Involvement of Active Oxygen Species in Degradation of Light-Harvesting Proteins under Light Stresses†

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ABSTRACT: This paper presents evidence for light-mediated degradation of isolated light-harvesting proteins (Lhc2) and involvement of oxygen free radicals in the process. The time course of light harvesting photodestruction is much slower than that of D1 protein (requiring hours for complete breakdown). By use of mass spectrometry and amino acid sequencing, it has been revealed that the primary cleavages take place in the hydrophilic portion of the NH_2 region where oxygen-containing radicals attack randomly and not at specific sites. Moreover, these chlorophyll binding proteins are completely fragmented. From the effectiveness of scavengers and the preliminary electron paramagnetic resonance measurements reported, it appears that singlet oxygen is involved as a short-lived species, and hydroxyl and alkoxyl radicals act at higher light intensity or over a longer time, whereas hydrogen peroxide and superoxide anions are not observed. Antenna proteins appear more resistance to photodestruction in their monomeric form than in trimeric form, while minor antenna are highly sensitive. However, the organization of both minor and major proteins in the photosystem II supracomplex affords some photoprotection. Interestingly, leaves exposed to strong light contained degraded major antenna, unlike those kept in the dark, which is consistent with studies on the illumination of isolated proteins, supporting the hypothesis that active oxygen species play a role *in vivo* in the short-term acclimative adaptation of plants.

It has long been accepted that strong illumination of oxygenic photosynthetic organisms results in loss of their photosynthetic ability (*1*). The most studied and documented event is the photoinactivation of PSII[1] and specific degradation of the D1 protein of the photochemical reaction center (photoinhibition) (*2*−*4*), while limited evidence has been reported on PSI photoinhibition (*5*) and degradation of light harvesting proteins (*6*). With respect to the cause of D1 degradation, three different hypotheses have been put forward. Some authors suggest that singlet oxygen ($^1O_2$) and its related oxidizing species cause irreversible damage of the reaction center, inducing the degradation of the D1 protein (*7*). Illumination initially affects the acceptor side of PSII (acceptor-side photoinhibition), causing the triplet state of P680 to be formed, which subsequently reacts with oxygen to generate toxic $^1O_2$; the electron flow is blocked but the primary photochemical process goes on (*8*). In PSII subcomplexes, various fragments of the D1 protein can be observed after strong illumination (*9*). The primary cleavage site varies depending on illumination conditions; however, a fragment of 23 000−24 000 (*10*) is considered to be the primary degradation product (*3*).

Studies suggest that serine-type protease(s) intrinsically present in PSII preparations, and possibly a component of PSII itself, catalyze(s) this degradation (*3*). The possible role of the FtsH protease, an ATP-dependent zinc metalloprotease, has recently been hypothesized (*11*, *12*). In this case, the primary light-induced cleavage product of the D1 protein is a 23 000 fragment, similar to the one observed upon reaction of oxygen radicals (*13*). Moreover, GTP bound to chloroplast thylakoid membrane is required for light-induced multienzyme degradation of D1 protein (*14*, *15*), and the identification of a novel light-inducible chloroplast protease complex, associated with thylakoid membranes, has recently been reported (*16*).

Finally, some authors have proposed a modified model in which $^1O_2$ or oxidizing species generated inside the reaction center alter the conformation of the D1 protein and expose the otherwise protected cleavage region(s) of D1, so as to render it susceptible to degradation by the putative protease(s) (*17*).

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[1] Abbreviations: ADMR, absorbance-detected magnetic resonance; APMSF, (4-amidinophenyl)methanesulfonyl fluoride; BBY, photosystem II membranes; Car, carotenoid; Chl, chlorophyll; CP, chlorophyll protein; DABCO, 1,4-diazabicyclo[2.2.2]octane; DM, *n*-dodecyl β-D-maltoside; DMPO, 5,5-dimethyl-1-pyrroline N-oxide; DMPO-OH, hydroxyl radical adduct of 5,5-dimethyl-1-pyrroline N-oxide; DMPO-R, carbon-centered radical adduct of 5,5-dimethyl-1-pyrroline N-oxide; EPR, electron paramagnetic resonance; ESI, electrospray ionization; FDMR, fluorescence-detected magnetic resonance; HPLC, high-performance liquid chromatography; LHC, light harvesting complex; MES, 2-[*N*-morpholino]ethanesulfonic acid; MS, mass spectrometry; OD, optical density; OG, *n*-octyl β-D-glucopyranoside; PAGE, polyacrylamide gel elctrophoresis; PMSF, phenylmethanesulfonyl fluoride; PS, photosystem; Q_y, first excited singlet state of chlorophyll *a*; RIC, reconstructed ion chromatogram; RP, reversed phase; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; TEMP, 2,2,6,6-tetramethylpiperidine; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; TFA, trifluoroacetic acid; Tricine, *N*-[2-hydroxyl-1,1-bis(hydroxylmethyl)-ethyl]glycine; Tris, tris(hydroxymethyl)aminomethane.

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Although there is general consensus that the primary target of photoinhibition is the reaction center of PSII, few studies have focused on degradation of light harvesting proteins during the acclimative adaptation of plants.

It has been suggested that a proteolytic activity is involved in the degradation of the major light-harvesting chlorophyll *a*/*b*-binding protein of photosystem II (LHCII) when the antenna size of photosystem II is reduced upon acclimation of plants in response to exposure of leaves to increased irradiance (*18*). This acclimative response shows a delay of 2 days after transfer of the leaves to strong light. However, to date, the identity of such a protease and the location of the substrate recognition site for the regulatory protease remain unknown, especially for antenna proteins. On the other hand, evidence that the proteases recently discovered for degradation of D1 protein may also act on light harvesting proteins has not been reported. Conversely, the possibility that ^{3}Chl, *in vitro* and in antenna complexes, may interact with molecular oxygen via intersystem crossing (*19*) and subsequently interact with antenna proteins has never been taken into account. In photosynthesis, excitation of pigments [chlorophylls (Chl) or carotenoids (Car)] leads efficiently to a free-energy stabilizing charge separation. This charge separation may cause the formation of unwanted and potentially dangerous chlorophyll triplet excited states (^{3}Chl) by intersystem crossing. These states are so high in energy that they can react with triplet ground-state oxygen to form singlet excited oxygen. Indeed, the lifetime of ^{3}Chl is substantially shortened in the presence of oxygen (*20*), and in the absence of a competitive compound one possible decay pathway involves the $^3Chl-{}^3O_2$ interaction. This could results in the formation of extremely reactive singlet oxygen, which can damage pigments and proteins of any chlorophyll protein. Recently triplet Chl, probably generated far from the reaction center, has been detected (*21*). Thus, the formation of singlet oxygen by this pathway could provide, at least in principle, an attractive mechanism for light-induced damage to the light harvesting proteins. However, this possibility has not yet been investigated.

In the present study, the possible involvement of active oxygen species in the *in vitro* degradation of the LHCII proteins was investigated with isolated monomeric, trimeric, and BBY subcomplexes. 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.

MATERIALS AND METHODS

Isolation of the PS II Major and Minor Antenna Systems by Sucrose-Gradient Ultracentrifugation. The light-harvesting complex was isolated from the PSII membranes as previously described (*22*) with the following modifications: PSII membranes were pelleted by centrifugation at 10 000*g* for 5.0 min at 4 °C, suspended in B3 (50 mM MES, pH 6.3, 15 mM sodium chloride, and 5 mM magnesium chloride) buffer at 1.0 mg/mL chlorophyll and then solubilized by adding 1% (w/v) *n*-dodecyl β-D-maltoside (DM). 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 model Centricon T-1080 ultracentrifuge equipped with a model TST 41.14 rotor at 39 000 rpm for 18 h at 4 °C. Green bands were harvested with a syringe. The SDS−PAGE 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 (*22*). These bands were used for HPLC analysis without any further treatment.

HPLC Separations. PSII antenna proteins were separated by a reversed-phase column under the following experimental conditions. Both analytical and semipreparative scale separations were carried out with the same volatile mobile phase system consisting of trifluoroacetic acid in a water−acetonitrile mixture with increasing acetonitrile content during gradient elution. The Vydac C-4 columns were preequilibrated with 40% (v/v) aqueous acetonitrile solution containing 0.1% (v/v) TFA and samples were eluted by a gradient with 0.1% (v/v) TFA. Gradient consisted of 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 a 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.

High-Performance Liquid Chromatography and Electrospray Mass Spectrometry. The RP-HPLC-ESI-MS experiments were carried out with a Beckman (Fullerton, CA) Gold Nouveau system, consisting of two Model 126 solvent delivery pumps and a Model 168 diode array detector. Samples were introduced onto the column by a Model 210A sample injection valve with a 100-μL sample loop. ESI-MS was performed by splitting the column effluent so that 50 μL/min flowed into the ESI ion source of a triple quadrupole API 2000 (Applied Biosystems) or an ion trap Esquire 3000 plus (Bruker Daltonik, Germany). For HPLC-MS analysis with pneumatically assisted electrospray ionization, a spray voltage of 4 kV and a nebulizer gas pressure of 15 psi were employed, and mass spectra were recorded by scanning from 400 to 3000 mass-to-charge ratios. The separations were performed on a 250 × 4.6 mm Vydac protein C-4 column (The Separation Group, Hesperia, CA), packed with 5-μm porous butyl silica particles. The Vydac C-4 columns were preequilibrated with 39% (v/v) aqueous acetonitrile solution containing 0.05% (v/v) TFA, and samples were eluted with a gradient from 39% to 69% (v/v) acetonitrile in 45 min. After the separation, the column was washed with 95% acetonitrile in 0.05% TFA for 10 min before reequilibration at 39% acetonitrile for 10 min.

Amino Acid Analysis. The amino acid sequence was determined by automated Edman degradation on a Perkin-Elmer model AB476A sequencer. Samples separated on Tris−Tricine SDS−PAGE were electroblotted onto a poly(vinylidene difluoride) membrane (Problott, Perkin-Elmer) and stained with Coomassie Brillant Blue. Bands of interest were excised and directly analyzed.

Preparation of Monomeric Major Antenna and Apoprotein. LHCII trimers were isolated from spinach according to Krupa et al. (*23*). LHCII monomers were generated by incubation of the trimeric complex with 3 μg of phospho-

lipase A_2/mL from bee venom (Sigma) in a medium containing 0.6% (w/v) octyl glucoside (OG) with a chlorophyll (Chl) concentration of 1 mg/mL at room temperature overnight according to Nussberger et al. (*24*). After incubation, the samples were loaded onto a 5−20% sucrose gradient and centrifuged at 200 000*g* for 18 h at 4 °C (*25*). 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) sodium dodecyl sulfate (SDS) and dialyzed against 1% (w/v) OG overnight.

Trypsin Digestion. A mixture of major antenna (50 μL), contained in sucrose ultracentifuge band 3, was digested with pure trypsin (5 μL) at a final concentration of 0.01 mg/mL at pH 7.5. After 30 s of incubation at 25 °C, the sample was loaded onto a reversed-phase column, so that digestion was stopped by the run starting, since trypsin, being more hydrophilic, elutes with the front of the mobile phase.

Protease Inhibitors. Protease inhibitors used were PMSF (phenylmethanesulfonyl fluoride), 1 mM; APMSF [4-amidinophenylmethanesulfonyl fluoride], 0.1 mM and aprotinin, 0.1 mg/mL.

Photoinhibitory Light Treatments. Photoinhibition was performed in BBY and LHCII samples diluted with the corresponding buffer to 0.2 and 0.055 mg of chlorophyll/mL, respectively, and exposed to 1000 μmol m^{-2} s^{-1} from a HQI-T 250 W lamp in a temperature-controlled glass cuvette at 4 °C, with gentle stirring. The scavengers used were histidine at a concentration of 10 mM and DABCO at a concentration of 1 mM for $^1O_2$ (*26*), and *n*-propyl gallate at 1 mM for •OH and alkoxyl radicals (*27*), and SOD and catalase at 100 μg/mL for O_2^- and H_2O_2, respectively.

Electrophoresis. Denaturing sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was carried out in 12−17% acrylamide gradient gels (20 cm × 16 cm × 1.5 mm) containing 7 M urea, on a Protean II Bio-Rad gel electrophoresis system. Gels were run at room temperature (20 °C) for 16 h at constant current of 20 mA in 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/v) methanol−glacial acetic acid−water mixture, containing 0.1% (w/v) Coomassie Blue.

EPR. X-band EPR spectra were recorded from 50 μL aliquots by a Bruker ECS-106 spectrometer at room temperature using 9.45 GHz microwave frequency, 16 mW microwave power, and 100 kHz modulation frequency (*28*). Singlet oxygen was detected by following the formation of TEMPO, a stable nitroxide radical yielded in the reaction of singlet oxygen with the sterically hindered amine TEMP by EPR spectroscopy (*29*). Samples containing 10 mM TEMP were subjected to photoinhibition for 2 h, and nitroxide radicals produced were extracted into ethyl acetate as described (*30*). DMPOs were used as •OH radical trapping reagents. EPR spectra were measured at room temperature with a microwave power of 16 mW and 100 kHz modulation frequency.

RESULTS

To verify if strong illumination induces degradation of the light harvesting proteins, as observed for core proteins, we have investigated the effect of light on both isolated and aggregated PSII antenna proteins from spinach and pea, using a number of techniques, including HPLC, SDS electrophoresis, mass spectrometry, and EPR. We found that separation of proteins by reversed-phase HPLC, previously developed in our laboratory (*31*, *32*), was particularly useful. Besides being more effective than SDS−PAGE electrophoresis, it also allows quantitative evaluation of the relative amount of each protein component. This is calculated from the area underlying each HPLC peak and the fact that the antenna proteins are strongly conserved, so they may be assumed to have similar optical extinction coefficients. Moreover, HPLC coupled on-line with a mass spectrometer interfaced with an electrospray ion source (ESI-MS) allows the molecular weight of proteins to be determined in both native and nonnative conditions.

In all experiments performed, the material used was previously dialyzed to remove any free chlorophyll from the solution. Moreover, preliminary experiments were performed in the presence and in the absence of a mixture of protease inhibitors in order to rule out the involvement of proteases in the phenomena described later. However, the results obtained show that illumination affects the antenna proteins differently under low light intensity compared with high light intensity. The two sets of experiments are discussed in separate sections, since they probably involve different mechanisms. In the first part, data showing the effect of low light intensity (100 μmol m^{-2} s^{-1}) are presented.

Illumination at Low Light Intensity. We started with the major antenna proteins (band 3 from sucrose-gradient ultracentrifugation), which are present in this band in trimeric form. We have recently presented data showing that the major antenna proteins can be well separated by reversed-phase HPLC into four distinct peaks: Lhcb2, Lhcb3, and two isoforms of Lhcb1: Lhcb1.1 and Lhcb1.2 (Figure 1a). The identification and subsequent assignment of each HPLC peak was performed by coupling the HPLC outlet with a mass spectrometer (HPLC-ESI-MS) equipped with an electrospray ion source (*33*), which was particularly suitable for identification of isomeric Lhcb1 antenna proteins (*34*). The M_r measured was consistent with that expected from the DNA sequences: 24 757 for Lhcb2, 24 310 for Lhcb3, and 24 940 and 25 014 for the two isoforms Lhcb1.1 and Lhcb1.2, respectively (Figure 1a). Recently, it has been reported that these two isoforms show slightly different amino acid sequences in the NH_2-terminal region (*35*). Rapid trypsin digestion of the protein mixture (30-s incubation) results in a decrease of the area of some peaks (Lhcb1 and Lhcb2), while new peaks are detected at longer elution times (Figure 1b). This is due to the removal of a short portion of the hydrophilic NH_2-terminal region, confirmed by amino acid sequencing of the digested protein products (manuscript in preparation), which renders the residual protein, containing three transmembrane α-helixes, more hydrophobic. The shift at longer times allows the shortened protein to be distinguished from the native one. For comparison, Figure 1 also contains the HPLC chromatogram where the same proteins are exposed to gentle illumination (100 μmol m^{-2} s^{-1}) at room temperature in the presence of protease inhibitors for 3 h (Figure 1c). It is apparent that in the latter case LHCII proteins also show lower peaks than the control and a small shift toward higher elution times occurs. Comparing these

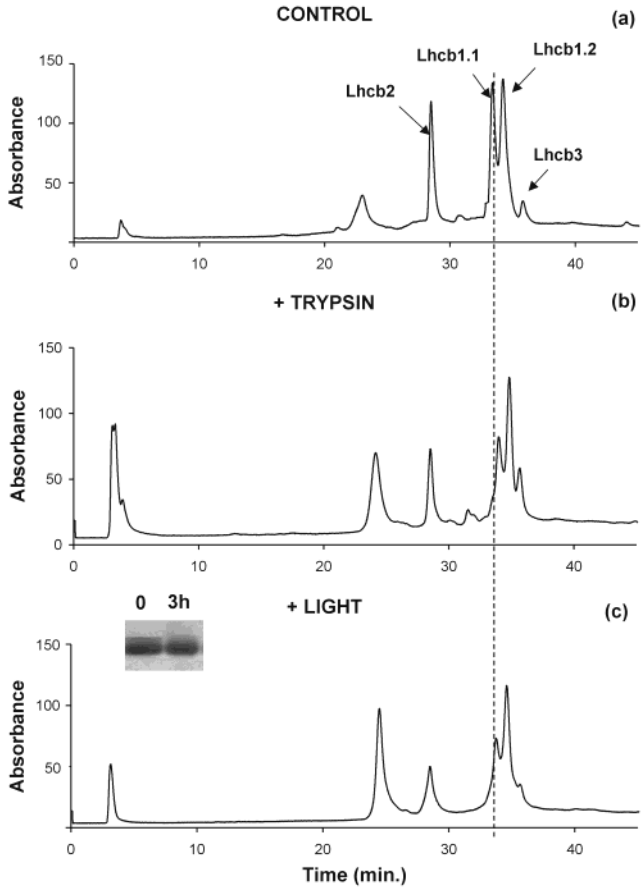

FIGURE 1: HPLC chromatograms of the major spinach antenna proteins: (a) control, (b) after 30 s of incubation at 25 °C with trypsin, and (c) upon illumination at low light (100 μmol m^{-2} s^{-1}) for 3 h. Inset of panel c reports SDS−PAGE of proteins. UV detection was at 214 nm.

with the digested protein, it is reasonable to assume that illumination of antenna protein may also cause short fragments to be removed. Interestingly, SDS−PAGE of both digested and illuminated proteins (inset of Figure 1c) does not show any changes in the electrophoretic mobility of the treated LHCII nor a significant reduction in Coomassie staining, suggesting that SDS−PAGE is not sufficiently sensitive to reveal small changes in proteins. However, the significant optical decrease observed following tryptic digestion and upon light illumination seems to depend greatly on the peptidic fragment(s) removed and does not reflect a proportionate decrease in the total protein. This is due to the fact that aromatic amino acids contribute most to the optical absorption reading of a protein, and since the external hydrophilic portions of the protein contain some of these, their removal results in a marked fall in optical absorption, as recently observed with phycobilisome proteins (*36*). However, to demonstrate unequivocally that the changes observed in the HPLC chromatogram of light-exposed antenna proteins, is really due to removal of the hydrophilic NH_2-terminal region, we coupled the HPLC on-line with a mass spectrometer. Figure 2 compares the reconstructed ion chromatogram, based on the individual ESI-MS spectra of the major antenna recorded before (panel a) and after (panel b) exposure of the mixture to 100 μmol m^{-2} s^{-1} for 3 h at room temperature (similar conditions as in Figure 1c). It can be observed that most of the reconstructed ionic currents in the ESI-MS peaks reported in Figure 2a are concomitant with those observed by UV detection. However, RIC reveals a significant decrease of the ionic current intensity corresponding to the antenna protein peaks, which is not revealed by SDS−PAGE but agrees with the optical decrease observed. This would imply that ionizable amino groups are essentially located on the external part of the protein. Interestingly, a new peak at short elution time (3.59 min) also appears, which was not revealed in HPLC. Deconvolution analysis of the ESI spectra of this new peak reveals that it contains a mixture of short peptides (data not shown). Deconvolution analysis of the ESI spectra relative to those main peaks corresponding to the antenna proteins reveals that proteins are partially fragmented. Figure 3 compares the deconvolution analysis performed on native protein (panel a) with those illuminated for short (3 h) and longer times (8 h) (panels b and c, respectively). It may observed that native Lhcb2 and Lhcb1 proteins have molecular masses of 24 757, 24 939, and 25 014, respectively, but after short illumination these fall to 24 401 and 24 073 for Lhcb2, 24 523 and 24 398 for Lhcb1.1, and 24 583 for Lhcb1.2. Interestingly, Lhcb3 does not show significant alteration under this gentle light stress. Upon longer illumination time, most of the proteins have lost part of the NH_2-terminal region, with the RIC peaks strongly reduced and all proteins shortened, reaching the

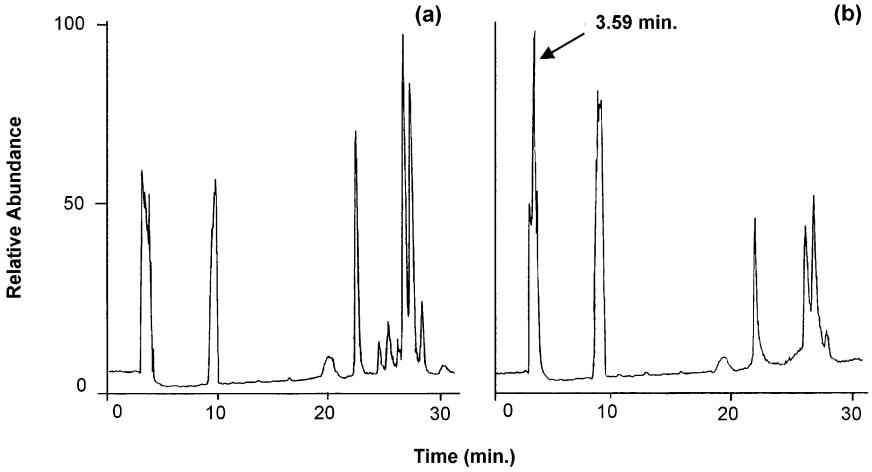

FIGURE 2: Reconstructed ion chromatogram of the spinach major antenna proteins: (a) control and (b) after illumination at low light (100 μmol m^{-2} s^{-1}) for 3 h.

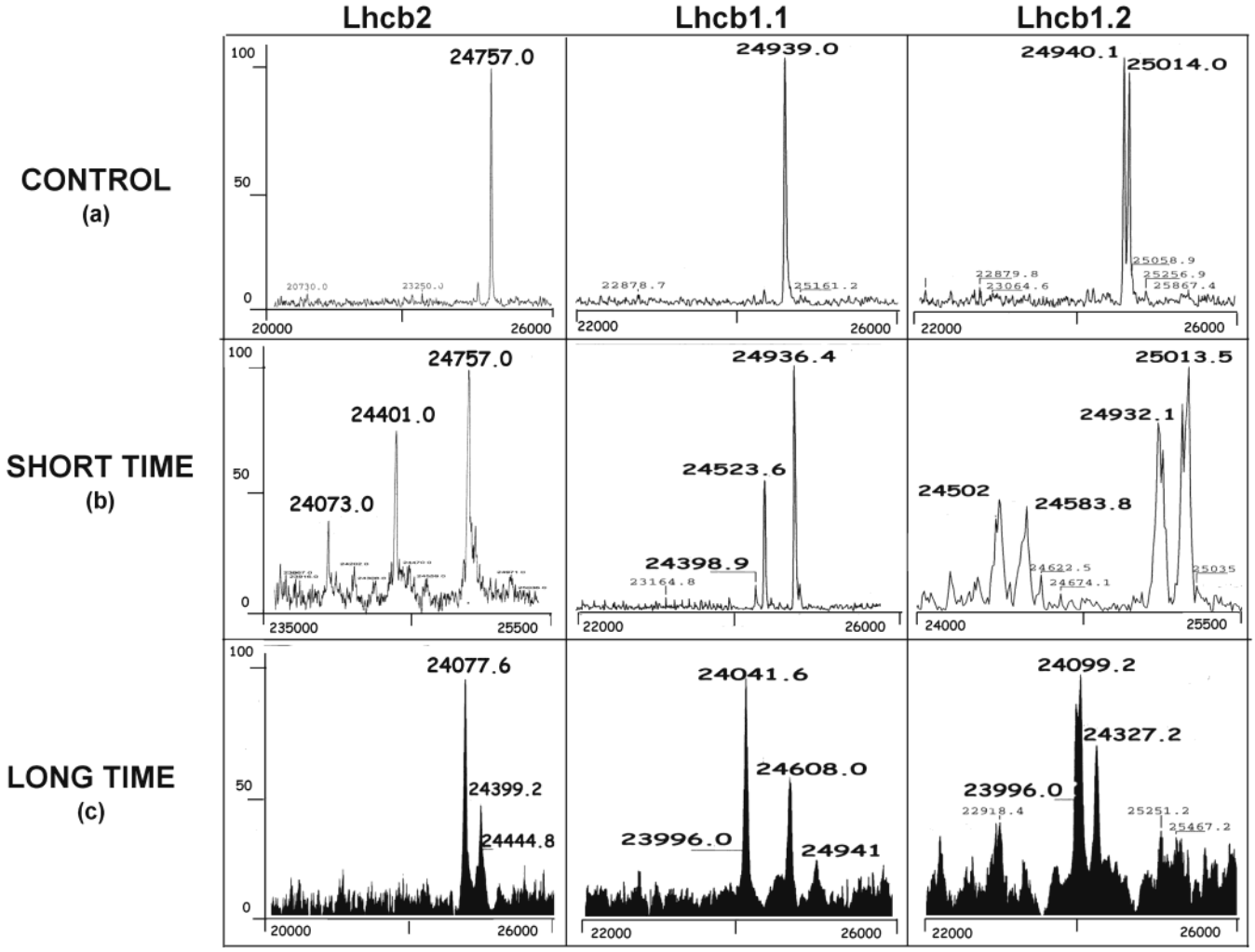

FIGURE 3: Deconvoluted spectra obtained from individual ESI-MS spectra of the major spinach antenna proteins: (a) control; (b, c) subjected to low light intensity (100 μmol m^{-2} s^{-1}) for (b) 3 or (c) 8 h.

values of 24 077 for Lhcb2, 24 041 for Lhcb1.1, and 24 099 for Lhcb1.2 (see Figure 3c). Accordingly, native proteins (having molecular masses of 24 757, 24 939, and 25 014, respectively) have completely disappeared at this time. On the contrary, SDS−PAGE does not show any significant reduction in Coomassie staining, confirming that all proteins are present but probably truncated of a few amino acids. Amino acid microsequencing was performed on these truncated proteins in order to elucidate where cleavage has occurred and confirm the hypothesis that cleavage occurs in the external regions of the protein. It is well-known that the native major Lhcb1 and Lhcb2 antenna proteins have blocked NH_2 termini and so cannot be sequenced, yet there was no chemical block on these fragmented proteins and their resulting microsequences are displayed in Table 1. Upon lining the microsequences up with sequences reported for Lhcb2 (tomato) (*37*) and Lhcb1 (spinach) (*35*, *38*), the truncated Lhcb1 protein starts at position nine and Lhcb2 at position six. This corresponds to a fragment of molecular weight 894 missing from Lhcb1.1 and one of 682 from Lhcb2, according to the molecular masses measured by ESI-MS of 24 041 and 24 099 for Lhcb1 and 24 077 for Lhcb2. Thus it may be reasonably concluded that the primary cleavage of the native protein occurs only at the NH_2 terminus. When data are collected at different illumination times and compared, it is clear that fragmentation takes place randomly (see Table 1). Sometimes, at longer times, values of 19 364 and 19 402 are recorded for Lhcb1 and Lhcb2, respectively. It is worth remarking that these M_r correspond to the portion containing the first transmembrane segment of the major LHCII, as confirmed by the amino acid sequencing (data not shown). This evidence indicates that, under this soft illumination and longer times, the complete hydrophilic portion of antenna protein is removed.

Illumination at 100 μmol m^{-2} s^{-1} of the major PSII antenna prepared in monomeric form, obtained by phospholipase digestion of the trimeric forms (*24*), shows slower degradation with respect to trimeric aggregation. This aspect will be more apparent at higher illumination intensity (see later). Finally, BBY left at room temperature under 100 μmol m^{-2} s^{-1} light for 24 h shows a significant decrease only in the peak corresponding to CP29.

Interestingly, the results reported for spinach major antenna and BBY were also observed in pea, suggesting that this may represent the situation in most plant species.

Illumination at High Light Intensity. To get more information on the different behavior of each protein, we have performed an analytical study subjecting the same spinach mixture of major antenna proteins to a higher illumination intensity of 1000 μmol m^{-2} s^{-1}. At this light intensity, in

Table 1: Alignment of the N-Terminal Microsequences Determined on Major Antenna Proteins[a]

Protein	N-terminal blockage of native protein	N-terminal sequences	Molecular masses measured by ESI-MS Native	Illuminated
		a b 10 20		
Lhcb2	**yes**	**R // R // TVK -- SAPQSIWYGEDRPKYLGPFSEOTPS**	24 757	
		XAPQSLXYGPD		24 077
		c d e f 10 20		
Lhcb1.1	**yes**	**RK // S // A // G // KPK -- TVQSGSPWYGPDRVKYLGPFS**	24 939	
		TVQSGSPXYGPDRVKYXL		24 041
		10 20		
Lhcb1.2	**yes**	**RK // T // A // G // KPK -- TVQSSSPWYGPDRVKYLGPFS**	25 014	
		TVQSGSPXYGPDRVKY		24 099

[a] Samples were illuminated at 100 μmol m^{-2} s^{-1} for 8 h. Sequences of native protein were deduced from genes cloned for Lhcb1 spinach (accession number P12333 in SWISS-PROT) and Lhcb2 tomato (accession number P10708 in SWISS-PROT). Italic letters indicate the initial amino acid of the fragments, which were deduced from the protein molecular masses measured at different illumination times: (*a*) corresponding to the molecular mass of 24 558; (*b*) to 24 404−24 406; (*c*) to 24 603, 24 605, 24 608; (*d*) to 24 502−24 508; (*e*) to 24 449; (*f*) to 24 327.

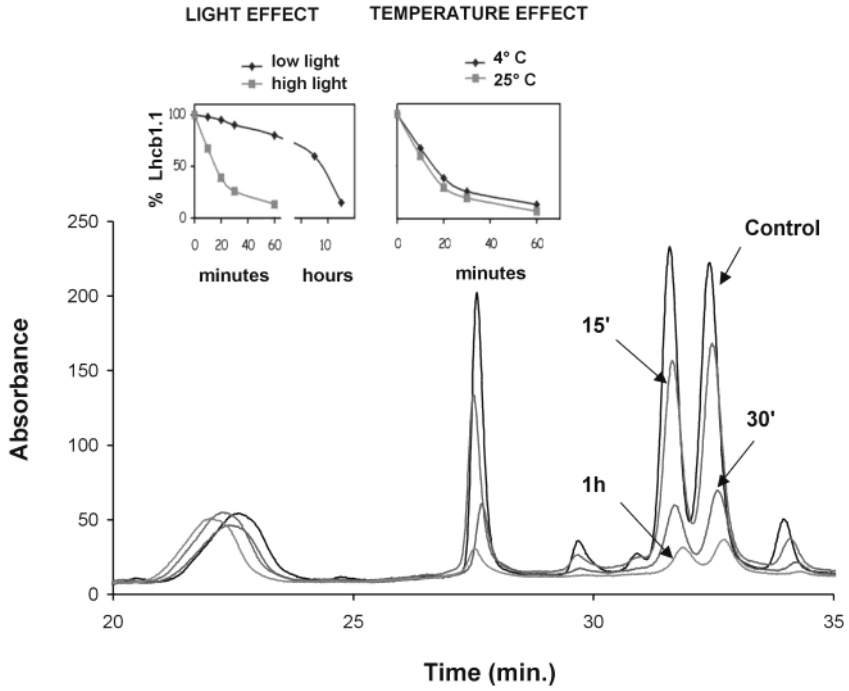

FIGURE 4: HPLC chromatograms recorded at different times from major antenna exposed to strong light (1000 μmol m^{-2} s^{-1}). The left inset reports the percentage decrease of area underlying the Lhcb1.1 peak recorded at 4 °C upon both low (100 μmol m^{-2} s^{-1}) and high (1000 μmol m^{-2} s^{-1}) light intensity. The right inset reports the percentage decrease of area underlying the Lhcb1.1 peak recorded at 4 °C and 25 °C upon high light intensity.

fact, the phenomenon observed takes place quickly, and it is possible to analyze and make comparisons between different antenna proteins. The left inset of Figure 4 shows, for example, the percentage decreases in the area underlying Lhcb1.1 as a function of the two different light intensities of 100 and 1000 μmol m^{-2} s^{-1}, both measured at 4 °C. The time course of falling UV detection is 4 times faster when the proteins are illuminated at 1000 μmol m^{-2} s^{-1}. On the contrary, the right inset shows the slight temperature influence on the process.

Figure 4 superimposes chromatograms recorded from a mixture of major antenna when subjected to strong light illumination at different times. At this light intensity all antenna proteins are affected, including Lhcb3, although to different extents. However, since the antenna have a strongly conserved amino acid sequence, integration of the area under each peak can be used to compare the time course of UV decline observed for each major antenna. Figure 5a shows that when antenna proteins are in trimeric aggregation, the time course is in the order Lhcb1.1 > Lhcb2 > Lhcb1.2 > Lhcb3. Interestingly, after 1 h of illumination the optical absorption of each protein was almost 20%, indicating that all proteins have lost the NH_2 terminus. Moreover, after this treatment first aggregates appeared and the Coomassie staining was significantly reduced. Thus under high light intensity the degradation process is faster and involves not only the hydrophilic part of proteins but also the transmembrane α-helical structures. However, the complete destruction

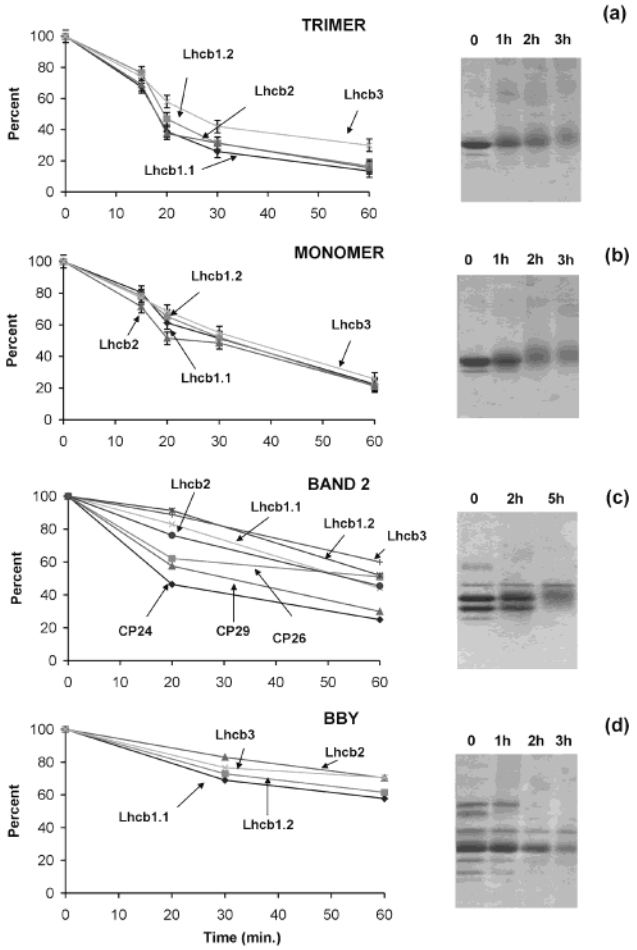

FIGURE 5: Percentage optical decreases of the peaks recorded in HPLC when major antenna in trimeric form (a) or in monomeric form (b), sucrose band 2 (c), and BBY complex (d) are exposed to strong light (1000 μmol m^{-2} s^{-1}). Right panels report SDS−PAGE of the same samples.

of protein required more than 3 h of exposure, with formation of higher aggregates, as reported for D1 protein (*13*).

Influence of the Protein's Conformational State. When trimeric major antenna are transformed into their monomeric form, they show a greater resistance to decreasing optical absorption (Figure 5b). SDS−PAGE confirms the UV observations. It is of interest that Lhcb2 antenna is the most labile antenna protein in monomeric form, the order being Lhcb2 > Lhcb1.1 > Lhcb1.2 > Lhcb3.

Illumination of sucrose band 2 containing both major and minor antenna (CP29, CP26, and CP24) in monomeric form confirmed first (Figure 5c) that the monomeric major antenna show higher resistance to degradation and also that their time course is unaffected by the presence of minor antenna. In addition, the minor antenna are more sensitive to degradation, CP24 being the first protein attacked, followed by CP29 and CP26.

Interestingly, both major and minor antenna organized in the native form of PSII (BBY) show a significantly higher resistance (Figure 5d), suggesting that assembly in the PSII supercomplex offers some sort of photoprotection.

HPLC-ESI-MS analysis of antenna extracted from leaves taken in the dark shows the molecular weight of native protein without any fragmented protein. In contrast antenna extracted from leaves exposed to 1000 μmol m^{-2} s^{-1} for 3 h have a deconvolution analysis of the ESI-MS spectra similar to those reported for isolated antenna upon illumination at low light intensity. Figure 6 reports the reconstructed ionic chromatogram, based on the individual ESI-MS spectra of the major antenna recorded from leaves exposed to 1000 μmol m^{-2} s^{-1} for 3 h at room temperature. Insets report the deconvolution analysis of the ESI spectra of each peak. It can be observed that, besides the native protein, also fragmented proteins are present, as observed *in vitro*. Moreover, calculation from the measured molecular masses confirmed the putative cleavage sites in the NH_2-terminal region. In addition, being all these calculated cleavage sites not Lys-Arg-dependent, the involvement of usual proteases can be excluded.

This evidence indicates that the phenomenon described also occurs in intact leaves, therefore, strong light has the same effect *in vivo* as *in vitro*: it causes the removal of short fragments from native antenna proteins.

Active Oxygen Determination. To further investigate to 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 4 h. The initial optical absorption as well as initial protein molecular weight does not change before or after illumination when measured by HPLS-ESI-MS. This could indicate a possible role of chlorophylls in inducing the observed fragmentation. In addition, exposure of the native major antenna to light in the absence of oxygen does not result in any fragmentation, nor does it when ascorbic acid is added to the mixture in the presence of oxygen. Thus both chlorophyll and oxygen seem to play a role in the effects reported above. Since it has been hypothesized in the case of D1 protein that oxygen is converted to singlet state due to a spin conversion during the energy transfer upon light absorption, we have subjected the antenna proteins to different light intensities in the presence of different active oxygen scavengers and subsequently analyzed the products using HPLC and EPR instruments. The scavengers used were catalase (for H_2O_2), SOD (for O_2^-), histidine and DABCO (for $^1O_2$; *26*), and *n*-propyl gallate (for •OH and alkoxyl radicals; *27*). By quantification of the area under the HPLC peaks, it can be seen in Figure 7b (after 30 min of illumination) that *n*-propyl gallate efficiently protects (70%) the protein against photodestruction, while histidine is less efficient (60%) and DABCO even less (40%). With a mixture of *n*-propyl gallate and histidine, one should expect the complete suppression of the protein degradation, whereas only 85% of the protein is protected. This seems to suggest that the protein site, where the first oxygen is produced, is not free to chemicals.

Figure 7a reports the SDS−PAGE of the same proteins analyzed by HPLC but subjected to 2 h of illumination. A quantitative estimation by Coomassie staining of protein present confirms what was observed by optical absorption: *n*-propyl gallate protects the protein against photodestruction more efficiently than histidine and DABCO.

To determine the identity of the oxygen-containing radicals involved in the photodestruction, preliminary EPR experiments were performed on proteins illuminated in the presence of TEMP or DMPO. TEMP is used to detect singlet oxygen. If present, TEMP is transformed into TEMPO, a stable

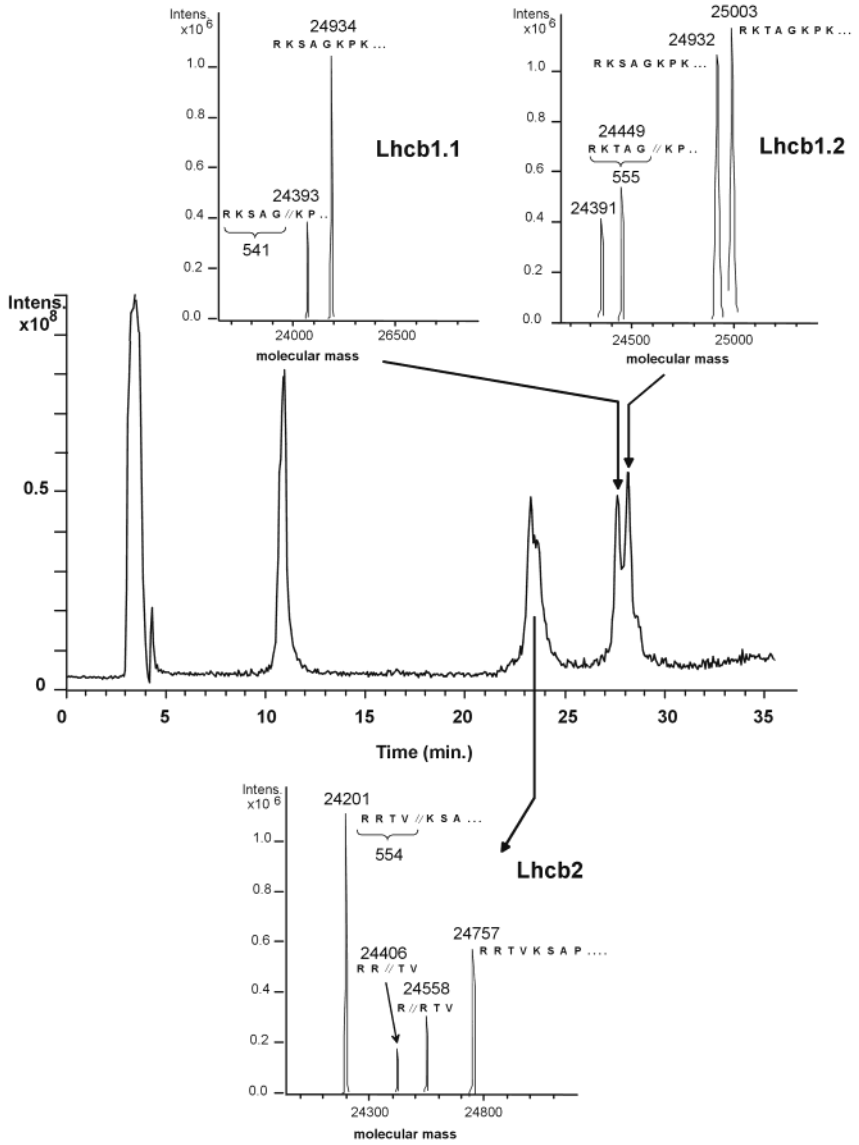

FIGURE 6: Reconstructed ion chromatogram of the spinach major antenna recorded from leaves exposed to 1000 μmol m^{-2} s^{-1} for 3 h at room temperature. Insets report the deconvolution analysis of the ESI spectra of each peak.

nitroxide radical, which can be detected by EPR spectroscopy (*29*). In contrast, DMPO is a spin trap of •OH and alkoxyl radicals, giving rise to DMPO-OH, which gives a characteristic EPR signal (*39*). No marked singlet oxygen was produced in isolated LHCII preparations during the first 25−30 min of high-intensity illumination, but a significant increase in production is observed after 2 h. Interestingly, free and solubilized chlorophyll molecules produced a short, temporary burst of singlet oxygen at the beginning of illumination for 2−3 min. Figure 8b shows the EPR spectra of the nitroxide radical (TEMPO) produced from the reaction of TEMP and singlet oxygen in isolated LHCII preparations when they are exposed to low light intensity. Figure 8d shows EPR spectra recorded when trimeric antenna are subjected to a light intensity of 1000 μmol m^{-2} s^{-1} in the presence of the scavenger DMPO for 10 min. The spin adduct (a_N = a^{β}_H = 14.8 G) is assigned to DMPO-OH by referring to the reported constant (*40*), indicating that under this light intensity the prevalent active oxygen form is •OH. Thus, these preliminary experiments suggest that oxygen radicals are involved in the degradation of antenna proteins and that different radicals may be prevalent depending on the light intensity used or exposition time.

DISCUSSION

In this paper we have reported that active oxygen is involved in damage to isolated light-harvesting proteins (Lhc2). It is well established that fragmentation of D1 is induced by light, and although various fragments may be formed depending on the illumination conditions, a single fragment of 23 000−24 000 found both *in vivo* (*10*) and *in vitro* is considered to be the primary degradation product (*3*). Some authors have demonstrated that this fragment arises from oxygen radical attack (*13*), while others attribute its formation to proteolytic cleavage by chloroplast proteases (*11*, *12*). However, the latter explanation is based on the existence of light-inducible proteases (*16*) or hypothetical damage to D1 already before proteolytic cleavage (*11*, *17*), confirming that the mere presence of proteases is not sufficient to explain the onset of degradation.

In the case of antenna proteins, we are at the beginning of this discussion. In fact, whereas the D1 fragment is easily

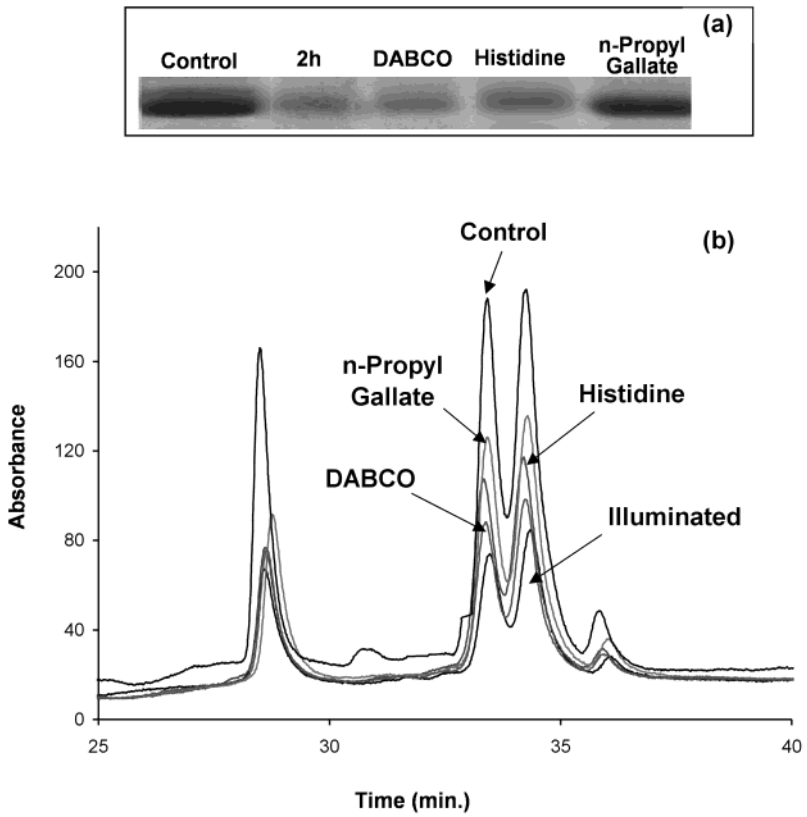

FIGURE 7: Protective effect of different oxygen scavengers on major antenna subjected to strong light (1000 μmol m^{-2} s^{-1}): (a) SDS−PAGE of major antenna in the presence or absence of different oxygen scavengers for 2 h at 4 °C; (b) HPLC chromatograms of the same samples but illuminated for 30 min. UV detection was at 214 nm.

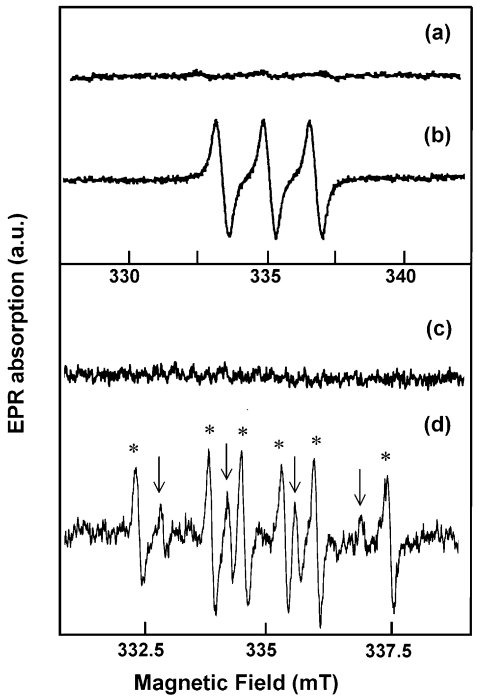

FIGURE 8: EPR spectra of major antenna (a, c) before light exposure or (b, d) when the samples, containing 10 mM TEMP (b) or 80 mM DMPO (d), were subjected to photoinhibition. Asterisks mark EPR spectra of DMPO-R ($a_N = 14.8$ G, $a^{\beta}_H = 22.6$ G); downward arrows indicate spectra of DMPO-OH ($a_N = a^{\beta}_H = 14.8$ G).

visualized by SDS−PAGE and a significant smearing and mobility shift of Coomassie-stained bands is also observed (*41*, *42*), in the case of light harvesting proteins its degradation is more difficult to reveal. Studies by Lindahl et al. (*18*) showed that degradation of antenna shows a delay of 2 days after transfer of the leaves to strong light. Our study *in vitro* shows that degradation of isolated antenna proteins takes place in a short time. It involves initial removal of the NH_2-terminal region, detectable only by HPLC and mass spectrometry, and subsequently degradation of the entire polypeptide chain into end products observable also by SDS−PAGE. However, when only a small part of the native protein is removed it escapes SDS−PAGE detection, explaining why nobody has previously reported this occurrence. In reversed-phase HPLC this truncated protein elutes after the native one, since it is more hydrophobic. Mass spectrometry and amino acid sequencing show unequivocally that before the entire protein is degraded, fragmentation of the NH_2-terminal region takes place randomly. This result suggests that this area is probably the first to come into contact with reactive species generated by photodamage or even that it is in this area that they are produced. At longer times degradation involves the entire hydrophilic NH_2 region, reducing the protein to around 19 364 for both Lhcb1 and Lhcb2, which corresponds to the beginning of the first α-helix of protein, inaccessible to any external agents. Similarly, in D1 the cleavage takes place in the loops connecting the five transmembrane α-helices. In any case cleavage of the internal α-helix transmembrane of D1 has never been reported, even when free Chl and its derivatives were incorporated into SDS micelles as photosensitizers (*13*). This is related to the fact that D1 contains five transmembrane α-helices and only two chlorophyll molecules that, although requiring less energy to form singlet oxygen, cannot contribute much to further degradation, whereas the preliminary degradation of antenna proteins

delivers many free chlorophylls that can rapidly render the reaction autocatalytic. This may explain why upon strong illumination intensity (1000 μmol m^{-2} s^{-1}) the antenna proteins are completely degraded into small fragments, as may be observed by mass spectrometry and by the Coomassie staining of SDS−PAGE-separated proteins.

Obviously the degradation here reported is a phenomenon observed *in vitro* and it is not necessarily occurring *in vivo*. However, the new evidence that emerges from our data is that isolated antenna proteins can be partially or completely degraded by a process that is light-induced and cannot be explained by the action of proteases. This process is in fact temperature-independent and the first cleavage of the NH_2 terminus occurs randomly. Moreover, addition of protease inhibitors does not block the process. In contrast, active oxygen species have been clearly revealed by EPR and the process is inhibited by oxygen scavengers, which represent direct evidence that oxygen species are actively involved. Accordingly, apoprotein illuminated at high light intensity for a long time does not give rise to any fragmentation.

Reactive Oxygen Species. As judged from the effectiveness of active oxygen scavengers, the species that participate in damaging proteins are $^1O_2$, •OH, and alkoxyl radicals, while H_2O_2 and O_2^- seem not to be involved. The protective effect of *n*-propyl gallate (70%), histidine (60%), and DABCO (40%) with respect to SOD and catalase was also observed with D1 in the presence of RC complexes (*13*), which are subjected to donor-side photoinhibition. From our preliminary EPR measurements, low illumination intensity produced prevalently $^1O_2$ forms, while at high intensity •OH and alkoxyl radicals are the main forms observed. This suggests that singlet oxygen is the source of the other radical species. It is well-known that •OH can be generated by secondary reactions of H_2O_2, O_2^-, and $^1O_2$, while alkoxyl radicals arise from reactions of $^1O_2$ and •OH with organic molecules (*43*, *44*). In fact, when aromatic and sulfur-containing residues of proteins (*26*) are attacked by $^1O_2$ and •OH, radicals located on their side chains are generated, which react with other susceptible residues to form aggregates by covalent cross-links (*45*). Aggregation predominates when the rate of cleavage of peptide bonds proceeds slowly, as in the case of D1, while the light harvesting antenna proteins are completely destroyed and no significant protein aggregation is observed. In fact, in strong light or at longer times when antenna proteins are significantly degraded, EPR measurement revealed the presence of both •OH and alkoxyl radicals. In the antenna proteins, it is reasonable to suppose that preliminary degradation of LHCII releases a large number of chlorophyll molecules that act as photosensitizers to generate further singlet oxygen (*44*). Moreover, recent results demonstrate that the transmembrane helical structure of light harvesting proteins for both PSI and PSII is destroyed by strong light treatment within a relatively short time (*46*). Thus, the higher oxygen radical production, related to free chlorophylls, and the low α-helix content (*46*, *47*) may contribute to complete degradation of LHC.

Our EPR measurement revealed the presence of singlet oxygen in isolated antenna proteins after hours at low light intensity and after the first 30−40 min at high illumination, in agreement with that reported by Hideg and Vass (*48*). Recently, three previously undescribed triplet populations at emission wavelengths characteristic of PSII chlorophyll/protein complexes have been revealed by FDMR (*21*): two are associated with the inner core pigments, while one is probably generated by external antenna complex(s). There is now strong evidence that much light-induced damage in isolated thylakoids and intact algal cells occurs via Chls that are energetically uncoupled from the main antenna matrix (*49*−*51*). The existence of a minor population of triplet in light harvesting proteins with respect to core proteins is probably due to the efficient quenching of triplet chlorophyll and/or singlet oxygen by carotenoids (*19*). However, it is not surprising that a chlorophyll distant from carotenoids may transfer its energy to oxygen, giving rise to singlet oxygen. In particular, the T−S spectra of the Car triplets showed prominent absorption changes in the Chl *a* Q_y region, prompting researchers to associate Chl *a* with the transfer of energy to nearby Car triplets (*52*). However, with FDMR a Chl *a* triplet not quenched by Cars was observed (*53*). A recent paper reported that the LHCII mutants missing Chls A2, B2, A4, and B3 had an enhanced resistance to strong illumination in the presence of oxygen (*54*). In particular, the A4 chlorophyll is located toward the stromal surface, where the N-terminal region of light harvesting proteins is exposed. Thus, it is possible that the active oxygen species may be produced near the N-terminal of LHC. Interestingly, the mixture of *n*-propyl gallate and histidine suppressed the degradation phenomenon only in part (85%), confirming that active oxygen is formed inside the protein and both scavengers are not able to suppress the active oxygen species present in the surrounding medium, similarly to what is observed when RC complexes were illuminated (*13*). In this way DABCO, a potent singlet oxygen quencher, is not able to inhibit completely the antenna degradation, especially at high light intensity where more than one active oxygen species are formed.

Aggregation State of Proteins. All antenna seem to be amenable to degradation. At present, it is not clear whether the process starts in a particular antenna protein and then other intrinsic proteins are consequently cleaved. Lhcb3 seems to be the most resistant to degradation, but this protein lacks the first 10 amino acids found in Lhcb1 and Lhcb2 proteins; therefore, its resistance may be only apparent. Nevertheless, when major antenna proteins are in trimeric form, as in sucrose ultracentrifugation band 3, or assembled in the PSII complex, Lhcb1.1 and/or Lhcb1.2 are the first to be affected. In particular, by comparison of the time courses of Lhcb1.1 and Lhcb1.2 degradation, it appears that the former is a likely candidate for the initial oxygen radical producer. ADMR experiments are in progress to confirm this hypothesis. On the other hand, the monomeric form showed a higher resistance to fragmentation than the trimeric form. Still, minor antenna show a significant sensitivity to fragmentation with respect to major antenna proteins. In particular, CP24 is the most readily attacked antenna, even though CP29 contains the longest hydrophilic NH_2 region. The different rates of LHCII fragmentation observed require a more accurate analysis, since this behavior is attributable to different stoichiometries of the carotenoid pigments bound to several antenna proteins, as well as to different conformational states of the proteins. In this connection, a recent paper (*55*) reported that changes in the protein conformation induced by binding of carotenoids with distinct molecular structure, rather than different photochemical properties of

individual carotenoid species, are involved in the quenching phenomena associated with LHC proteins. Moreover, both the FDMR (*53*) and ADMR (*56*) signals showed that the efficient transfer of triplet from Chl to Car is strongly dependent on the LHCII aggregation state. This has been explained either by an increased intertrimer triplet transfer (*56*) or by a redistribution of the triplets over the two central xanthophylls that are present in each LHCII monomeric subunit (*57*).

In any case the most relevant aspect of the data reported is that both the major and minor antenna proteins assembled in the PSII complex show strong resistance to photoinhibition, suggesting that the *in vivo* organization affords a sort of some photoprotection.

Physiological Considerations. From a physiological point of view, a finding that deserves particular attention, together with the above photoprotection related to PSII organization, is that leaves subjected to strong light illumination showed partially degraded LHCII. This degradation show Lys-Arg-independent cleavage sites, in agreement with what is found upon illumination of isolated proteins. Thus the present study suggests that also *in vivo* active oxygen may play a role in the degradation of antenna proteins, such as during the short-term acclimative adaptation of plants. In the case of D1 it is generally accepted that active oxygen cannot degrade the entire protein to amino acids, but modification of proteins by active oxygen is known to increase their susceptibility to proteases in the chloroplast (*4*, *58*), prompting the hypothesis that complete degradation can be accomplished by the concerted actions of active oxygen and the proteases that digest abnormal proteins. Thus it may be speculated that, upon illumination, phosphorylation induces dissociation of the assembled light harvesting proteins (*59*) and prolonged illumination induces active oxygen production on the dissociated antenna proteins and subsequent attack of their NH_2-terminal region. This is in agreement with recent evidence that aggregation of trimeric LHCII into macroaggregates reduces the triplet yield several times (*60*, *61*). Nevertheless, the NH_2-terminal region is important for trimerization or higher organization of antenna proteins (*62*), so that the removal of this part induces permanent PSII disassembly. Its subsequent lateral movement to the nonappressed thylakoid regions (*63*), where most of the chloroplast proteases are present (*11*, *12*, *16*), could expose the damaged antenna protein to complete proteolytic digestion.

Obviously more experiments are required to better clarify the exact mechanism that brings the light harvesting protein to complete destruction, a mechanism not yet discovered for the core proteins although many investigations have been undertaken. ADMR and EPR measurements are in progress to identify the chlorophyll(s) involved in the active oxygen production, with the final aim of producing recombinant light-harvesting proteins that show a higher resistance in the acclimative adaption to light stress.

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