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Characteristic changes of function and structure of Photosystem II during strong-light photoinhibition under aerobic conditions

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Abstract

The effects of strong-light photoinhibition under aerobic condition on the function and structure of Photosystem (PS) II were studied by measuring flash-induced changes of chlorophyll fluorescence yield, thermoluminescence glow curves, Fourier transform infrared (FTIR) spectra and the amounts of the PS II proteins, including the D1 and D2 proteins, in PS II membranes. Aerobic photoinhibition resulted in decrease in light-induced FTIR difference spectra of PS II. The intensity of the positive band at 1479 cm^{-1} due to a semiquinone anion in the Q_A pocket decreased with the same kinetics as the inhibition of O_2-evolving activity. In an early stage of photoinhibition, the very fast decaying component of flash-induced fluorescence relaxation due to rapid electron transfer from Q_A^- to Q_B was eliminated, and the intensity of thermoluminescence band due to $S_2Q_B^-$ charge recombination decreased concomitant with appearance of another band due to $S_2Q_A^-$ charge recombination. These results suggested that the impairment of Q_B function preceded the loss of Q_A function and O_2 evolution. The aerobic photoinhibition also induced a decrease in the F_m level of fluorescence, but its course was much faster than the loss of O_2 evolution. Degradation of the D1 and D2 proteins followed the loss of O_2 evolution but it did not induce clear changes in whole structure of PS II as detected by FTIR even after prolonged photoinhibition. Based on these results, the functional and structural changes of PS II by the aerobic photoinhibition were discussed, in relation to the primary event of photoinhibition.

Keywords: Photosystem II; Photoinhibition; FTIR; Thermoluminescence; Plastoquinone; D1 protein

1. Introduction

Illumination of oxygen-evolving photosynthetic organisms with strong light results in the loss of photosynthetic activity by a process known as photoinhibition (for reviews see Ref. [1–5]). It has been widely accepted that the primary site of photoinhibition is Photosystem (PS) II when light is the sole source of stress. The loss of PS II activity is followed by degradation of the D1 protein of PS II reaction center [6,7]. It is believed that light-induced degradation of the D1 protein occurs even under weak light condition, which is overcome by a recovery process involving de novo synthesis of the D1 protein, so that no marked inhibition of the photosynthetic activity is manifested [6,8,9]. The very rapid turnover of this D1 protein found under illumination may be a reflection of these photoinhibition/recovery processes.

Events of photoinhibition have been extensively studied using various in vivo and in vitro systems [1–5]. Through these studies, it has been established that the acceptor side of PS II is primarily damaged in samples retaining a healthy electron donation activity from water to PS II, whereas the donor side of PS II is preferentially damaged in samples having impaired O_2-evolution activity. Since O_2 evolution is functional in normal plant materials, the damage of the acceptor side of PS II is predominantly responsible for the loss of photosynthetic activity under strong light.

As to the acceptor-side photoinhibition, a detailed molecular mechanism of its primary step has been proposed based on in vitro analyses of the effects of strong-

Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FTIR, Fourier transform infrared; Mes, 4-morpholineethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PS, Photosystem; Q_A, primary quinone acceptor of Photosystem II; Q_B, secondary quinone acceptor of Photosystem II; F_o, F_v, F_m, initial, variable and maximum level of fluorescence.

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light under anaerobic condition on the acceptor side reaction of PS II [10,11]. Illumination of thylakoids in the absence of oxygen induces complete inhibition of O_2 evolution due to the formation of an inactive state of Q_A that is attributed to stabilization of doubly reduced Q_A by protonation. Since the primary charge separation in PS II is not affected under this condition, further illumination of the inhibited PS II facilitates the formation of the triplet state of reaction center chlorophylls (P680) [11]. These mechanisms led them to expect that a highly reactive singlet oxygen would be produced when the photoinhibitory treatment is carried out in the presence of oxygen which would result in the degradation of PS II proteins [11,12]. In fact, no degradation of the D1 protein has been reported in the anaerobic photoinhibition except for a special case [13]. In the anaerobic photoinhibition, the level of Chl fluorescence from PS II shows characteristic changes; the initial fluorescence (F_o) increases as high as to the maximum fluorescence (F_m) level that decreases with time of photoinhibition; the variable fluorescence (F_v) level decreases markedly as a consequence [10,11,14,15].

Under aerobic condition, the process of photoinhibition is, however, rather complicated, still being a matter of debate. Much evidence shows that the primary charge separation between pheophytin and P680 remains active after complete inhibition of O_2 evolution in a similar manner as the anaerobic photoinhibition [16–19]. However, the aerobically photoinhibited PS II differs from anaerobically photoinhibited PS II: the former PS II shows only very small chlorophyll triplet signal measured even in the absence of oxygen [15,18,19]. Damage of the reaction center will be faster under the conditions which permit efficient production of Chl triplet, since the triplet state formed on reaction center chlorophylls [20] will yield reactive singlet oxygen and damages the reaction center. Hence, the small triplet signal is consistent with the unexpected long survival of the capability of primary charge separation in aerobically photoinhibited PS II, and suggests that the singlet oxygen may not be the predominant cause of the aerobic photoinhibition. The decrease in the EPR signal arising from $Q_A^- Fe^{2+}$ has been reported to closely correlate with the loss of O_2 evolution [18,19]. This has been interpreted to mean that impairment of Q_A function is responsible for the incapability of O_2 evolution in the aerobic photoinhibition as well. It has, however, been reported that the aerobic photoinhibition affects the non-heme iron of PS II: the intensity of the $g = 8$ EPR signal from the non-heme iron decreases in parallel with the loss of O_2 evolution [21,22]. Moreover, the functional loss of the non-heme iron by introducing a mutation into His-268 of the D2 protein has resulted in the block of electron transfer from Q_A^- [23]. It is, therefore, possible that the impairment of the function of the non-heme iron is the cause for the inhibition of O_2 evolution and the loss of $Q_A^- Fe^{2+}$ EPR signal despite the presence of Q_A^-. In addition, the effect of the aerobic photoinhibition on fluorescence induction properties is known to be quite different from that of the anaerobic photoinhibition; the aerobic photoinhibition results in little increase in the F_o level but a large decrease in the F_m level [10,15,16]. Thus, in the aerobic photoinhibition, there remain many ambiguities as to the initial step and the primary site of photoinhibition, and also the properties of the photoinhibited PS II as well.

This study aims at understanding the functional and structural changes occurring in PS II during the aerobic photoinhibition. For this purpose, we probed PS II by means of FTIR spectroscopy, thermoluminescence and fluorescence kinetics, and characterized the changes induced by photoinhibition. The results provided the direct evidence that the loss of Q_A function is the cause for the impairment of O_2 evolution by the aerobic photoinhibition. We also show that the structural changes of PS II protein accompanying the damage of the PS II proteins including the D1 and D2 proteins are much smaller than usually expected.

2. Materials and methods

PS II membranes capable of O_2 evolution were prepared from spinach according to the methods described [24], and then stored in liquid N_2 until use. The membranes were washed twice with a medium containing 400 mM sucrose, 20 mM NaCl, 40 mM Mes-NaOH (pH 6.5) and finally suspended in the same medium. Illumination for photoinhibition was performed aerobically at a Chl concentration of 250 μg Chl/ml. The membrane suspension stirred in a glass cylinder of 2.5 cm in internal diameter was exposed to white light from a 500 W halogen lamp. The sample temperature was controlled at 20° C by circulating water in the jacket of the glass cylinder. The light intensity was 200 mW/cm^2 (< 800 nm) at the surface of the glass cylinder. After illumination, the photoinhibited membranes were incubated at 0° C in the dark for more than 2 h, and then subjected to various measurements. No further changes in O_2 evolution activity and the amount of the D1 protein were detected during the dark incubation. All procedures were carried out under dim green safe light unless otherwise noted.

For FTIR measurements, the sample membranes were precipitated by centrifugation at $150\,000 \times g$ for 15 min, and the pellet was pressed between a pair of BaF_2 plates. The sample amount was adjusted so that the absorbance of the amide I peak (1657 cm^{-1}) was between 0.5 and 1.0. FTIR spectra were measured on a JEOR JIR-6500 spectrophotometer equipped with a MCT detector (EG and G Jadson IR-DET 101) as described previously [25]. The sample temperature was controlled at 210 K with a temperature control system (Oxford DN1704 with ITC-4). Light-induced difference spectra were obtained by subtraction between the two single-beam spectra recorded before and

after illumination. Light illumination was performed for 5 s with red light (20 mW/cm^2, > 620 nm) passing through several layers of heat-cut filters. Each single-beam spectrum was an average of 300 scans (150 s accumulation). The spectral resolution was 4 cm^{-1}. For analysis of the amide I bands in the original (not difference) spectra, the water absorption around 1645 cm^{-1} overlapping on the amide I band was eliminated by subtraction of the buffer spectrum on the basis of the isolated water band around 2230 cm^{-1} (combination band). Second-derivative spectra were obtained with a Savitsky-Golay derivative function [26] for a five data points window.

For thermoluminescence measurement, 70 μl of the membrane suspension were directly applied on a 2 cm × 2 cm filter paper, then covered with a transparent plastic plate as described in [24]. The sample membranes were excited by a single-turnover saturating-flash from a xenon flash lamp (5 μs, 4 mJ, white light) and immediately frozen in liquid N_2. The light emission during heating the sample (0.8 C°/s on average) was recorded as a function of sample temperature.

For detection of rapid relaxation kinetics of chlorophyll fluorescence yield, a PAM fluorimeter (Waltz, Effeltrich, Germany) equipped with a DA 100 data acquisition system was used. Samples were excited with a single-turnover flash (8 μs), and the relaxation kinetics was monitored at 20 μs resolution with measuring light modulated at 100 kHz.

Oxygen evolution was measured with a Clark-type oxygen electrode at 25° C using 0.4 mM phenyl-*p*-benzoquinone as an exogenous electron acceptor.

PS II proteins were separated by SDS-PAGE using the buffer system of Laemmli with modifications as described [27]. The polyacrylamide concentration in the separation gel was 13%. After electrophoresis, the separated proteins were transferred to a nitrocellulose filter (Schleicher & Schuell, 0.2 μm) according to the method of Towbin et al. [28] with a semi-dry-type blot apparatus. After blotting, the filter was probed with polyclonal antibodies raised in rabbit against the spinach D1 and D2 proteins (kindly provided by Dr. M. Ikeuchi). The immunocomplex was further reacted with a goat antibody against rabbit IgG conjugated with alkaline phosphatase (Jackson ImmunoResearch), and visualized by the reaction with nitroblue tetrazolium and bromochloroindolyl phosphate as chromogenic substrate for alkaline phosphatase.

3. Results

Fig. 1A shows the effect of the aerobic photoinhibition on the relaxation kinetics of Chl *a* fluorescence after illumination with a saturating single-turnover flash. In control membranes, the fluorescence stayed at the F_0 level under weak modulated light, and instantly increased to reach the F_m level upon excitation by the saturating flash. The amplitude of the F_m level was almost the same as that found in the presence of DCMU, indicating that the F_m level reflects full reduction of Q_A. The F_m was followed by rapid relaxation due to the electron transfer from Q_A^- to Q_B [29]. It is of note that the rate was considerably slower in PS II membranes than in chloroplasts presumably due to

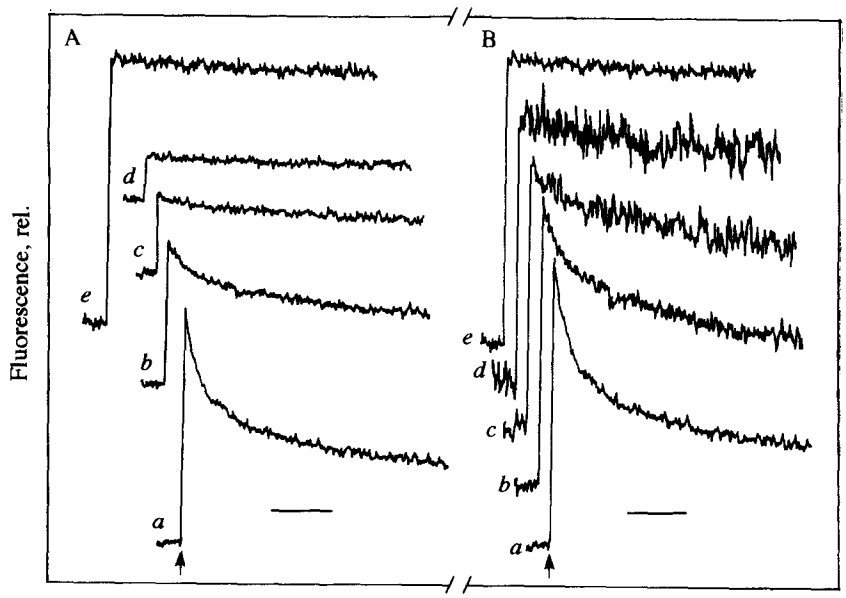

Fig. 1. Effect of photoinhibition on the relaxation kinetics of chlorophyll fluorescence. PS II membranes were illuminated with a single turnover flash and the change of fluorescence yield was measured. The membranes were photoinhibited for 0 min (a,e), 6 min (b), 20 min (c) and 40 min (d) at 20° C, and then the fluorescence relaxation was measured in the absence (a,b,c,d) and in the presence (e) of 10 μM DCMU. Each relaxation trace was presented before (panel A) and after (panel B) normalization at the maximum fluorescence intensity. A flash was represented by an arrow in trace a. The bar in the figure indicates a time of 2 ms.

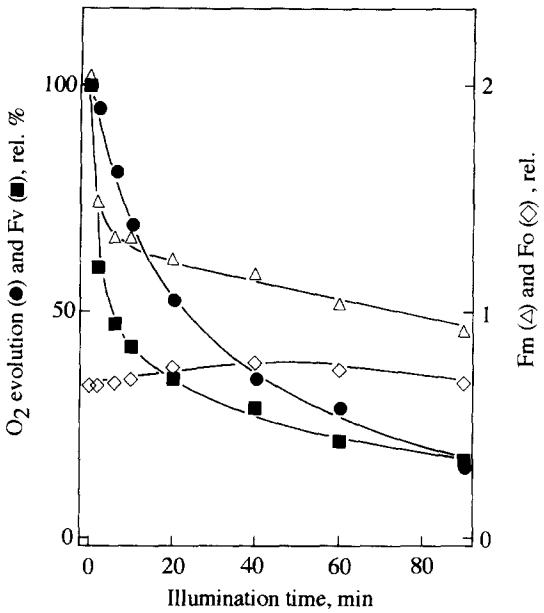

Fig. 2. Time-course of changes of O_2 evolution (●) and of fluorescence characteristics during photoinhibition. F_0 (◇), F_m (△) and F_v ($=F_m-F_0$) (■) were estimated by the single flash experiments as shown in Fig. 1. 100% O_2 evolution was 800 μmol O_2/mg Chl per h.

some modification of the acceptor side of PS II as commonly observed in detergent-solubilized PS II preparations [22]. The F_m level decreased with the time of photoinhibitory treatment and became about 50% of the control after 10 min of photoinhibition. Photoinhibition affected not only the fluorescence yield but also its decay kinetics, as clearly shown in Fig. 1B, in which the relaxation courses were presented after normalization of F_m intensities. The relaxation was composed of fast, slowly and very slowly decaying components. In the control membranes, the half decay times of the fast and slow components were about 1.0 and 30 ms, respectively. The relative amplitude of the fast decaying component decreased with the progress of photoinhibition, but its decay time did not much change. This indicates that the population of PS II with impaired electron transfer between Q_A and Q_B increased by photoinhibition. After 20 min photoinhibition, the fast decaying component was eliminated almost completely, exhibiting a profile as typically seen in the presence of DCMU which blocks the electron transfer between Q_A and Q_B.

Fig. 2 shows the changes of various parameters of fluorescence kinetics and oxygen evolution activity during the course of photoinhibition. The F_m level (open triangles) exhibited an initial rapid decreased followed by a later gradual decrease, but F_0 (open diamonds) kept a relatively constant level except for very faint increase transiently found after 40 min of photoinhibition. The F_v ($=F_m-F_0$) level (closed squares) rapidly decreased in the initial stage of photoinhibition; the F_v dropped to about 60% of the control level after 3 min photoinhibition. Notably, this drop was not accompanied by any inhibition of O_2 evolution. The results indicate that the F_v decrease is not directly related to the functional impairment of PS II by photoinhibition, but is attributable to a putative quencher or quenching state that accumulates during illumination by some side-path reactions.

As the fluorescence relaxation measurements (Fig. 1) suggested that photoinhibition resulted in interruption of the electron transfer between Q_A and Q_B, changes in properties of the acceptor side of PS II were studied by means of thermoluminescence as shown in Fig. 3A. In control membranes, a flash excitation induced a single-peaked thermoluminescence band. The peak temperature of the band was located at around 35 and 10° C in the absence and presence of DCMU, respectively. The charge pairs of $S_2Q_B^-$ and $S_2Q_A^-$ have been assigned as the origins of the 35 and 10° C bands, respectively [30]. Photoinhibition affected both the shape and intensity of the thermoluminescence band. In membranes photoinhibited for 7.5 min, the glow curve showed a distinct shoulder at around 10° C, and the shoulder became more pronounced with photoinhibition time, showing a comparable height with the $S_2Q_B^-$ band after 20 min photoinhibition. After 45 min photoinhibition, the glow curve showed a small single peak at around 10° C with nearly no contribution of the $S_2Q_B^-$ band. On the basis of its peak temperature, we may assume that the thermoluminescence band at 10° C found in the photoinhibited membranes is ascribed to the $S_2Q_A^-$ charge pair. This view is in good agreement with the result obtained from the fluorescence relaxation kinetics shown in Fig. 1, in which the very fast relaxation component due to the electron transfer from Q_A^- to Q_B was inhibited by photoinhibition.

Fig. 3B shows the changes in the relative fraction of PS II centers capable of emitting the $S_2Q_B^-$ band during the course of photoinhibition. A thermoluminescence glow curve given at an indicated photoinhibition time was deconvoluted into $S_2Q_A^-$ and $S_2Q_B^-$ components [31] (deconvolution not shown). The $S_2Q_B^-$ fraction thus obtained (open triangles) reflects the PS II capable of normal electron transfer from Q_A^- to Q_B. The result showed that this component decreased with photoinhibition time much faster than the loss of O_2 evolution activity (closed circles), indicating that the electron transfer from Q_A^- to Q_B is impaired preferentially in the initial stage of photoinhibition. The total thermoluminescence, the sum of the $S_2Q_A^-$ and $S_2Q_B^-$ components, was also plotted in Fig. 3B (open squares) as the amount of the active PS II emitting thermoluminescence bands. The total intensity decreased in good correlation with the loss of O_2 evolution activity.

Another feature of the glow curve characteristics of the photoinhibited membranes was a marked elevation of the base line at the high-temperature end. This is due to a thermoluminescence band peaking at around 70° C (data not shown). The intensity of this component linearly increased with illumination time for photoinhibition. Presumably, this band is attributable to Chl or its degradation product in non-physiological state produced by strong

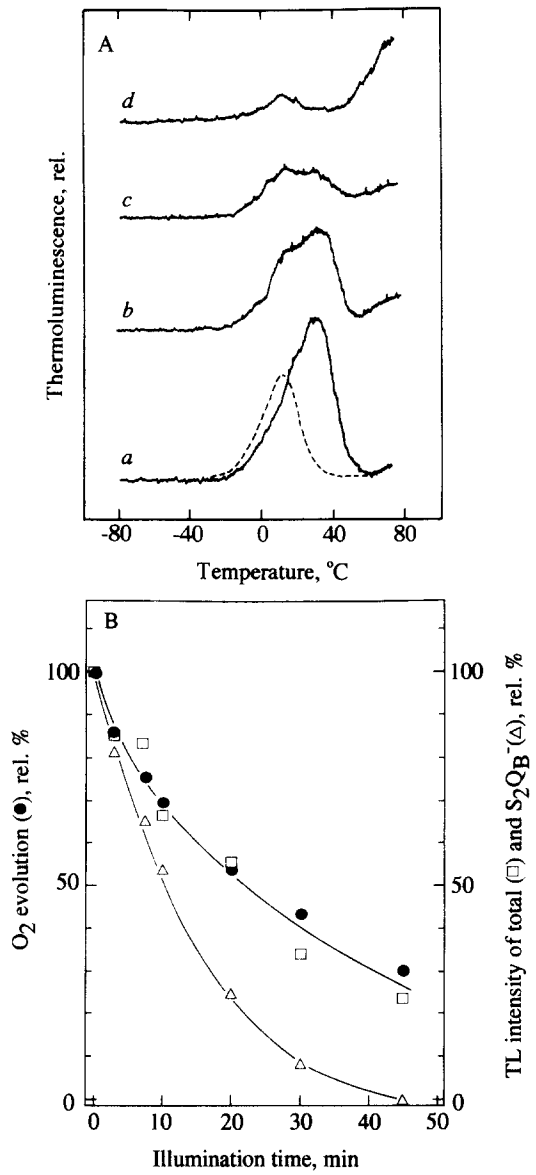

Fig. 3. (A) Effect of photoinhibition on thermoluminescence glow curves. PS II membranes were photoinhibited for 0 min (a), 7.5 min (b), 20 min (c) and 45 min (d) at 20° C. The broken glow curve on (a) was obtained in the presence 10 μM DCMU. The membranes were excited by a single flash at 5° C. (B) Time-course of change of O_2 evolution (●), total amounts of the charge pairs ($S_2Q_A^-$ plus $S_2Q_B^-$) responsible for thermoluminescence (□) and relative amount of the charge pair emitting $S_2Q_B^-$ thermoluminescence (△) during photoinhibition. The amount of the total and the $S_2Q_B^-$ charge pairs were estimated from the intensity of glow curves as described in the text.

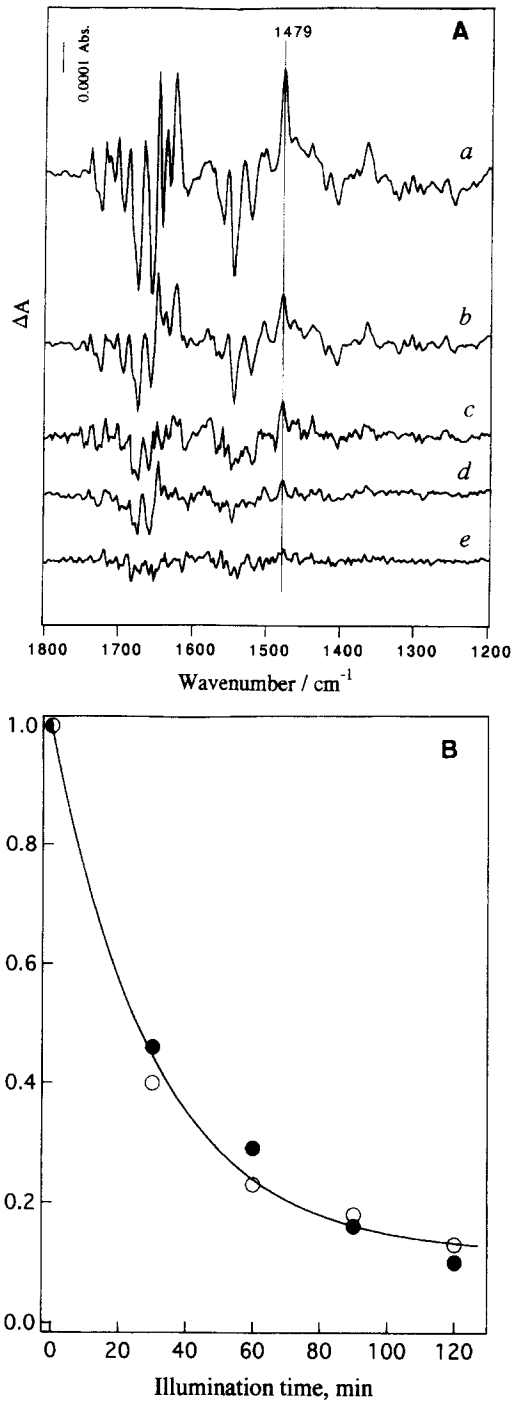

Fig. 4. (A) Effect of photoinhibition on the light-minus-dark FTIR difference spectra. PS II membranes were photoinhibited for 0 min (a), 30 min (b), 60 min (c), 90 min (d) and 120 min (e) at 20° C. The spectra were normalized on the basis of the area intensity of the amide I band at 1657 cm^{-1} in the original spectra. The position of the positive 1479 cm^{-1} band attributed to the CO stretch of Q_A^- is marked. The membranes were illuminated with continuous light for 5 s at 210 K. (B) Time-course of the changes of the amount of Q_A^- (●) and O_2 evolution activity (○). The amount of Q_A^- was estimated from the intensity of the band at 1479 cm^{-1}. 100% O_2 evolution was 600 μmol O_2 /mg Chl per h.

illumination. This might be related to the appearance of break-down fragments of the D1 protein during the course of photoinhibition (see Fig. 6), but the amount of Chl degradation seems to be very small, since no appreciable changes in absorption spectrum and Chl content could be detected during photoinhibition in this time range (data not shown).

Fig. 4A shows the effect of the aerobic photoinhibition on light-induced FTIR spectra. All the difference spectra

were normalized on the basis of the area intensity of the amide I band around 1657 cm^{-1} in the original (not difference) spectra after subtraction of the water contribution. Each sample was illuminated with continuous light at 210 K, and the difference spectrum after-minus-before illumination was recorded. At this low temperature, the electron transfer from Q_A^- to Q_B is blocked, and hence continuous-light illumination accumulates Q_A^- on the acceptor side, while on the donor side the S_2 state is accumulated in normally functioning PS II [25]. On the other hand, redox components, such as cytochrome *b*-559, Chl and carotenoid are oxidized by $P680^+$ instead of the Mn-cluster when the oxygen-evolving center is inactive [32,33]. Thus, the light-induced FTIR difference spectrum includes both the Q_A^-/Q_A and donor-side changes.

The FTIR difference spectrum of the control membranes was basically the same as our previous $S_2Q_A^-/S_1Q_A$ difference spectrum measured at 250 K in the presence of DCMU [25]. The bands in the spectrum include structural changes in the redox components and the changes in the protein moiety induced by the redox reactions. Among the many bands found in the spectrum, the positive 1479 cm^{-1} band has been assigned to the CO stretching mode of a semiquinone anion of the plastoquinone molecule in the Q_A pocket [34], while the negative 1404 cm^{-1} band has been ascribed to the S_2/S_1 change and tentatively assigned to the symmetric COO^- stretching mode of the carboxylate ligand of the Mn-cluster [25]. Since the 1479 cm^{-1} band has a strong intensity and is located at a position isolated from other intense bands in the region of 1700–1500 cm^{-1}, The amount of functional Q_A plastoquinone in the membranes can be directly probed by the use of this band as a marker. Upon photoinhibition, all the bands in the difference spectra decreased in their intensities, but spectral features basically did not change. In Fig. 4B, the intensity of the 1479 cm^{-1} band (closed circles) was plotted as a function of the time of photoinhibition together with the O_2-evolving activity (open circles). It was shown that the decrease course of the 1479 cm^{-1} band is in good agreement with that of the O_2-evolving activity.

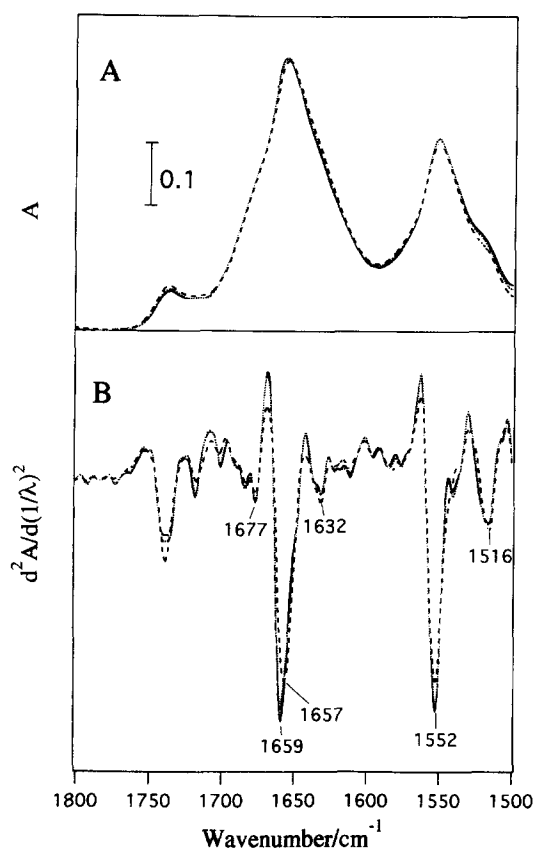

Fig. 5. Effect of photoinhibition on the FTIR spectra in the amide I and amide II regions. PS II membranes were photoinhibited for 0 min (solid curve), 60 min (dotted curve) and 240 min (dashed curve) at 20° C. Original spectra (panel A) and second-derivative spectra (panel B) were shown. Original spectra were normalized on the basis of the area intensities of the amide I bands.

Fig. 5 shows the effect of photoinhibition on FTIR spectra including the amide I (1700–1600 cm^{-1}) and amide II (1600–1500 cm^{-1}) regions (panel A) and their second-derivative spectra (panel B). The amide I mode of protein is attributed to C = O stretching vibration weakly coupled with C–N stretching and N–H bending vibrations in the backbone amide group, whereas the amide II mode is attributed to N–H bending strongly coupled with C–N stretching [35,36]. It has been known that amide I frequencies are quite sensitive to the secondary structures of proteins, and analysis of the amide I bands is useful to estimate the content of each conformation and to monitor the conformational changes of proteins [35,36]. The second-derivative spectrum of nonphotoinhibited membranes showed a strong negative peak at 1659 cm^{-1}, which is a typical frequency of an α-helical conformation [35,36], indicating that the major contribution of the secondary structures in the PS II proteins is α-helical structures. The small negative band at 1632 cm^{-1} can be ascribed to β-sheet structures [35,36]. The small intensity of this band indicates the minor content of and β-sheet structures. The second-derivative spectra in the amide I and amide II regions of the membranes exhibited almost no change after the photoinhibitory treatment for 60 min which caused the marked loss of oxygen-evolving activity as shown in Fig. 4B. After 240 min photoinhibition, the main amide I band downshifted by 2 cm^{-1} with slight decrease in intensity, but the intensity of the 1632 cm^{-1} band did not change.

Fig. 6 shows the change in the amounts of the D1 and D2 proteins as determined by immunoblotting, and the amounts of other PS II proteins as determined by Coomassie blue staining during the course of photoinhibition. The photoinhibitory treatment induced the loss of the D1 protein, and the degradation products with apparent molecular masses of 23 and 16 kDa became pronounced with the time of photoinhibition (panels A and B). Photoinhibition also led to the appearance of a band with apparent molecular mass of 41 kDa that has been ascribed to the aggregate between the D1 protein and the α-subunit

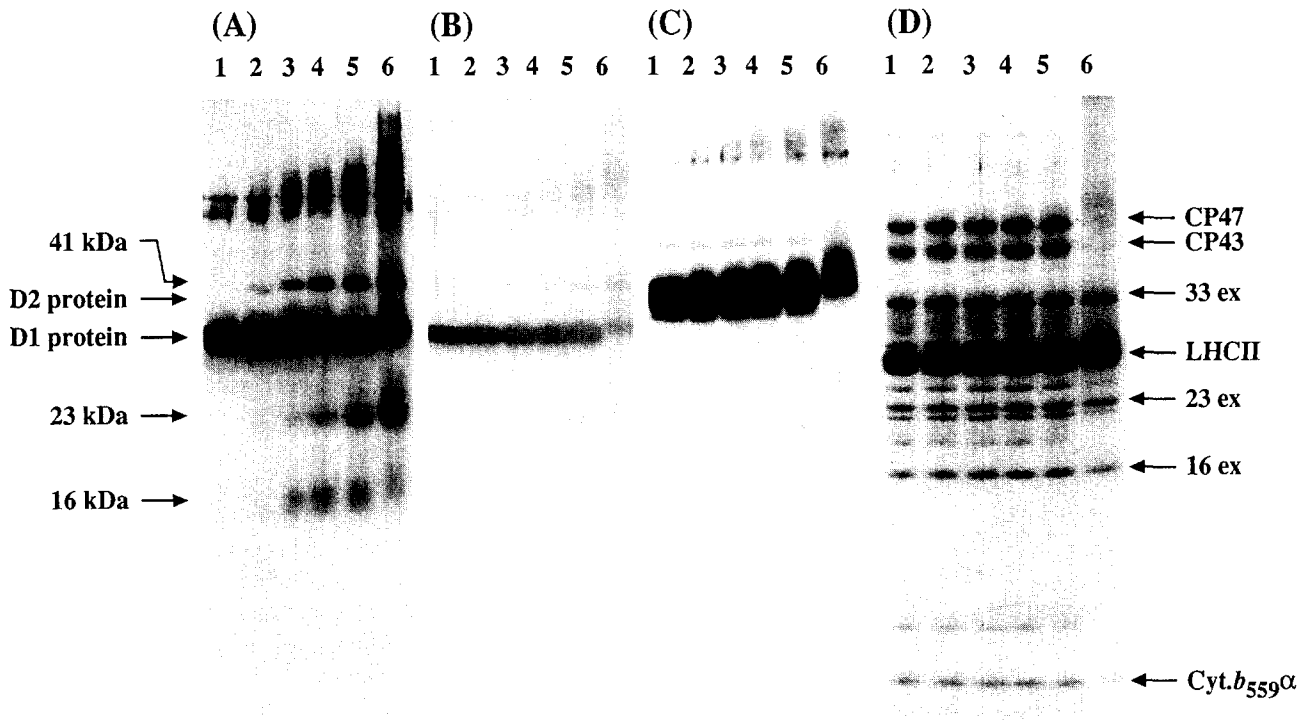

Fig. 6. Changes in protein composition of PS II membranes during photoinhibition. PS II membranes (0.75 μg of Chl per each lane) were resolved by SDS-PAGE, and stained with Coomassie blue (panel D) or subjected to immunoblot analysis with antisera raised with spinach D1 (panels A and B) and D2 (panel C) proteins. The membranes were photoinhibited for 0 min (1), 30 min (2), 60 min (3), 90 min (4), 120 min (5) and 240 min (6), respectively. Identical samples were loaded on the same SDS-PAGE gel for panels A–D. The change of the relative amount of the D1 protein is demonstrated in the panel B, while the degradation products of the D1 and D2 proteins are demonstrated by extension of the reaction time with Nitroblue tetrazolium and bromochloroindolyl in the panels A and C.

of cytochrome *b*-559 [37]. The loss of the D1 protein was, however, much slower than that of O_2 evolution, and a large amount of the native D1 protein was still preserved even after 60 min photoinhibition (panel B, lane 3). The D2 protein was much less susceptible to photoinhibition; no degradation product was detected after 60 min photoinhibition (panel C, lane 3) and then the degradation product at around 23 kDa started to appear after 90 and 120 min photoinhibition (panel C, lanes 4 and 5). After 240 min photoinhibition (panels A, B and C, lane 6), the amounts of the D1 and D2 proteins markedly decreased with the appearance of bands of their degradation products, accompanied by some and smear bands probably due to cross-linking products between PS II proteins including the D1 and D2 proteins in higher molecular mass regions.

The SDS-PAGE gel stained by Coomassie-blue showed almost no change in PS II membranes photoinhibited for 60 min (panel D, lane 3), but considerable loss of the polypeptide bands of LHC II, and α-subunit of cytochrome *b*-559, CP43 and CP47 occurred, and a mass of protein band could be seen on top of the separation gel (panel D, lane 6) in the membranes photoinhibited for 240 min. Since the change in the FTIR spectrum in the amide I and amide II regions was rather faint even after 240 min photoinhibiton (Fig. 5), the overall protein structure of the PS II complex does not much change even after the cleavage and/or aggregation of PS II proteins by severe photoinhibition. Presumably, the changes of the PS II proteins manifesting on SDS-PAGE profile are mostly attributable to those of the protein region protruding outside of the membrane, and thus the structural integrity of the PS II may not be much influenced even after prolonged photoinhibition.

4. Discussion

The present study has provided the direct evidence that the capability of photoreduction of the plastoquinone molecule at the Q_A pocket is impaired by strong-light photoinhibition under aerobic condition. The decrease in amount of the photoreducible Q_A as measured by 1479 cm^{-1} FTIR band closely matched the loss of O_2-evolving activity in the course of photoinhibition. This result implies that the loss of Q_A function is directly responsible for the loss of O_2 evolution in the photoinhibited PS II membranes under aerobic condition. Spectral features including the 1404 cm^{-1} band assigned as S_2/S_1 [25,33] did not change by photoinhibition, although the overall intensity of the spectrum decreased. Since impairment of the

electron donation from the Mn-cluster leads to oxidation of other redox components on the donor side of PS II instead of the Mn-cluster [32,33], no change in the spectral features may imply that the oxidation of these components, e.g., cytochrome *b*-559, Chl or carotenoid, does not contribute to the spectra. This result, therefore, indicates that the O_2-evolving Mn-cluster is active at least in the PS II unit that preserves active plastoquinone in the Q_A pocket. The decrease in the intensity of the FTIR difference spectra is due to the impairment of the stable charge separation between Q_A and the Mn-cluster, since the primary charge separation between pheophytin and P680 is hard to be inactivated by the aerobic photoinhibition [16–19]. The FTIR result is consistent with the report that photo-accumulation of the EPR signal arising from a magnetic interaction between Q_A^- and Fe^{2+} is inhibited by photoinhibition [18,19] and clearly show that the incapability of Q_A^- formation is the direct cause of the loss of this magnetically interacting signal and the capability of O_2 evolution.

At a first glance, the present result is consistent with the idea that the over-reduction of Q_A leads to the formation of doubly reduced Q_A, followed by the loss of the plastoquinone molecule from the Q_A pocket as proposed based on the results obtained from photoinhibition under anaerobic condition [11]. The loss of Q_A function by the anaerobic photoinhibition has been accompanied by the stable formation of Chl triplet because of the increased probability of primary charge separation between P680 and pheophytin [11,38]. In the photoinhibition under aerobic conditions, on the other hand, only a small amount of Chl triplet signal is induced in the photoinhibited sample, in which both the O_2 evolution and $Q_A^-Fe^{2+}$ EPR signal formation are severely inhibited even though the capability of the primary charge separation is still largely preserved [15,18,19]. This suggests that the properties of PS II photoinhibited under aerobic conditions are substantially different from those photoinhibited under anaerobic condition. It has been shown that the aerobic photoinhibition causes a conversion of redox potential of cytochrome *b*-559 from its high- to low-potential form with the same course as the inhibition of O_2 evolution [18]. Furthermore, ferricyanide-dependent formation of the $g = 8$ EPR signal reflecting the oxidized Fe^{3+} form of the endogenous non-heme iron between Q_A and Q_B was also inhibited in correlate with the inhibition of O_2 evolution in the aerobic photoinhibition [21,22]. Thus, we may consider that the aerobic photoinhibition affects not only Q_A but also other redox components in PS II.

The present study also showed that the aerobic photoinhibition modified the properties of PS II preceding the loss of O_2 evolution. The electron transfer beyond Q_A was interrupted in an early stage of photoinhibition as revealed by fluorescence relaxation kinetics (Figs. 1, 2) and thermoluminescence measurements (Fig 3). It is of note that the inhibition was an irreversible process, since the measurements were carried out after dark incubation of inhibited membranes for 2 h. It has been reported in *Chlamydomonus* cells [39] and pea leaves [40] that a downshift of the peak position of the thermoluminescence band due to recombination between Q_B^- and S states is induced in an initial stage of photoinhibition. This change is, however, reversible and has not been detected in isolated thylakoids [39,40]. It may, therefore, be rational to consider that the modified $S_2Q_B^-$ found in vivo systems is not the cause of the downshift we observed in the present study. As shown in Fig. 4, the fraction of PS II that undergoes normal electron transfer from Q_A to Q_B as estimated by the intensity of the $S_2Q_B^-$ band decreased much more quickly than the loss of O_2 evolution. This implies that the externally added artificial quinone efficiently accepts electrons by binding to the Q_B pocket in the photoinhibited PS II in which the endogenous electron transfer from Q_A to Q_B is interrupted, since phenyl-*p*-benzoquinone cannot directly accept electrons from Q_A^- without binding at the Q_B pocket. The lack of plastoquinone molecule in the Q_B pocket is thus likely to be responsible for the inhibition of the electron transfer beyond Q_A^- as found in the early stage of photoinhibition.

Irreversible loss or vacancy of plastoquinone molecule in the Q_B pocket may be explained by two alternatives: (i) plastoquinone molecules are broken down presumably due to reaction with oxygen and/or active-oxygen species that would be formed by electron donation from reduced plastoquinone to molecular oxygen; or (ii) the properties of the Q_B pocket are altered to reduce its affinity for a plastoquinone molecule. In thylakoid preparations, photoinhibition induces a decrease in the intensity of $S_2Q_B^-$ thermoluminescence band and the course of the decrease matches to the loss of O_2 evolution, but neither the peak temperature nor the band shape changes [14,41]. This indicates that the preferential loss of plastoquinone molecule from the Q_B pocket does not take place in case of thylakoids. When we take account of the much lower amounts of pooled plastoquinone molecules in detergent-treated PS II membranes than in thylakoid membranes, the former explanation seems to be more likely, although we cannot totally exclude the latter possibility.

Other changes found in a very early stage of photoinhibition are those in fluorescence properties. The decrease in the F_v level has always been found during photoinhibition both under aerobic and anaerobic conditions, and has been attributed to the formation of the modified Q_A by overreduction [14] or the formation of an unknown quencher on the donor side of PS II [15]. Our results have shown that the decrease in F_v is much faster than inactivation of the Q_A function or O_2 evolution (Fig. 2). It is likely that the F_v level decreases due to the formation of a certain quenching state that is not directly responsible for the inhibition of O_2 evolution. Judging from our results, this quenching state does not seem to influence the thermoluminescence yield, although we cannot give any further comments about its nature.

Aerobic photoinhibition is known to lead to the degradation of the D1 protein, but the degradation is much slower than the loss of O_2 evolution. It has been considered that the D1 protein degradation does not in itself require light but is triggered by some light-induced damage of the D1 protein [42]. As hardly any change in the FTIR spectra was found after 60 min photoinhibition, the postulated structural change that triggers the D1 protein degradation does not seem to be detected by FTIR. The postulated structural change might be too small to be detected by FTIR.

In vivo system, the degradation of the D1 protein by photoinhibition is followed by the recovery process in which the damaged D1 protein is replaced by a newly synthesized protein [6,7]. Experiments with whole thylakoids suggest that the protein subunits of the photoinhibited PS II are disassembled during migrating from the appressed to non-appressed region of thylakoids, and the damaged D1 protein is eliminated from the thylakoid membranes [43]. As shown in Figs. 5 and 6, the whole structure of the PS II complex did not change to any extent, even after photoinhibitory treatment for 240 min that led to a severe loss of the native D1 and D2 proteins together with many of PS II proteins on SDS-PAGE. This unexpected result may indicate that the protein subunits of the PS II complex are not dissociated even after severe photoinhibition. Assuming that the grana-lamella is enriched in our PS II membranes, this may imply that the PS II complex remains to be assembled unless the damaged PS II complex begins to migrate to the non-appressed thylakoid region. We note in this context that the loss of the PS II proteins on SDS-PAGE profile do not necessary imply the elimination of the protein from the PS II complex. This is compatible with our earlier observation that tryptic digestion of the PS II membranes at pH 6.5 induces a dramatic change on SDS-PAGE profile but do not affect O_2 evolution capability [44].

Exposure of the isolated PS II reaction center to strong-light illumination for 30 min has induced the changes in SDS-PAGE profile of the D1 and D2 proteins in a quite similar manner to those found in the PS II membranes photoinhibited for 240 min in the present study, but drastic changes in FTIR spectra has been reported [45]. Since the total amount of proteins per PS II unit in the reaction center preparation could be accounted to be up to 20% of that in the PS II membranes [46], the very small changes in the FTIR spectra after 240 min photoinhibition may lead to the conclusion that the structural change of the PS II reaction center induced by severe photoinhibition is much less manifest in the PS II membrane than in the isolated reaction center preparations. This may imply that the whole structure of the photoinhibited reaction center is not much distorted when it is preserved in the appressed thylakoid region and starts to change after isolation from other PS II proteins in the non-appressed thylakoid region.

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