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Formation of radicals from singlet oxygen produced during photoinhibition of isolated light-harvesting proteins of photosystem II

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Abstract

Electron spin resonance spectroscopy and liquid chromatography have been used to detect radical formation and fragmentation of polypeptides during photoinhibition of purified major antenna proteins, free of protease contaminants. In the absence of oxygen and light, no radicals were observed and there was no damage to the proteins. Similarly illumination of the apoproteins did not induce any polypeptide fragmentation, suggesting that chlorophyll, light and atmospheric oxygen are all participating in antenna degradation. The use of TEMP and DMPO as spin traps showed that protein damage initiates with generation of $^1O_2$, presumably from a triplet chlorophyll, acting as a Type II photosensitizer which attacks directly the amino acids causing a complete degradation of protein into small fragments, without the contribution of proteases. Through the use of scavengers, it was shown that superoxide and H_2O_2 were not involved initially in the reaction mechanism. A higher production of radicals was observed in trimers than in monomeric antenna, while radical production is strongly reduced when antennae were organized in the photosystem II (PSII) complex. Thus, monomerization of antennae as well as their incorporation into the PSII complex seem to represent physiologically protected forms. A comparison is made of the photoinhibition mechanisms of different photosynthetic systems.

Keywords: Reactive oxygen species; Light-harvesting complex; ESR; Protein degradation; Singlet oxygen; Photoinhibition

1. Introduction

Most of the chlorophyll on Earth is bound to a class of homologous proteins called light-harvesting proteins which are localized in the thylakoid membranes of plant chloroplasts. In higher plants, these proteins are nuclear encoded and divided into two groups, namely the major and minor antenna proteins, differing in their relative abundance, topological location, function and corresponding number of genes. The major antennae (LHCII) are the most abundant membrane protein complexes known and contain up to 50% of the total chlorophyll in the thylakoid membrane. They can be grouped into three protein families: Lhcb1, Lhcb2 and Lhcb3 [1]. Both major and minor antenna proteins (called Lhcb4, Lhcb5, Lhcb6) contain three transmembrane α-helices and bind chlorophyll *a*, chlorophyll *b* and xanthophyll as pigments. The largest difference between major and minor antennae is that the former are organized in trimers in their functional state, and dissociate into monomers under high light intensity, where they are reduced quantitatively by proteolysis [2] and/or chemical degradation [3]. In fact, although the primary function of photosynthetic light-harvesting complexes is the absorption of light and the transfer of the excitation energy to the photochemical reaction centers, they are also essential for regulation and distribution of excitation energy within the photosynthetic apparatus, and respond to both short- and long-term fluctuations in light intensity and quality [4–7]. The LHCII proteins carry out these regulatory functions through different mechanisms: phosphorylation, dissociation into monomers, migration from grana to stroma, binding of xantophyll, etc. (for a review see Ref. [8]). However, it is well known that when plants are exposed to higher light intensities, they become prone to photoinhibition [9] unless they activate mechanisms to decrease the light interception

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or dissipate the excess energy in a harmless way to avoid damage to the photosynthetic apparatus [10]. Although there is general consensus that the primary target of photoinhibition is the reaction center of photosystem II (PSII) [11,12] also the light-harvesting complexes are very susceptible to excess light, but so far only few studies on the antenna proteins have been reported. Recent work has shown that thylakoids isolated from *Phaseolus vulgaris* plants possess proteolytic activity directed against the LHCII, which is developmentally regulated and seems to originate in a peripherally bound protease [13–15]. A similar proteolytic activity directed against LHCII has been detected during high light acclimation of spinach leaves, and ascribes also to a protease peripherally bound to thylakoids [2,16]. A 68-kDa polypeptide, considered to be part of a light-inducible protease (SppA), isolated lately from thylakoids of *Arabidopsis thaliana* [17], has been suggested to be involved in the degradation of PSII antenna complexes. However, up to now the identity of the protease and the location of the substrate recognition site for the regulatory protease remain unknown.

As an alternative explanation, the possible involvement of reactive oxygen species in the in vitro degradation of the LHCII proteins was recently investigated with isolated monomeric, trimeric, and BBY systems [3]. We found that, in these isolated subcomplexes, active oxygen is involved in LHCII protein degradation by random cleavage, starting in the NH_2 terminal region and resulting in the complete destruction of the antenna proteins [3]. It is well known that a light-activated sensitizer can transfer energy from its triplet state to molecular oxygen with generation of singlet oxygen ($^1O_2$, Type II mechanism), or interact with solvent or substrates by electron or hydrogen transfer with generation of radicals (Type I mechanism) [18]. Chlorophyll may act as sensitizer to produce active oxygen species, since Chl triplets have been detected in many PSII enriched systems [19] and components, including outer [20–22] and inner antenna [23,24] complexes. Thus the formation of singlet oxygen via this pathway would provide an attractive mechanism for light-induced damage to the light-harvesting proteins, but this has never investigated in detail. The aim of the present study was to use spin trapping ESR spectroscopy to search for direct evidence that free radicals are formed in isolated light-harvesting proteins upon visible light irradiation.

2. Materials and methods

2.1. Materials

Magnesium chloride, sodium chloride, methanol and glacial acetic acid were obtained from Carlo Erba (Milan, Italy). Acrylamide, *N*,*N*′-methylene-bis-acrylamide, and all other reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Bio-Rad (Segrate, Italy). Sucrose, tricine, tris-(hydroxymethyl) aminomethane (Tris), *n*-dodecyl β-D-maltoside, *n*-octyl β-D-glucopyranoside (OG), 2[*N*-morpholino]-ethanesulfonic acid (MES), 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), as well as scavengers were obtained from Sigma (Milan, Italy).

2.2. Isolation of the PSII antenna systems by sucrose-gradient ultracentrifugation

The light-harvesting complex was isolated from the PSII membranes as previously described [25] with the following modifications: PSII membranes were pelleted by centrifugation at 10 000 × *g* for 5 min at 4 °C, suspended in B3 buffer (50 mM MES pH 6.3, 15 mM sodium chloride, 5 mM magnesium chloride) at 1.0 mg/ml chlorophyll and then solubilized by adding 1% (w/v) *n*-dodecyl β-D-maltoside. Unsolubilized material was removed by centrifugation at 10 000 × *g* for 10 min. The supernatant was rapidly loaded onto a 0.1–1.0 M sucrose gradient containing B3 buffer and 5.0 mM *n*-dodecyl β-D-maltoside. The gradient was then spun on a Kontron Centrikon T-1080 ultracentrifuge equipped with a TST 41.14 rotor at 39 000 rpm for 18 h at 4 °C. Green bands were collected with a syringe. The HPLC analysis (data not shown) of these green bands revealed that band 2 contained a mixture of the protein components of the major and minor PSII antenna systems, whereas band 3 essentially contained the protein components of the major PSII antenna system, as previously reported [25,26].

Monomeric major antenna proteins were generated by incubation of the trimeric complex with 3-μg phospholipase A2/ml according to Zolla and Rinalducci [3]. For preparation of LHCII apoprotein, the purified LHCII trimer was washed three times with 80% (v/v) acetone. After centrifugation the white pellet was solubilized in 0.1% (w/v) SDS and dialyzed against 1% (w/v) OG overnight.

2.3. Photoinhibitory light treatments

Photoinhibition was normally performed on LHCII samples diluted to 0.1 mg chlorophyll/ml, and exposed to different light intensities at room temperature. The scavengers used were histidine at a concentration of 10 mM, 1,4-diazabicyclo[2.2.2]octane (DABCO) 1 mM and sodium azide (NaN_3) at a concentration of 2 mM for $^1O_2$ [27,28]; *n*-propyl gallate 1 mM for hydroxyl and alkoxyl radicals [29]; superoxide dismutase (SOD) and catalase 100 μg/ml for $O_2^{-\bullet}$ and H_2O_2, respectively.

2.4. Electrophoresis

Denaturing SDS-PAGE was carried out in 12–17% acrylamide gradient gels (20 cm × 16 cm × 1.5 mm) containing 7 M urea, using a Protean II Biorad gel-electrophoresis system. Gels were run at 20 °C for 16 h at constant current of 20 mA using 25 mM Tris/192 mM glycine buffer, pH 8.8, containing 0.1% SDS. Gels were fixed and stained

for 3 h in a 5:1:4 (v/v) methanol–glacial acetic acid–water mixture, containing 0.1% (w/v) Coomassie blue.

2.5. Electron spin resonance spectroscopy

Samples for ESR measurements were prepared under dim light and typically contained LHCII diluted to 0.1 mg chlorophyll/ml and 80 mM DMPO (5,5-dimethyl-1-pyrroline *N*-oxide) in the B3 buffer. A volume of approximately 50 μl was drawn into glass capillaries, sealed and measured at room temperature with a Bruker ESP300 spectrometer equipped with a TE_{110}-mode resonator, using 10-mW power at 9.79 GHz. Spectra were recorded using 1.0-G modulation and 100-G scanning in 21 s. For kinetics experiments the signal intensity of a single line in the spectrum was monitored continuously for at least 22 min, using 2.1-G modulation and an instrument time constant of 1.3 s. Some experiments were made using gas-permeable TPX capillaries (Wilmad, Buena, NJ) to allow a controlled oxygen level in the sample. Samples were irradiated by light directly during the measurements with a Schott KL-1500 fiberoptics lamp giving nominal light intensities from 350 to 6500 μmol m^{-2} s^{-1} photosynthetically active radiation. These values should be considered relative; the effective light intensity is difficult to determine since 50% is cut off by the illumination window grid but incident light is reflected efficiently from all walls of the resonator. The light did not contain wavelengths below 330 nm, which eliminated the possibility of photochemical reactions of DMPO [30].

Detection of singlet oxygen was performed by following the formation of nitroxide radicals produced in illuminated LHCII samples containing 10 mM TEMP (2,2,6,6-tetramethylpiperidine) after extraction into ethyl acetate as described [3].

2.6. HPLC separations

PSII antenna proteins were separated by a reversed phase column under the following experimental conditions. The Vydac C-4 columns were pre-equilibrated with 40% (v/v) aqueous acetonitrile solution containing 0.1% (v/v) trifluoroacetic acid (TFA) and samples were eluted by a first linear gradient from 40% to 88% (v/v) acetonitrile in 90 min, followed by a second gradient segment from 88% to 100% (v/v) acetonitrile in 1 min. Finally 10-min isocratic elution with 100% acetonitrile was used for washing out hydrophobic contaminants of the PSII antenna system from the column. The flow rate was 1.0 ml/min.

3. Results and discussion

The light-harvesting complexes of PSII are known to be very susceptible to excess light, but so far only few studies on the molecular mechanism by which these antenna proteins are removed have been reported [2,3,15,16].

Fig. 1 shows the SDS-PAGE of a mixture of purified spinach major antenna proteins exposed to two different light intensities (100 and 1000 μmol m^{-2} s^{-1}) as a function of irradiation time at room temperature. Coomassie staining of the SDS-PAGE revealed that at low light intensity (Fig. 1A) the proteins showed a small increase in electrophoretic mobility which became more prominent at longer times, indicating a small light-induced loss of mass. On the contrary, at high light intensity (Fig. 1B) bands on the SDS gels appeared blurred already after the first hour; the decrease in electrophoretic mobility (2–3 h) was accompanied by a drastic decrease of Coomassie staining intensity, indicating that the proteins are destroyed. Thus it seems that at low light intensity a simple process was activated which became more complex and rapid at higher light intensities. However, in the absence of oxygen there were neither changes of the electrophoretic mobility nor any Coomassie staining decrease independent of the light intensity, suggesting that atmospheric oxygen played an essential role in the degradation. Similarly proteins kept in the presence of oxygen but in the dark did not shown any damage (data not shown), indicating that the phenomenon observed requires both light and atmospheric oxygen. To further investigate the process by which degradation takes place, we took the apoprotein of each major protein, obtained by treating native proteins with acetonitrile or acetone to remove all chlorophylls, and subjected it to an illumination intensity of 1000 μmol m^{-2} s^{-1} for 3 h. The SDS-PAGE of the apoprotein before or after illumination gives the same electrophoretic pattern, confirming the role for chlorophylls in inducing the fragmentation observed (Fig. 1B). In this connection, it is important to stress that in all experiments the material used was previously dialyzed to remove any

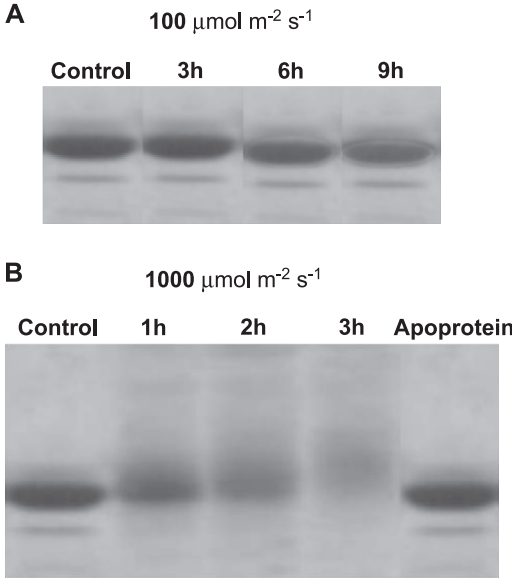

Fig. 1. SDS-PAGE of LHCII after prolonged exposure to low (A) or high intensity (B) illumination. The apoprotein sample was exposed to high intensity light for 3 h.

free chlorophyll from the solution, in order to avoid production of spurious radicals. Moreover, it should be emphasized that under our experimental conditions only bands corresponding to antenna protein were observed, and no other proteins, such as potential proteases, could be detected on the gels even after silver staining (not shown).

3.1. Formation of free radicals

To identify the reactive oxygen species responsible of protein degradation and the succession of the events which cause this degradation, isolated trimeric LHCII were illuminated with photoinhibitory light levels directly during measurements of the ESR spectra in the presence of TEMP or DMPO as spin traps. TEMP is used specifically to detect singlet oxygen: if it is present TEMP is transformed into TEMPO, a stable nitroxide radical, which can be detected by ESR spectroscopy [31]. Fig. 2A shows ESR spectra produced from the reaction of TEMP and singlet oxygen in isolated LHCII preparations when they are exposed to low light intensity. Under these conditions DMPO-HO$^{\bullet}$ seems not be produced in significant amounts, indicating that at low intensity light the prevalent reactive species found is singlet oxygen. It has been shown that at low light intensity the primary cleavages take place in the hydrophilic portion of the NH_2 region [3]. At higher light intensity the complete degradation of proteins is observed and consequently a complex radical production should be expected. Under these experimental conditions, TEMP is not sufficient to describe the total reactions that may occur; consequently, the use of the spin trap DMPO enabled us to identify the free radicals trapped during the photoinhibition process and discuss their possible role in the photodamage. Moreover, by this spin trap it has been possible to follow the kinetics of molecular photoinhibition which is induced by high light intensity.

The ESR spectra of oxygen-saturated samples containing trimeric LHCII and the spin trap DMPO showed only traces of radicals when kept in the dark; these signals could be assigned to DMPO impurities and radicals formed due to dim light exposure during sample preparation. When dark-adapted samples were irradiated continuously with high intensity white light, the formation of two different radical species could be observed (Fig. 2B). Initially the characteristic four-line signal of the hydroxyl adduct of DMPO was seen (DMPO-HO$^{\bullet}$; hyperfine splittings $a^N = a^H = 14.9$ G). Within 1 min, the six-line signal of a second radical appeared in the spectrum; from the splitting constants this radical could be identified as a trapped carbon-centered alkyl or alkoxyl species (DMPO-R$^{\bullet}$; hyperfine splittings $a^N = 15$ G, $a^H = 22.5$ G). Such radicals are typical of the small fragments produced during radical-mediated degradations of proteins and lipids, but unfortunately, a large variety of carbon-centered radicals give DMPO adducts with very similar ESR spectra, making it impossible to characterize the radical more precisely [32,33]. When the same experiments were carried out using LHCII apoprotein, there was no formation of radicals at all; only the trace amount of spurious radicals present initially in the dark-adapted sample could be detected and remained throughout the period of illumination (spectra not shown).

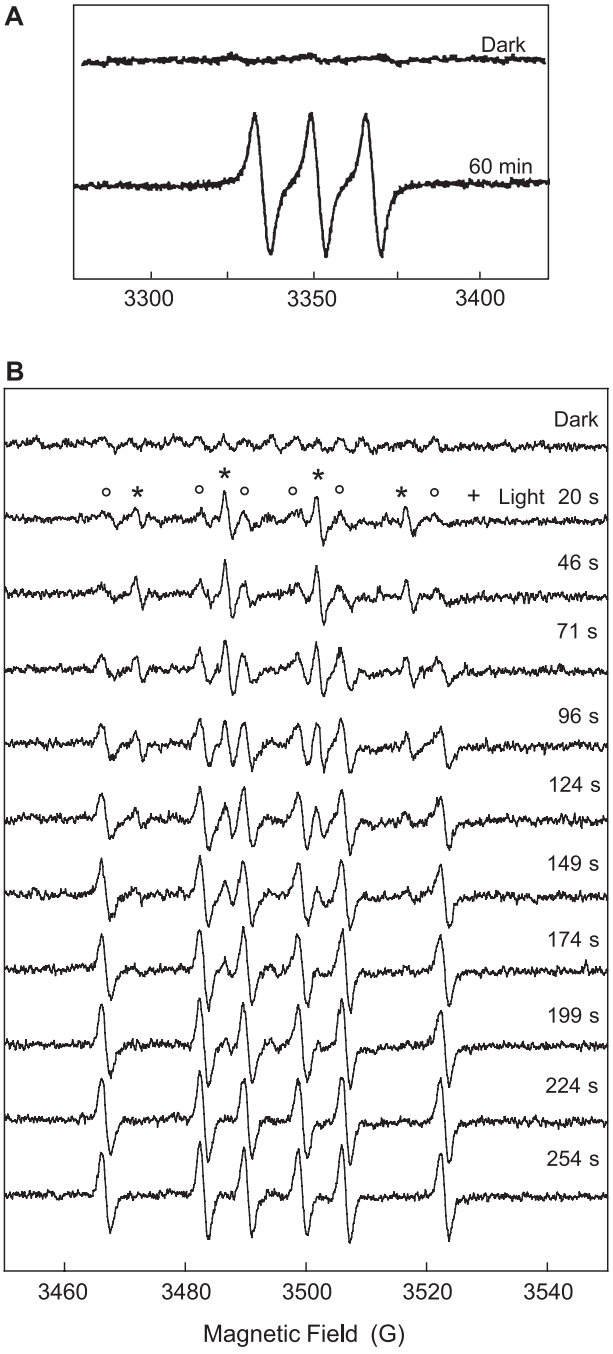

Fig. 2. ESR spectra of spin-trapped radicals generated during illumination of LHCII in the presence of 10 mM TEMP (A) or 80 mM DMPO (B). The duration of illumination indicated includes the scan time of the spectrum; the light intensity was 100 μmol m^{-2} s^{-1} in (A) and 3300 μmol m^{-2} s^{-1} in (B). The symbols indicate line components belonging to DMPO-HO$^{\bullet}$ (∗) and DMPO-R$^{\bullet}$ (○). The spurious signals seen in dark-adapted samples (top) remained constant for 30 min if the sample was not illuminated.

More information about the reaction mechanism was obtained from the behavior of formation and disappearance of the two radical types. In these measurements the size of a single peak in the ESR spectrum was monitored continuously; this procedure allowed precise determination of the reaction kinetics although only one radical species could be followed in each experiment (Fig. 3). At high light intensities the DMPO-HO$^{\bullet}$ signal reached a plateau within seconds after the onset of irradiation; this type of kinetics is very common for free radicals in solution and is normally due to reactions involving two radicals. When the radical concentration increases the reaction between them, tipically dismutation or dimerization, becomes more and more frequent, and at the plateau value the rate of formation is identical to the rate of disappearance [34]. Surprisingly, after a few minutes the DMPO-HO$^{\bullet}$ signal disappeared rapidly, indicating that the steady-state conditions were no longer maintained. This suggested that a component necessary for DMPO-HO$^{\bullet}$ formation was no longer available. Since the LHCII chlorophylls maintained their characteristic green color and appeared still intact, it is reasonable to assume that the rapid decay of the DMPO-HO$^{\bullet}$ signal was due to the consumption of all oxygen in the sample (see below). Upon extensive irradiation at high light levels the samples became yellow and eventually white as a result of photobleaching, however, this phenomenon did not occur under the experimental conditions selected for the radical studies reported in this work.

3.2. Sources of the DMPO-HO$^{\bullet}$ signal

What is the origin of the DMPO-HO$^{\bullet}$ radical? The detection of DMPO-HO$^{\bullet}$ does not necessarily mean that the photoinhibitory process involves hydroxyl radicals directly; apart from free HO$^{\bullet}$ trapped by DMPO, there are various potential sources for the ESR signal of DMPO-HO$^{\bullet}$, such as hydroxyl, peroxide and superoxide radicals. In fact, it is well known that DMPO-HO$^{\bullet}$ may be formed as a result of a Fenton-type reaction involving H_2O_2, or by decomposition of the DMPO adduct with superoxide, DMPO-$O_2^{-\bullet}$. The lack of a visualized DMPO-OOH spin adduct signal could not exclude the production of $O_2^{-\bullet}$; since the direct ESR detection of DMPO-OOH is difficult because of the short lifetime of the adduct. However, the addition of both SOD, a specific and efficient catalyzer of $O_2^{-\bullet}$ disproportionation, and catalase, a specific scavenger for H_2O_2, did not influence the intensity of the DMPO-HO$^{\bullet}$ adduct. It can be deduced from these results that DMPO-HO$^{\bullet}$ was not generated from decomposition of DMPO-OOH. It is known that DMPO reacts directly with $^1O_2$ through a complex mechanism that leads to the creation of both DMPO-HO$^{\bullet}$ and free hydroxyl radicals [28]:

$$\mathrm{DMPO} + {}^1\mathrm{O}_2 \rightarrow [\mathrm{DMPO} - {}^1\mathrm{O}_2]$$

$$[\mathrm{DMPO} - {}^1\mathrm{O}_2] \xrightarrow{\mathrm{H}^+} \mathrm{DMPO-HO}^{\bullet} + \mathrm{HO}^{\bullet} \qquad \text{(a)}$$

$$\mathrm{DMPO} + \mathrm{HO}^{\bullet} \rightarrow \mathrm{DMPO-HO}^{\bullet}$$

Thus in agreement with the results found at low light intensity using TEMP, singlet oxygen appears to be the starting point of the photoinhibition process also at high light levels. In order to corroborate this hypothesis, specific $^1O_2$ quenchers have been used. Sodium azide [28] even at low concentrations caused large decreases in the intensity and formation rate of the DMPO-HO$^{\bullet}$ signal (Fig. 4), supporting the hypothesis of a direct reaction between $^1O_2$ and DMPO. Unfortunately, histidine, the most common scavenger used as specific for singlet oxygen [35], is not suitable for ESR studies since the light-dependent degradation of the resulting imidazole peroxide causes the formation of radical species, which are trapped by DMPO [35].

On the other hand, it cannot be excluded that HO$^{\bullet}$ normally plays a role in this process, but unfortunately it is not easy to use scavengers such as mannitol to distinguish whether the observed DMPO-HO$^{\bullet}$ has its origin in $^1O_2$ or free HO$^{\bullet}$, since the reaction between $^1O_2$ and DMPO produces HO$^{\bullet}$ directly and the radicals cannot be detected without the addition of DMPO. In any case the experiments with scavengers confirm that $^1O_2$ is the starting point of the photoinhibition process by energy transfer from the triplet chlorophyll (sensitizer) to oxygen.

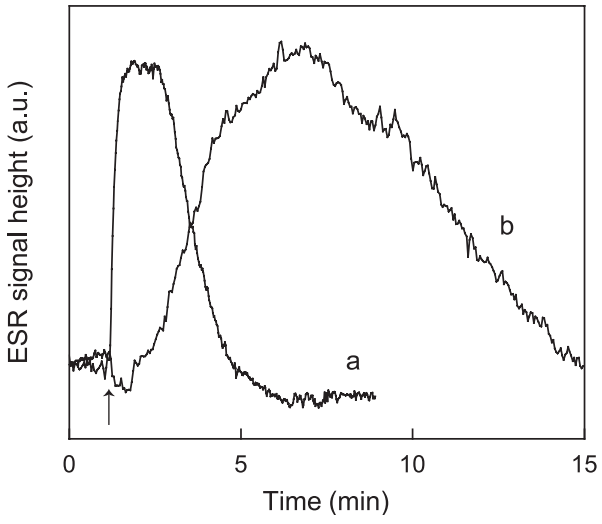

Fig. 3. Kinetics of the formation of DMPO-HO$^{\bullet}$ (a) and DMPO-R$^{\bullet}$ (b) upon illumination of LHCII. The light was turned on at the point indicated by the arrow; the light intensity was 3300 μmol m^{-2} s^{-1}.

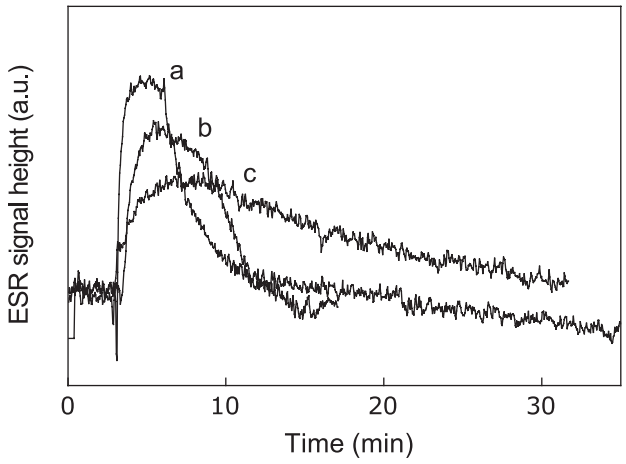

Fig. 4. Kinetics of the formation of DMPO-HO$^{\bullet}$ upon illumination of a LHCII sample in the presence of azide. (a) Control; (b) 1 mM NaN_3; (c) 5 mM NaN_3. The light intensity was 3300 μmol m^{-2} s^{-1}.

3.3. Effects of the light intensity and molecular oxygen on the DMPO-HO$^{\bullet}$ adduct

Normally the mechanism of a sensitizer (Sens) is explained based on the scheme proposed by Foote [18]:

$$\begin{array}{c} \text{Sens} \\ \downarrow h\nu \\ \underset{(\text{Sens}^{\bullet},\ \text{HO}^{\bullet},\ \text{O}_2^{-\bullet},\ \text{etc...})}{\text{Radicals or Radical Ions}} \leftarrow \text{Sens}^* \xrightarrow{\text{O}_2} {}^1\text{O}_2 \end{array} \qquad \text{(b)}$$

Sens* can either react with the substrate or solvent (Type I) or with molecular oxygen (Type II). Often the effects of photosensitization can be ascribed to a combination of Type I and Type II reactions.

In order to establish the type of physiological mechanism involved in the case of LHCII, we tested the role of the light and molecular oxygen. To verify the influence of light on the singlet oxygen production, we measured the kinetics of DMPO-HO$^{\bullet}$ formation at different light intensities (Fig. 5) considering that the spontaneous decay of the DMPO-HO$^{\bullet}$ radical is known to be much slower than the time course measured in these experiments. The possibility of a disappearance of DMPO-HO$^{\bullet}$ adduct due to a light-dependent secondary reaction is evident; in fact, the initial rate of radical generation, the plateau reached, the duration of the signal and the rate of its disappearance were all found to be proportional to the irradiation intensity. This result suggests that the DMPO-HO$^{\bullet}$ radical was able to react directly with ^{3}Chl. This was confirmed in additional experiments in which the light was turned off during the measurement (Fig. 5, inset): the radical decayed slowly and when the light was turned on again the signal returned to the previous level.

To confirm that in our case Sens* is shifted towards to singlet oxygen formation, we studied the effect of oxygen using gas-permeable capillaries that allowed ESR measurements under nitrogen atmosphere or with a continuous supply of air (Fig. 6). No radical production at all could be detected in anaerobiosis; in contrast, the kinetics changed when oxygen was constantly available. The initial phase of DMPO-HO$^{\bullet}$ formation was not affected; however, the signal was no longer transient but decayed very slowly over a period of 20–30 min. From these experiments it is evident that the rapid disappearance of DMPO-HO$^{\bullet}$ observed in the previous experiments was caused by complete consumption of oxygen in the samples, and the results also are consistent with the reaction between singlet oxygen and DMPO being the source for the DMPO-HO$^{\bullet}$ radical. Since in the absence of oxygen, where $^1O_2$ cannot be generated, there is no damage to the

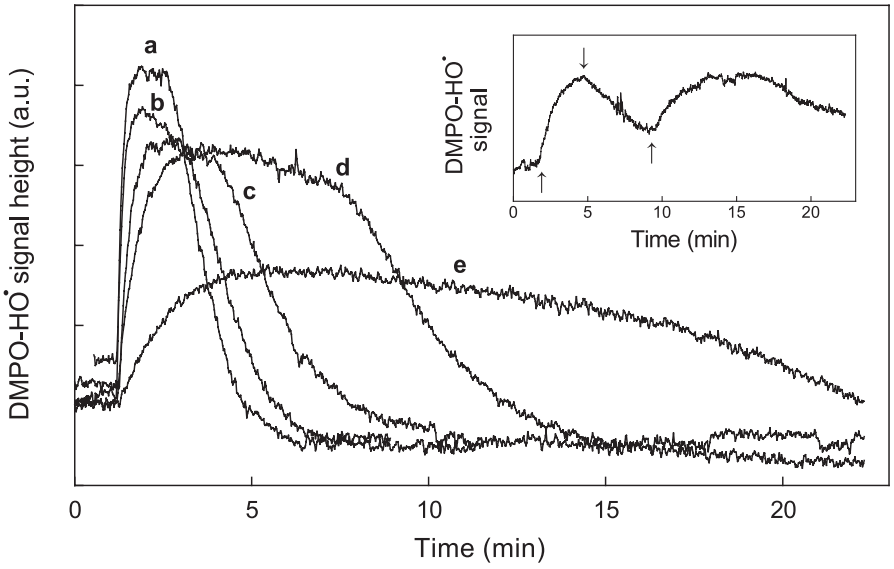

Fig. 5. Effect of the photoinhibitory light intensity on the kinetics of the formation of the DMPO-HO$^{\bullet}$ signal. The light intensities were 3300 (a), 2500 (b), 1200 (c), 350 (d) and 180 (e) μmol m^{-2} s^{-1}. The inset shows the curve obtained in an experiment in which the light was turned on (↑) and off (↓) to demonstrate the slow decay of the DMPO-HO$^{\bullet}$ signal in the dark.

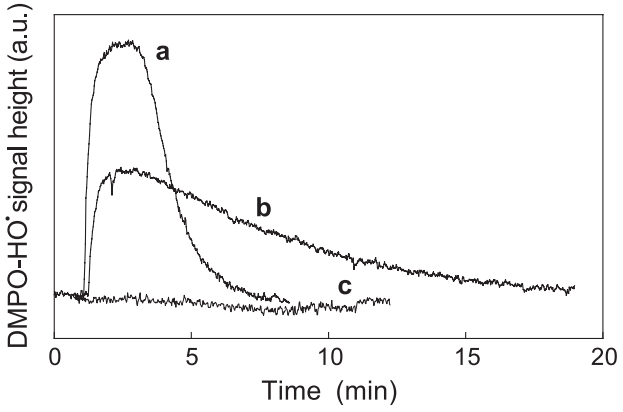

Fig. 6. Kinetics of the formation of DMPO-HO• upon illumination of a LHCII sample contained in a glass capillary (a), or in a oxygen-permeable TPX-capillary with a surrounding flow of air (b). The light intensity was 3300 μmol m^{-2} s^{-1}. The signal intensity in (b) is lower because the TPX-capillary holds a smaller volume and is less transparent to light. Trace (c) was obtained under anaerobic conditions, using the TPX-capillary with a surrounding flow of N_2.

proteins and no formation of radicals, we conclude that the physiological event in LHCII photoinhibition appears exclusively Type II. The absence of this signal in anaerobiosis and its enhanced duration when oxygen is continuously supplied demonstrate the importance of molecular oxygen in the mechanism, while a direct interaction between LHCII and DMPO does not seem possible.

Only in the presence of DMPO do we observe a Type I reaction in the form of spin elimination of DMPO-HO• by the sensitizer [36,37] (in this case a triplet Chl), which explains why the decay of the ESR signal is slower for the samples illuminated at low light intensity. On the contrary, in the case of phycocyanin irradiation also a Type I mechanism involving superoxide was observed [38].

3.4. Sources of the DMPO-R• signal

If only Type II mechanism physiologically exists, the DMPO-R• radical appears to be generated by direct reaction of $^1O_2$ with LHCII targets, presumably amino acid moieties. The DMPO-R• radical was not formed initially, it started to accumulate slowly within a minute and then increased in parallel with the DMPO-HO• decay, reaching a maximum once all DMPO-HO• had disappeared. As seen from Fig. 3, the DMPO-R• radical was relatively stable and decayed slowly over a period of 15–20 min. Unfortunately it is not possible to prove the nature of these DMPO-R• radicals only by the ESR spectrum, since many alkyl/alkoxyl radicals give almost identical spectra [32,33]. Actually the six-line signal most likely contains components from different radical species that all give similar spectra. When trapped by DMPO these radicals give rise to spectra with narrow line widths, indicating that they are low molecular weight compounds, which is consistent with small fragments of proteins or amino acids.

In conclusion, the photoinhibitory process in case of light-harvesting proteins can be described as a linear sequence of reactions:

$$\mathrm{Chl} \xrightarrow{h\nu} {}^3\mathrm{Chl} \xrightarrow{O_2} {}^1O_2 \xrightarrow{\text{Protein}} \text{Amino acids}^{\bullet}$$

Thus, photoinhibitory damage to LHCII initiates with generation of a triplet chlorophyll with the subsequent formation of singlet oxygen, which can attack nearby peptide strands directly. This process eventually leads to the complete destruction of the antenna proteins into small fragments [3]. It should be noticed that the results presented here do not exclude the participation of proteases under physiological conditions. However, our results demonstrate that the proteolytic activity observed during photoinhibition of light-harvesting complexes can be fully explained without the contribution of proteases.

The generation of ROS during photoinhibition has been reported for various other photosynthetic systems, and surprisingly it seems that each system has a unique pattern of radical formation. In plant thylakoids, formation of singlet oxygen has been found to be involved in acceptor-side photoinhibition of PSII and in LHCII photoinhibition; in contrast, $^1O_2$ was not detected during donor-side PSII or PSI photoinhibition [35,39]. Also phycobiliprotein produced $^1O_2$ in a Type II process, but in this case also a Type I reaction involving superoxide was observed [38,40]. Although donor side PSII photoinhibition does not require the presence of oxygen [41,42], the pattern of radical formation looks much like the pattern for LHCII described in this work, with initial formation of DMPO-HO• and successive appearance of a DMPO-R• species. In acceptor side PSII photoinhibition, the six-line radical signal is the first spectrum to appear, whereas this radical was not seen at all after irradiation of phycocyanin [38]. The responses of different types of photosynthetic units to excess light are summarized in Table 1. At the moment it can be concluded

Table 1
Responses of different types of photosynthetic units to light excess

	$^1O_2$	First radical	Second radical	Protein damage	References
LHCII	+	DMPO-HO•	DMPO-R•	small fragments	[3]
PSII acceptor	+	DMPO-R•	DMPO-HO•	D1 N-terminal fragment	[35,41]
PSII donor	–	DMPO-HO•	DMPO-R•	D1 C-terminal fragment	[35,41]
PSI	–	n.d.	n.d.	LHCI and PsaA/B degradation	[39,51]
PC	+	DMPO-HO•	none	PC degradation	[38,40,52]

that there does not seem to be any common pathway of photoinhibition, each system responds in a specific way.

With the final aim to demonstrate that what was revealed by DMPO actually reflects what may occur upon illumination of light-harvesting proteins, we performed SDS-PAGE analysis of antennae irradiated in the presence of scavengers for different reactive oxygen species (Fig. 7). The scavengers used were catalase (for H_2O_2), SOD (for $O_2^{-\bullet}$), histidine, DABCO and NaN_3 (for $^1O_2$; [27,28]), and *n*-propyl gallate (for $HO^\bullet$ and alkoxyl radicals; [29]). A quantitative estimation by Coomassie staining of protein shows that after 2 h of illumination, *n*-propyl-gallate efficiently protects (70%) the protein against photodestruction, while histidine and NaN_3 are less efficient (60%) and DABCO even less (40%). However, the lack of effects seen for SOD and catalase, both in terms of protein degradation and in the spin trapping experiments, confirm that H_2O_2 and $O_2^{-\bullet}$ do not play any role during the initial steps of photoinhibition. These reactive oxygen species may instead appear later in the process, when extensive degradation of the peptides occurs. Thus, it seems that degradation of LHCII proteins may occur through direct attack on aromatic amino acids of antenna by the singlet oxygen produced upon illumination of chlorophylls. In agreement with the results reported for LHCII degradation the addition of propyl gallate resulted in a lower amount of detectable DMPO-$R^\bullet$ radical, consistent with the function of propyl gallate as an alkoxyl scavenger [29] (Fig. 8).

3.5. Monomers and trimers

Since different aggregation states of LHCII represent a physiologically relevant situation which happens under high light intensities in vivo, we tested the influence of the conformational state of the protein on the radical production. We have recently presented data showing that the major antenna proteins can be well separated into four distinct peaks and identified by reversed phase HPLC-ESI-MS: Lhcb2, Lhcb3 and two isoforms of Lhcb1: Lhcb1.1 and Lhcb1.2 [43]. Integration of the area underlying each chromatographic peak allows quantitative estimation of the relative amount of each protein component. Thus, comparing the optical absorption decreases of each peak, we can determine for each protein its sensitivity to degradation

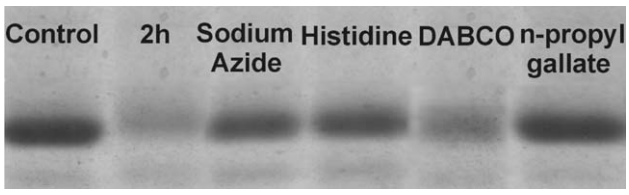

Fig. 7. Effects of different scavengers on LHCII exposed to high intensity illumination for 2 h, as measured by SDS-PAGE. The concentrations of the scavengers were 2 mM azide, 10 mM histidine, 1 mM DABCO and 1 mM *n*-propyl gallate.

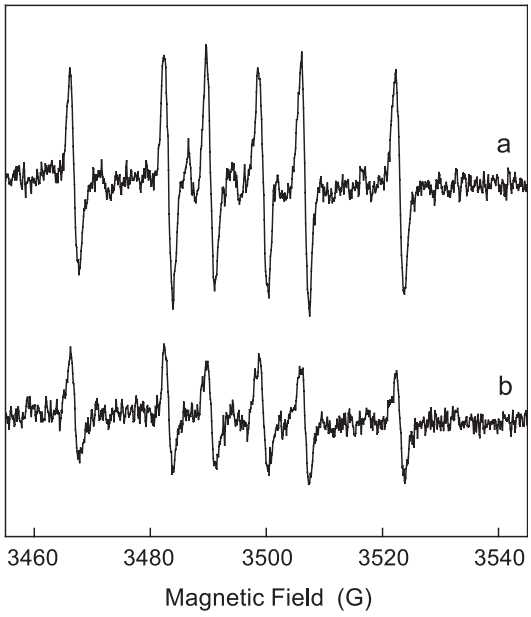

Fig. 8. Spectra of DMPO-$R^\bullet$ measured after 3 min illumination in the absence (a) or presence of 2 mM *n*-propyl gallate (b). The light intensity was 3300 μmol m^{-2} s^{-1}.

when it is in a monomeric, trimeric LHCII complex or organized in the native form of PSII (BBY). Fig. 9A and B reports the degradation of Lhcb1.1 and Lhcb1.2 in the three aggregation states. It can seen that in both cases the monomeric form shows a higher resistance to protein degradation than trimers, whereas the PSII complex offers LHCII some sort of photoprotection. Consequently, generation of DMPO-$R^\bullet$ adduct should reflect the protein fragmentation process just reported. In agreement, Fig. 9C shows kinetic measurements of DMPO-$R^\bullet$ signal formed by monomer and trimer LHCII, at the same chlorophyll concentration, when exposed to high light intensity. It can be seen that the maximum ESR signal height is reached after 5 min of illumination in trimeric LHCII, but after 15 min in monomeric LHCII, suggesting that the former is degraded more rapidly. Interestingly, BBY complexes produce a significative amount of DMPO-$R^\bullet$ only after longer times (data not shown).

At this point the open question is: are more singlet oxygen produced in trimers than in monomers or do these two forms show different sensitivity to attack? Since in a previous paper we demonstrated that protein degradation, although toward the NH_2 terminal region, is due to a nonspecific cleavage, there is no reason to think that monomeric and trimeric antennae present different sensitivity to oxygen radicals attack. On the other hand, Fig. 9D reveals that after 2 min of irradiation, DMPO-$HO^\bullet$ is already present in trimeric antenna but completely absent in monomers, corroborating that the anticipated and higher production of DMPO-$R^\bullet$ observed in trimers may reflect a more rapid production of singlet oxygen in trimers than monomers. A lower radical production rate in illuminated mono-

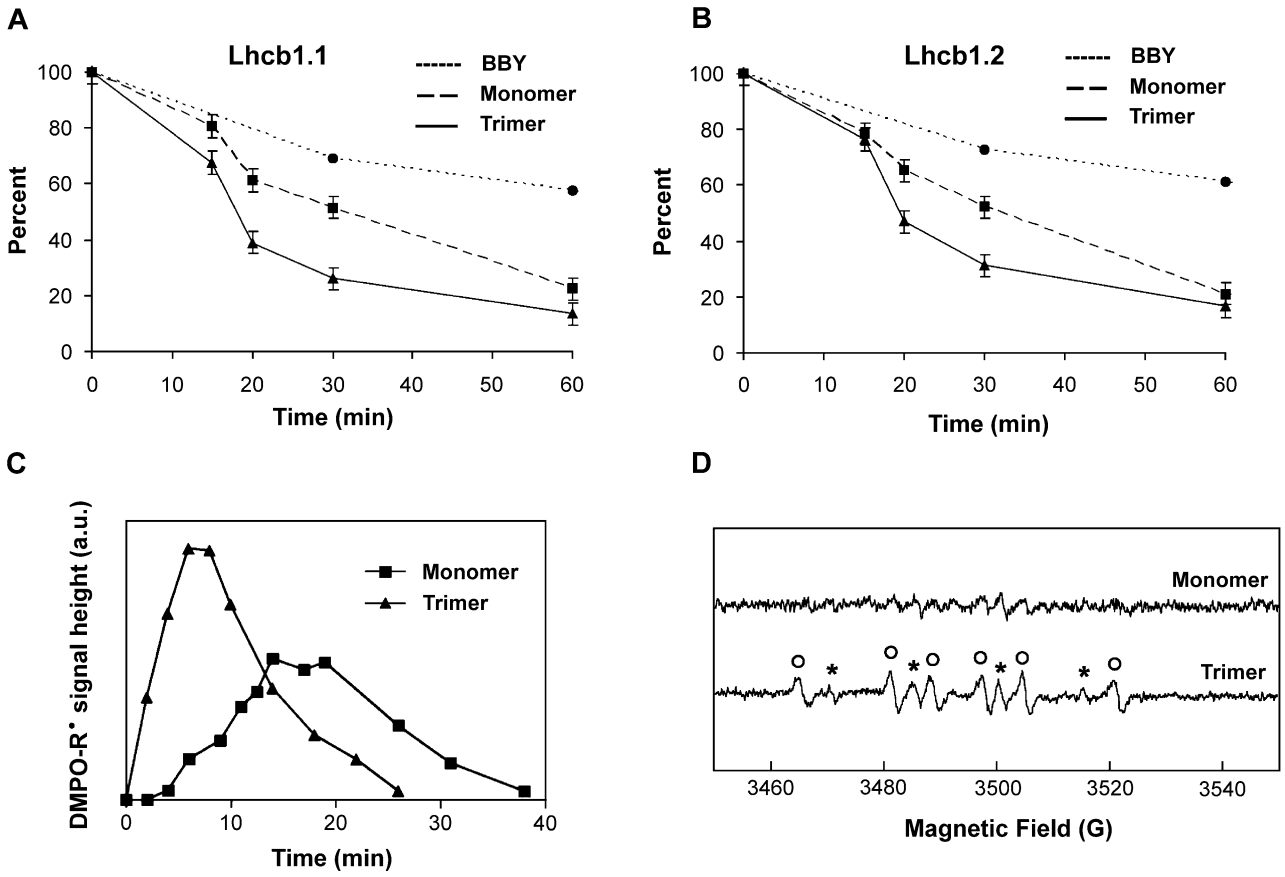

Fig. 9. Protein degradation and relative oxygen radical production from antenna proteins exposed to high light intensity. Panels A and B: Percentage optical decreases of the isomeric forms Lhcb1.1 (A) and Lhcb1.2 (B) determined on major antennae organized in trimers, monomers or present in BBY complex. Panel C: Kinetics of the DMPO-R$^{\bullet}$ formation upon illumination of a trimer or monomer LHCII sample. Panel D: ESR spectra of spin-trapped radicals after 2 min of irradiation. Symbols refer to Fig. 2.

mer LHCII was in agreement with a recent study demonstrating their higher capability of quenching of chlorophyll *a* singlet excited state. The data presented by Garab et al. [44] show that dynamic properties (most probably for steric reasons) of monomeric LHCII permit the formation of quencher more readily than in the presumably more compactly organized trimers. Furthermore, photogeneration of quenchers is a fast reaction that competes with other excitation–relaxation pathways [45]. It can also be speculated that uncoupled chlorophylls that have been detected in photoinhibited thylakoids [46] originate from monomerized LHCII representing a protective quenched state of the antenna proteins as response to high light stresses. It is now established that the PSII light-harvesting system can exist in two different states in vivo: an unquenched state that is functional in light-harvesting and a quenched state, induced under excess light conditions, that dissipates the excess absorbed excitation energy [47]. By use of an in vitro system of purified LHCII components, a model for the in vivo nonphotochemical quenching (NPQ), it was found that trimeric LHCII did not quench to the same extent or as rapidly as either its monomeric form or the minor, monomeric complexes (CP29 and CP26) [48,49].

What was observed in in vitro studies could represent what occurs in vivo. In a recent paper, in fact, has been for the first time reported detection of $O_2^{-\bullet}$ and $^1O_2$ by fluorescent sensors in spinach leaves exposed to either excess photosynthetically active radiation or UV-B [50], which may support the hypothesis of oxygen radicals involvement in antenna degradation. Moreover, our data revealed that monomerization of antenna, usually observed under high light irradiation [7,44], could represent an important physiological plant response to light stress since in this way the trimeric form is reduced [7,44] because it may produce dangerous oxygen radicals. Monomers represent also the more diffusive species for migration of antennae from the appressed (grana) to non-appressed (stroma) thylakoid membrane, where separated antenna, showing the transmembrane portion partially denaturated [3], are finally digested by proteases.

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