Evidence That Cytochrome b_{559} Protects Photosystem II against Photoinhibition†

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ABSTRACT: Light that exceeds the photosynthetic capacity of a plant can impair the ability of photosystem II to oxidize water. The light-induced inhibition is initiated by inopportune electron transport reactions that create damaging redox states. There is evidence that secondary electron transport pathways within the photosystem II reaction center can protect against potentially damaging redox states. Experiments using thylakoid membranes poised at different ambient redox potentials demonstrate that light-induced damage to photosystem II can be controlled by a redox component within the reaction center [Nedbal, L., et al. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89,* 7929−7933]. The rate of photoinhibition is slow when the redox component is oxidized, but increases by more than 10-fold when the redox component is reduced. Here, using spinach thylakoid membranes, we provide evidence that the redox component is cytochrome b_{559}, an intrinsic heme protein of the photosystem II reaction center. The results support a model in which the low-potential (LP) form of cytochrome b_{559} protects photosystem II by deactivating a rarely formed, but hazardous redox state of photosystem II, namely, P680/Pheo$^-$/Q_A^-. Cytochrome b_{559}LP is proposed to deactivate this potentially lethal redox state by accepting electrons from reduced pheophytin. The key observations supporting this proposal are as follows: (1) The oxidation−reduction potential of cytochrome b_{559}LP is in the range predicted by redox titrations of photoinhibition. (2) If cytochrome b_{559}LP is reduced prior to illumination, the rate of photoinhibition is fast, whereas if the cytochrome is oxidized prior to illumination, the rate of photoinhibition is slow. (3) Irradiation of thylakoid membranes results in the photoreduction of cytochrome b_{559}LP, which is followed by the loss of water oxidation capacity. (4) The photoreduction of cytochrome b_{559}LP in continuous light precedes the reversible loss of the variable fluorescence, which monitors the photoreduction of pheophytin, supporting the proposal that electrons can flow from pheophytin to cytochrome b_{559}LP. (5) As predicted by the model, the protective pathway is effective over a broad range of light intensities, offering protection from 500 to 20 000 μmol of photons m^{-2} s^{-1}.

Photosystem II (PSII)[1] is a membrane-bound protein complex that converts light energy into chemical free energy by transferring electrons from water to plastoquinone, resulting in the release of molecular oxygen into the atmosphere. In plants the PSII reaction center can turn over at rates that exceed 200 electrons per second. Because each reaction center is served by an antenna system containing hundreds of pigment molecules, the intensity of light required to saturate electron transport is often much less than that of full sunlight (Lee & Whitmarsh, 1989). Consequently, it is not uncommon for PSII to operate at light intensities that far exceed the electron transport capacity of the enzyme. Under these conditions light-induced damage can occur, leading to irreversible loss of the oxygen-evolving capacity of the reaction center (Aro et al., 1993; Prasil et al., 1992). Plants are particularly susceptible to photoinhibitory damage when exposed to high light in conjunction with heat, cold, drought, or other environmental stress (Baker et al., 1988), although photoinhibition has been reported to occur under conditions that are optimal for photosynthesis and at light levels below light saturation (Ögren & Sjöstrom, 1990).

Plants respond to excess light at different organizational levels to protect against photoinhibition. At the whole plant level, leaf angles can be adjusted to control the amount of absorbed light (Björkman & Powles, 1982), while at the cell level, chloroplasts can orient in a manner that alters the amount of light intercepted [reviewed by Haupt and Scheuerlein (1990)]. Within chloroplasts, molecular processes have evolved to protect photosystem II against damage due to excess light. One such process involves the antenna system, which can divert exciton energy away from photosystem II reaction centers (Demmig-Adams, 1990). Observation of a correlation between the capacity to dissipate exciton energy and the presence of deepoxified xanthophylls has prompted different molecular mechanisms for converting exciton energy into heat, thereby decreasing the amount of energy reaching photosystem II (e.g., Crofts & Yerkes, 1994; Horton

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[1] Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Cyt, cytochrome; Cyt b_{559}HP, high-potential form of cytochrome b_{559} (midpoint potential between 320 and 400 mV); Cyt b_{559}LP, low-potential form of cytochrome b_{559} (midpoint potential between 20 and 100 mV); HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-morpholinoethanesulfonic acid; P680, the primary donor of photosystem II; Pheo, pheophytin; Q_A, primary plastoquinone acceptor of photosystem II; Q_B, secondary plastoquinone acceptor of photosystem II; PSII, photosystem II.

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& Ruban, 1992). This process seems to be ubiquitous in plants and appears to play an essential role in allowing plants to maintain high rates of photosynthesis over a wide range of light conditions.

In addition, there is evidence that secondary electron transfer reactions within photosystem II may protect against photodamage [reviewed in Whitmarsh and Pakrasi (1995)]. Recently we showed that the light-induced damage to PSII can be controlled by a redox component within the reaction center (Nedbal et al., 1992; Whitmarsh et al., 1994). The redox component exerts remarkably strong control over the rate of photoinhibition. Adding a single electron to the component causes the rate of light-induced PSII damage to increase by more than an order of magnitude. The redox behavior of the component indicates that it could be the low-potential form of Cyt b_{559}, a heme protein intimately associated with the photosystem II reaction center [reviewed by Cramer et al. (1993), Shuvalov (1994), and Whitmarsh and Pakrasi (1995)]. We proposed that Cyt b_{559}LP acts as a safety valve, protecting PSII against photoinhibition by deactivating a potentially damaging anionic state. As a working model, we suggested that upon formation of the potentially damaging state P680/Pheo$^-$/Q_A^- an electron can be transferred from pheophytin to Cyt b_{559}LP, thereby protecting other redox components of PSII from the strong reducing power of Pheo$^-$. Support for this model is provided by the demonstration that Pheo$^-$ can reduce Cyt $b_{559}LP^{ox}$ in isolated reaction centers (Barber & De Las Rivas, 1993). Here we describe experiments designed to elucidate the mechanism of photoprotection within PSII and provide evidence that the low-potential form of Cyt b_{559} plays a key role in protecting the reaction center from excess light.

MATERIALS AND METHODS

Membrane Isolation. Thylakoid membranes were isolated from market spinach as described elsewhere (Whitmarsh & Ort, 1984). The membranes were used within 6 h of isolation and were stored on ice in a medium containing 0.2 M sorbitol, 2 mM $MgCl_2$, fatty acid-free bovine serum albumin (0.5 mg/mL), and 5 mM HEPES/NaOH (pH 7.5) at a chlorophyll concentration of 1−2 mM. Experiments were done at 18 °C using thylakoid membranes resuspended in a reaction medium consisting of 0.2 M sorbitol, 10 mM KCl, 2 mM $MgCl_2$, fatty acid-free bovine serum albumin (0.5 mg/mL), and 20 mM HEPES/NaOH for pH values of 7 and above and 20 mM MES/NaOH for pH values below 7. Further additions to the reaction medium are indicated in the following and in the figure legends. The chlorophyll concentration was determined according to extinction coefficients determined by (Porra et al., 1989).

Removal of O_2 from the Reaction Medium. For experiments done under anaerobic conditions, the reaction medium was bubbled with high-purity argon for 5 min in a cuvette that could be made gas-tight. Thylakoids were then added to the reaction medium. Unless indicated otherwise, the concentration of oxygen was decreased further by adding glucose oxidase (50 units/mL), glucose (1 or 10 mM), and catalase (1000 units/mL). After these additions, air bubbles were removed from the reaction mixture, the cuvette was made air-tight, and the samples were gently stirred in darkness for 5 min before measurements. Using a Clark-type electrode (YSI/5331 Oxygen Probe, Yellow Springs, OH), we showed that the oxygen concentration was less than 1 μM. In some cases the reaction medium was not made air-tight, but was kept under a positive pressure of argon.

Redox Titrations. The redox state of Cyt b_{559} was determined by difference absorbance spectroscopy at various ambient redox potentials (Dutton, 1978). Thylakoid membranes were suspended at a chlorophyll concentration of 40 μM in 16 mL of reaction medium with 2 μM nigericin in a redox cuvette. The cuvette was placed in a DW2 spectrophotometer operated in dual-wavelength mode (half-bandwidth 2 nm). The reaction medium was deoxygenated prior to titration by bubbling with high-purity argon gas, which was kept at positive pressure in the cuvette during the titration. The ambient redox potential of the reaction mixture was measured by using a platinum electrode in combination with a calomel reference electrode (Radiometers K101 and K401, respectively, Copenhagen, Denmark). The following redox mediators were added to the assay medium: 1,2-naphthoquinone, 1,4-naphthoquinone, duroquinone (each at 20 μM), and 2,5-dihydroxybenzoquinone (5 μM). Sodium dithionite [dissolved in 0.1 M Tricine (pH 8.8) before addition] or ferricyanide was used to adjust the ambient redox potential of the medium. The sample was allowed to equilibrate for 10 min, and absorbance spectra were taken in the α-band region of Cyt b_{559}. Because at ambient potentials below 0 mV Cyt b_6 starts to become reduced, it was necessary to deconvolute the spectra of Cyt b_{559} and Cyt b_6 (Rich & Bendall, 1980). This was done by solving two simultaneous equations as described here. The midpoint potential of Cyt b_{559}LP was determined by using the Nernst equation for a one-electron component giving the best fit to the data (FIG P software from Biosoft, Cambridge, U.K.).

Absorption Spectroscopy. The redox states of cytochromes *f*, b_{559}, and b_6 were determined by absorption spectroscopy using a dual-wavelength spectrophotometer (DW2 Aminco/SLM, Rochester, NY) modified by OLIS (Bogart, GA). Chemical difference spectra were measured by taking the difference in absorbance in the α-band region of the cytochromes at various ambient redox potentials controlled by chemical additions. Light-induced difference spectra were measured by taking the difference in absorbance before and after illumination with various doses of white actinic light. Scattering of the high-intensity actinic light prevented us from recording spectra during illumination. Actinic light was provided by a 250 W tungsten−halogen lamp and was controlled by an electronic shutter (UniBlitz SD-10, Rochester, NY). Infrared radiation was removed from the light by a heat-reflecting filter (03 MHG 009, Melles Griot, Irvine, CA) and a heat-blocking filter (CS 1-75, Kopp Glass Inc., Pittsburgh, PA). The path length of the irradiance through the sample was 1 cm. The reference wavelength was 570 nm and the half-bandwidth was 2 nm. Under some experimental conditions two cytochromes (either Cyt b_{559}-HP and Cyt *f* or Cyt b_{559}LP and Cyt b_6) contribute to the spectrum. In these cases, the relative contribution of each cytochrome was determined by deconvolution of the spectrum into its component parts using two simultaneous equations (Whitmarsh & Ort, 1984) based on the following differential extinction coefficients and relative absorbance values: for Cyt b_{559}, $\Delta\epsilon_{560-570} = 17.5$ mM^{-1} cm^{-1} (Cramer et al., 1986), $\Delta\epsilon_{554-570} = 7.7$ mM^{-1} cm^{-1}, and $\Delta\epsilon_{563.5-570}$ mM^{-1} cm^{-1} = 12.8; for Cyt *f*, $\Delta A_{560-570}/\Delta A_{554-570} = 0.25$; and for Cyt b_6, $\Delta A_{560-570}/\Delta A_{563.5-570} = 0.56$ [see Poulson (1995) further details and references]. For spectra containing

Cyt b_{559}HP and Cyt *f*, the following equation gives the concentration (in micromolar) of the high-potential form of Cyt b_{559}:

$$[\text{Cyt } b_{559}\text{HP}] = 64(\Delta A_{560-570}) - 16(\Delta A_{554-570})$$

For spectra containing Cyt b_{559}LP and Cyt b_6, the following equation gives the concentration (in micromolar) of the low-potential form of Cyt b_{559}:

$$[\text{Cyt } b_{559}\text{LP}] = 97(\Delta A_{560-570}) - 54(\Delta A_{564-570})$$

Rate of Oxygen Evolution. The rate of oxygen evolution was measured with a Clark-type electrode using saturating red light provided by two tungsten–halogen lamps placed on either side of the cuvette as described elsewhere (Nedbal et al., 1991). Ferricyanide (800 μM) and 2,5-dimethyl-*p*-benzoquinone (400 μM) were used as electron acceptors. Dimethyl-*p*-benzoquinone was used as the electron acceptor because it selects for active photosystem II complexes (Nedbal et al., 1991). Experiments done using 2,6-dichloro-*p*-benzoquinone, which accepts electrons from active and inactive photosystem II centers, gave essentially the same results (data not shown). For measurements, thylakoids were removed from an anaerobic cuvette that had been exposed to various doses of white light. The thylakoids were reoxygenated by gentle bubbling with air before measuring the rate of oxygen evolution.

Fluorescence Measurements. Chlorophyll fluorescence from thylakoid membranes was measured with a Walz fluorometer (Walz PAM 101, Germany). This instrument detects the modulated measuring beam and is insensitive to nonmodulated actinic illumination. Thylakoids were suspended under anaerobic conditions and dark adapted for 5 min, after which the initial fluorescence level (F_o) was measured using a low-intensity measuring beam modulated at 1.6 kHz. The sample was then irradiated with 10 000 μmol m^{-2} s^{-1} white light, and the measuring beam modulation was simultaneously increased to 100 kHz. The maximum fluorescence (F_m) was recorded during the period of illumination. The variable fluorescence (F_v) was calculated using the following equation: $F_v = F_m - F_o$.

Photoinhibition at High and Low Ambient Redox Potentials. Thylakoid membranes were suspended in reaction medium poised at an ambient redox potential above or below that of Cyt b_{559}LP essentially as described previously (Nedbal et al., 1992). Photoinhibition was measured as the rate of loss of the variable fluorescence, which is correlated with the loss of water oxidation (Nedbal et al., 1992). The reaction mixture consisted of thylakoid membranes at a chlorophyll concentration of 10 μM in reaction medium (pH 6.0) containing 2 μM nigericin that had been bubbled with high-purity argon for 10 min. For the high ambient redox potential experiments, 10 μM either 1,2-naphthoquinone ($E_{m,6}$ = +195 mV) or 1,4-naphthoquinone ($E_{m,6}$ = +120 mV) was added to the medium. For the low ambient redox potential experiments, either 2-hydroxy-1,4-naphthoquinone ($E_{m,6}$ = −77 mV) or anthraquinonesulfonate ($E_{m,6}$ = −165 mV) was added to the reaction medium. Ferricyanide or dithionite was then added to adjust the ambient redox potential. Samples were allowed to equilibrate for 10 min in the dark. The samples were then illuminated for 10 min with white light at different intensities while the variable fluorescence was measured.

RESULTS

Redox Titration of Cyt b_{559}LP. Potentiometric titrations of thylakoid membranes in high light reveal that a one-electron redox component controls the rate of light-induced inhibition of PSII in thylakoid membranes (Nedbal et al., 1992). To test whether the low-potential form of Cyt b_{559} could account for the redox behavior of the controlling component, we determined the equilibrium midpoint potential of Cyt b_{559}LP. In thylakoid membranes isolated from spinach, the midpoint potential of Cyt b_{559}LP at pH 7.5 is +62 ± 20 mV. From pH 6 to 8 the midpoint is nearly pH independent ($E_{m,6}$ = +73 ± 11 mV, $E_{m,6.7}$ = 59 ± 18 mV, $E_{m,8}$ = 65 ± 23 mV). For each value the average midpoint potential (±standard deviation) was determined for at least five different membrane preparations. These results are similar to those of Rich and Bendall (1980), who found that Cyt b_{559}LP titrates as a one-electron component in lettuce thylakoids with a midpoint potential of +20 mV that is pH independent, and to the results of Horton and Croze (1977), who determined a midpoint potential at pH 7.8 of 77 mV in pea thylakoids. It should be noted that reported values for the midpoint potential of Cyt b_{559}LP in thylakoid membranes range from about 20 to 120 mV and in some membrane preparations show a pH-dependent midpoint potential (Whitmarsh & Pakrasi, 1995).

Continuous Illumination Drives the Reduction of Cyt b_{559}LP. Chemical difference spectra show that, in dark-adapted thylakoids, the low-potential form of Cyt b_{559}LP is oxidized and the high-potential form is reduced under anaerobic or aerobic conditions (data not shown). Figure 1A shows that illumination of thylakoids drives the photoreduction of Cyt b_{559}LP and Cyt b_6. The deconvoluted spectrum shown in Figure 1B shows that 90% of Cyt b_{559}-LP is reduced in the light. A requirement for the photoaccumulation of reduced Cyt b_{559}LP is anaerobic conditions. If oxygen is present, no net photoreduction of Cyt b_{559}LP is observed. This is likely due to the fact that oxygen can act as a chemical oxidant of Cyt b_{559}LP$^-$, Pheo$^-$, or possibly Q_A^-. We found that the addition of molecular oxygen in the dark to an anaerobic sample containing reduced Cyt b_{559}-LP resulted in the oxidation of the cytochrome within 1 min (data not shown). Under the same experimental conditions, the high-potential form of the cytochrome was not oxidized. Figure 2 shows that the Q_B site inhibitor, DCMU, does not block the photoaccumulation of reduced Cyt b_{559}LP, although it lowers the amount of Cyt b_{559}LP photoreduced to about 30% of that observed in its absence. As expected, DCMU completely inhibits the photoreduction of Cyt b_6 (Jones & Whitmarsh, 1988).

It has been suggested that light may create reducing equivalents in the oxygen-scrubbing system, for example, via the flavin in glucose oxidase (V. McNamara, personal communication). To test this possibility, the oxygen-scrubbing system was illuminated for 15 min, after which thylakoids were then added to the preilluminated reaction medium. Chemical difference spectra showed that Cyt b_{559}-LP remained oxidized under these conditions, indicating that the oxygen-scrubbing system does not create a reductant capable of chemically reducing Cyt b_{559}LP. In addition, because flavins absorb blue light, we showed that red light was sufficient to drive the photoreduction of Cyt b_{559}LP (data not shown). These results indicate that anaerobicity is the

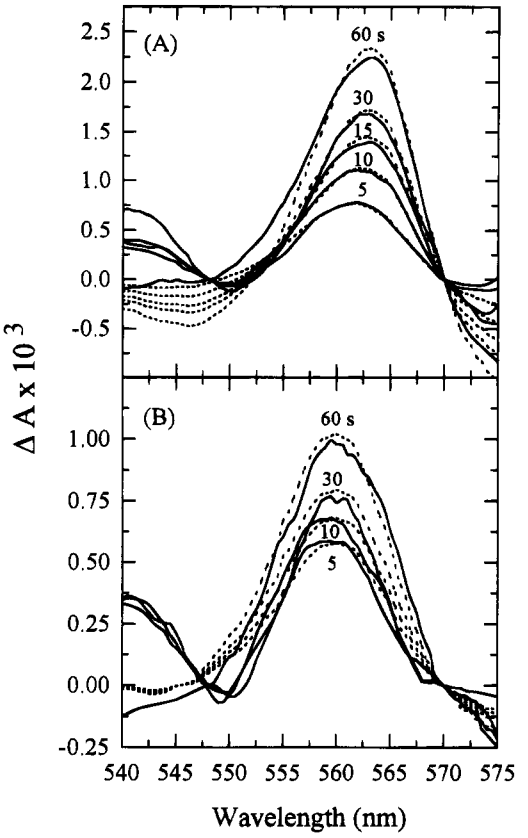

FIGURE 1: (A) Photoaccumulation of reduced Cyt b_{559}LP and Cyt b_6 in thylakoid membranes under anaerobic conditions in the absence of added redox mediators. Thylakoids were suspended in reaction medium (pH 7.5) at a chlorophyll concentration of 50 μM and illuminated for various periods with white light at an intensity of 10 000 μmol m^{-2} s^{-1}. Spectra were recorded before and after illumination. The contributions of Cyt b_{559}LP and Cyt b_6 to the spectrum were determined using the equation described in Materials and Methods. The sum of the contribution of Cyt b_{559}LP and Cyt b_6 is shown as a dashed line. (B) Deconvolution of the light-induced spectrum shown panel A revealing the contribution of Cyt b_{559}LP.

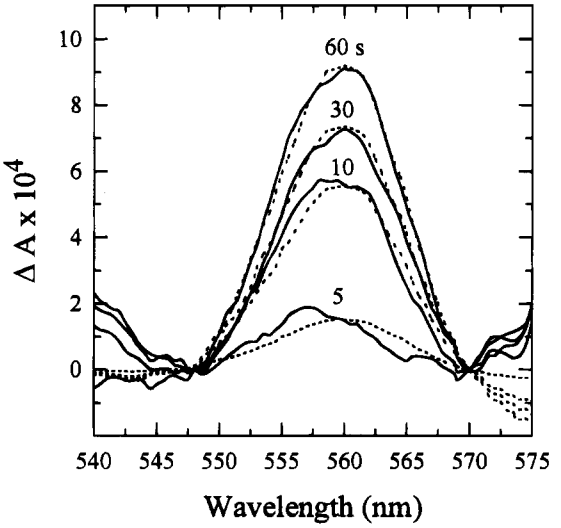

FIGURE 2: Photoaccumulation of reduced Cyt b_{559}LP in thylakoid membranes measured in the presence of DCMU (15 μM). Thylakoid membranes were illuminated under anaerobic conditions without added redox mediators as described in Figure 1A.

critical requirement to observe the photoreduction of Cyt b_{559}-LP.

Photoreduction of Cyt b_{559}LP Precedes the Loss of Water Oxidation in High Light. To investigate the relationship

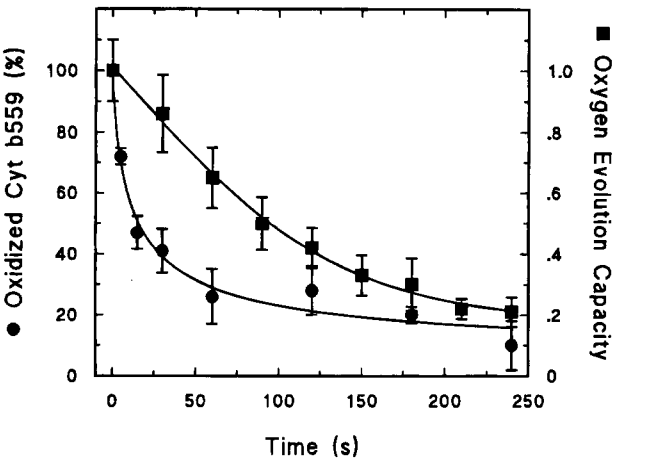

FIGURE 3: Time course of light-induced reduction of Cyt b_{559}LP (●) and the loss of oxygen evolution capacity (■) in isolated thylakoid membranes. These measurements were done using thylakoid membranes under anaerobic conditions in the absence of redox mediators. The membranes were exposed to 10 000 μmol of photons m^{-2} s^{-1} of white light for the time periods indicated. Each point is the average (±SD) of at least four measurements. The control rate of oxygen evolution was 112 ± 12 mmol of O_2 (mol of Chl)$^{-1}$ s^{-1}.

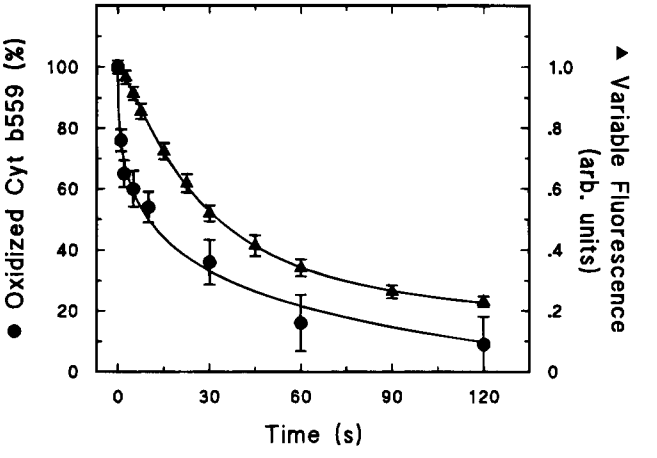

FIGURE 4: Time course of the light-induced reduction of Cyt b_{559}-LP (●) and the loss of the variable fluorescence (▲) in thylakoid membranes. Thylakoid membranes were suspended in anaerobic reaction medium and exposed to various doses of white light as described in Figure 1A. Further details are given in the text.

between the redox state of Cyt b_{559}LP and photoinhibition, we measured the loss of water oxidation in continuous light under the same conditions that we used to measure the photoreduction of Cyt b_{559}LP. Figure 3 shows that photoaccumulation of reduced Cyt b_{559}LP precedes the loss of water oxidation. Cyt b_{559}LP is photoreduced with a half-time of approximately 10 s, while the loss of water oxidation exhibits a half-time near 100 s. Addition of the uncoupler nigericin (2 μM) to the reaction medium did not alter these results (data not shown).

Photoreduction of Cyt b_{559}LP Precedes the Reduction of Pheophytin Monitored by the Loss of Variable Fluorescence. To test whether the photoaccumulation of reduced Cyt b_{559}-LP is coupled to the photoaccumulation of reduced pheophytin, we monitored the redox state of pheophytin by measuring chlorophyll fluorescence. Klimov et al. (1985) showed that reduced pheophytin quenches the variable fluorescence. We used this relationship to monitor the redox state of pheophytin in continuous illumination. Figure 4 shows the time course for the reversible loss of variable

Table 1: Proportion of High- and Low-Potential Cyt b_{559} in Isolated Spinach Thylakoids after 0, 1, 2, and 4 min Preillumination with 10 000 μmol of Photons m^{-2} s^{-1} under Anaerobic Conditions[a]

illumination time (min)	Cyt b_{559}HP (%)	Cyt b_{559}LP (%)
0	72 ± 7	28 ± 7
1	72 ± 4	28 ± 3
2	66 ± 3	34 ± 3
4	62 ± 7	38 ± 7

[a] The amount of Cyt b_{559} was measured using absorption spectroscopy as described in the text. The contributions of Cyt b_6 and Cyt f were determined as described in Materials and Methods. Cyt b_{559}HP was determined by the amount reduced by hydroquinone, and Cyt b_{559}LP was determined by the amount reduced by dithionite. As described in the text, over 90% of the low-potential form of the cytochrome titrated as a one-electron component with a midpoint potential near +60 mV. The total amount of Cyt b_{559} present in the membranes is 1 Cyt b_{559} heme per 238 ± 36 Chl (average ± standard deviation of measurements based on 33 thylakoid preparations). The values shown in the table are the average ± the standard deviation (or measurement error if larger) calculated using three or more thylakoid preparations.

fluorescence and the photoreduction of Cyt b_{559}LP in thylakoid membranes illuminated by high-intensity white light under anaerobic conditions. The kinetic traces show that the photoaccumulation of reduced Cyt b_{559}LP ($t_{1/2}$ = 10 s) precedes the photoaccumulation of reduced pheophytin ($t_{1/2}$ = 35 s).

Estimation of the Concentration of High- and Low-Potential Cyt b_{559} in Thylakoids. In thylakoid membranes, Cyt b_{559} exists in at least two different thermodynamic states: a high-potential form ($E_{m,7}$ = 360−400 mV) and a low-potential form (Whitmarsh & Pakrasi, 1995). In spinach thylakoids, we find that the low-potential form has a midpoint potential near 60 mV that is nearly pH independent. Table 1 shows that in spinach thylakoid membranes approximately 72% of Cyt b_{559} is in the high-potential form and the remaining 28% is in the low-potential form. The total amount of Cyt b_{559} present in the membranes is 1 Cyt b_{559} heme per 228 ± 34 Chl. We found that over 90% of the low-potential form of Cyt b_{559} titrates as a homogeneous component with a single midpoint potential. The high-potential form of Cyt b_{559} also titrates as a homogeneous component (Horton et al., 1976). These results indicate that intermediate forms of Cyt b_{559} observed in some membrane preparations (e.g., Horton & Croze, 1977; Ortega et al., 1988) are likely due to isolation procedures or to the way the thylakoids were treated after isolation and are not normally present (less than 10%). To determine whether excess light irreversibly converted high-potential Cyt b_{559} to the low-potential form, we determined the relative concentrations of Cyt b_{559}LP and Cyt b_{559}HP in thylakoid membranes before and after irradiation with various doses of light. Table 1 shows that a 1 min exposure of thylakoids to white light under anaerobic conditions does not irreversibly convert Cyt b_{559}HP to Cyt b_{559}LP. The illumination and cytochrome assay were done in the absence of oxygen. After longer illumination times, there was conversion of some high-potential Cyt b_{559} (less than 15%) to the low-potential form.

Effect of Light Intensity on the Rate of Photoinhibition at High and Low Ambient Redox Potentials. The redox component controlling the rate of photoinhibition is able to protect against damage over a broad range of light intensities. Figure 5 shows that the oxidized component protects at light intensities as high as 20 000 μmol of photons m^{-2} s^{-1}. Photoinhibition was measured by the loss of variable

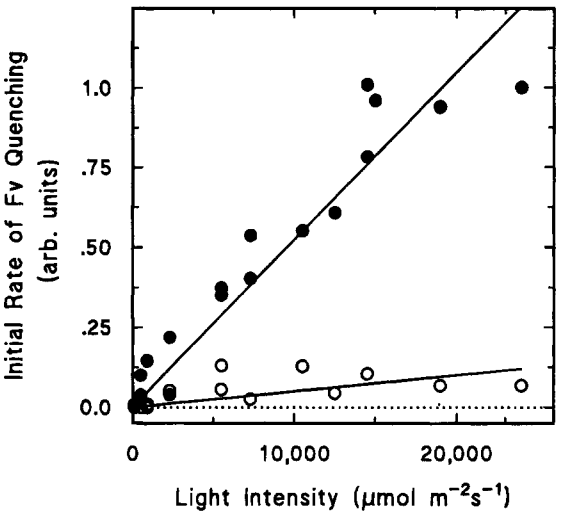

FIGURE 5: Effect of light intensity on the rate of variable fluorescence quenching at high (+100 to +225 mV) (○) and low (−100 to −190 mV) (●) ambient redox potentials. Experimental details are described in Materials and Methods. The data shown were obtained using three different thylakoid preparations.

fluorescence, which is related to the accumulation of reduced pheophytin (see above) and the loss of water oxidation (Nedbal et al., 1992). At low ambient redox potentials (−100 to −190 mV), the rate of photoinhibition is rapid and increases as the illumination intensity increases. However, at high ambient redox potentials (+100 to +225 mV), the rate of photoinhibition is slow and shows almost no increase as the light intensity increases by 40-fold.

DISCUSSION

The study was initiated to test the hypothesis that Cyt b_{559} protects PSII by deactivating a rarely formed but potentially damaging stable state of $Pheo^-$ (Nedbal et al., 1992; Whitmarsh et al., 1994). Normally $Pheo^-$ is short-lived, transferring its electron to Q_A within a few hundred picoseconds (Eckert et al., 1988). However, a long-lived $Pheo^-$ can be created when the intensity of the irradiation exceeds the capacity of the electron transport apparatus, resulting in a state in which both pheophytin and Q_A are reduced ($Y_Z/P680^+/Pheo^-/Q_A^-$). While this state can decay by back-reaction between $P680^+$ and $Pheo^-$, there is a low probability that $P680^+$ will be reduced by the secondary donor, Y_Z. Depending on the S state of the water-oxidizing apparatus, this reaction can result in the stable state $P680/Pheo^-/Q_A^-$ (Allakhverdiyev et al., 1988; Klimov et al., 1985, 1986; Rutherford & Zimmerman, 1984). Because $Pheo^-$ is a strong reductant (Rutherford et al., 1981), it has been proposed to initiate photoinhibition, for example, by overreducing Q_A (Styring et al., 1990; van Mieghem et al., 1989; Vass et al., 1992). Vass et al. (1992) have provided evidence that in excess light $Pheo^-$ can reduce Q_A^-, resulting in Q_AH_2, which debinds from PSII. In the absence of Q_A, the back-reaction between $Pheo^-$ and $P680^+$ has a greater probability of forming chlorophyll triplet (van Mieghem et al., 1989). Interaction between the P680 triplet and oxygen is proposed to lead to the formation of singlet oxygen (Durrant et al., 1990; Telfer et al., 1994; Vass et al., 1992), an energetic radical that triggers reactions that lead to the loss of water oxidation and the eventual removal of the D1 polypeptide from the reaction center.

A model showing how Cyt b_{559}LP can protect PSII from light-induced damage by $Pheo^-$ is shown in Figure 6 (Nedbal

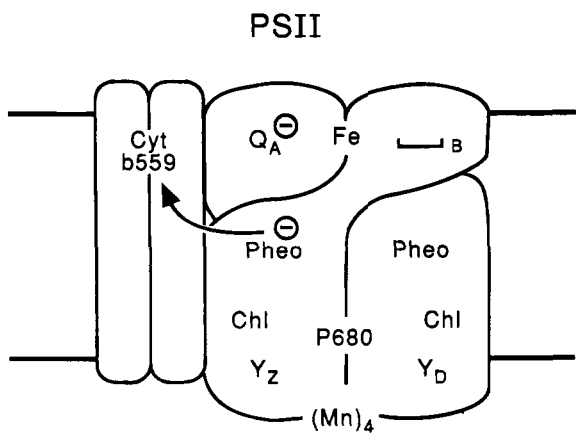

FIGURE 6: Electron pathway in PSII proposed to protect against damaging anion radicals created by excess light. The role of Cyt b_{559}LP is to accept an electron from pheophytin when PSII is trapped in the redox state (P680/Pheo$^-$/Q_A^-).

et al., 1992; Whitmarsh et al., 1994). The central idea is that oxidized Cyt b_{559}LP deactivates the redox state P680/Pheo$^-$/Q_A^- by accepting an electron from Pheo$^-$. This model is based on the observation that the redox state of a one-electron component can change the rate of light-induced inhibition of PSII by more than 10-fold (Nedbal et al., 1992). When the redox component is oxidized the rate of photoinhibition is slow. The redox component controlling photoinhibition exhibits an apparent midpoint potential of 20 mV that is nearly pH independent.

Comparison of the redox behavior of PSII prosthetic groups indicates that the low-potential form of Cyt b_{559} is the most likely candidate for the redox component controlling the rate of photoinhibition (Nedbal et al., 1992). Other candidates with midpoint potentials in the 0−100 mV range include free plastoquinone and the bound quinones Q_B and Q_A. Free plastoquinone in the membrane can be eliminated because its midpoint potential is strongly dependent on pH (Golbeck & Kok, 1979). Q_B can be discounted because DCMU, which displaces plastoquinone from the Q_B site, does not alter the redox control of photoinhibition (Nedbal et al., 1992). The couple Q_A/Q_A^- is unlikely because Q_A is reduced by the actinic light throughout the titration of photoinhibition (Nedbal et al., 1992). The redox couple Q_A^-/Q_A^{2-} is not a likely candidate because it has a low midpoint potential (E_m < −350 mV) (van Mieghem et al., 1989) nor is a protonated state of a reduced form of Q_A because it would be pH dependent.

To compare the redox behavior of Cyt b_{559}LP with that of photoinhibition, we measured the midpoint potential of the cytochrome under conditions similar to those used to measure the redox behavior of photoinhibition, except that the titrations of the cytochrome were done in the dark. We found an essentially pH-independent midpoint potential near 60 mV for low-potential Cyt b_{559}. This is in the range expected for the redox component controlling photoinhibition. Considering the inherent uncertainty in measuring midpoint potentials of membrane-bound components, and the fact that the redox behavior of photoinhibition was measured in the light, the 40 mV difference is not significant.

Direct evidence for the involvement of Cyt b_{559}LP in protecting PSII is provided by comparing the rate of photoaccumulation of reduced Cyt b_{559}LP and the rate of photoinhibition. According to the model (Figure 6), as long as Cyt b_{559}LP remains oxidized there should be little photoinhibition. However, reduction of Cyt b_{559}LP would lead to the photoaccumulation of Pheo$^-$, which subsequently triggers the loss of water oxidation. We tested this hypothesis by illuminating thylakoid membranes in the absence of an electron acceptor under anaerobic conditions. As shown in Figure 3, the photoaccumulation of reduced Cyt b_{559}LP precedes the loss of water oxidation. About 90% of Cyt b_{559}LP is reduced in the light with a half-time of 10 s, while under the same experimental conditions the loss of the water-oxidizing capacity of PSII occurs with a half-time of 100 s.

We monitored the photoaccumulation of reduced pheophytin by measuring the loss of variable fluorescence. Figure 4 shows that the loss of the variable fluorescence ($t_{1/2}$ = 35 s) occurs after the reduction of Cyt b_{559}LP and prior to the loss of water oxidation. By assuming that the decline in the variable fluorescence is primarily due to the production of Pheo$^-$, Figure 4 confirms the prediction that Pheo photoaccumulation occurs subsequent to the photoreduction of Cyt b_{559}LP and precedes the irreversible loss of water oxidation. Barber and De Las Rivas (1993) have provided strong evidence for the reduction of Cyt b_{559} by Pheo$^-$ in a PSII reaction center preparation. In a photoaccumulation experiment using optical spectroscopy to monitor the redox states of Pheo and Cyt b_{559}, they demonstrated full reduction of Cyt b_{559}LP by pheophytin with a half-time of approximately 15 s. An important feature of this observation is that the reaction center preparation that they used did not contain Q_A. Additional evidence that the Cyt b_{559}LP can be reduced by Pheo$^-$ is provided by the effect of DCMU. We found that DCMU lowered the amount of Cyt b_{559}LP that could be photoreduced. In thylakoid membranes under anaerobic conditions about 30% of the total amount of Cyt b_{559}LP was photoreduced after 1 min of illumination (Figure 2). The decreased amount of photoreducible Cyt b_{559}LP matches the effect of DCMU on the photoreduction of Pheo$^-$ under anaerobic conditions (Klimov, 1985). The effect of DCMU on the amount of photoreduced Cyt b_{559}LP is also in accordance with reports that DCMU partially inhibits photoinhibition under anaerobic conditions (Arntz & Trebst, 1986) and the observation that DCMU slows the formation of stable forms of Q_A^- (Kirilovsky et al., 1994).

The photoprotective model shown in Figure 6 requires at least one Cyt b_{559}LP heme per reaction center. Experiments using thylakoid membranes in the dark reveal that 28% of the total amount of Cyt b_{559} is low potential (Table 1). Even if there are two Cyt b_{559} hemes per PSII, which in itself is a controversial question [reviewed by Whitmarsh and Pakrasi (1995)], the amount of Cyt b_{559}LP detected in the dark is short by a factor 2. In view of this, it has been suggested that Cyt b_{559}HP may be reversibly converted to the low-potential form in the light (Nedbal et al., 1992; Barber & De Las Rivas, 1993). In thylakoid membranes we found that there is very little irreversible conversion of the high-potential form to the low-potential form of cytochrome b_{559} measured before and after light treatment (Table 1), but it must be kept in mind that we have no information about the midpoint potential of the cytochrome during illumination.

Because our model protects against a low quantum yield reaction, it predicts that redox control of photoinhibition should extend over a wide range of light intensities. To test this prediction, we measured the loss of variable fluorescence in isolated thylakoids at high and low redox potentials as a function of actinic light intensity. The results show redox control of photoinhibition at actinic light intensities from 500

to 20 000 μmol m^{-2} s^{-1} white light (Figure 5).

In our model we have not identified an oxidant for Cyt b_{559}LP. Likely candidates include molecular oxygen, plastoquinone, Cyt b_{559}HP, or a redox component on the donor side of PSII. Although reduced Cyt b_{559}LP is readily oxidized by the addition of molecular oxygen (data not shown), it is not known whether this reaction occurs *in vivo*. Under physiological conditions, Cyt b_{559} may be protected from oxidation by oxygen to avoid the formation of superoxide, which can lead to deleterious reactions. The notion that plastoquinone could act as an oxidant is supported by the observation that plastoquinone in thylakoid membranes can reduce the high-potential form of Cyt b_{559} (Whitmarsh & Cramer, 1978).

An attractive feature of the photoprotective model described here is that it accounts for the slow turnover of Cyt b_{559} observed under physiological conditions (e.g., Canaani & Havaux, 1990; Whitmarsh & Cramer, 1977). It must be kept in mind that the photoaccumulation experiments described here determine the time course of the accumulation of a product in continuous light, in our case reduced Cyt b_{559}LP, and are not a measure of the pairwise rate of electron transfer. We expect the pairwise rate of electron transfer from pheophytin to Cyt b_{559} to be rapid, but because the probability of the reaction is low, it would be difficult to detect. According to our model the time course of the photoreduction of Cyt b_{559}LP in the light is dictated by the quantum yield for the formation of a stable $Pheo^-$, a low quantum yield process (Klimov et al., 1977). We estimate that the probability that an electron will be transferred to Cyt b_{559}LP is less than 1 in 500 and could be as low as 1 in 2000. As a consequence, even in high light the turnover number of Cyt b_{559}LP is expected to be low, less than one per second. Our model differs from the proposal that the function of Cyt b_{559} is to dissipate energy by cyclic electron transport [discussed by Whitmarsh and Pakrasi (1995)]. In these energy dissipation models, electron flow through Cyt b_{559} would need to be rapid for the electron transport cycle to offer protection. Our data indicate that Cyt b_{559} turns over very slowly in continuous light in response to a rarely formed, but dangerous redox state.

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