Light-induced degradation of cytochrome *b*559 during photoinhibition of the photosystem II reaction center

José M. Ortega*, Mercedes Roncel, Manuel Losada

Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla y CSIC, Americo Vespucio s/n, 41092 Sevilla, Spain

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Abstract The behaviour of cytochrome (cyt) *b*559 during acceptor- and donor-side photoinhibition has been investigated in oxygen-evolving and non-evolving photosystem II (PSII) membranes. Strong illumination at 20°C under aerobiosis induced a strong decrease in the absorbance of the cyt *b*559 α-band in the two preparations. This absorbance decline was observed only in non-oxygen-evolving PSII samples when illumination was performed under aerobiosis but at 4°C, or under anaerobiosis at 20°C. These results suggest that acceptor-side photoinhibition induces the degradation of cyt *b*559 by a mechanism related to an enzymatic reaction mediated by singlet oxygen. Donor-side photoinhibition may induce, however, a non-enzymatic photocleavage of the protein.

Key words: Cytochrome *b*559; Degradation; Photoinhibition; Photosystem II

1. Introduction

Cytochrome (cyt) *b*559 is an intrinsic component of the photosystem II (PSII) reaction center (RC), whose structure and function are not known (for a review, see [1]). It has been proposed to be a heme-bridged protein heterodimer with two subunits, α and β, of 9 and 4 kDa, respectively, encoded by *psb*E and *psb*F genes. Each polypeptide chain forms a transmembrane α-helix, the heme being perpendicular to the membrane plane and close to the stromal side [2]. A peculiar property of this cyt is that it exhibits several redox potential forms, usually a labile high-potential (HP) form (midpoint redox potential (E'_m) = +380 mV) and a stable low-potential (LP) form (E'_m = +140 mV, pH > 7.6) [3,4]. Although widely investigated, the physiological role of cyt *b*559 remains unclear [1]. Over the past 10 years, the participation of cyt *b*559 in redox mechanisms to protect PSII against donor- and acceptor-side photoinhibition has been proposed [5–8].

Donor-side photoinhibition occurs when the rate of electron donation by the water oxidation system is lower than the rate of electrons leaving PSII. This would lead to an increase in the lifetime of the primary donor of PSII, $P680^+$, a species able to damage chromophores and proteins in its surrounding [9]. Acceptor-side photoinhibition occurs under high-light conditions which can fully reduce the plastoquinone pool, the primary quinone acceptor (Q_A) becoming doubly reduced and protonated and subsequently vacating its binding site. Under such conditions, there is an increase in the probability of charge recombination of the primary radical pair $P680^+$ pheophytin$^-$, leading to the triplet state of P680 (^{3}P680), which can interact with oxygen, forming highly reactive singlet oxygen species [10]. Both processes inhibit the PSII activity and eventually lead to the proteolytic degradation of D1 protein [9,11,12]. HP and LP forms of cyt *b*559 could participate in protective mechanisms involving redox cycles which short-circuit acceptor- and donor-sides of PSII, so decreasing, on one hand, the possibility of photoaccumulating highly oxidizing species (HP form, [5]) and, on the other hand, competing with recombination reactions leading to the formation of ^{3}P680 (LP form, [6]). Barber and De Las Rivas [7] proposed a 'molecular switch' model in which cyt *b*559 could protect against both donor- and acceptor-side photoinhibition by reversible conversion between HP and LP forms. In a different model [1,13], the cyt *b*559 redox state regulates the formation of a fluorescence quencher (chlorophyll $(Chl)_z^+$) which disipates excitation energy, limiting photodamaging reactions. In this hypothesis, cyt *b*559 switches between HP and LP forms in order to turn the protective mechanism off and on.

Understanding the behavior of cyt *b*559 during acceptor- and donor-side photoinhibition seems to be necessary in order to clarify the proposed role of cyt *b*559 in protective mechanisms. Several authors have reported that photoinhibition induces changes in the redox properties of cyt *b*559 [14–18]. Styring et al. [14] have observed oxidation of the HP form during aerobic photoinhibition of thylakoids. Anaerobic photoinhibition of thylakoids induced, however, photoreduction of the LP form [15]. The conversion of the HP to LP form has been observed during photoinhibitory illumination in thylakoids under aerobiosis [14,16] or anaerobiosis [15,16] and in manganese (Mn)-depleted PSII membranes [17,18]. Although the cleavage of some PSII polypeptides (D1, D2, CP43) during photoinhibition is relatively well documented, not much information, and in some cases contradictory, is available about the behaviour of the cyt *b*559 content. Whereas Iwasaki et al. [18] reported no changes of the content of cyt *b*559 during strong illumination in Mn-depleted PSII membranes, Allakhverdiev et al. [19] have observed more recently, by using also Mn-depleted PSII samples, a decrease by 30% of the cyt *b*559 content after 6 min of photoinhibition. The aim of this work is to study the changes in cyt *b*559 properties induced by donor- and acceptor-side photoinhibition. The most outstanding effect observed is an important decrease of the

*Corresponding author. Fax: (34) (9544) 60065.
E-mail: jmortega@cica.es

Abbreviations: Chl, chlorophyll; cyt, cytochrome; DCPIP, 2,6-dichlorophenol-indo-phenol; E'_m, midpoint redox potential; HP and LP, high- and low-potential forms of cyt *b*559; Mn, manganese; PAGE, polyacrylamide gel electrophoresis; PSII, photosystem II; P680, primary donor of PSII; Q_A, primary quinone acceptor; Q_B, secondary quinone acceptor; RC, reaction center; SDS, sodium dodecyl sulfate; TL, thermoluminescence

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content of cyt *b*559 in PSII membranes under both photoinhibitory conditions.

2. Materials and methods

2.1. Preparation of PSII membranes

Oxygen-evolving PSII-enriched membranes were prepared from market spinach by the method of Kuwabara and Murata [20]. Non-oxygen-evolving PSII membranes were prepared by a treatment with hydroxylamine (NH_2OH) according to Iwasaki et al. [18]. Oxygen evolution activity was less than 5% of non-treated PSII samples.

2.2. Photoinhibitory treatment

Photoinhibition was carried out aerobically at 20°C by exposing PSII samples (50 μg/ml Chl) to strong heat-filtered white light (5000 μE/m^2/s) for various times and under continuous slow stirring. Buffers were 50 mM MES-NaOH (pH 6.5) and 50 mM MES-NaOH (pH 6.5), 10 mM NaCl and 0.4 M sucrose (buffer A), for oxygen-evolving and NH_2OH-treated PSII samples, respectively. Anaerobiosis required in some experiments was achieved by addition of 10 mM glucose, 0.2 mg/ml glucose oxidase and 0.2 mg/ml catalase, followed by a 10 min dark incubation before light treatment. Samples for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were frozen and stored at −80°C. All other analyses were performed immediately after photoinhibitory treatment. For any given experiment, control samples were kept in the dark under conditions identical to illuminated samples.

2.3. Electron transport measurements

Oxygen evolution was determined polarographically at 20°C with a Clark-type oxygen electrode by using saturating white light. Assay medium was 50 mM MES-NaOH (pH 6.5), 0.1 M sucrose, 5 mM $CaCl_2$ and 5 mM $MgCl_2$, with 0.4 mM 2,5-dimethyl-1,4-benzoquinone and 1 mM ferricyanide as electron acceptors. Electron transport in NH_2OH-treated PSII membranes was determined spectrophotometrically at 20°C by monitoring the absorbance decrease at 600 nm induced by illumination with blue light (100 μE m^{-2} s^{-1}). Assay medium was buffer A with 1 mM $MnCl_2$ as electron donor and 40 μM 2,6-dichlorophenol-indo-phenol (DCPIP) as electron acceptor. Photoinhibited samples were dark-adapted for 20 min on ice, pelleted by centrifugation and resuspended in the assay media at 50 μg Chl ml^{-1} before measurements.

2.4. Thermoluminescence (TL) measurements

TL was measured using a home-built set-up (for basis description, see [21]). The sample cuvette consisted of a horizontal chamber (2 cm Ø) with a copper film on the bottom. A double-stage Peltier plate (model DT 1089-14, Marlow Industries, USA) was mounted below the chamber for temperature regulation. The Peltier element was cooled by a temperature-controlled bath. PSII samples were adsorbed on a piece of filter (0.45 μm, Whatman) that was pressed against copper film, after adding 50 μl water to improve thermal conduction. Samples were dark-incubated for 2 min at 20°C, cooled to −10°C and illuminated with a saturating single-turnover flash (Walz, Germany) through a fiber optic. Light emission was recorded during warming samples from −10 to 70°C at a heating rate of 0.5°C/s by using a Hamamatsu H5701-50 photomultiplier. Signal recording, temperature regulation and signal analysis were performed using a program designed by Dr J.-M. Ducruet. Photoinhibited samples were dark-adapted for 20 min on ice prior to measurements.

2.5. Optical measurements

Optical measurements were performed at 20°C in a SLM Aminco DW-2000 UV-VIS spectrophotometer in a 3 ml cuvette containing PSII suspensions (50 μg Chl ml^{-1}). The redox state and contents of HP and LP forms of cyt *b*559 were determined from the absorbance decrease at 559–570 nm induced by addition of 1 mM ferricyanide (HP_{red}) and from the absorbance increase resulting from sequential reduction with 4 mM hydroquinone (HP_{ox}) and 2 mM dithionite (LP_{ox}). The total content of cyt *b*559 was either estimated from the absorbance difference at 559–570 nm between the fully reduced (hydroquinone and dithionite) and fully oxidized (ferricyanide) state or from the reduced (dithionite, 2 mM) minus oxidized (ferricyanide, 1 mM) absorbance difference spectrum, applying a $\Delta\varepsilon$ (559.5–570 nm) of 17.5 mM^{-1} cm^{-1} [1]. Optical measurements were done immediately after photoinhibitory treatment.

2.6. Gel electrophoresis and immunoblotting

PSII proteins were resolved by SDS-PAGE on 12–17% linear acrylamide gradient gels containing 6 M urea, according basically to Laemmli [22]. Detergent and lipids were previously removed by extraction for 1 h at −20°C with an acetone-ethanol (1:1) solution. The resolved proteins were either stained with Coomassie brilliant blue or electroblotted and identified as [23], by using a polyclonal antibody raised against D1 protein. Immunodecorated blots quantification was performed by combining a scanner and a Sparc-SUN computer station using the Bio Image program (Millipore).

3. Results

Oxygen-evolving (active) and non-evolving PSII-enriched membranes have been exposed to strong illumination in order to generate both acceptor- and donor-side photoinhibition. In active PSII membranes, experiments were done in the absence of artificial electron acceptors, conditions in which acceptor-side photoinhibition is expected to occur after strong illumination [9]. In non-oxygen-evolving PSII samples, obtained by removal of the Mn cluster with a NH_2OH treatment, donor-side photoinhibition is induced under illumination [19,24,25].

Fig. 1A shows the time course of PSII electron transport activity in oxygen-evolving (active) and NH_2OH-treated PSII-enriched membranes exposed to strong illumination under aerobiosis at 20°C. Illumination of active PSII samples in the absence of artificial electron acceptors induced the progressive inhibition of oxygen evolution (apparent inhibition half-time of about 10 min), the activity being decreased by 85% after 60 min of illumination. A much more rapid decay of PSII activity and a higher extent of photodamage was observed in NH_2OH-treated membranes. In these samples, PSII activity, measured as the rate of DCPIP reduction by using $MnCl_2$ as electron donor, was totally inhibited after 20 min of illumination, showing an inhibition half-time of about 2 min. Dark-incubated controls (Fig. 1A, closed symbols) showed no decrease of electron transport activity.

The effects of photoinhibitory illumination on the electron transport capacity of PSII have also been analyzed by measuring its TL emission after the charge separation induced by a flash (Fig. 1B). These light emissions are related with the processes of charge recombination between quinones Q_A and secondary quinone acceptor (Q_B) and S_2 or S_3 states of the Mn cluster. Dark-adapted active PSII membranes (Fig.

Table 1
The content of cyt *b*559 in PSII membranes exposed to photoinhibitory illumination

Photoinhibitory treatment[a]	Content of cyt *b*559 (%)[b]
Active PSII	
Aerobiosis, 20°C	68
Aerobiosis, 4°C	100
Anaerobiosis, 20°C	93
NH_2OH PSII	
Aerobiosis, 20°C	45
Aerobiosis, 4°C	63
Anaerobiosis, 20°C	70

[a]Photoinhibitory treatment of oxygen-evolving (active PSII) and non-evolving (NH_2OH PSII) PSII samples was carried out during 60 min under aerobiosis or anaerobiosis at the indicated temperature, as described in Section 2.
[b]The total content of cyt *b*559 was estimated as described in Section 2.

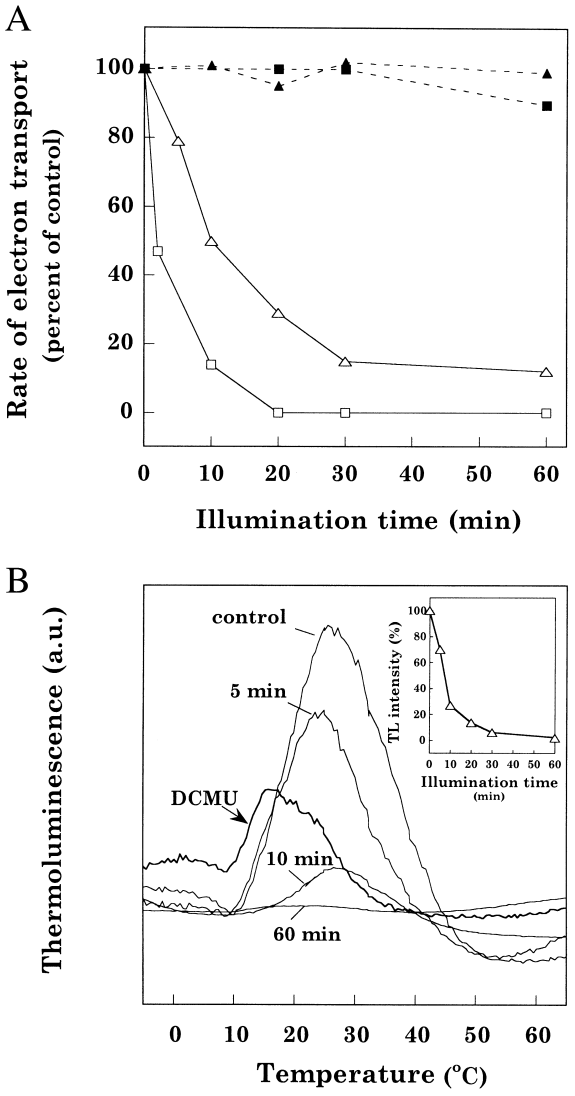

Fig. 1. (A) Time course of electron transport activity in oxygen-evolving (triangles) and NH_2OH-treated (squares) PSII samples during photoinhibition. PSII samples were photoinhibited (open symbols) or incubated in the dark (closed symbols) as described in Section 2. Electron transport values for controls were 310 μmol O_2/mg Chl/h and 570 μmol $DCPIPH_2$/mg Chl/h for active and for NH_2OH-treated PSII samples, respectively. (B) TL emission from control and photoinhibited oxygen-evolving PSII membranes after one flash at −10°C. Control, dark-adapted PSII sample; DCMU, dark-adapted PSII sample incubated for 10 min with 20 μM DCMU. 5, 10, 60 min, PSII samples photoinhibited for these times. The inset shows time course of the TL signal during photoinhibitory treatment. a.u., arbitrary units.

1B, control) showed a typical TL curve, with maximal emission at 28°C. This is the so-called B-band, which reflects $S_2Q_B^-$ and $S_3Q_B^-$ recombination processes [26]. The addition of DCMU significantly decreased the B-band signal and generated another band with a maximal emission at 13°C which can be assigned to the Q-band, reflecting $S_2Q_A^-$ charge recombination [26]. Photoinhibitory treatment (5, 10 and 60 min) induced a progressive decrease of the B-band area, which was not compensated by an increase of the Q-band. The time course of photoinhibition of the TL signal from B-band (inset of Fig. 1B) showed a decay curve with an apparent half-time of 8 min, the signal being totally inhibited after 30 min of illumination. These results suggest that photoinhibitory treatment induces the production of inactive PSII RC, in which Q_A and Q_B functions are quickly destroyed [27].

The effect of photoinhibitory illumination on the relative content of D1 protein in the membrane has been investigated by immunoblotting (Fig. 2). Fig. 2 shows the immunoblots (Fig. 2A) and quantification of the D1 content (Fig. 2B) of active and NH_2OH-treated PSII samples incubated for various periods of time under photoinhibitory illumination. In NH_2OH-treated PSII samples, the D1 content rapidly decreased under illumination, being almost completely lost (90%) after 20 min of photoinhibition. In active PSII samples, however, less than 20% of D1 degradation could be detected after 20 min of illumination, but the protein abruptly decreased by 80% after 30 min.

The effects of donor- and acceptor-side photoinhibition on cyt *b*559 have been investigated by measuring the changes in the α-band of cyt *b*559 absorption spectra after photoinhibitory treatment in active and NH_2OH-treated PSII samples

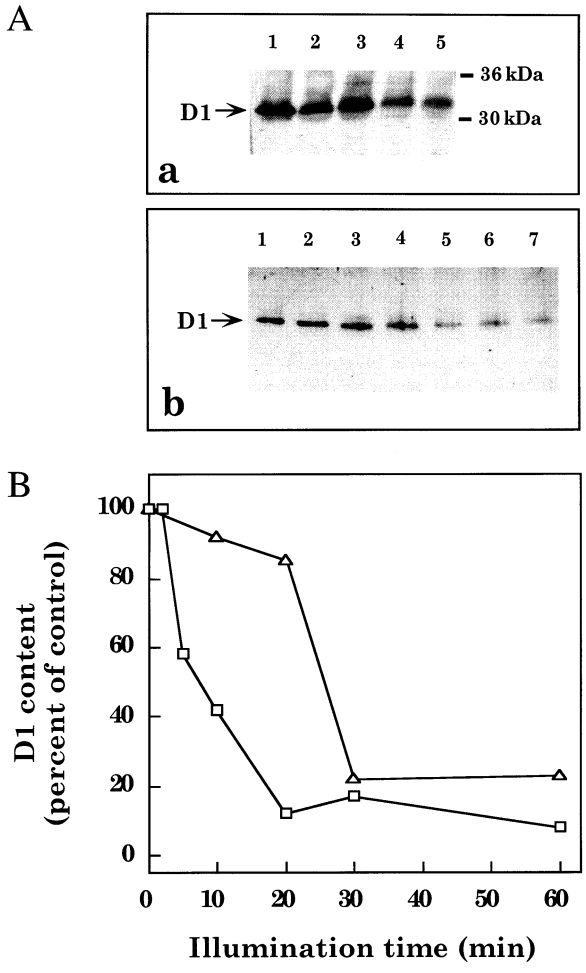

Fig. 2. Light-induced degradation of D1 protein in oxygen-evolving (active) and NH_2OH-treated, PSII-enriched membranes. (A) Immunoblots with rabbit antiserum anti-D1 in control and photoinhibited PSII samples. a, active PSII samples. Lanes: 1, dark control; 2–5, PSII samples illuminated for 10, 20, 30 and 60 min, respectively. b, NH_2OH-treated PSII samples. Lanes: 1, dark control; 2–7, PSII samples illuminated for 2, 5, 10, 20, 30 and 60 min, respectively. 8 μg Chl was used per lane. (B) Quantitative plot of the D1 protein content determined by immunoblotting in active (triangles) and NH_2OH-treated PSII samples (squares).

(Figs. 3 and 4, and Table 1). Fig. 3A shows the absorbance changes at 559–570 nm induced by the addition of ferricyanide (F), hydroquinone (H) and dithionite (D) in oxygen-evolving PSII samples incubated for 60 min at 20°C in the dark and under photoinhibitory illumination. Upon addition of ferricyanide to a dark-incubated sample (left trace), the initial reduced HP form of cyt *b*559 became oxidized. It could then be reduced by hydroquinone. A further addition of dithionite resulted in the reduction of the oxidized LP form. Thus, dark-incubated PSII samples contained 50% cyt *b*559 in its oxidized LP form and 50% in its reduced HP form. After 60 min of illumination (Fig. 3, right trace), cyt *b*559 became fully oxidized and the resulting oxidized species was now mainly LP form (the HP content decreased by 60% while the LP content was not significantly affected). Interestingly

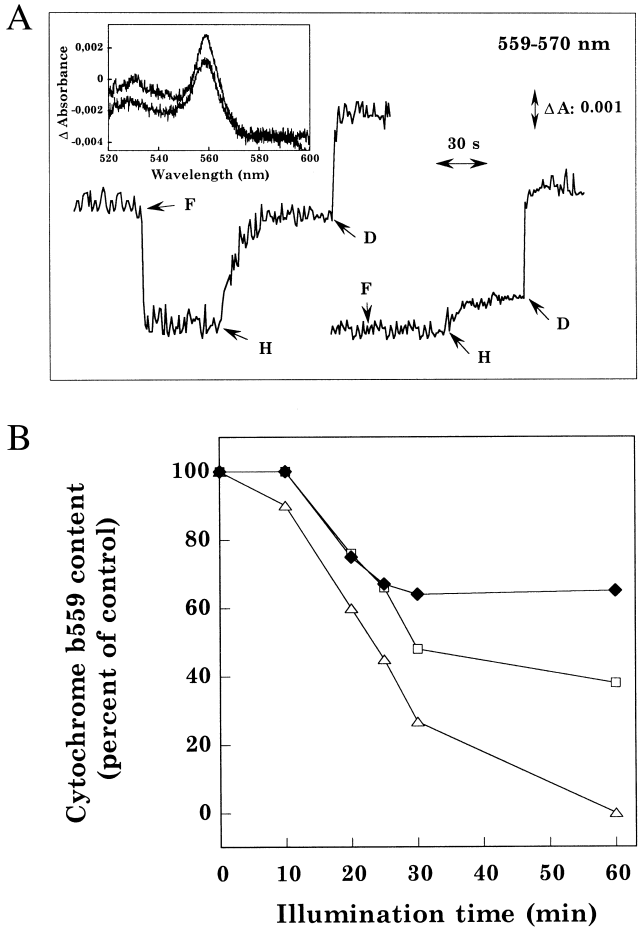

Fig. 3. (A) Chemical-induced absorbance changes at 559–570 nm in oxygen-evolving PSII samples incubated for 60 min at 20°C in the dark (left trace) and under photoinhibitory illumination (right trace). Ferricyanide (F, 1 mM), hydroquinone (H, 4 mM) and dithionite (D, 2 mM) were added as pointed out by arrows. The inset shows the dithionite minus ferricyanide absorbance difference spectra in the α-band region of cyt *b*559 in these PSII samples incubated for 60 min in the dark (upper trace) and under strong illumination (lower trace). (B) Time course of the cyt *b*559 content (content of total protein, ♦; content of HP form, □ and content of reduced form, △) during photoinhibitory treatment in active PSII samples. Experiments analogous to those of A were carried out at a different time of illumination. The content of total cyt *b*559, of the HP form and of the reduced form were determined as described in Section 2 and the values corresponding to 100% were 107, 55 and 60 nmol cyt *b*559, respectively.

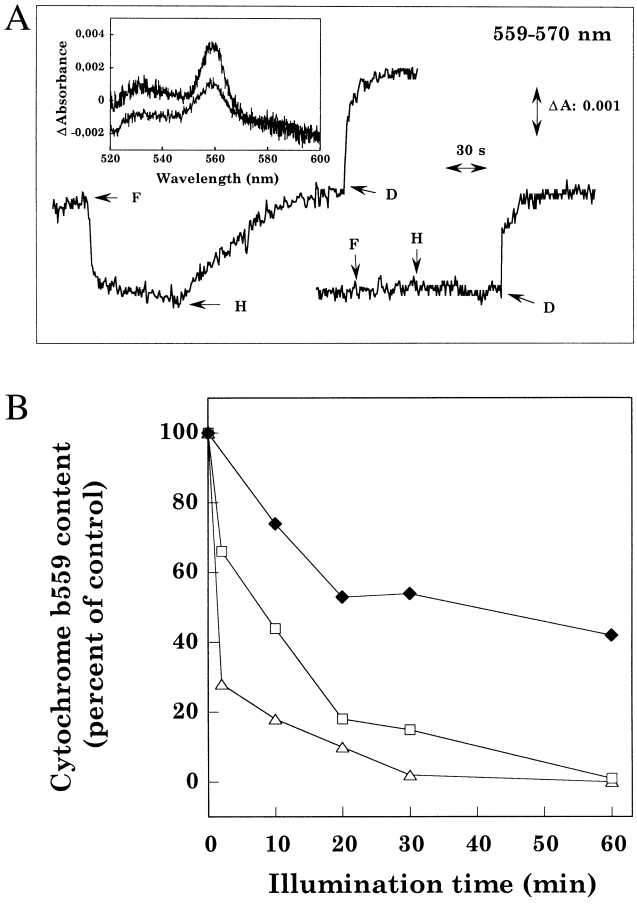

Fig. 4. (A) Chemical-induced absorbance changes at 559–570 nm in NH_2OH-treated PSII samples incubated for 60 min at 20°C in the dark (left trace) and under photoinhibitory illumination (right trace). Ferricyanide (F, 1 mM), hydroquinone (H, 4 mM) and dithionite (D, 2 mM) were added as pointed out by arrows. The inset shows the dithionite minus ferricyanide absorbance difference spectra in the α-band region of cyt *b*559 in these PSII samples incubated for 60 min in the dark (upper trace) and under strong illumination (lower trace). (B) Time course of the cyt *b*559 content (content of total protein, ♦; content of HP form, □ and content of reduced form, △) during photoinhibitory treatment in NH_2OH-treated PSII samples. Experiments analogous to those of A were carried out at a different time of illumination. The content of total cyt *b*559, of the HP form and of the reduced form were determined as described in Section 2 and the values corresponding to 100% were 89, 43 and 39 nmol cyt *b*559, respectively.

enough, the total content of cyt *b*559, inferred from the absorbance difference at 559–570 nm between the fully oxidized and fully reduced state of cyt, decreased by 32% after 60 min illumination. The chemical difference spectra in the cyt α-band region of these PSII samples incubated for 60 min in the dark or under strong illumination (inset of Fig. 3A) corroborate that the absorbance decline observed at 559–570 nm corresponds in fact to cyt *b*559. Fig. 3B shows the time course of the cyt *b*559 content (protein, HP form and reduced form) during photoinhibitory illumination of active PSII samples under aerobiosis at 20°C. Photoinhibitory treatment induced the progressive decrease of the three above-mentioned parameters, with very similar decay curves (apparent half-times of about 18–20 min). If photoinhibitory treatment was carried out in active PSII samples during 60 min under aerobiosis but at 4°C, or under anaerobiosis at 20°C, the total content of cyt

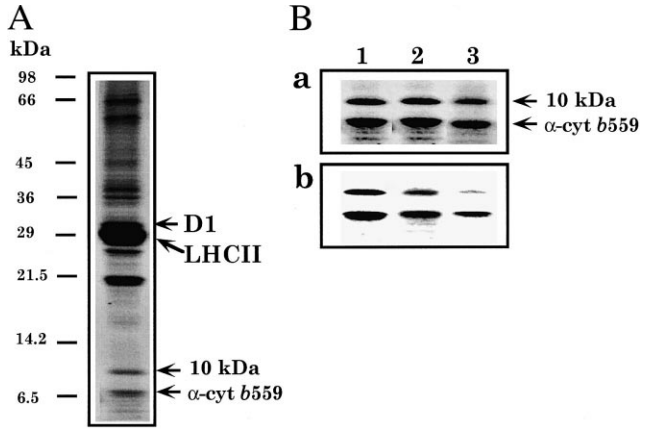

Fig. 5. SDS-urea-PAGE analysis of the effect of strong illumination on the content of the α-subunit of cyt *b*559 in oxygen-evolving (active) and NH_2OH-treated, PSII-enriched membranes. (A) Coomassie-stained SDS-urea-PAGE of a not photoinhibited active PSII sample. (B) Coomassie-stained SDS-urea-PAGE of the lower portion of the gels in active (a) and NH_2OH-treated (b) PSII samples exposed to different times of photoinhibitory illumination. Lanes: 1, dark control; 2 and 3, PSII samples illuminated for 10 and 60 min, respectively. 8 μg Chl was used per lane.

*b*559 (Table 1) and the contents of HP and LP forms (data not shown) did not change significantly.

NH_2OH-treated PSII membranes incubated for 60 min at 20°C in the dark contained 50% cyt *b*559 in its oxidized LP form and 50% in its reduced HP form, that was initially oxidized by ferricyanide (Fig. 4A, left trace). After 60 min of illumination, reduced HP cyt *b*559 became fully oxidized and the resulting oxidized species was the LP form (Fig. 4, right trace). The content of the HP form was abolished, whereas the LP one did not change significantly and the total content of cyt *b*559 decreased by 55% after this treatment. The chemical difference spectra in the cyt α-band region of these PSII samples incubated for 60 min in the dark or under illumination (inset of Fig. 4A) also showed that the absorbance decrease at 559–570 nm induced by illumination can be clearly attributed to cyt *b*559. Fig. 4B shows the behavior of cyt *b*559 (contents of protein, HP form and reduced form) during photoinhibitory treatment in NH_2OH-treated PSII samples. While the contents of total protein and the HP form did decline with similar kinetic curves (half-times of about 8–10 min), a much more rapid disappearance of the reduced form was detected during the treatment (half-time of about 2 min). NH_2OH-treated PSII samples photoinhibited during 60 min under aerobiosis but at 4°C, or under anaerobiosis at 20°C, showed a minor decrease of the cyt *b*559 content, by 37 and 30%, respectively (Table 1).

The effects of photoinhibitory illumination on the content of cyt *b*559 have also been analyzed by SDS-urea-PAGE (Fig. 5). Fig. 5A shows the polypeptide composition of a not illuminated oxygen-evolving PSII sample. Our analysis was focused on the lower portion of the gels, between the 14.2 and 6.5 kDa molecular mass standards, because we are mostly interested in the fate of cyt *b*559 during photoinhibition. The two more prominent Coomassie-stained bands of this region, with apparent molecular masses of 10 and 9 kDa, can be assigned to the *psb*R gene product and the α-subunit of cyt *b*559, respectively [28]. An apparent decrease in the total content of the α-subunit of cyt *b*559 (the lower band), and also of *psb*R polypeptide, was detected in active PSII samples (Fig. 5B, a) and, in a higher extent, in NH_2OH-treated PSII samples (Fig. 5B, b) after photoinhibitory illumination.

4. Discussion

The main objective of this work was to investigate the effect of photoinhibition on cyt *b*559 properties. Acceptor- and donor-side photoinhibition have been generated, respectively, by exposing oxygen-evolving and non-evolving PSII membranes to strong illumination under aerobiosis (see Figs. 1 and 2). Under both conditions, an important decrease in the absorbance of the cyt *b*559 α-band has been observed (Figs. 3 and 4) and assumed to be a consequence of a light-induced degradation of the polypeptide chains of the protein. This is supported by the decline in the content of α-subunit of cyt *b*559 observed in Coomassie-stained SDS-urea-PAGE of photoinhibited samples (Fig. 5). The behavior of the cyt *b*559 content during photoinhibition was yet not investigated in depth. Recently, Allakhverdiev et al. [19] have reported a decrease by 30% of the cyt *b*559 content after 6 min of photoinhibition in Mn-depleted PSII membranes. Barbato et al. [29] have demonstrated the accumulation of a 41 kDa cross-linked product between D1 and the α-subunit of cyt *b*559 during photoinhibition. This phenomenon seems not to be related to the absorbance decrease in the cyt *b*559 α-band observed by us, since they concluded that the linkage reaction does not perturb the cyt structure sufficiently to affect heme absorption at 560 nm.

Under acceptor-side photoinhibition, the generated singlet oxygen triggers the enzymatic process of D1 degradation, a reaction that is blocked at low temperatures [9,11]. Cyt *b*559 degradation was not observed when acceptor-side photoinhibition was carried out under anaerobiosis or under aerobiosis but at a low temperature (Table 1). Thus, cyt *b*559 degradation also seems to be related to an enzymatic process mediated by singlet oxygen. Taking into account the similar kinetics for D1 and cyt *b*559 degradation (Figs. 2B and 3B), one possibility is that cyt *b*559 cleavage is a consequence of the PSII RC disintegration process initiated by D1 degradation. It cannot be excluded, however, that cyt *b*559 cleavage might be an independent enzymatic process with a similar triggering. Under donor-side photoinhibition, it has been described that an increase of the lifetime of $P680^+$ initiates the process leading to enzymatic degradation of D1 [9]. Interestingly enough, we also observed a partial degradation of cyt *b*559 at low temperatures under such conditions (Table 1). This result can be interpreted by assuming that under donor-side photoinhibition, cyt *b*559 can be degraded by a non-enzymatic reaction, i.e. a direct photocleavage due to the extraction of electrons by oxidative species generated under these conditions ($P680^+$, Chl^+). The decrease of cyt *b*559 degradation observed when photoinhibition was carried out under anaerobiosis (Table 1) seems to indicate that a singlet oxygen-mediated cleavage of cyt *b*559 is also occurring in NH_2OH-treated PSII samples.

Several authors have reported that the most important effect of photoinhibition on cyt *b*599 is the conversion of the HP form to lower potential forms [14–18]. The reversible conversion between HP and LP forms has been proposed as being the crucial part of the models involving cyt *b*559 in protective

mechanisms against photoinhibitory damage [1,7,8,13]. Under both donor- and acceptor-side photoinhibition, an important decrease in the content of the HP form has been observed (Figs. 3 and 4). It is very difficult to know if this phenomenon corresponds to a real conversion between HP and LP forms, because a parallel degradation of the protein also occurs. On the contrary, the results seem to indicate that under our experimental conditions, the decrease of the HP content can be due to specific degradation of the population of HP cyt *b*559 and not due to its conversion to the LP form: (i) the kinetics of decrease of the HP content and degradation of cyt *b*559 were very similar (Figs. 3B and 4B), (ii) the amounts of HP content disappeared after 60 min of photoinhibition were similar to that of the cyt *b*559 content being degraded (Figs. 3B and 4B), (iii) the LP contents did not change significantly after photoinhibitory treatments (Figs. 3A and 4A).

Summarizing, the most outstanding effect on cyt *b*559 of photoinhibitory treatment in PSII membranes has been its partial degradation. The existence of two different mechanisms of light-induced degradation of cyt *b*559 occurring under photoinhibitory conditions is suggested: (i) a degradation related to an enzymatic reaction mediated by singlet oxygen under acceptor-side photoinhibition and (ii) a non-enzymatic direct photocleavage under donor-side photoinhibition. The possibility is considered that cyt *b*559 might be the primary target for the singlet oxygen or for the damaging oxidizing species generated during acceptor- and donor-side photoinhibition, respectively.

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