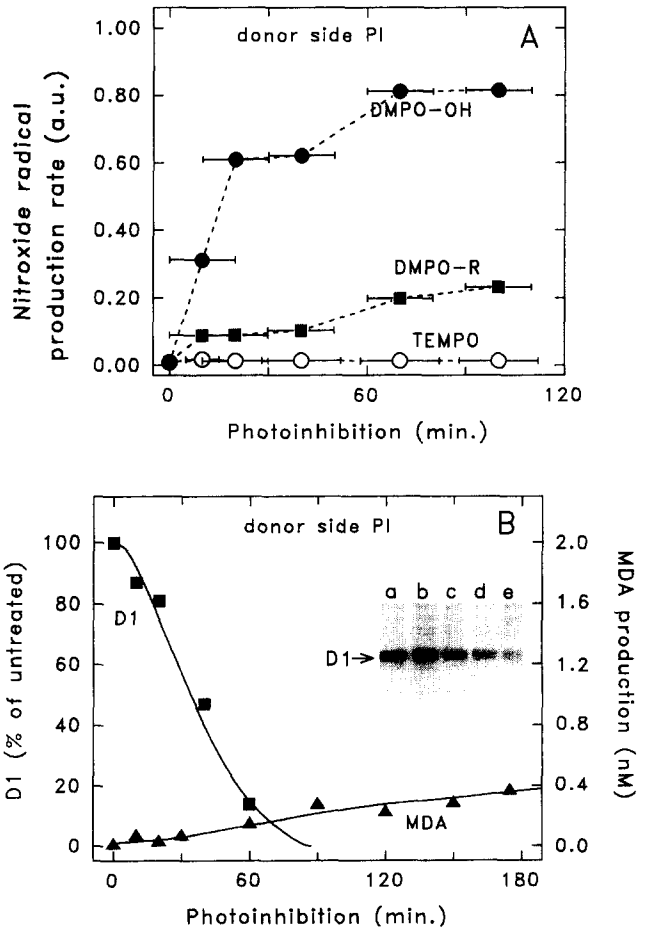

Fig. 4. (A) Production rates of TEMPO (○), DMPO-OH (●) and a carbon centred free radical adduct of DMPO (■, DMPO-R) in Tris pre-treated spinach thylakoids exposed to photoinhibition. (B) The kinetics of D1 protein degradation (■ and MDA production (▲) in these samples. Data in (B) were obtained from samples which were kept in the dark for 60 min (a), photoinhibited for 10 (b), 20 (c), 40 (d) or 60 (e) min. Other details are the same as for Fig. 2.

this constant rate was maintained for the rest of the process (Fig. 4A). Some carbon centred radicals appear later (full squares in Fig. 4A), 50–60 min after the onset of illumination, when MDA formation also becomes detectable (Fig. 4B). D1 degradation starts after 10–20 min of illumination, when hydroxyl radical production has already reached an intense rate, and continues under the conditions of the high, constant level of OH radical formation.

The degradation of D1 protein was about four times faster in Tris-washed thylakoids than in untreated ones (compare Figs. 2B and 4B). This ratio is smaller than the one reported by Jegerschöld et al. [26], which could be explained by the lower light intensity applied in our experiments. In agreement with the results of Hundal [51], we also found that photoinhibition was accompanied by MDA formation in its later phase. Examining both acceptor- and donor-side-induced photodamage, we found that MDA production was smaller in the latter case (compare Figs. 2B and 4B).

D1 protein degradation during donor-side-induced photoinhibition is probably initiated by the presence of long lived, oxidizing radicals (Tyr_Z^+ and $P680^+$) at the donor side of PS II [25–30]. In order to investigate the possible connection between the presence of long lived Tyr_Z^+ ($P680^+$) and hydroxyl radical production as well as D1 protein degradation we modified the extent of electron donation to the donor side of Tris-washed thylakoids. In one experiment we used Mn^{2+}, which acts as an electron donor to PS II, most likely *via* Tyr_Z^+, whose lifetime is therefore decreased [25]. Manganese ions (added as $MnCl_2$), delayed both D1 degradation and hydroxyl radical formation (Fig. 5). The opposite effect was observed when the manganese ions which are loosened and inactive in oxygen evolution but not completely removed from their binding site during the Tris-washing were extracted with EDTA. The addition of EDTA increases the lifetime of Tyr_Z^+ in Tris-treated samples (Vass, I. and Ghanotakis, D., unpublished) and in our experiment it accelerated the damage of D1 protein as well as the increase of OH* production rate (Fig. 5). The above results indicate that D1 degradation and OH* production are both influenced by the rate of electron donation to Tyr_Z^+ and $P680^+$.

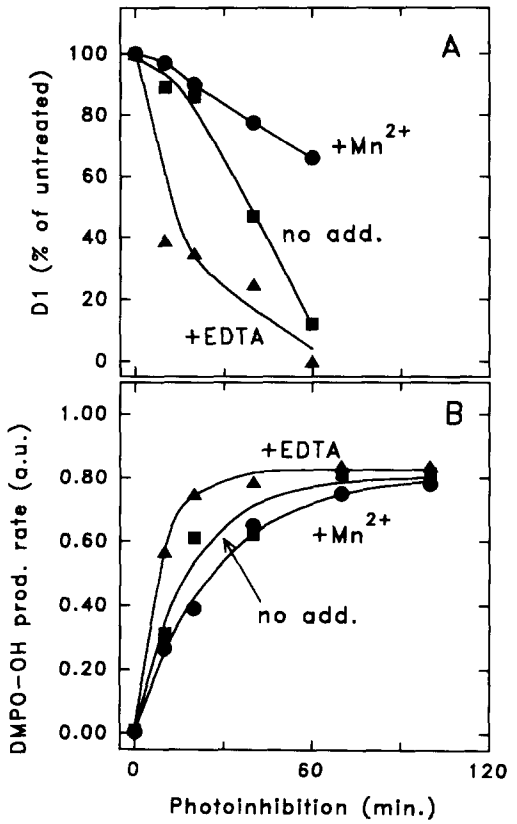

Fig. 5. Comparison of (A) D1 protein degradation and (B) hydroxyl radical production rate in Tris pre-treated thylakoids during photoinhibition in the presence of no additional chemicals (■), 20 mM EDTA (▲) or 5 mM $MnCl_2$ (●).

4. Discussion

In addition to our earlier results demonstrating the production of singlet oxygen in photodamaged thylakoids [19,52] we show that $^1O_2$ is only produced if photoinhibition is initiated by the impairment of PS II acceptor side: there is no detectable $^1O_2$ production in Tris-washed thylakoids undergoing donor-side-induced photoinhibition (Figs. 2A and 4A). This is consistent with the model deriving singlet oxygen in thylakoids from the reaction between ground state oxygen ($^3O_2$) and the triplet reaction centre chlorophyll ($^3P680$) formed as a result of acceptor-side damage [10–13]. In donor-side-photoinhibited samples the dominant light-induced state of P680 is not the triplet but the radical cation state ($P680^+$) [25–30]; hence, the above singlet oxygen yielding reaction is not likely to occur. This difference between the two types of photodamage has been supported by the observation that donor-side-induced photoinhibition does not require the presence of oxygen [32,33] and it is now further confirmed by our data demonstrating that the anaerobic condition suppresses radical formation during acceptor-side-induced photoinhibition but it has little effect on the donor-side-induced process (Fig. 1).

Our experiments with the spin trap DMPO prove that both acceptor-side- and donor-side-induced photoinhibition are accompanied by free radical production (Fig. 1). This is in agreement with earlier suggestions that free radicals may be involved in the photodamage of D1 [22–24]. Various oxygen radicals (hydroxyl, peroxide, superoxide) form nitroxides with DMPO which have characteristic, well-distinguishable EPR spectra [40,41,44]. This way, the use of this spin trap enabled

us to identify the radicals trapped during the process. The hyperfine splitting constants from EPR spectra of the spin trapped DMPO adducts demonstrate that while donor-side-induced photoinhibition was dominated by hydroxyl radical formation, acceptor side induced photoinhibition initiated the production of carbon centred, probably alkyl or hydroxyalkyl, radicals (Fig. 1). These were among the ones found earlier in chloroplasts exposed to an other type of stress: methylviologen poisoning [53,54].

The superoxide radicals suggested to damage hydroxylamine pre-treated, photoinhibited PS II membranes in a study by Chen et al. [31] were not detected in our experiment. Because various spin adducts of DMPO may be converted to DMPO-OH by chemical or enzymatic processes [55], it can not be excluded that some other oxygen radicals, e.g., superoxide, are also present but they are detected as DMPO-OH. However, this process can not appear as a major artifact, because DMPO is reportedly applicable for the detection of superoxide in thylakoids, if these radicals dominate the process [53]. This way, while our spectra demonstrate that donor-side-induced photoinhibition is dominated by hydroxyl radicals, the contribution of other free radicals (of which carbon centred ones are directly detected) can not be excluded either. Our observation of DMPO-OH during donor-side-induced photoinhibition suggests that the failure of its detection in the acceptor-side-induced process is not due to a limited access of DMPO to the production site, as suggested in a forthcoming publication of Shiraishi et al. [56], but rather to the much lower production rate of OH* during acceptor-side-induced photodamage, as compared to other radicals.

Comparing the kinetics of D1 damage and the yield of DMPO adducts demonstrates that, during the progress of both types of photoinhibition, the production rate of the dominant free radical increases prior to the loss of D1 (Figs. 2 and 4). This implies that the radicals are formed in PS II centres damaged by photoinhibition and may be involved in the sequence of events leading to D1 degradation. This is supported by the observation that radical production during acceptor-side-induced photoinhibition requires oxygen (Fig. 1A), similarly to singlet oxygen formation and D1 degradation [3,10], while donor-side-induced D1 damage [32,33] and free radical production (Fig. 1B) proceeds in the absence of oxygen. Participation of free radicals in damaging the D1 protein would also be in line with earlier reports on the partial protection of D1 against photoinhibition by free radical scavengers [22–24].

In acceptor-side photoinhibition, singlet oxygen or its products were suggested to initiate the D1 protein degradation [10,19,21]. The data presented here show that the degradation process is accompanied mainly with carbon centred radicals, contrary to other suggestions on the participation of hydroxyl radicals [57]. Since high yields of radical production were observed earlier than the start of D1 protein degradation but later than the onset of singlet oxygen production, it is possible that the radicals are produced in a radical cascade initiated by $^1O_2$ and participate in the attack on D1. This is supported by our observation that the attack of $^1O_2$ on histidine yields carbon-centred radicals in a model experiment (Fig. 3). In the histidine molecule $^1O_2$ is captured by the imidazole ring resulting in the formation of an unstable *trans*-annular peroxide which quickly decomposes [58]. These data imply that the carbon centred radical trapped in the model experiment (also detected from $^1O_2$ + imidazole, not shown) is a breakdown product of histidine. This way, the observation of this radical in thylakoids during acceptor-side-induced photoinhibition suggests the following model: Acceptor-side impairment of PS II results in the formation of ^{3}P680 [10–13] and consequently $^1O_2$ [10,16,17]. Singlet oxygen attacks crucial histidine residues of the reaction centre (e.g., those which bind chlorophylls). The breakdown of histidine yields carbon centred radicals and results in a (possibly conformational) modification of the reaction centre making D1 accessible to proteolytic degradation. The carbon-centred radicals may participate in the further fragmentation of the D1 protein, but the precise mechanism of this process has yet to be established.

This model provides a straightforward explanation for the specific bleaching of P680 observed in isolated reaction centre complexes during photoinhibition [21,28] as a consequence of the damage in the ligating histidine residues. On the other hand, a direct attack of $^1O_2$ on P680 which has been suggested as the triggering event of D1 degradation [21,59] parallel to the damage of histidines can not be excluded, either. However, the similarity of this reaction (involving illuminated chlorophyll, oxygen and amino acids) to the ones used in model experiments in which the photodynamically generated singlet oxygen is always likely to react with proteins than with its substrate dye ([58] and references therein) favours the former possibility: bleaching of the reaction centre chlorophyll as a consequence of amino acid damage.

In Tris-washed thylakoids photoinhibition is dominated by hydroxyl radicals. Because the most characteristic feature of donor-side-induced photodamage is the formation of the unusually long-lived, strong oxidizing radicals ($P680^+$ and Tyr_Z^+) [25–30], it has been suggested that they could initiate the damage via oxidizing nearby amino acid residues or electron transport components [30]. A plausible explanation of our data is that the observed hydroxyl radicals are produced in these oxidizing reactions. However, while there are several reports on hydroxyl radical attack on proteins

(for review see Ref. [60]) making them susceptible to proteolytic hydrolysis [61,62], we have failed to find reference to cation radical attack on amino acids yielding hydroxyl radicals. Nevertheless, our data demonstrate that hydroxyl radicals are produced and the time-course of their production rate, as well as that of D1 degradation, was delayed by the increase and accelerated by the decrease in the life-time of the oxidizing cation radicals (Fig. 5). Further implication of the connection between the two features is that neither hydroxyl radical production (Fig. 1B) nor D1 protein degradation is suppressed in the absence of oxygen [32,33]. These similarities suggest that hydroxyl radicals may be involved in the process targeting the protein for degradation and perhaps also participate in the breakdown process.

The exact mechanism of free radical attack on D1 is not yet understood. Recent experiments with free radicals generated by radiolysis show that protein damage by radicals can lead to unfolding as a result of amino acid oxidation, decarboxylation or deamination [60–62]. This way, the free radicals generated during photoinhibition may promote the exposure of D1 to the proposed protease [4]. However, it is noteworthy that OH* production is driven at high rates even in the late phase of photoinhibition when most of the D1 protein is already damaged. Under these conditions no primary charge separation and therefore neither Tyr_Z^+ nor $P680^+$ formation is expected. A possible explanation is the onset of other hydroxyl radical yielding reactions as a consequence of a more general membrane photodamage in reaction centres lacking D1. The presence of more complex radical sources in this late phase than in the earlier (D1 protein degrading) stage of photoinhibition is supported by the appearance of the donor-side-induced carbon centred radicals (Fig. 4B) (which, contrary to the ones dominating the acceptor-side-induced process are also produced under anaerobic conditions, Fig. 1B) and by the increase in the amount of damaged DMPO molecules (data not shown).

It is a common characteristic of both acceptor- and donor-side-induced photoinhibition that the first part of the process, in which D1 is degraded to about 60–70%, features only one type of free radical: radicals other than the dominant one are only detected in the later phase of damage (Figs. 2 and 4). Because MDA production (an indicator of lipid peroxide radicals and other membrane degradation products) is also characteristic to this later phase, we assume that the reactions yielding several different types of radicals (as it is discussed above, DMPO-OH can show some superoxide or peroxide, too) are associated with a more complex, more general membrane damage, possibly involving lipids or other proteins, too. The observed difference in MDA production between samples undergoing acceptor- and donor-side-induced photoinhibition may imply that the pathway of this late damage is different in the two types of sample. However, because the MDA assay is easily affected by the chemistry of the sample (e.g., the presence of strong oxidizers [46]), it can not be excluded that the data are distorted by artifacts more dominant in samples suffering donor-side-induced photodamage.

The difference in the pathway of radical reactions, featuring singlet oxygen and the carbon centred radical in acceptor-side-induced photoinhibition in contrast to $P680^+$ and hydroxyl radicals in donor-side-induced photoinhibition might be correlated with the different pattern of D1 protein fragments observed in the two types of photodamage [63,64].

The appearance of free radicals prior to the onset of D1 protein degradation but following singlet oxygen or $P680^+$ formation implies that these radicals are involved in the events leading to the degradation of the D1 protein in both types of photodamage, as intermediates. However, the possibility that the observed free radicals are produced in the photoinhibited centres, independently from or as by-product of D1 protein degradation can not be excluded either.

Acknowledgements

This work was supported by research grants from the Hungarian Academy of Sciences (OTKA/III-888, OTKA/6241) and UNIDO-ICGEB(GE/GLO/89/001 No. 91/54). We thank Dr. Roberto Barbato (Department of Biology, University of Padova, Italy) for his gift of the antibodies and Professor Kálmán Hideg (Central Research Laboratory of Chemistry, Medical University of Pécs, Hungary) for providing us high purity TEMP and DMPO.

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