Two-Step Mechanism of Photodamage to Photosystem II: Step 1 Occurs at the Oxygen-Evolving Complex and Step 2 Occurs at the Photochemical Reaction Center†

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ABSTRACT: Under strong light, photosystem II (PSII) of oxygenic photosynthetic organisms is inactivated, and this phenomenon is called photoinhibition. In a widely accepted model, photoinhibition is induced by excess light energy, which is absorbed by chlorophyll but not utilized in photosynthesis. Using monochromatic light from the Okazaki Large Spectrograph and thylakoid membranes from *Thermosynechococcus elongatus*, we observed that UV and blue light inactivated the oxygen-evolving complex much faster than the photochemical reaction center of PSII. These observations suggested that the light-induced damage was associated with a UV- and blue light-absorbing center in the oxygen-evolving complex of PSII. The action spectrum of the primary event in photodamage to PSII revealed the strong effects of UV and blue light and differed considerably from the absorption spectra of chlorophyll and thylakoid membranes. By contrast to the photoinduced inactivation of the oxygen-evolving complex in untreated thylakoid membranes, red light efficiently induced inactivation of the PSII reaction center in Tris-treated thylakoid membranes, and the action spectrum resembled the absorption spectrum of chlorophyll. Our observations suggest that photodamage to PSII occurs in two steps. Step 1 is the light-induced inactivation of the oxygen-evolving complex. Step 2, occurring after step 1 is complete, is the inactivation of the PSII reaction center by light absorbed by chlorophyll. We confirmed our model by illumination of untreated thylakoid membranes with blue and UV light, which inactivated the oxygen-evolving complex, and then with red light, which inactivated the photochemical reaction center.

Photosystem II (PSII),[1] a critical component of the photosynthetic machinery, is inactivated by strong light (*1*). In the currently accepted hypothesis for the mechanism of photodamage to PSII, excess light energy, which is absorbed by photosynthetic pigments and cannot be utilized efficiently in photosynthesis, produces reactive oxygen species (ROS) (*2*−*4*) and/or overreduces Q_A, the primary electron acceptor plastoquinone (*2*, *5*), which results in damage to PSII. However, in a recent study of the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter, *Synechocystis*), we found that oxidative stress due to H_2O_2 and singlet oxygen inhibited the repair of photoinactivated PSII but did not inactivate PSII directly (*6*, *7*). Other kinds of stress, such as salt stress and cold stress, also inhibited repair but did not accelerate photoinactivation (*8*, *9*). We also found that the rate of electron transport through PSII had no effect on the photodamage to PSII (*10*). These observations led us to examine the mechanism of the photoinactivation of PSII using monochromatic light.

Hakala et al. (*11*) proposed another hypothesis for the mechanism of photoinhibition of PSII, namely, that photoinhibition is caused by a process that is independent of electron transport. They postulated that the earliest step in photoinhibition is the release of a manganese ion from PSII.

In this study, we examined the photoinduced damage to the photochemical reaction center and to the oxygen-evolving complex separately using monochromatic light generated by the Okazaki Large Spectrograph (*12*, *13*). Our results suggest that the initial target of photodamage is the oxygen-evolving complex and that a second subsequent event occurs at the photochemical reaction center, after the oxygen-evolving complex has been photoinactivated.

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[1] Abbreviations: DCIP, dichlorophenolindophenol; DPC, diphenylcarbazide; PSII, photosystem II; ROS, reactive oxygen species.

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EXPERIMENTAL PROCEDURES

Strain and Culture Conditions. The thermophilic cyanobacterium *Thermosynechococcus elongatus* was provided by M. Ikeuchi (University of Tokyo, Tokyo, Japan). Cells were cultivated at 47 °C under white light at 50 μE m^{-2} s^{-1} in BG11 medium, as described elsewhere (*14*), which had been supplemented with 20 mM TES-KOH (pH 8.2), with aeration by air that contained 1% CO_2 until the optical density at 730 nm reached 0.8−1.2.

Preparation of Thylakoid Membranes. Cells were harvested by centrifugation at 7500*g* for 15 min at room temperature. After suspension in a solution of 1.0 M glycine betaine, 0.4 M sorbitol, 20 mM HEPES-NaOH (pH 7.0), 15 mM $CaCl_2$, 15 mM $MgCl_2$, and 1 mM 6-amino-*n*-caproic acid at a concentration of 700 μg of Chl/mL, cells were passed through a French pressure cell (SLM Instruments, Urbana, IL) at 160 MPa. The homogenate was centrifuged at 4000*g* for 10 min at 4 °C to remove cellular debris. Thylakoid membranes were collected by centrifugation at 20000*g* for 60 min at 4 °C and then suspended in medium A [1.0 M glycine betaine, 40 mM MES-NaOH (pH 6.5), 15 mM $CaCl_2$, 15 mM $MgCl_2$, and 10 mM NaCl]. After centrifugation at 20000*g* for 60 min at 4 °C, collected thylakoid membranes were resuspended in medium A at a concentration of 5 μg of Chl/mL, frozen in liquid nitrogen, and stored at −80 °C.

Thylakoid membranes that were defective in the oxygen evolving activity of PSII were prepared by treating thylakoid membranes with a high concentration of Tris buffer as follows. Thylakoid membranes corresponding to 2.4 mg of chlorophyll were collected by centrifugation at 20000*g* for 60 min at 4 °C and suspended in 100 mL of a solution of 1 M Tris-HCl (pH 8.0), 3 mM EDTA, 15 mM $CaCl_2$, and 15 mM $MgCl_2$. The suspension was incubated on ice for 20 min in the dark and then centrifuged at 20000*g* for 1 h at 4 °C. The pelleted thylakoid membranes were resuspended in medium A and centrifuged at 20000*g* for 60 min at 4 °C. The Tris-treated thylakoid membranes were collected and resuspended in medium A at a concentration of 5 μg of Chl/mL, frozen in liquid nitrogen, and stored at −80 °C.

Measurements of Photosynthetic Activity. PSII activity was monitored by assessment of the light-induced transport of electrons from H_2O to dichlorophenolindophenol (DCIP) or from diphenylcarbazide (DPC) to DCIP. The reduction of DCIP was monitored spectrophotometrically in terms of the decrease in absorption at 580 nm with a spectrophotometer (HITACHI-557, Hitachi, Tokyo, Japan) (*15*). The reaction mixture contained thylakoid membranes that corresponded to 5 μg of chlorophyll, 80 μM DCIP, and 0.5 mM DPC as appropriate; the latter two chemicals were added just before the measurement of PSII activity. The spectrophotometer was equipped with a slide projector lamp in combination with a red cutoff filter (VR-67, Toshiba, Tokyo, Japan, which provided red light with a photon flux density of 1000 μmol m^{-2} s^{-1}). The photodetector of the spectrophotometer was protected by a 4-96 filter (Corning, Corning, NY).

Measurement of Photodamage to PSII and Action Spectra. Monochromatic light with a half-bandwidth of 10 nm was provided by the Okazaki Large Spectrograph (*12*, *13*). To measure the photodamage to PSII, thylakoid membranes were illuminated at 20 °C for designated periods of time with the monochromatic light. Each action spectrum was generated by plotting the reciprocal of the photon flux density that reduced the PSII activity to 50% of the original. Thylakoid membranes were illuminated for 20 min at 20 °C with light at each wavelength at four different densities of photon flux, which was adjusted by use of a convex lens and neutral-density filters.

Western Blotting Analysis. Western blotting analysis was performed as described previously (*16*, *17*).

RESULTS AND DISCUSSION

Photodamage to PSII by Monochromatic Light. Studies of the characteristics of photodamage to PSII require that PSII should be very stable and should respond only to light and not to other factors, such as a change in temperature and proteolysis during photodamage or subsequent measurements of electron transport activity. Therefore, we chose thylakoid membranes from the thermophilic cyanobacterium *T. elongatus* for our analysis. We monitored the activity of the oxygen-evolving complex by quantitating electron transport from H_2O to DCIP, and we monitored the activity of the reaction center in the absence of water oxidation by quantitating electron transport from DPC to DCIP. There was no decrease in these activities over the course of 2 h at 20−25 °C in darkness. Therefore, we chose 20 °C as the temperature of photodamage treatment and 25 °C for measurements of activity. At all other times, the thylakoid membranes were kept at 0−4 °C or were stored at −80 °C. First, we compared the rates of photodamage to PSII induced by light of various wavelengths by monitoring electron transport from H_2O to DCIP and electron transport from DPC to DCIP in untreated (intact) thylakoid membranes (Figure 1). The latter reaction bypasses the oxygen-evolving complex and provides an index of the activity of the reaction center of PSII (*18*). Figure 1 shows that the rate of transport of electrons from H_2O to DCIP decreased much more rapidly than that from DPC to DCIP at 310, 350, 430, and 500 nm, whereas both reactions were impaired at approximately the same rate at 680 nm and in white light. These observations indicated that the oxygen-evolving complex was more sensitive than the PSII reaction center to light in the UV and blue regions.

To examine whether the photodamage to the PSII reaction center corresponded to loss of the capacity for electron transport from H_2O to DCIP or from DPC to DCIP, we performed Western blotting to monitor the integrity of the D1 protein, a key component of the PSII reaction center that is prone to photodamage. We performed this analysis after untreated thylakoid membranes had been exposed to light at 430, 500, and 680 nm at a photon flux density of 500 μmol m^{-2} s^{-1} for 20 and 40 min (Figure 2). During exposure to light at 20 °C, the level of the D1 protein remained unchanged (data not shown) even though the transport of electrons from H_2O to DCIP and from DPC to DCIP ceased (see Figure 1). This phenomenon corresponded to a situation in which the PSII reaction center is functionally inhibited but the (photodamaged) D1 protein is not degraded (*19*). To accelerate the degradation of the D1 protein in photodamaged PSII, we incubated thylakoid membranes at 50 °C for 60 min in darkness. Figure 2 shows that, under these conditions, the D1 protein was degraded after illumination of thylakoid

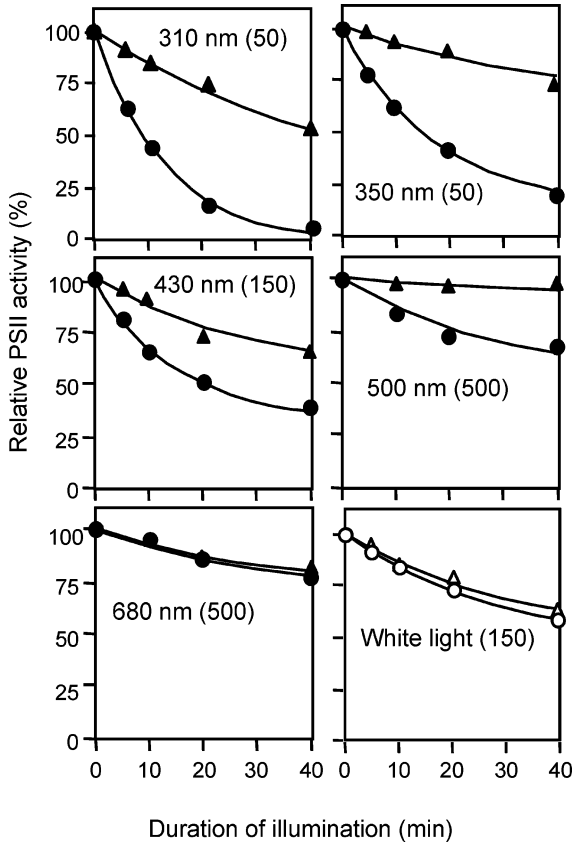

FIGURE 1: Photodamage to PSII in untreated thylakoid membranes by monochromatic light at various wavelengths. Thylakoid membranes were incubated at 20 °C for designated periods of time under monochromatic light at 310, 350, 430, 500, and 680 nm, or under white light from incandescent lamps at the photon flux density in micromoles per square meter per second that is indicated in parentheses. The activity of PSII was measured at 25 °C: (●) electron transport from H_2O to DCIP and (▲) electron transport from DPC to DCIP. Absolute activities before photodamage were 42 and 50 μmol of DCIP reduced (mg of chlorophyll)$^{-1}$ h^{-1} for the transport of electrons from H_2O to DCIP and for that from DPC to DCIP, respectively.

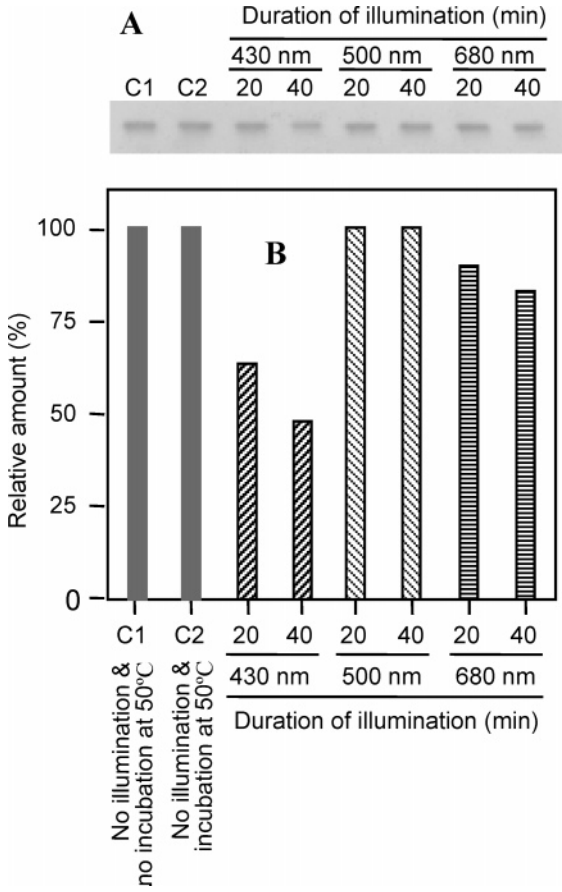

FIGURE 2: Degradation of the D1 protein after photodamage to PSII by monochromatic light. Untreated thylakoid membranes were exposed to monochromatic light at 430, 500, and 680 nm at a photon flux density of 500 μmol m^{-2} s^{-1} for 20 or 40 min. Then they were incubated at 50 °C for 60 min in darkness: (A) Western blotting analysis of the D1 protein with antibodies against the D1 protein and (B) quantitation of the results of Western blotting analysis. C1, thylakoid membranes before illumination; C2, thylakoid membranes before illumination that had been incubated at 50 °C for 60 min in darkness.

membranes with monochromatic light at 430 nm and also, but to a lesser extent, at 680 nm but not with light at 500 nm. Thus, the photoinduced damage to the D1 protein followed the photoinactivation of electron transport from DPC to DCIP, suggesting that the extent of damage to the PSII reaction center was reflected by the inhibition of electron transport from DPC to DCIP, but not from H_2O to DCIP.

Action Spectra of Photodamage to PSII. We compared the action spectrum of photodamage to PSII, as reflected by the decrease in the rate of electron transport from H_2O to DCIP, with the absorption spectrum of untreated thylakoid membranes. In so doing, we examined the kinetics of the photodamage and established that the photodamage followed first-order kinetics (data not shown). We also found that measurement of the dependence of photodamage to PSII on photon flux density (plotted on a logarithmic scale) yielded sigmoidal curves (data not shown). The reciprocal of the photon flux density that reduced the measured activity of PSII to 50% of the original level was taken as the effectiveness of light at that wavelength to cause damage.

The action spectrum of photodamage to PSII, as reflected by electron transport from H_2O to DCIP in untreated thylakoid membranes (Figure 3A), revealed a sharp increase in effectiveness at lower wavelengths in the UV region, as well as sharp but relatively small peaks at 400, 440, and 490 nm. Beyond 500 nm, there were very small peaks at 550, 620, and 690 nm. This action spectrum is very different from the absorption spectrum of thylakoid membranes (Figure 3C) and suggests that light absorbed by chlorophyll was ineffective in inducing the primary event in the photoinactivation of PSII.

Jones and Kok (*20*) were the first to investigate the action spectrum of photodamage to PSII in the visible and UV regions, which they determined from measurements of electron transport from H_2O to DCIP in thylakoid membranes from spinach leaves. The action spectrum was similar to the action spectrum shown here in Figure 3A. Jung and Kim (*21*) and Tyystjärvi et al. (*22*) also generated an action spectrum of the photodamage to PSII by visible light using materials and methods similar to those used by Jones and Kok (*20*). The action spectra that they reported were essentially similar to that shown here in Figure 3A, although the fine structures are not identical. These findings suggest that, not only in cyanobacteria but also in higher plants, the primary event in photoinactivation does not involve the light absorbed by chlorophyll.

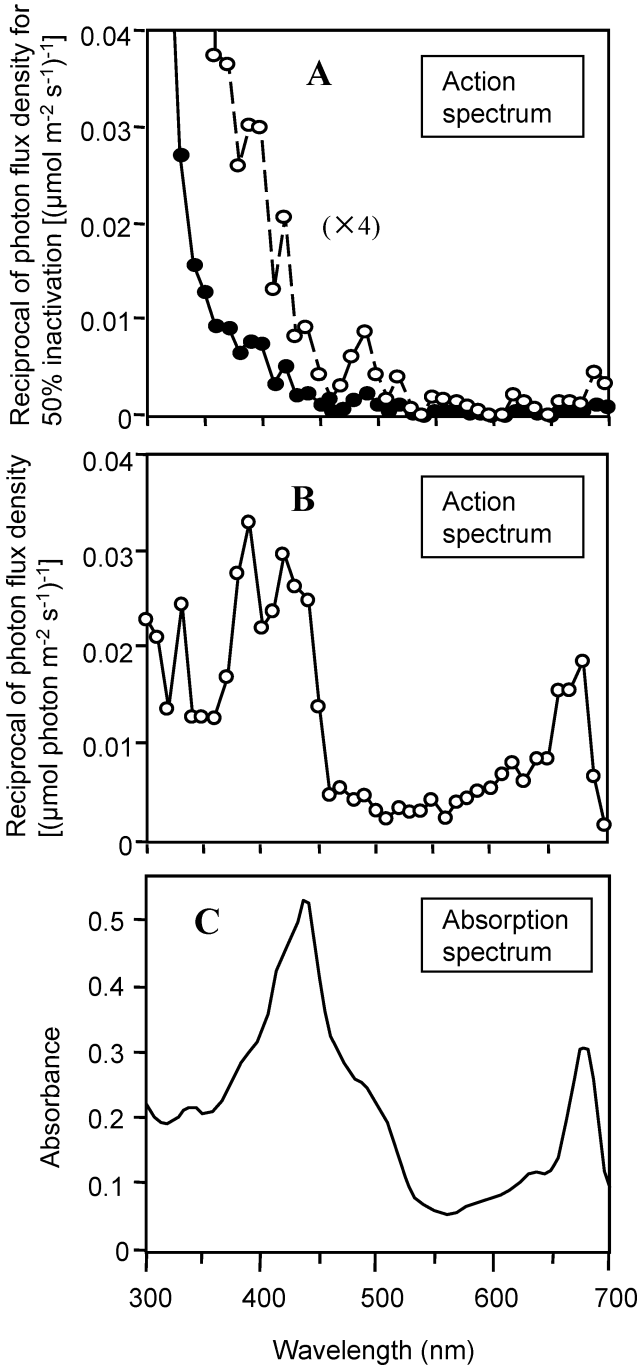

FIGURE 3: Action spectra of photodamage to PSII in untreated and Tris-treated thylakoid membranes. (A) Electron transport from H_2O to DCIP in untreated thylakoid membranes. (B) Electron transport from DPC to DCIP in Tris-treated thylakoid membranes. (C) Absorption spectrum of untreated thylakoid membranes suspended at 5 μg of chlorophyll/mL. Two and three independent experiments were performed to determine the action spectra in panels A and B, respectively. In each case, essentially the same results were obtained, and the data presented are the averages of results of the replicate experiments.

The action spectrum in Figure 3A is similar to the absorption spectrum of a dimer of Mn(III/IV) in a model compound (*23*, *24*), suggesting that the Mn cluster in the oxygen-evolving complex might be the light sensitizer in the primary event in the photodamage to PSII. Renger et al. (*25*) observed that irradiation with UV light of membrane fragments from spinach that included PSII decreased the amount of Mn in PSII and interrupted electron transport from

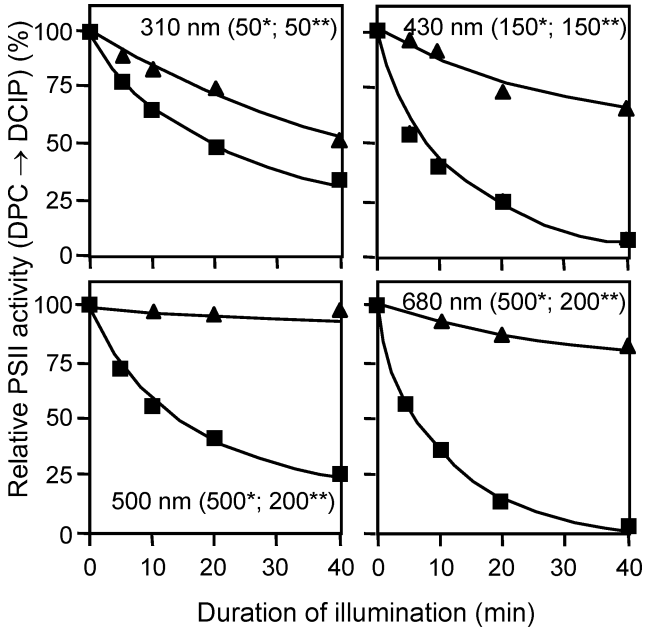

FIGURE 4: Photodamage to PSII by monochromatic light at various wavelengths. Untreated thylakoid membranes and Tris-treated thylakoid membranes were incubated at 20 °C for designated periods of time under monochromatic light at 310, 430, 500, and 680 nm at the photon flux density in micromoles per square meter per second that is indicated in parentheses (one asterisk, untreated; two asterisks, Tris-treated thylakoid membranes). The transport of electrons from DPC to DCIP was monitored at 25 °C: (▲) untreated thylakoid membranes and (■) Tris-treated thylakoid membranes.

H_2O to DCIP. Hakala et al. (*11*) observed that irradiation with UV and strong visible light released manganese ions in parallel with the inactivation of electron transport from H_2O to DCIP. They suggested that the primary event in photodamage to PSII by UV-B light involves, at least, the Mn cluster of the oxygen-evolving complex.

Next, we compared the rate of photoinactivation of electron transport from DPC to DCIP in Tris-treated thylakoid membranes in which the oxygen-evolving complex had been completely destroyed (*18*) with the rate in untreated thylakoid membranes (Figure 4). At all wavelengths that were examined, the former rate was higher than the latter, a result that was consistent with previous reports that the so-called "donor-site photodamage", which is monitored in Tris-treated thylakoid membranes, occurs more rapidly than the photodamage in untreated thylakoid membranes (*26*−*29*). Moreover, the dependence on wavelength of photoinactivation of electron transport from DPC to DCIP was very different from that of the electron transport from H_2O to DCIP: the photoinactivation was driven by visible light, and UV light was less effective. This difference is clearly seen in Figure 3B, in which the action spectrum of photodamage to PSII in Tris-treated thylakoid membranes is similar to the absorption spectrum of thylakoid membranes (Figure 3C) in the visible light region, but different from the action spectrum of photodamage to untreated thylakoid membranes (Figure 3A). In addition, we observed individual peaks at 330 and 390 nm in the UV-B region. This result suggests that there might be another photosensitizer for the photoinactivation of the transport of electrons from DPC to DCIP in Tris-treated thylakoid membranes.

Two-Step Model of Photodamage to PSII. Our findings suggest that photodamage to PSII might involve two steps.

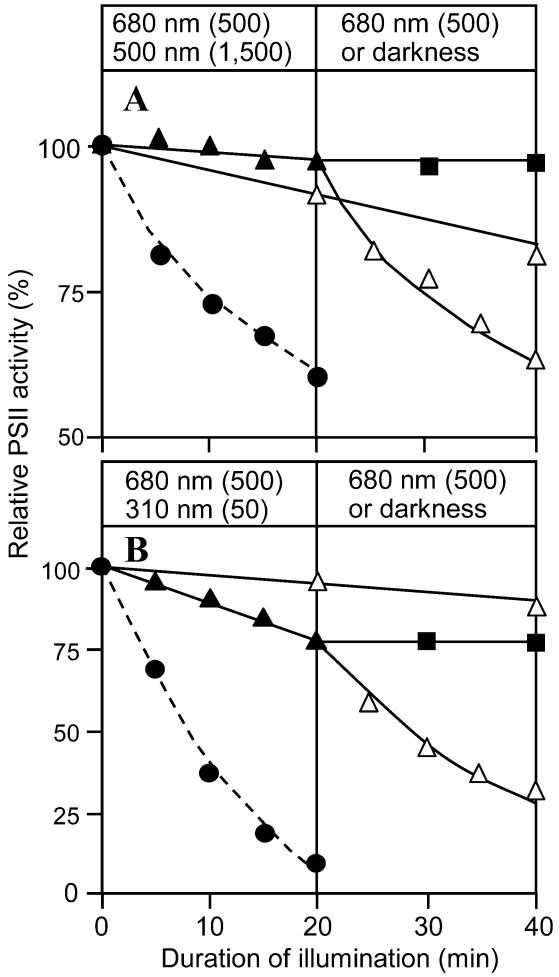

FIGURE 5: Selective effects of light at three wavelengths on PSII in untreated thylakoid membranes. (●) Changes in the activity of electron transport from H_2O to DCIP in light at 500 or 310 nm. Changes in the activity of electron transport from DPC to DCIP in light at 500 or 310 nm (▲), in light at 680 nm (△), and in darkness (■). (A) Exposure to light at 500 nm for 20 min and then to light at 680 nm or darkness. (B) Exposure to light at 310 nm for 20 min and then to light at 680 nm or darkness. The photon flux density of monochromatic light is indicated in parentheses.

Step 1 is the inactivation of the oxygen-evolving complex by UV and/or blue light, while step 2 is the inactivation of the PSII reaction center by light absorbed by photosynthetic pigments and occurs after the oxygen-evolving complex has been inactivated. To evaluate this hypothesis, we illuminated untreated thylakoid membranes with light at a wavelength (500 nm) that was very effective in inactivating the oxygen-evolving complex but had little effect on the photochemical reaction center of PSII. Then we illuminated these same membranes with light at a wavelength (680 nm) that was very effective in inactivating the photochemical reaction center but had little effect on the oxygen-evolving complex. We monitored the photodamage to PSII in terms of electron transport from H_2O to DCIP and from DPC to DCIP (Figure 5A). Electron transport from H_2O to DCIP was sensitive to light at 500 nm, while that from DCP to DCIP was not. However, light at 680 nm, which had little impact on electron transport from DPC to DCIP in untreated thylakoid membranes, was very effective after thylakoid membranes had been illuminated at 500 nm. Our observations suggest that it was only after the oxygen-evolving complex had been inactivated by light at 500 nm that the PSII reaction center became sensitive to light at 680 nm. We obtained essentially the same results with light at 310 nm instead of 500 nm (Figure 5B).

Our observations provide strong evidence for a model in which the photodamage to PSII occurs in two steps. Step 1 is the inactivation of the oxygen-evolving complex by blue and/or UV light, and step 2 is the inactivation of the PSII reaction center by red and/or blue light, which occurs only after the oxygen-evolving complex has been inactivated. Our model is similar to that proposed by Hakala et al. (*11*), who studied thylakoid membranes from a higher plant. Therefore, it is very likely that our two-step model of photodamage to PSII is of general relevance.

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