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Generation of reactive oxygen species upon strong visible light irradiation of isolated phycobilisomes from *Synechocystis* PCC 6803

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ABSTRACT

The mechanism of photodegradation of antenna system in cyanobacteria was investigated using spin trapping ESR spectroscopy, SDS-PAGE and HPLC-MS. Exposure of isolated intact phycobilisomes to illumination with strong white light (3500 μmol m^{-2} s^{-1} photosynthetically active radiation) gave rise to the formation of free radicals, which subsequently led to specific protein degradation as a consequence of reactive oxygen species-induced cleavage of the polypeptide backbone. The use of specific scavengers demonstrated an initial formation of both singlet oxygen ($^1O_2$) and superoxide (O_2^-), most likely after direct reaction of molecular oxygen with the triplet state of phycobiliproteins, generated from intersystem crossing of the excited singlet state. In a second phase carbon-based radicals, detected through the appearance of DMPO-R$^.$ adducts, were produced either via O_2^- or by direct $^1O_2$ attack on amino acid moieties. Thus photo-induced degradation of intact phycobilisomes in cyanobacteria occurs through a complex process with two independent routes leading to protein damage: one involving superoxide and the other singlet oxygen. This is in contrast to the mechanism found in plants, where damage to the light-harvesting complex proteins has been shown to be mediated entirely by $^1O_2$ generation.

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1. Introduction

In oxygenic photosynthesis generation of harmful reactive oxygen species (ROS) occurs during photochemical energy conversion and especially when absorption of light energy exceeds the capacity for its utilization in the photosynthetic apparatus. ROS-induced oxidative damage to both photosynthetic reaction centers and peripherical light-harvesting structures may contribute to a light-induced decrease in photosynthetic activity known as photoinhibition, however the mechanisms behind this process are still much debated [1–6]. To survive excessive illumination several protective strategies have evolved in plants, algae and cyanobacteria. ROS-mediated damage has been extensively studied for the D1 protein of plant photosystem II (PSII), leading to the suggestion that serine-type proteases intrinsically present in PSII preparations, and possibly a component of PSII itself, catalyze D1 protein degradation [2]. Many reports now confirm that the ATP-dependent zinc metalloprotease FtsH is responsible for the primary cleavage of the D1 protein in thylakoids of higher plants and cyanobacteria [7–11]. In this scenario the role of ROS has been proposed to consist in triggering D1 degradation by changing its conformation and rendering it susceptible to the proteases [2]. In contrast to this hypothesis an alternative mechanism has been suggested by Miyao and co-workers, who demonstrated direct chemical cleavage of

the D1 protein by ROS [12,13]. Interestingly, recent studies are in line with this view; in fact, many D1 (and also D2) fragments identified during high-light treatments of cells, photosynthetic membranes and isolated reaction center components from the cyanobacterium *Synechocystis* 6803 were found simply to be results of the direct cleavage by ROS, without the assistance of any real protease(s) [14]. Similarly the selective ROS-induced fragmentation of PSII light-harvesting proteins has been demonstrated to cause in a short time the complete degradation of isolated antenna proteins into small peptide fragments [15,16], although the participation of proteases in this process in intact membrane systems is also well documented [17–21]. ROS-induced damage depends on chlorophyll molecules acting as photosensitizers under excess light, with energy transferred from a triplet state chlorophyll to molecular oxygen to yield singlet oxygen ($^1O_2$), a potent oxidizer. It is generally accepted that singlet oxygen can react spontaneously with proteins to cause both amino acid oxidation and larger-scale structural changes such as polypeptide chain scission and cross-linking as a result of a variety of reactions involving ROS and peptide-based radicals [22–24].

In cyanobacteria light is not captured by LHCII, but by large membrane-extrinsic complexes, the phycobilisomes (PBSs), which are attached to the outer surface of thylakoid membranes. These complexes are highly ordered, supramolecular assemblies of $5–20 \times 10^6$ Da, and consist of phycobiliproteins, which carry covalently linked bilins (open-chain tetrapyrrole chromophores), and linker peptides, which are required for the organization of the phycobilisome. On the basis of their visible absorption properties, phycobiliproteins have been

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assigned to four classes, phycoerythrocyanins (PECs), phycoerythrins (PEs), phycocyanins (PCs), and allophycocyanins (APCs). Each phycobiliprotein is composed of two different subunits, α and β, that associate to form a monomer. Monomers form disk shaped trimers ($\alpha_3\beta_3$), and hexamers ($\alpha_6\beta_6$). These oligomers are the building units for the assembly of phycobilisomes (reviews [25,26]). In general, phycobilisomes consist of two morphologically distinct substructures, the core and peripheral rods. The core is composed primarily of allophycocyanins and is in direct contact with thylakoid membranes. Multiple sets of rods radiate from the core, which are made up of phycocyanins, phycoerythrins, and/or phycoerythrocyanins. The membrane/phycobilisome association is mediated by a large chromoprotein present within the phycobilisome core, which also has linker polypeptide features; it is referred to as the anchor protein or core–membrane linker polypeptide (reviews [25,26]). From here, the energy is transferred to the chlorophylls of the inner chlorophyll antenna, CP43, and CP47 (containing chlorophyll a and carotenoids) and to reaction center II. Phycobilisomes can also transfer energy to photosystem I [27,28]. Studies on the effects of high light on cyanobacterial system are still scarce, but some photoprotective mechanisms mediated by phycobilisomes, such as non-photochemical quenching [29], have recently been described. The involvement of phycobiliproteins in ROS production suggests that the bilin pigments may act as photosensitizers and produce $^1O_2$ (Type II mechanism), or alternatively give rise to photo-induced formation of superoxide and hydroxyl radicals (Type I mechanism) [30], but little information on these photoacclimative processes has been available so far.

The aim of the present study was to investigate the effects of high-intensity irradiance in damaging phycobiliproteins. By using spin trapping ESR spectroscopy we obtained direct evidence that free radicals are formed when intact isolated phycobilisomes are subjected to illumination with strong white light, and specific protein degradation as a consequence of a ROS-induced cleavage of the polypeptide backbone was demonstrated by SDS-PAGE and HPLC-MS.

2. Materials and methods

2.1. Phycobilisome preparation

Synechocystis PCC 6803 was grown at 37 °C at a light intensity of 50 μmol m^{-2} s^{-1} in BG11 medium [31]. Phycobilisome complexes were prepared according to Ajlani et al. [32]. One liter cultures were harvested and washed twice in 0.8 M potassium phosphate buffer, pH 7.0 (KP). Cells were resuspended in KP containing 1 mM phenylmethanesulfonyl fluoride, 1 mM sodium azide and 2 mM EDTA, then vortexed twice for 1 min with half the volume of glass beads (0.17 mm diameter). Triton X-100 was added to the broken cells to a final concentration of 2% (v/v). After incubation for 30 min with occasional shaking, unbroken cells and debris were removed by centrifugation at 15,000 × g for 20 min at 20 °C. One milliliter of supernatant was loaded onto sucrose step-gradients in 12 ml ultracentrifuge tubes prepared as follows: 1 ml of 2.0 M, 3 ml of 1.0 M, 2.5 ml of 0.75 M, 2.5 ml of 0.5 M, and 2 ml of 0.25 M sucrose solution in 0.8 M KP. The gradients were spun 16 h in a Beckman SW41 rotor at 35,000 rpm at 20 °C and the blue layer containing the phycobilisome was removed. Protein concentration of phycobilisomes was estimated according to Lowry et al. [33].

2.2. Light treatment

Phycobilisome preparations were illuminated for different periods of time with continuous stirring at 4 °C using strong white light (3500 μmol m^{-2} s^{-1}) from a 250-W
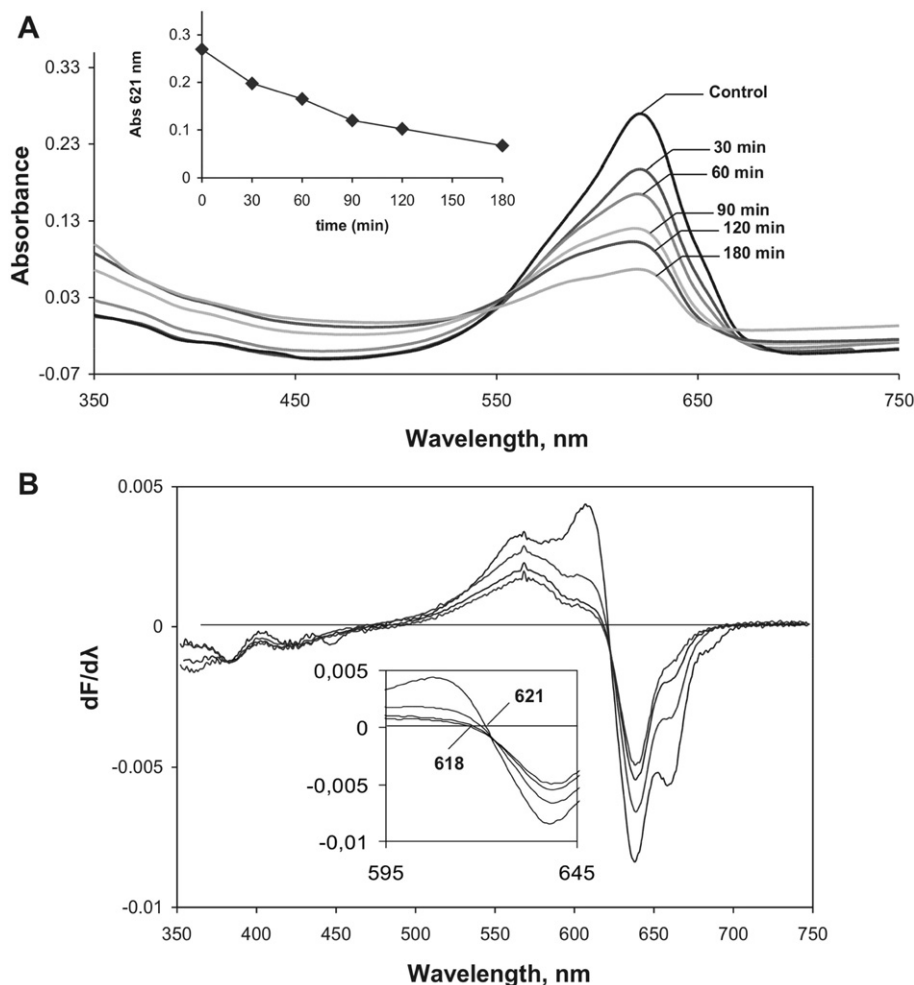
Fig. 1. (A) Room temperature absorption spectra of phycobilisomes exposed to strong visible light for different times. Inset shows the percentage variation of the absorbance at 621 nm. (B) First derivative of the absorption spectra of irradiated phycobilisome complexes. Inset shows the red region of the first derivative.

quartz/halogen projector lamp. When indicated, 1:25 volume of a standard protease inhibitor solution (Protease Inhibitor Cocktail, Roche, Mannheim, Germany) or various active oxygen scavengers was added before illumination. 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 $^1O_2$ [34,35]; superoxide dismutase (SOD) and catalase 100 μg/ml for O_2^- and H_2O_2, respectively.

2.3. Spectroscopic analysis

Room temperature absorption spectra were recorded with a Varian Cary 4 spectrophotometer. EPR spectra were 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. 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 eliminate the possibility of photochemical reactions of DMPO [36].

2.4. High-performance liquid chromatography and electrospray-mass spectrometry

Protein separation by HPLC was performed using a reverse phase Vydac Protein C-4 column (250×4.6 mm I.D.) packed with 5 μm porous butyl silica particles [37,38]. This column was operated at a flow rate of 1 ml/min for optimum separation efficiency. All solutions were filtered through a Millipore (Milan, Italy) type FH 0.5-μm membrane filter and degassed by bubbling with helium before use. Optimization of chromatographic separations was performed using a Beckman (Fullerton, CA, USA) System Gold system, consisting of a Model 126 solvent delivery pumps and a Model 168 UV diode-array detector. Samples were introduced onto the column by a Model 210A sample injection valve with 50 μl sample loop. The Vydac C-4 columns were pre-equilibrated with 20% (v/v) aqueous acetonitrile solution containing 0.05% (v/v) trifluoroacetic acid, and samples were eluted by a linear gradient from 20% to 100% (v/v) acetonitrile in 60 min, followed by a 10-min isocratic elution with the eluent containing 100% acetonitrile. The 1 ml/min flow through the analytical column was split post-column, with 50 μl/min entering the mass spectrometer ion trap Esquire 3000 plus (Bruker Daltonik, Germany), and 950 μl/min going to the UV and fluorescence detector. For analysis with pneumatically assisted ESI, an electrospray voltage of 4 kV and a nitrogen

sheath gas flow were employed. The temperature of the heated capillary was set at 300 °C. Protein mass spectra were recorded by scanning between 500 and 2000 amu.
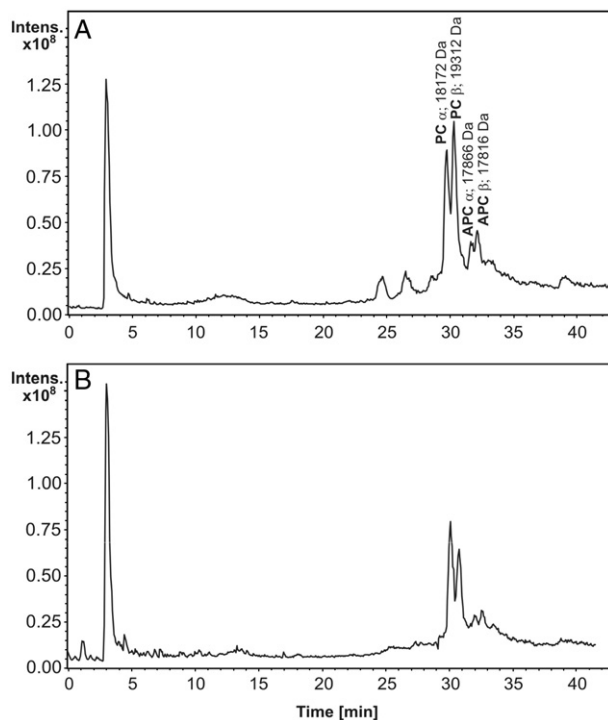
2.5. Electrophoresis

Phycobilisome proteins were separated using 12.5% SDS-PAGE according to Laemmli [39]. For phycobilisome loading, the absorbance of the samples was measured at 620 nm, and an amount of biliproteins equivalent to OD$_{620}$ = 1.5 was loaded per well. For protein visualization, gels were stained with colloidal Coomassie Brilliant Blue G (Sigma) [40].

3. Results

To investigate the photochemistry of phycobilisomes under high irradiance, the isolated complex from *Synechocystis* 6803 was exposed to high level of visible light (from 1000 to 3500 μmol m^{-2} s^{-1} photosynthetically active radiation) for different times. Initially the effects on spectral properties were analyzed, and since light intensities lower than 3000 PFD (photon flux density) did not influence significantly the phycobilisome absorption characteristics, a level of 3500 μmol m^{-2} s^{-1} was chosen for further experiments. Fig. 1A shows the time course changes in the room temperature absorption spectra of isolated phycobilisomes during illumination at 3500 PFD. The spectra measured in untreated phycobilisome preparations showed the typical absorption peak at 621 nm of phycocyanin, the major component of the phycobilisome antenna system. As the time of illumination increased the peak declined; about 75% had disappeared when phycobilisomes were irradiated for 3 h (inset of Fig. 1A). In addition, the maximum peak position was shifted by 3 nm towards shorter wavelengths after 2 h of illumination. This becomes particularly clear from the changes in first derivative spectra (Fig. 1B), which also show the gradual time-dependent alteration in the position of the major peak in the red region (Fig. 1B inset). Thus, irradiation of phycobilisomes with strong white light leads to progressive loss of absorbance. This bleaching of the chromophore takes place without significant changes in the position
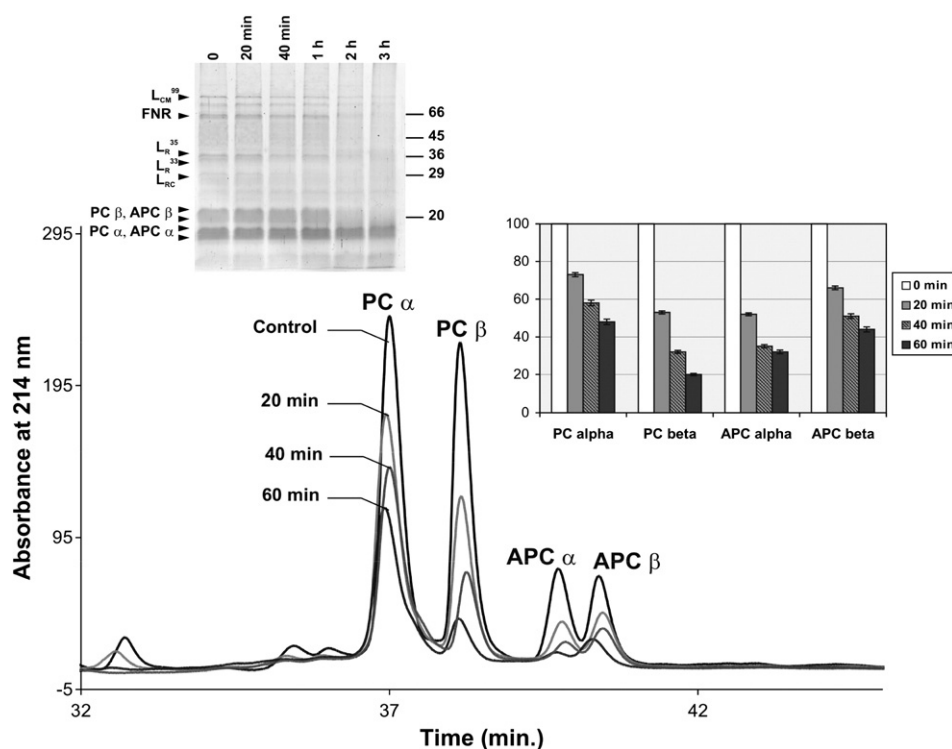
Fig. 2. HPLC chromatograms recorded at 214 nm from *Synechocystis* phycobilisomes exposed to strong white light for different periods of time. Phycobilisome samples (200 μl, initial A_{621} = 0.8) were injected onto a C-4 reverse phase column following the experimental conditions reported in Materials and methods. Left inset displays the SDS-PAGE analysis of phycobilisomes exposed to strong visible light for the indicated time periods. Right inset shows the percentual optical decreases of the peaks recorded in HPLC. PC, phycocyanin; APC, allophycocyanin. Absorbance units: mAU.
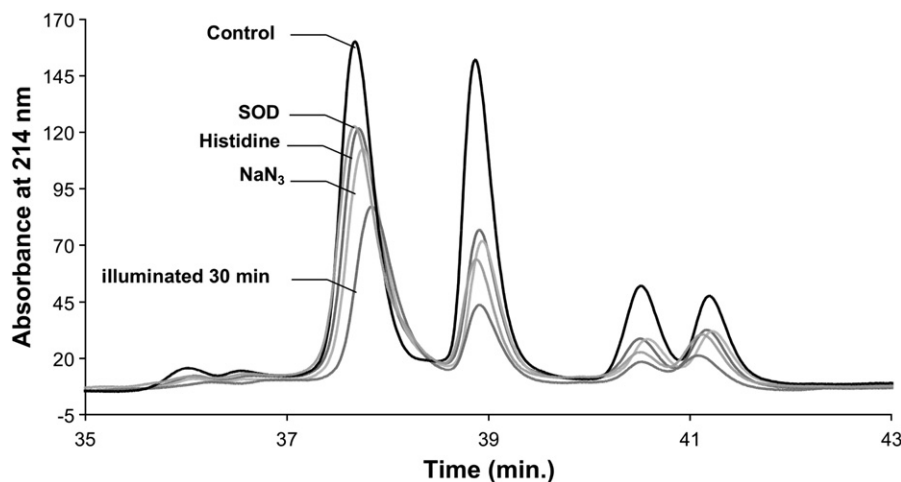
Fig. 3. Total ion chromatograms of *Synechocystis* phycobilisomes: (A) control and (B) after illumination at 3500 μmol m^{-2} s^{-1} for 30 min. Molecular weights obtained by deconvoluting the ESI spectra are also reported.

underlying each chromatographic peak are plotted as a function of irradiation time. It can be seen that phycocyanin-β and allophycocyanin-α were the phycobiliproteins mainly affected, and to a quite similar extent. Analysis of apoprotein photodestruction was performed by SDS-PAGE (left inset of Fig. 2) and the significant decrease in Coomassie staining over time indicated polypeptide backbone cleavage. Concomitantly a slight decrease in the electrophoretic mobility and a protein smear below the original phycobiliprotein bands appeared, whereas high molecular mass aggregates accumulated after more prolonged illumination, albeit in low yields. We also made experiments in which a protease inhibitor cocktail was added to the sample before irradiation, and no protective effect on the photodegradation process was observed (not shown). On the contrary, in anaerobic conditions there were neither detectable changes of the electrophoretic mobility nor any decrease in staining, suggesting that atmospheric oxygen played an essential role in the degradation. By comparing results from HPLC and SDS-PAGE, it can be inferred that the optical reduction does not reveal the true extent of the protein degradation, leading to erroneously high estimates of protein loss. Instead the rapid UV absorption decrease may be ascribed to the direct damages of aromatic amino acids and bilin, similarly to the photodestruction of phycobiliproteins by UV-B radiation [38,41,42]. To determine more exactly the nature and degree of protein degradation we coupled the HPLC on-line with a mass spectrometer. Fig. 3 compares the total ion chromatogram (TIC) based on the individual ESI-MS spectra of the phycobiliproteins recorded for both control and treated (3500 μmol m^{-2} s^{-1} for 30 min) samples. A significant decrease in the ionic current intensity corresponding to the allo- and phycocyanin peaks can be seen, in agreement with the optical decrease observed by HPLC. No new peaks were detected at lower elution times, while the front peak of the chromatogram increased, suggesting formation of very small amino acid fragments. On the other hand, deconvolution analysis of the ESI spectra relative to the main peaks belonging to biliproteins gave molecular weights corresponding to intact native proteins [37,38]. Thus no truncated forms were detectable; this implies that a degradation mechanism of the "all-or-none" type is involved.

All these events are a common manifestation of the action of reactive oxygen species on protein photodestruction, however in order to know the molecular mechanisms involved we exposed the phycobilisomes to strong white light in the presence of different reactive oxygen scavengers and subsequently analyzed the degradation products with HPLC (Fig. 4). The scavengers employed were histidine, DABCO and NaN$_3$ for ^{1}O$_2$ [34,35], and superoxide dismutase and catalase for O$_2^-$ and H$_2$O$_2$, respectively. The results showed a protective effect on the part of ^{1}O$_2$ quenchers, in particular histidine

and shape of the bands, and without the appearance of new bands in the visible region of the spectra. This indicates that the conjugation of the bilin moiety is considerably reduced as a consequence of the oxidative process. To determine whether also the polypeptide composition of phycobilisomes was affected by high-light exposure, intact phycobilisomes were separated by both HPLC and SDS-PAGE. Fig. 2 superimposes chromatograms of phycobilisomes exposed to strong white light illumination (3500 μmol m^{-2} s^{-1}) at different times. At the light intensity used, all phycobiliproteins were affected, although to different extents. Since the biliproteins have a strongly conserved amino acid sequence, integration of the area underlying each peak can be used to compare the time course observed for each phycobilisome component [38]. This quantitative estimation is shown in the right inset of Fig. 2 where the percentage decreases in the area
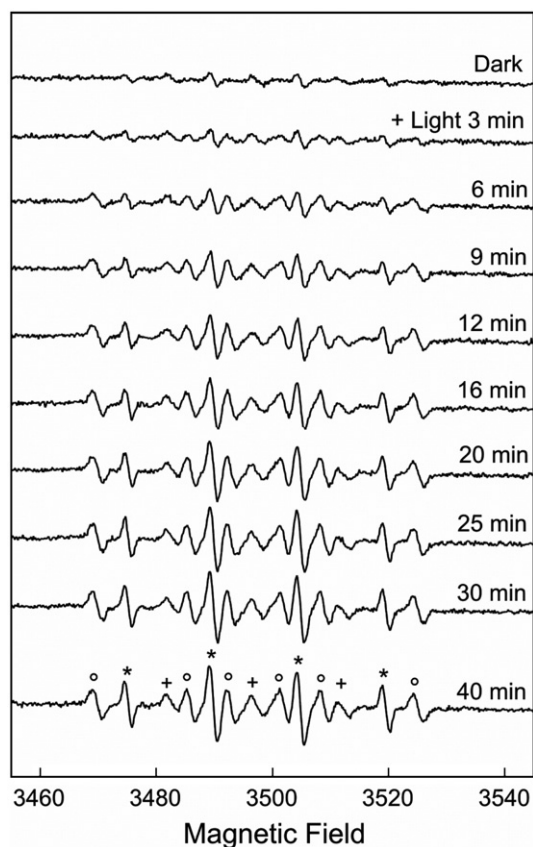

Fig. 4. HPLC chromatograms recorded at 214 nm from phycobilisomes subjected for 30 min to strong visible light in the presence or absence of different oxygen scavengers. Absorbance units: mAU.

Fig. 5. ESR spectra of spin-trapped radicals generated during illumination of *Synechocystis* phycobilisomes in the presence 80 mM DMPO. The duration of illumination indicated includes the scan time of the spectrum. The symbols indicate line components belonging to DMPO-OH· (*) and DMPO-R· (○); an unidentified DMPO radical adduct (+) was also detected.

Fig. 6. Kinetics of the formation of DMPO-OH· (■) and DMPO-R· (▲) upon illumination of intact phycobilisomes. The light was turned on after 3 min of data acquisition.

and sodium azide, against photodestruction. Also SOD turned out to be an efficient scavenger, whereas catalase had no effect on the UV signal intensity (data not shown). To establish the identity of the reactive oxygen species responsible for protein degradation, and the succession of the events which cause this degradation, isolated phycobilisomes were illuminated with photoinhibitory light levels directly during ESR measurements in the presence of DMPO as spin trap.

When an oxygen-saturated aqueous solution containing phycobilisomes and DMPO (80 mM) was irradiated continuously with strong visible light, the formation of two different radical species could be observed. Initially a four-line ESR spectrum was clearly seen with an intensity ratio of 1:2:2:1 and hyperfine splittings $a^N = a^H = 14.9$ G (Fig. 5). This characteristic spectrum was attributed to the hydroxyl adduct of DMPO (DMPO-OH·). Within 3 min, the six-line signal of a second radical appeared exhibiting the peaks and hyperfine splitting characteristics of spin trap-carbon centered alkyl or alkoxyl species (DMPO-R·, $a^N = 16.0$, $a^H = 23.2$ G). Curiously, also a third ESR signal (marked with a cross in Fig. 5) was detected, but unfortunately we were not able to identify these radicals solely on the basis of the spectra from their DMPO adducts. This signal could be due to spurious radicals of spin trap degradation products or to a different carbon-centered DMPO radical adduct partially masked by the other predominant signals. More information about the reaction mechanism was obtained from the kinetic evaluation of the formation of the two main radicals detected (Fig. 6). It can be seen that both DMPO-OH· and DMPO-R· adducts reached a plateau within 40 min after the onset of irradiation, although their relative signal intensity was different. This type of kinetics is very common for free radicals in solution; when the radical concentration increases the reaction between them (typically
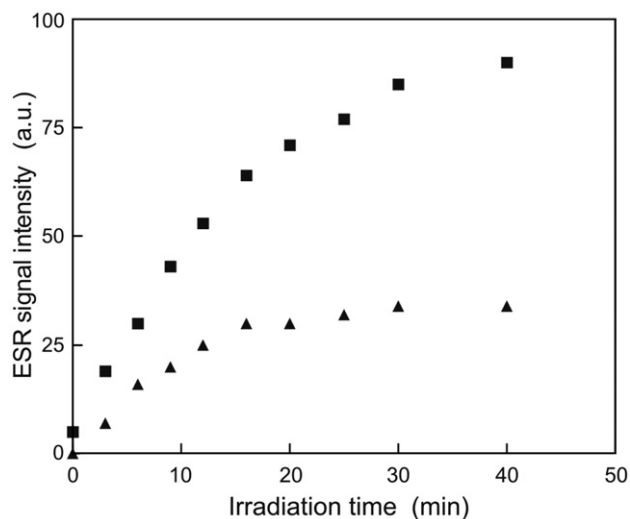
dismutation or dimerization) becomes more and more frequent, and at the plateau level the rate of formation is identical to the rate of disappearance [43]. To understand the potential sources for the detected ESR signals, specific quenchers of singlet oxygen (NaN$_3$ and DABCO) were used. In the presence of sodium azide (2 mM), the signal intensity of the DMPO-OH· adduct was about 30% lower than that of the sample without scavenger, while in the presence of DABCO (50 mM) it was only 20% lower (data not shown). Unfortunately histidine, the most effective scavenger and considered specific for singlet oxygen [44], 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 [44]. As the use of $^1O_2$ scavengers only partially prevents formation of the DMPO-OH· adduct, the involvement of $O_2^{-·}$ and H_2O_2 in the production of free OH· was investigated by adding SOD and catalase. Interestingly, the presence of SOD in the system significantly inhibited the formation of the DMPO-OH· adduct and eliminated about 40% of the ESR signal, whereas addition of catalase had no effect, at least within the reaction times examined (data not shown).
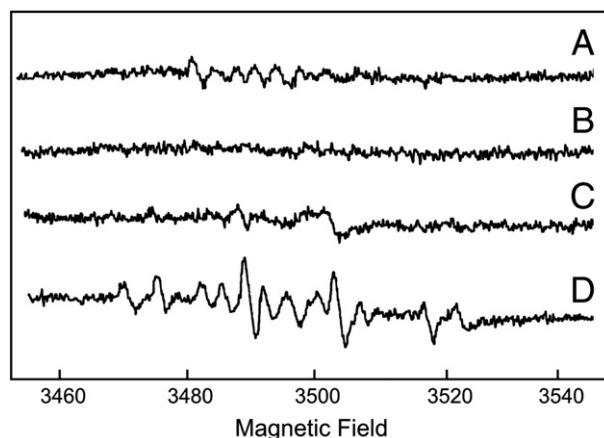
Fig. 7. ESR spectra of DMPO-trapped radicals produced by irradiation of a phycobilisome sample contained in a gas-permeable TPX-capillary under anaerobic conditions. Spectra were measured during dark-adapted conditions (A) and after illumination for 15 min (B) and 60 min (C). A control experiment shows the spectrum after 15 min illumination of an identical sample contained in a gas-permeable TPX-capillary with a surrounding flow of air (D).

To confirm the prominent role of O_2 in the mechanism of photosensitization of phycobilisomes, we studied the effect of oxygen by using gas-permeable capillaries that allowed ESR measurements under nitrogen atmosphere or under a continuous flow of air (Fig. 7). In the absence of oxygen none of the DMPO adducts observed in Fig. 5 could be detected. After prolonged irradiation a low intensity signal from a different radical was observed (Fig. 7C); the broad central line suggested that this species was not a small DMPO adduct but perhaps a phycobilin- or protein-based radical. The lack of spectral features did not allow any assignment of this radical; however, it was not seen when the same experiment was done with a continuous supply of air. In this case the spectra showed the formation of the same radicals as in Fig. 5. This result also excludes that the consumption of oxygen in the sample could be the reason why the curves in Fig. 6 tend to level off at longer reaction times.

4. Discussion

4.1. Reactive oxygen species

Oxygen is potentially toxic for all organisms, but is a particular problem for photosynthetic oxygen-producing cells, in which the generation of various ROS such as singlet oxygen ($^1O_2$), the superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^.$) during photosynthetic light reactions has been demonstrated [45]. These species may cause oxidative damage to components of photosynthetic apparatus including pigments, lipids and proteins [46]. In particular, proteins bearing colored prosthetic groups, such as phycobiliproteins, can be both the source and target of $^1O_2$ with consequent loss of the macromolecular biological functions [22]. No studies had yet been published on the photosensitized formation of reactive oxygen species in intact phycobilisomes. Moreover, the role of oxygen active species in photodegradation has been only partially studied so far, and only for isolated biliproteins [47–49]. Previous evidence on photogeneration of $^1O_2$ by isolated phycobiliproteins comes from indirect methods, either measurements of secondary DMPO-$OH^.$ production as follows

$$DMPO + {}^1O_2 \rightarrow [DMPO - {}^1O_2] \overset{H^+}{\rightarrow} DMPO - OH^\bullet + OH^\bullet \qquad (1)$$

$$OH^\bullet + DMPO \rightarrow DMPO - OH^\bullet \qquad (2)$$
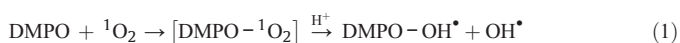
or by spectroscopically monitoring the photobleaching of phycobiliproteins by the $^1O_2$ generated [48,50]. More recently, Paul et al. [49] investigated the photodynamic generation of $^1O_2$ by phycocyanins by using EPR-spin trapping (TEMPL-assay). Our findings clearly demonstrate the photogeneration of reactive oxygen species from whole phycobilisomes and, on the basis of our experimental results, the pathways most likely involved can also be defined.

Normally the excited state (a singlet or, more commonly, a triplet) of a photosensitizer (PS*) contributes to free radical production either in a one-electron oxidation-reduction reaction (Type I photochemistry), generating several radical ions (e.g. PS^-, O_2^-, $OH^.$ etc.), or through energy transfer to ground state oxygen (Type II photochemistry), generating singlet oxygen [30]. In the case of isolated phycocyanin the effects of photosensitation have been ascribed to a combination of both Type I and Type II mechanisms [47,48,50]. In agreement, our studies indicate that the triplet state of phycobiliproteins, generated from intersystem crossing of the excited singlet state, is the inducer of both $^1O_2$ and O_2^-. To this end, we demonstrated that the DMPO-$OH^.$ adduct generated partially from the direct reaction between the spin trap and singlet oxygen, according to Eqs. (1) and (2); in fact the DMPO-$OH^.$ signal was effectively reduced by the treatment with $^1O_2$ quenchers. On the other hand, there can be several other potential sources for the ESR signal of DMPO-$OH^.$, such as free hydroxyl, peroxide and superoxide

radicals. The lack of visualized DMPO-OOH spin adduct signal does not exclude the production of O_2^-. Indeed, the direct ESR detection of DMPO-OOH is still difficult because of the short lifetime of the adduct in protic solvents where this adduct rapidly decomposes to DMPO-$OH^.$ [51,52]. Thus, the confirmation of superoxide generation was obtained by adding SOD, a specific and efficient catalyzer of O_2^- disproportionation, and observing the consequent inhibition of the DMPO-$OH^.$ signal in the ESR spectra. The evidence mentioned above suggests that production of reactive oxygen species in irradiated phycobilisomes proceeds via both Type I and Type II mechanisms. It is unlikely that the superoxide observed is produced directly from singlet oxygen as no other examples of direct conversion of $^1O_2$ to O_2^- have been reported in the literature, and furthermore such a mechanism would be expected to give rise to superoxide formation also in plants where only $^1O_2$ (a Type II mechanism) could be detected [16]. The reason for this difference between photoinhibition in plants and in cyanobacteria is not clear; one possibility is that the open structure of the bilin chromophore, compared to the more stable closed ring arrangement of chlorophyll, results in a higher tendency of the bilin excited state to lose an electron to molecular oxygen. In the absence of oxygen neither $^1O_2$ nor O_2^- could be detected, but a weak EPR signal from a more immobilized species might indicate the formation of a $PC^{+.}$ radical.

4.2. Protein degradation

Characterization of the oxidative damage to proteins by electrophoretic methods led to identification of the following modifications: (i) mobility shift of the protein bands on polyacrylamide gel; (ii) formation of discrete protein cross-links of two proteins (adduct formation); (iii) formation of large cross-linking products (aggregation); and (iv) formation of discrete protein fragments [53]. $^1O_2$ has high reactivity toward specific amino acid residues such as His, Trp, Met and Cys [46]. H_2O_2 is only a weak oxidizing and reducing agent and is in general only poorly reactive. According to Stadtman [54] hydrogen peroxide does damage proteins indirectly by generating hydroxyl radicals via the Fenton reaction in the presence of metal ions bound to proteins, but other reactions may also be involved [55]. These radicals are the most reactive oxygen species known, with a highly positive redox potential. Indeed, their attack leads to cleavage of peptide bonds, cross-linking and aggregation reactions. Superoxide, by comparison, is far less reactive with non-radical species: the direct biological damage is highly selective and often involves its reaction with other radicals [46]. Protonation of superoxide yields the hydroperoxyl radical with increased reactivity.

The results show that both the formation of radicals and the degradation of proteins proceed via two different mechanisms: one involving superoxide and one initiated by singlet oxygen formation. Our observations that dismutation of O_2^- with SOD partially inhibited generation of $OH^.$ indicate that O_2^- is involved in the production of free hydroxyl radicals, but it is unlikely that the reaction pathway includes free H_2O_2, since scavenging of free H_2O_2 with catalase seems not to influence the production of $OH^.$, at least during the initial steps of photoinhibition. The explanation of these findings could be that $OH^.$ is produced either directly from O_2^-, bypassing dismutation to H_2O_2, or indirectly via reduction of O_2^- forming a bound peroxide that is insensitive to catalase. As for thermodynamic reasons the direct reduction of O_2^- to $OH^.$ is unfavourable, the formation of bound peroxide is the more likely explanation. It is well known that formation of bound peroxides on both the polypeptide backbone (at α-carbon positions) and the side chains mediates the radical-induced fragmentation processes of proteins by generation of carbon-centered radical species [56–58].

On the other hand, the results also demonstrate that damage to the proteins can be prevented in part by specific $^1O_2$ scavengers, which have no effects on superoxide. This suggests a direct reaction between $^1O_2$ and susceptible amino acids leading to the formation of bound

peroxides or carbon-centered radicals, also here without any requirement for the formation of an intermediate hydroxyl radical species. Our experiments clearly demonstrate that both of these pathways, either via superoxide or by direct $^1O_2$ attack, can lead to the formation of DMPO-R$^.$ adducts, in both cases as a consequence of the reactive oxygen species attack to amino acid moieties. Such reactive species probably play an important role in the initiation and propagation of radical chain reactions, giving rise to complete degradation of the parent protein and other nearby biological targets. Obviously the degradation of phycobiliproteins reported here is a phenomenon observed *in vitro* that may not necessarily occur *in vivo*. However, the new evidence that emerges from our data is that isolated phycobilisomes can be partially or completely degraded by a process that is light-induced and cannot be explained by the action of proteases. In fact, as judged from the lack of suppressive effect of protease inhibitors and the efficient protection by active oxygen scavengers, this protein fragmentation represents a consequence of the action of ROS generated during illumination. Thus our results substantiate the ability of ROS to cause direct scission of biliprotein peptide bonds, without the involvement of proteases. Of course this does not exclude the participation of proteases under physiological conditions, where the complete degradation of damaged phycobilisomes complexes can be accomplished by the concerted actions of active oxygen species and proteolytic enzymes. One may speculate that a decrease in the antenna size of cyanobacterial thylakoids is one of the photoacclimation steps activated under strong illumination, where photo-induced damage and degradation by ROS of antenna binding proteins contribute to the photoinhibition process. Indeed, ROS generation and light-induced degradation has been reported for other photosynthetic proteins, each of them with a unique pattern of radical formation and breakdown products. The most interesting comparison is with the D1 protein, where involvement of active oxygen species in the damage and cleavage of the protein, as a result of high-intensity irradiation, have been extensively investigated. In this case only the formation of a few well-defined degradation products is observed, as reviewed by Aro et al. [2] and Yamamoto [3]. A substantial difference exists with the degradation process of antenna system where unspecific and random cleavages occur, directly visible as a smear in the SDS-PAGE gel. Also the ESI-MS data confirm that a more extensive degradation phenomenon takes place in phycobilisomes, where the proteins are completely destroyed by an "all-or-none" mechanism. Whether this process propagates from a single specific cleavage site or can be triggered through a multitude of initial ROS targets remains to be established.

5. Conclusions

The results presented here provide evidence that light-induced degradation of intact phycobilisomes in cyanobacteria occurs through a complex mechanism with two independent routes leading to damage protein, one involving superoxide and the other singlet oxygen. This is in contrast to the mechanism found in plants, where damage to the light-harvesting complex proteins has been shown to be mediated entirely by $^1O_2$ generation. The difference may be a consequence of the open structure of the bilin chromophore compared to the more stable closed ring arrangement of chlorophyll, this aspect is yet to be studied. Although the formation of spin trap adducts of the hydroxyl radical is observed during the process for both cyanobacteria and plants, there is no evidence for the direct involvement of hydroxyl radicals or Fenton-type reactions in the initial reactions of photoinhibition.

Acknowledgments

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