Fluorescence Quenching by Chlorophyll Cations in Photosystem II[†]

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ABSTRACT: Although fluorescence is widely used to study photosynthetic systems, the mechanisms that affect the fluorescence in photosystem II (PSII) are not completely understood. The aim of this study is to define the low-temperature steady-state fluorescence quenching of redox-active centers that function on the electron donor side of PSII. The redox states of the electron donors and acceptors were systematically varied by using a combination of pretreatments and illumination to produce and trap, at low temperature, a specific charge-separated state. Electron paramagnetic resonance spectroscopy and fluorescence intensity measurements were carried out on the same samples to obtain a correlation between the redox state and the fluorescence. It was found that illumination of PSII at temperatures between 85 and 260 K induced a fluorescence quenching state in two phases. At 85 K, where the fast phase was most prominent, only one electron-transfer pathway is active on the donor side of PSII. This pathway involves electron donation to the primary electron donor in PSII, P680, from cytochrome b_{559} and a redox-active chlorophyll molecule, Chl_Z. Oxidized Chl_Z was found to be a potent quencher of chlorophyll fluorescence with 15% of oxidized Chl_Z sufficient to quench 70% of the fluorescence intensity. This implies that neighboring PSII reaction centers are energetically connected, allowing oxidized Chl_Z in a few centers to quench most of the fluorescence. The presence of a well-defined quencher in PSII may make it possible to study the connectivity between antenna systems in different sample preparations. The other redox-active components on the donor side of PSII studied were the O_2-evolving complex, the redox-active tyrosines (Y_Z and Y_D), and cytochrome b_{559}. No significant changes in fluorescence intensity could be attributed to changes in the redox state of these components. The fast phase of fluorescence quenching is attributed to the rapid photooxidation of Chl_Z, and the slow phase is attributed to multiple turnovers providing for further oxidation of Chl_Z and irreversible photoinhibition. Significant photoinhibition only occurred at Chl concentrations below 0.7 mg/mL and above 150 K. The reversible oxidation of Chl_Z in intact systems may function as a photoprotection mechanism under high-light conditions and account for a portion of the nonphotochemical fluorescence quenching.

Fluorescence is used to probe a wide variety of photosynthetic systems from isolated proteins to whole organisms including single cell algae and plants. Many properties can be studied by fluorescence spectroscopic techniques. These range from detailed studies of energy and electron transfer in isolated proteins, where fluorescence is used as a direct probe of the excitation dynamics (van Gorkom, 1986; Schatz et al., 1988), to studies of plant stress levels, where fluorescence is used as a probe of the photosynthetic viability of the plant (Renger & Schreiber, 1986). However, the mechanisms that affect fluorescence on a molecular level are not yet completely understood.

It has been known for a long time that the redox state of the electron acceptor, Q_A, in photosystem II (PSII)[1] affects the chlorophyll (Chl) fluorescence intensity (Duysens & Sweers, 1963). When Q_A is reduced, quenching of the excitation by charge separation is blocked, and an increase in the fluorescence yield is observed. It has also been proposed that the redox state of the primary electron donor of PSII (P680) can influence the fluorescence of PSII. This proposal stemmed from the observation that samples in which cytochrome b_{559} (Cyt b_{559}) was preoxidized by treatment with ferricyanide prior to freezing have a lower peak fluorescence intensity at 77 K than samples with Cyt b_{559} prereduced (Okayama & Butler, 1972). It was observed that the changes in fluorescence intensity did not follow the reduction of Q_A (then known as C-550), but rather followed the oxidation of Cyt b_{559} (Butler, 1972). Okayama and Butler (1972) suggested that a relatively strong oxidant on the donor side of PSII is oxidizing Cyt b_{559}; this oxidant would be left in an oxidized state in ferricyanide-treated samples, and might act as a fluorescence quencher. Butler and co-workers proposed that $P680^+$ is the strong oxidant, and that it acts as a fluorescence quencher (Butler et al., 1973). Light-induced quenching of fluorescence was also observed by Kyle et al. (1983) at 77 K in chloroplast fragments and whole leaves. The quenching was observed preferentially at PSII emission wavelengths, and was attributed to quenching in PSII reaction centers due to reduction of pheophytin.

Since the work of Butler and co-workers and Kyle et al., a more complete picture of the electron-transfer reactions in PSII has been developed. A representation of the current picture of the electron-transfer reactions in PSII is shown in Figure 1. In this scheme, the primary electron-transfer

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[1] Abbreviations: Chl, chlorophyll; Chl_Z, a redox-active chlorophyll in photosystem II; Cyt b_{559}, cytochrome b_{559}; DCBQ, 2,5-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DMSO, dimethyl sulfoxide; EPR, electron paramagnetic resonance; OEC, O_2-evolving complex; Pheo, pheophytin; PSII, photosystem II; P680, primary electron donor in photosystem II; Q_A, quinone A; Q_B, quinone B; Y_D, tyrosine D; Y_Z, tyrosine Z.

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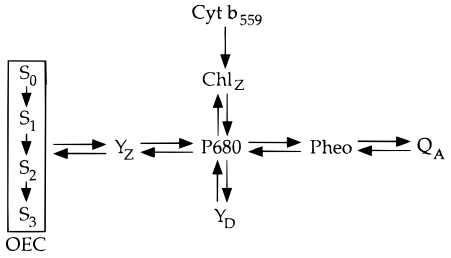

FIGURE 1: Electron-transfer pathways in PSII where P680 is the primary electron donor. Pheo is a pheophytin molecule, Q_A is a bound quinone molecule, Y_D is a redox-active tyrosine, Chl_Z is a redox-active chlorophyll molecule, Cyt b_{559} is cytochrome b_{559}, Y_Z is a redox-active tyrosine on the main electron-transfer pathway, and S_i are the S states of the O_2-evolving complex. The double arrows indicate the reversibility of electron-transfer pathways.

pathway is from the O_2-evolving complex (OEC) to Q_A. The charge transfer reactions are initiated when light energy is absorbed in the antennas and transferred to P680; the excitation energy on P680 then produces an electron transfer to Q_A via a pheophytin molecule, Pheo. The positive charge left on P680 is then rereduced by the S states via a redox-active tyrosine residue, Y_Z. In addition to the primary electron-transfer pathway, two secondary electron-transfer pathways are depicted in Figure 1. These are electron donation from a second redox-active tyrosine molecule, Y_D, and donation from a redox-active Chl molecule, Chl_Z. Oxidized Chl_Z can be rereduced by Cyt b_{559}. Multiple turnovers of the reaction center occur as Q_A is oxidized by a second quinone molecule, Q_B (not pictured in Figure 1).

Control of the electron-transfer reactions in PSII is possible with the use of different illumination temperatures, along with reductive and oxidative reagents (de Paula et al., 1985; Buser et al., 1990; Miller & Brudvig, 1991; Buser et al., 1992). At room temperature, the primary electron-transfer pathway involving the S states dominates. Continuous illumination at 0 °C results in the complete oxidation of Y_D, and the resulting $Y_D^{\bullet}$ EPR signal can be used as a spin standard. At 200 K, the electron-transfer reactions are essentially limited to one turnover, since the proton-coupled electron transfer from Q_A to Q_B is extremely slow or blocked entirely at this temperature. Oxidation of Y_D, also proton-coupled, is very slow at 200 K, and the dominant electron-donation pathway is from a single advance of the S states, S_1 to S_2. It is possible to produce this reaction in high yield by using samples dark-adapted at 0 °C. These samples are homogeneously in the S_1 state prior to illumination, and would undergo a transition from S_1 to S_2 upon 200 K illumination. At progressively lower temperatures below 200 K, there is a competition between the formation of the S_2 state and the secondary electron-transfer pathway involving Cyt b_{559} and Chl_Z such that with decreasing temperature the secondary electron-transfer pathway becomes more important. Below 100 K, this secondary electron-transfer pathway is the only one active on the donor side of PSII. $P680^+$ oxidizes Chl_Z which is rereduced by Cyt b_{559} in centers that contain reduced Cyt b_{559}. In centers where Cyt b_{559} is in an oxidized state when the sample is frozen, Chl_Z is stably oxidized. P680 does not form a stable radical even upon illumination at 77 K, as was previously proposed (Okayama & Butler, 1972); rather $P680^+$ is always reduced by the Cyt $b_{559}-Chl_Z$ pathway or by charge recombination. Also, reduced pheophytin is not formed under 77 K illumination as proposed by Kyle et al. (1983). With this new understanding of the electron-transfer reactions in PSII, coupled with the observation that Cyt b_{559} reduces the quenching species (Butler et al., 1973), one would predict that the relatively strong oxidant functioning as a fluorescence quencher observed by Butler and co-workers (Okayama & Butler, 1972; Butler et al., 1973) is Chl_Z^+, and that the light-induced fluorescence quenching observed by Kyle et al. (1983) was due to the formation of Chl_Z^+ instead of reduced pheophytin.

Given the extensive use of fluorescence spectroscopy in the study of photosynthetic systems, it is important that a more complete understanding of the physiological and physical mechanisms controlling the fluorescence be obtained. In order to understand better the mechanisms of fluorescence quenching in PSII, a study of the effect that the redox-active components of PSII have on its fluorescence emission was undertaken. By taking advantage of methods to prepare PSII in well-defined stable redox states used to elucidate the electron-transfer pathways described above, we were able to study the effect of each redox component on the fluorescence intensity. EPR and fluorescence measurements were carried out on the same samples, thus ensuring that a good correlation between the redox state and the fluorescence intensity could be made.

In an initial description of this work, we reported that PSII fluorescence was quenched under illumination at cryogenic temperatures, and that significantly more quenching occurs under conditions where Chl_Z^+ is formed versus oxidized Y_D (Schweitzer & Brudvig, 1995). However, sufficient work had not yet been done to ascertain what role the other redox-active components in PSII may play in the fluorescence quenching. In this paper, we build on the previous work by ascertaining the effect of other PSII redox centers on the fluorescence, and by explaining the biphasic character of the induction of the fluorescence quenching. The stable chlorophyll cation radical (Chl_Z^+) is shown to be a potent quencher of PSII fluorescence, and all other redox components studied are found to have no effect on the fluorescence. The fluorescence quenching is induced in a fast and a slow phase. The fast phase is due to rapid oxidation of Chl_Z, and the slow phase is a combination of multiple turnovers providing for further oxidation of Chl_Z and photoinhibition.

MATERIALS AND METHODS

Sample Preparations. PSII membranes were prepared from market spinach and stored in the dark at 77 K, in buffer containing 30% v/v ethylene glycol, 15 mM NaCl, and 20 mM tricine at pH 8.0, and at a chlorophyll concentration of 2−5 mg/mL (Berthold et al., 1981; Beck et al., 1985). PSII membrane samples were then diluted to a Chl concentration of 0.02−0.2 mg/mL in the same buffer, and an internal fluorescence standard (Schweitzer & Brudvig, 1996) was added at 0.3 mM (except data presented in Figure 2). All samples were at pH 8.0 unless noted otherwise. Samples at pH 6.0 used 20 mM MES as the buffer instead of tricine. Non-O_2-evolving Mn-depleted samples were obtained by treating PSII membranes with 5 mM hydroxylamine (Tamura & Cheniae, 1987; Miller & Brudvig, 1989).

Fluorescence and EPR measurements were carried out on the same sample in order to obtain a good correlation between the redox state present and the fluorescence

intensity. EPR measurements are typically done at concentrations of a few milligrams per milliliter of Chl, while fluorescence measurements are ideally done at concentrations of a few tens of micrograms per milliliter or lower. The Chl concentration is kept low in fluorescence measurements in order to avoid problems with reabsorption of the fluorescence. However, EPR measurements with PSII samples at a few tens of micrograms per milliliter are virtually impossible. In an effort to strike a balance between these two considerations, an intermediate Chl concentration of 0.2 mg/mL was used for these experiments. At 0.2 mg/mL Chl, there was some reabsorption of the fluorescence particularly on the short wavelength side of the spectrum, but an easily reproducible measure of the fluorescence intensity was still possible. At higher concentrations, it became increasingly difficult to measure the fluorescence intensity reproducibly, most likely due to inhomogeneities in the sample (Schweitzer & Brudvig, 1996). Also at 0.2 mg/mL Chl, an accurate quantitation of the organic radical EPR signal from PSII was possible. However, it was not possible to see the EPR signals from the metal centers in PSII at this concentration. In order to ascertain the potential role of redox-active components not visible by EPR spectroscopy at low concentration, we relied on the clear picture of the electron-transfer pathways and the methods of controlling the redox state of PSII (de Paula et al., 1985; Buser et al., 1990, 1992; Miller & Brudvig, 1991).

Illuminations for the combined EPR−fluorescence experiments were carried out with a 50 W quartz−tungsten halogen lamp (700 W/m^2). $Y_D^{\bullet}$ was formed by illumination for 2−6 min at 0 °C followed by 15 s of dark and then freezing in liquid nitrogen. In order to trap $Y_Z^{\bullet}$, a sample was first illuminated at 0 °C for 6 min in order to induce $Y_D^{\bullet}$ fully, followed by a brief illumination (5 s) at room temperature, and then a dry-ice/acetone bath was raised to freeze the sample while still under illumination (Tang et al., 1996). Finally, the sample was quickly wiped and immediately plunged into liquid nitrogen in the dark. The entire procedure after the initial 0 °C illumination took approximately 20 s. When doing fluorescence and EPR measurements on the same samples, the samples were kept frozen by quickly transferring them between the cryostats and liquid nitrogen. In this way, the same state was assayed in both measurements.

The excitation light used for the fluorescence measurements was of sufficient intensity ($\leq$50 W/m^2) that a maximum fluorescence was achieved within a few seconds of illumination at which time the intensity was recorded. Q_A was assumed to be entirely reduced under these conditions for all fluorescence measurements.

O_2-Evolution Rate Measurements. The O_2-evolution rate measurements were carried out as described in Beck et al. (1985) with the following changes. Measurements were made with a Clark-type electrode (YSI 5331 standard oxygen probe) connected to an amplifier (YSI 5300 two-channel biological oxygen monitor) and mounted into a home-built sample cell. The cell was equipped with a tapered light pipe to concentrate the light from a 1000-W quartz−halogen lamp filtered by a heat-absorbing (Schott KG-5) and long-pass filter (Oriel LP 610). It was possible to obtain saturating light for sample concentrations up to 20 μg/mL of Chl under these conditions. The temperature was held constant at 25 °C by water from a temperature-controlled circulating bath (Neslab Endocal RTE-9DD) flowing through the block of the sample cell. All O_2-evolution rate measurements were taken with 10 μg of Chl, and 25 μL each of 100 mM ferricyanide and 25 mM DCBQ (DMSO solution) in 2.4 mL of buffer containing 20 mM MES, 15 mM NaCl, and 10−20 mM $CaCl_2$ at pH 6.0. The Chl concentration measurements were done spectrophotometrically (Arnon, 1949).

The experimental protocol for measuring the amount of photoinhibition induced by illumination at low temperature was as follows. A PSII membrane sample was divided into two parts, one of which was illuminated in an EPR tube in the glass cryostat used for the fluorescence measurements. The other part was kept in the dark on ice during the illumination. The O_2-evolution rate of both samples was measured, and the evolution rate of the illuminated sample was scaled to that of the sample kept in the dark. The standard deviation of three measurements is shown as error bars in the figures. A second measurement of the control sample was also made, after freezing and thawing it in the dark, to verify that the freezing itself did not inhibit the sample. Incubating the sample at 200 K in the dark for as long as 140 min had no effect on the fluorescence intensity nor the O_2-evolution rate. Typical O_2-evolution rates were 300−450 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1} for untreated samples.

Ethylene glycol alone was used as a cryoprotectant except for samples with 50% or greater v/v cryoprotectant. For the 50, 55, and 60% cryoprotectant samples, 30% v/v ethylene glycol and 20, 25, and 30% v/v glycerol were used, respectively. Higher cryoprotectant concentrations were not used to avoid damage of PSII due to the cryoprotectant alone (Hillier et al., 1997). At the cryoprotectant concentrations used, little or no loss of O_2-evolution rate was observed in samples kept on ice in the dark for several hours.

Fluorescence Measurements. In order to obtain an accurate measure of the relative fluorescence in different frozen samples, an internal chelated terbium fluorescence standard was used which does not affect the O_2 evolution of PSII up to 1 mM (Schweitzer & Brudvig, 1996). The Chl fluorescence intensity was scaled to that of the standard, making it possible to eliminate effects of the differing optical properties of the samples due to differences in freezing. When using the terbium−chelate complex, it was not possible to use colored oxidants, since the oxidants interfered with the emission standard. Control of the redox state of PSII in this work was obtained through a combination of prereduction with ascorbate, depletion of Mn, and use of different illumination temperatures.

Fluorescence intensity measurements were made with a Perkin Elmer fluorescence spectrophotometer (Model 650-40) equipped with a home-built nitrogen-flow glass cryostat. The ratio of the Chl fluorescence (λ_{ex} = 468 nm, λ_{em} = 685 nm) to the Tb^{3+} emission (λ_{ex} = 310 nm, λ_{em} = 547 nm) of the internal standard was measured at four different positions on the sample, and an average of the intensity ratio was used to represent the fluorescence intensity. The excitation and emission slit widths were 2 and 10 nm, respectively. Each of the four measurements was made as quickly as possible with a minimum exposure to the excitation light so that the redox state of PSII was not significantly affected by the measurements. The measurements were, however, of sufficient length to form Q_A^- and represent measurements of F_{max}. The time-dependent fluorescence measurements were

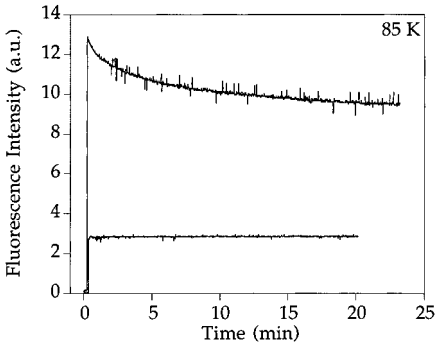

FIGURE 2: Time-dependent fluorescence intensity during illumination at 85 K of PSII membrane samples reduced with 15 mM ascorbate (upper) and oxidized with 2 mM hexachloroiridate (lower) at 0.02 mg/mL Chl, pH 8.0, and 30% ethylene glycol. The samples did not contain the terbium internal standard, and the fluorescence of two matched samples was not scaled.

recorded with a program written with LabView (National Instruments) on a MacIntosh SE. Only the excitation beam illuminated the sample during the time-dependent fluorescence measurements with the spectrophotometer conditions the same as above.

EPR Measurements. EPR measurements were carried out on a home-built X-band spectrometer interfaced to a Macintosh IIx computer and equipped with an Oxford ESR-900 liquid-helium cryostat. All EPR spectra were taken at 15 K, 1.8 μW, 9.07 GHz, 3280 G field center, 100 G sweep width, and a 4 G modulation amplitude. $Y_D^\bullet$ induced by 0 °C illumination was taken to be one electron spin, and was used as a spin standard (Babcock et al., 1983; Miller & Brudvig, 1991). A spectrum of the empty cavity was subtracted from all spectra. A double integral of the first-derivative spectrum was used to quantitate the amount of organic radical present. An uncertainty of ±10% was typical for the EPR measurements.

RESULTS

As shown in Figure 2, the fluorescence intensity of a chemically preoxidized PSII sample was significantly quenched relative to a prereduced sample when illuminated at 85 K. Previous measurements under these conditions have shown that illumination at 85 K results in the quantitative oxidation of Chl_Z and Cyt b_{559} in the oxidized and reduced samples, respectively (de Paula et al., 1985). These results provide a strong indication that Chl_Z^+ is a potent quencher of fluorescence. However, time-dependent changes in fluorescence quenching also occurred and were most pronounced in the reduced sample. The quenching induced by the fluorescence excitation light occurred in two phases which are clearly visible in untreated samples at 85 K, where a rapid quenching of the fluorescence occurs in the first few minutes, and a slow phase occurs over a period of many hours (Figure 3). At higher temperatures, both the rate and the amount of the fast and slow phases increase, such that at 200 K the amplitude of the fluorescence has dropped to 20% of its original value after 2 h of illumination. In order to understand the processes that are involved with the formation of the quenching state, it is useful to consider the slow and fast phases separately. We first consider the fast phase, and then the slow phase.

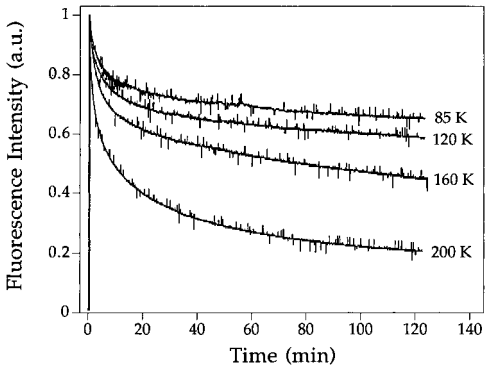

FIGURE 3: Time-dependent fluorescence intensity during illumination of untreated PSII membrane samples at 0.2 mg/mL Chl, pH 8.0, and 30% ethylene glycol. The individual traces were taken at the indicated temperature on different samples and scaled to their peak intensity.

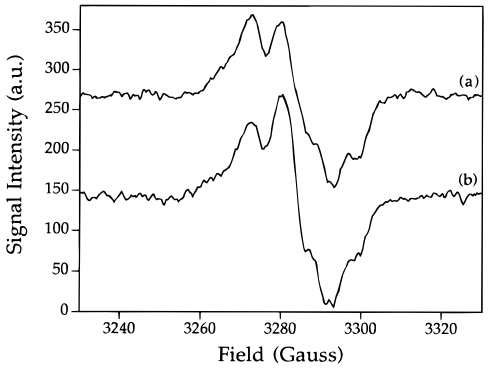

FIGURE 4: EPR spectra of the organic radical region of sample 1 from Table 1. (a) An untreated PSII sample at 0.2 mg/mL Chl was illuminated for 6 min at 0 °C to induce $Y_D^\bullet$ fully, and then (b) illuminated at 77 K for 1 min.

The Fast Phase of Fluorescence Quenching

The fact that the fluorescence quenching occurs at cryogenic temperatures suggests that the Cyt b_{559}/Chl_Z electron-donation pathway of PSII is involved in the quenching, because at 85 K other electron-donation pathways are known to be inactive and more complicated chemistries are unlikely to occur. Also, the fast phase is more prominent than the slow phase at 85 K, indicating that it is the result of an electron-transfer reaction involving the Cyt b_{559}/Chl_Z pathway. A series of experiments was carried out examining the effect that Cyt b_{559}, Chl_Z, and the other redox-active components on the donor side of PSII have on the fluorescence intensity.

Oxidized Chl_Z. An untreated PSII sample was illuminated at 0 °C, producing fully oxidized Y_D as is seen in Figure 4a. The fluorescence intensity was then measured at 85 K. Thereafter, the sample was reilluminated at 77 K for 1 min, and both the fluorescence intensity and EPR spectrum were remeasured. The relative intensities of the fluorescence and EPR signals are listed in Table 1. Under these conditions, Chl_Z was oxidized in 10% of the samples, and the fluorescence intensity was quenched by 26%.

When samples illuminated at 77 K are warmed to room temperature, Chl_Z^+ is quickly reduced, while Cyt b_{559} can remain oxidized for long periods of time. This suggests that the fluorescence quenching should be reversed when the sample is briefly warmed to room temperature, if Chl_Z^+ is the quenching species. In order to test this, an untreated

Table 1: Data from Fluorescence and EPR Measurements of the Same Sample[a]

sample	illumination sequence	fluorescence intensity	$Y_D^\bullet$	Chl_Z^+ [b]	$Y_Z^\bullet$ [b]
1. untreated	6 min, 0 °C	1.00	1.00	−	
	1 min, 77 K	0.74	1.00	0.10	
2. untreated	5 min, 0 °C	1.00	1.03	−	
	90 s, 200 K	0.29	1.00	0.17	
	thaw in dark	0.90	0.97	−	
3. ascorbate treated	dark-adapted		0.63		
	20 s, 200 K	0.94	0.67		
4. ascorbate treated	60 s, 77 K	1.06	0.63		
5. untreated	dark-adapted	1.00	0.43		
	6 min, 0 °C	1.60	1.00		
6. Mn-depleted	2 min, 0 °C	0.72	1.00		−
	2 min, 0 °C while freezing	0.81	1.00		0.67
7. untreated	5 min, 0 °C	1.00	1.00	−	
	20 min, 200 K	0.12	1.00	0.27	
8. deoxygenated	5 min, 0 °C	1.00	1.00	−	
	20 min, 200 K	0.34	1.00	0.11	

[a] Fluorescence was measured at 200 K, and EPR spectra were measured at 15 K. All samples were PSII membranes at 0.2 mg/mL Chl in 30% v/v ethylene glycol buffer. See text for details of the illumination protocol for each sample. EPR spectra were measured before and after the fluorescence measurement for samples 5 and 6. Errors in all measurements are approximately ±10%. [b] Chl_Z^+ and $Y_Z^\bullet$ amounts were estimated by integrating the organic radical region of the spectrum and subtracting one spin for $Y_D^\bullet$.

PSII sample (sample 2, Table 1) was first illuminated at 0 °C for 5 min to induce $Y_D^\bullet$ fully, then illuminated for 90 s at 200 K to induce a quenching state, and finally thawed briefly to relax the quenching state and refrozen. The fluorescence intensity and EPR spectra were measured after each manipulation. The initial fluorescence intensity after the 0 °C illumination was set to a value of 1.00, and the initial and postthawing areas of the EPR spectra were averaged to represent one spin of $Y_D^\bullet$ (Table 1). After the 200 K illumination, the fluorescence intensity was quenched to 29% of the initial value, and the area of the organic radical increased by 17%. Although it was not possible to deconvolute the percentage of $Y_D^\bullet$ and Chl_Z^+ present in the spectra, the increase in area in the EPR spectrum could be assigned to Chl_Z^+ based on the spectral line shapes which showed the second hyperfine peak of $Y_D^\bullet$ to be significantly larger than the first (data not shown). Also, the only other interfering organic radical would be oxidized Y_Z, and it is unlikely that oxidized Y_Z was stably formed under these conditions. Thawing and refreezing the sample produced a recovery in the fluorescence intensity to 90% of its initial value, and the area of the EPR spectrum relaxed to its original value.

This experiment shows that the fluorescence quenching induced by brief illumination at cryogenic temperatures is reversible, and indicates that Chl_Z^+ is the quenching species. The fast phase of the fluorescence quenching in Figure 3 can be accounted for by rapid photooxidation of Chl_Z. The yield of Chl_Z^+ produced in the experiments reported here is relatively low, because chemical oxidants were not used to preoxidize Cyt b_{559}. As shown in Figure 2, the amount of quenching induced in a chemically preoxidized sample is greater. However, it is also likely that complete oxidation of Chl_Z is not necessary to achieve the maximum amount of quenching, since 17% of oxidized Chl_Z is sufficient to quench the fluorescence by 71%. Although Chl_Z^+ is clearly a quencher, we also want to ascertain whether other redox centers in PSII contribute to quenching.

Oxidized Cytochrome b_{559}. Two identical samples containing 15 mM sodium ascorbate were incubated for 30 min in the dark in order to prereduce Cyt b_{559} before freezing. The EPR spectrum for one of the samples was measured in a dark-adapted state (sample 3 in Table 1) to establish how much $Y_D^\bullet$ was present prior to illumination. This sample was illuminated for 20 s at 200 K to advance the S states from S_1 to S_2, producing the S_2 state Cyt $b_{559}{}^{red}Q_A^-$ (see the introduction). The fluorescence intensity and the EPR spectrum were then measured. The amount of $Y_D^\bullet$ present in the sample was essentially unchanged by the 200 K illumination, being 63 and 67% before and after the illumination, respectively. The second sample (sample 4, Table 1) was illuminated at 77 K before doing the fluorescence and EPR measurements. Though it was not possible to measure directly the amount of oxidized Cyt b_{559} induced by the 77 K illumination, the charge-separated state produced under these conditions is known to be S_1 Cyt $b_{559}{}^{ox}Q_A^-$ (see the introduction). The fluorescence intensity was very similar for the two samples, indicating that Cyt b_{559} does not affect the fluorescence intensity.

Oxidized Y_D. In order to ascertain whether $Y_D^\bullet$ is a fluorescence quencher in PSII, an untreated sample (sample 5 in Table 1) was dark-adapted for 30 min before freezing in liquid nitrogen in the dark. The fluorescence intensity was measured and set to a value of 1.00. The sample was then illuminated at 0 °C for 6 min to oxidize Y_D fully, and the fluorescence intensity was remeasured, producing a relative value of 1.60. The EPR spectra were measured before and after each fluorescence measurement. The amount of $Y_D^\bullet$ present in the sample increased from about 43% to an assumed 100% after the illumination.

The 0 °C illumination more than doubled the amount of $Y_D^\bullet$ present in the sample, and produced an increase in the fluorescence intensity of 60%. This makes it clear that $Y_D^\bullet$ is not a fluorescence quencher. The increase in fluorescence intensity after the 0 °C illumination may be due to the formation of a small amount of Chl_Z^+ during the illumination used to measure the fluorescence at 200 K. Illumination at 0 °C is known to increase the amount of reduced Cyt b_{559} present in the sample (Buser et al., 1992). This would result in the formation of less Chl_Z^+ upon illumination at 200 K, thereby producing a higher fluorescent state.

Oxidized Y_Z. Oxidized Y_Z can be trapped in Mn-depleted samples frozen under illumination (Tang et al., 1996). In order to test whether $Y_Z^\bullet$ acts as a fluorescence quencher, the fluorescence intensity of a Mn-depleted sample was compared after illumination at 0 °C (2 min) to induce $Y_D^\bullet$ fully, and after illumination to trap $Y_Z^\bullet$, sample 6 in Table 1. A Mn-depleted sample was used because the lifetime of $Y_Z^\bullet$ is too short to allow it to be trapped in O_2-evolving samples. The fluorescence intensity was 72% after the 0 °C illumination, and 81% after being frozen under illumination (Table 1). The fluorescence was scaled to an initial value measured from this sample in a dark-adapted state prior to the illumination steps. The EPR measurements of the organic radical region of the spectrum show that approximately 70% oxidized Y_Z was trapped when frozen under illumination (Table 1). The tyrosine radical EPR signal was observed to decrease from 1.7 before to 1.4 spins after the second fluorescence measurement. This decrease is probably

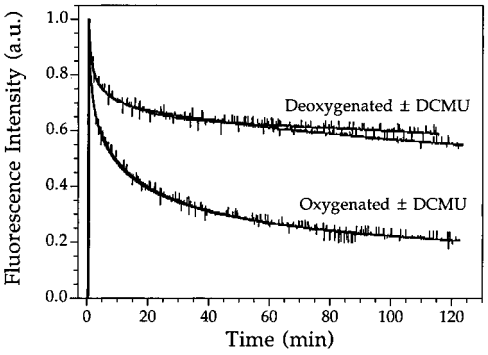

FIGURE 5: Time-dependent fluorescence intensity during illumination at 200 K of PSII membrane samples at 0.2 mg/mL Chl, 30% ethylene glycol, pH 8.0. The traces are from separate samples, treated as indicated and scaled to the maximum intensity.

due to the reduction of $Y_Z^{\bullet}$ during the fluorescence measurement at 200 K. We have observed a slow decay of $Y_Z^{\bullet}$ at 200 K which is attributed to $Y_Z^{\bullet}Q_A^-$ charge recombination. However, the fluorescence intensity was not significantly changed when the sample was frozen under illumination. This result strongly indicates the oxidation state of Y_Z does not influence the fluorescence intensity.

Mn S States. Illumination at 77 K of samples prereduced with ascorbate leaves the OEC in the S_1 state (sample 4, Table 1), while illumination at 200 K advances the S_1 state to S_2 (sample 3, Table 1). The fluorescence intensities of these two were essentially the same, indicating that the states S_1 and S_2 have the same effect on the fluorescence quenching. Also, the light-induced quencher is formed in Mn-depleted samples at 77 K in much the same way as in O_2-evolving samples. These observations rule out any significant participation of the S states in the quenching process.

The Slow Phase of Fluorescence Quenching

In order to understand better the slow phase of the induction of fluorescence quenching, the formation of a quenching state was compared in four samples illuminated at 200 K (Figure 5). Deoxygenating a sample, by three freeze−pump−thaw cycles, resulted in a significant reduction in the slow phase and a corresponding decrease in the yield of Chl_Z^+ during illumination, samples 7 and 8 in Table 1. This experiment shows that O_2 was acting as an electron acceptor, providing for multiple turnovers of PSII which resulted in a higher yield of Chl_Z^+ and a greater yield of fluorescence quenching. Experiments with higher sample concentrations of a few milligrams per milliliter Chl did not show evidence of multiple turnovers at 200 K (data not shown). This was probably due to the low relative concentration of O_2 to PSII at higher PSII concentrations. In either oxygenated or deoxygenated samples, the addition of DCMU had little or no effect on the time course of fluorescence quenching, indicating that electron transfer to Q_B is not involved in the formation of the quenching state.

Part of the slow phase of quencher formation is due to multiple turnovers of PSII resulting in further oxidation of Chl_Z, but the slow phase was not completely eliminated upon deoxygenation (Figure 5). Also, while the fluorescence quenching in samples that were exposed to brief illumination was completely reversible upon thawing the sample, long illumination resulted in partially irreversible quenching. The fluorescence intensity of a sample illuminated for 1 h at 200 K was 10% of the preillumination value, and the intensity only recovered to 40% of the preillumination value after thawing and refreezing. This compares with a recovery of 90% of the fluorescence upon thawing and refreezing subsequent to a 90 s illumination at 200 K, sample 2 in Table 1.

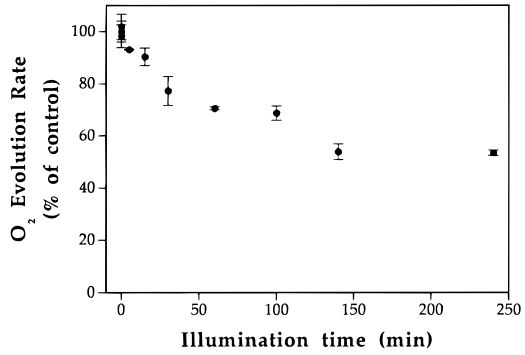

FIGURE 6: Relative O_2-evolution rate of PSII membrane samples at 0.2 mg/mL Chl, 30% ethylene glycol, and pH 8.0 after illumination at 200 K for different lengths of time. The O_2-evolution rate was scaled to that of an untreated sample which was left in the dark on ice during the illumination.

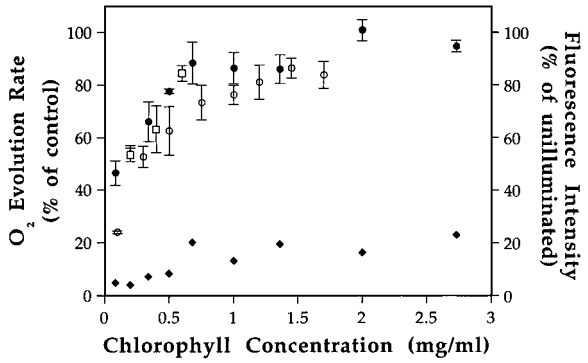

FIGURE 7: Effects of ethylene glycol, pH, and Chl concentration on the O_2-evolution rate and fluorescence intensity of PSII membrane samples illuminated for 140 min at 200 K. The O_2-evolution rate in samples with different percent ethylene glycol and pH are presented: 30% and pH 8.0 (solid circles), 20% and pH 8.0 (open circles), and 30% and pH 6.0 (open squares). The relative fluorescence intensity (scaled to the preillumination intensity) of samples with 30% ethylene glycol and pH 8.0 is also plotted (solid diamonds).

The irreversible formation of a fluorescence quenching state was found to correlate with an irreversible loss of O_2-evolution activity. Figure 6 shows the remaining O_2-evolution activity in a series of samples which were illuminated at 200 K for different lengths of time. After 140 min of illumination, the O_2-evolution rate was reduced to roughly 50% of the control. Longer illumination up to 4 h did not produce any further photoinhibition. Deoxygenating the sample in the dark prior to illumination at 200 K produced the same amount of photoinhibition as an untreated sample (data not shown).

The Chl concentration had a significant effect on the irreversible loss of O_2-evolution activity and the fluorescence (Figure 7). The fluorescence intensity was quenched in samples at high chlorophyll concentration to a level approximately 20% of the unilluminated value. At lower chlorophyll concentrations, more quenching was induced, which correlates with a dramatic increase in the amount of photoinhibition induced at chlorophyll concentrations below about 0.7 mg/mL. With increasing Chl concentration, the

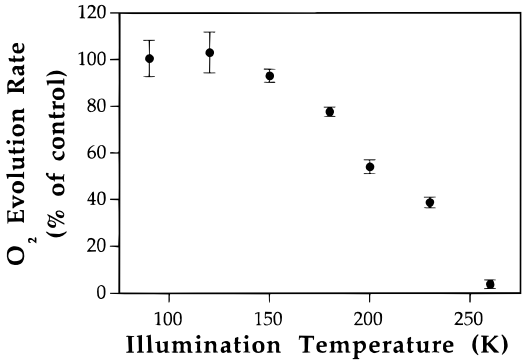

FIGURE 8: Relative O_2-evolution rate of PSII membrane samples at 0.2 mg/mL Chl, 30% ethylene glycol, and pH 8.0 illuminated at different temperatures for 140 min.

state of the sample changed from a snow to a glass, suggesting that ice crystal formation could play a role in the irreversible effects of illumination. Therefore, the O_2-evolution activity and fluorescence intensity were measured in a series of samples with 15−60% cryoprotectant and varying Chl concentrations after illumination for 140 min at 200 K (data for 20 and 30% ethylene glycol are shown in Figure 7). Ice crystal formation appears not to be related to the irreversible loss of O_2 evolution, because samples at 0.2 mg/mL Chl with different amounts of cryoprotectant showed similar inhibition of O_2 evolution. Simply freezing and thawing a sample also had no effect on the O_2-evolution rate, except at low cryoprotectant concentrations (<15%). We also examined the effect of pH on the irreversible changes, because pH has been found to have a significant effect on fluorescence quenching at physiological temperatures. However, no effect of pH was observed (Figure 7).

In Figure 8, the relative O_2-evolution rate is plotted for samples illuminated for 140 min at different temperatures. Below about 150 K, there was little or no inhibition produced by the illumination. With increasing temperature above 150 K, the amount of photoinhibition induced increases until at 260 K virtually no measurable O_2 evolution was observed. It is likely that the photoinhibition processes and irreversible fluorescence quenching observed at cryogenic temperatures are the same as those observed at room temperature (e.g., Chen et al., 1995), and that the decrease in photoinhibition at lower temperatures is due to a decrease in the mobility of some chemical species important to the inhibition reaction.

DISCUSSION

Four observations combine to give very strong support that Chl_Z^+ is a potent quencher of Chl fluorescence in PSII. First, there is a strong correlation between the presence of Chl_Z^+ and fluorescence quenching (Table 1). Second, the quenching state is formed at 85 K where the Cyt b_{559}−Chl_Z electron-transfer pathway dominates (Figure 3). Third, the fluorescence quenching is reversible (Table 1). And fourth, other redox-active species were not found to affect the fluorescence intensity (Table 1).

Long illuminations (∼45 min) of PSII at 200 K result in the fluorescence intensity being quenched to levels as low as 4−7% of the initial value. The induction of the fluorescence quenching occurs in an initial fast phase followed by a slower phase which is temperature-dependent (Figure 3). The fast phase of the fluorescence quenching induced at 85 K is due to the rapid oxidation of Chl_Z, and the slow phase is due to multiple turnovers of PSII providing for further oxidation of Chl_Z. At temperatures above 150 K, the fast phase is the same as at 85 K, and the slow phase is a combination of multiple turnovers of PSII and an irreversible photoinhibition reaction.

A possible mechanism of quenching by Chl_Z^+ would be that energy transfer occurs from the antenna Chl to Chl_Z^+ where it relaxes nonradiatively. The absorption spectrum of Chl cation radicals in solution (Hoshino et al., 1981) indicates that a reasonably good overlap would exist between the emission spectrum of the antenna Chl molecules (685 nm) and the absorption spectrum of higher excited states of the Chl radical (broad peak at 730 nm). Good energy transfer to Chl_Z^+ from the antenna should, therefore, be possible. Once the excitation reaches Chl_Z^+, it would very quickly relax to its lowest excited state at ∼813 nm (Hoshino et al., 1981), where it probably relaxes nonradiatively since fluorescence from Chl_Z^+ has not been detected. This would prevent the excitation from being detected in the fluorescence measured at 685 nm or from promoting an electron-transfer reaction. Back energy transfer from the Chl radical would be prevented since there would be little overlap from between the first excited-state emission spectrum of Chl_Z^+ and the absorption spectrum of the antenna Chl. In this way, Chl_Z^+ would effectively drain excitation energy out of the antenna.

The additional nonradiative−deexcitation pathway due to Chl_Z^+ would be expected to shorten the fluorescence lifetime of PSII. Indeed, the lifetime of one decay component of spinach PSII membranes was observed to decrease from 1.7 ns to 650 ps, and the yield associated with this component decreased by a factor of 4 when the pH was lowered from 6.5 to 4 (Bruce et al., 1997). The lifetime shortening and the associated decrease in the fluorescence yield were shown to be due to quenching by a chlorophyll cation radical by observing absorption changes at 820 nm. This study assigned the quenching to be from $P680^+$ as opposed to Chl_Z^+. It would be very difficult experimentally to distinguish whether the quenching was from $P680^+$ or Chl_Z^+, since the spectral characteristics of these two species should be essentially identical. However, $P680^+$ is a stronger oxidant than Chl_Z^+, and one would, therefore, expect that the redox equilibrium would favor the oxidation of Chl_Z, even at room temperature where these experiments were done.

The presence of a potent fluorescence quencher in PSII has implications on previous work done on the excitation dynamics in PSII. Our work indicates that only samples with a highly intact OEC and fully reduced Cyt b_{559} would not contain some Chl_Z^+ when illuminated at 200 K or lower temperatures. Previous EPR experiments show that relatively brief illuminations (about a few minutes) at 200 K of dark-adapted PSII produce the charge-separated state, S_2 P680 Q_A^-, and little or no Chl_Z^+ is formed (de Paula et al., 1985). With EPR spectroscopy, it is typical to be able to quantitate the amount of Chl_Z^+ present in a sample to within an accuracy of about 10%, so that small amounts of Chl_Z^+ (approximately a few percent) would not be easily detected by this technique. Fluorescence, on the other hand, is a much more sensitive technique, and oxidation of small amounts Chl_Z by very brief illumination (∼5 s) at 200 K results in some fluorescence quenching. So the presence of even small amounts Chl_Z^+ must be taken into account in fluorescence measurements at cryogenic temperatures, particularly below

110 K where the Cyt b_{559}−Chl_Z electron-transfer pathway dominates.

The identification of Chl_Z^+ as a fluorescence quencher also has implications for interesting future experiments. One could take advantage of the quenching effect by designing experiments in which samples had different amounts of Chl_Z^+ and compare the excitation dynamics in these samples. In this way, Chl_Z^+ could be used as a probe of excitation energy transfer between different PSII complexes. A comparison of the 85 K fluorescence spectra of oxidized and reduced samples of PSII membranes, prepared as in Figure 2, shows that the emission is significantly quenched at all emission wavelengths (unpublished data). This shows that the Chl molecules are energetically well connected throughout the antenna. Also, the fact that small amounts of oxidized Chl (∼15%) can quench significant amounts of the fluorescence (70%) already has implications about the connectivity of the antenna molecules in PSII membranes, namely, that Chl radicals in one PSII center can quench fluorescence from other PSII centers. This would imply that antenna systems from neighboring PSII are energetically connected, and that excitation energy from an antenna could be used for productive photochemistry by any one of many neighboring PSII centers.

The mechanisms of energy dissipation in plants under high-light conditions are being actively researched [see Long et al. (1994) and Horton et al. (1996) and references cited therein]. Nonphotochemical quenching within the antenna of PSII is thought to account for a considerable amount of the total quenching via the xanthophyll cycle (Demmig-Adams, 1990). In addition, quenching mechanisms within the PSII reaction center have been considered (Schreiber & Neubauer, 1990; Krieger et al., 1992). Although our work does not address the question of whether fluorescence quenching due to Chl_Z^+ is important under physiological conditions, several observations suggest that Chl_Z^+ could contribute to nonphotochemical fluorescence quenching. It has been found that acidification of thylakoid membranes causes an increase in nonphotochemical fluorescence quenching [reviewed in Schreiber and Neubauer (1990) and Horton et al. (1996)]. Increased quenching at low pH fits with a role of Chl_Z^+ in nonphotochemical fluorescence quenching because formation of Chl_Z^+ was found to be enhanced at low pH in PSII membranes at room temperature (Buser et al., 1992). In addition, Crofts and Horton (1991) found that low pH-dependent quenching in PSII particles required oxidizing conditions, which would cause oxidation of Cyt b_{559} and promote formation of Chl_Z^+. However, alternative explanations of pH-dependent quenching have been proposed including charge recombination between $P680^+$ and Q_A^- (Krieger et al., 1992) or aggregation of the LHC II (Mullineaux et al., 1993). It is not possible to rule out either of these explanations given the current understanding of quenching mechanisms in PSII. However, a shortening of the fluorescence lifetime and a drop in the overall fluorescence intensity were also seen in nonreduced versus reduced chlorosomes and bacteriochlorophyll *c* oligomers (van Noort et al., 1997). In this case, the shortening of the fluorescence lifetime and the drop in the fluorescence intensity may be ascribed to excited-state quenching by bacteriochlorophyll *c* radicals.

While it is not possible from this work to say if the fluorescence quenching due to Chl_Z^+ is important under physiological conditions, there is at least one situation that occurs *in vivo* where it would be advantageous to have a donor-side fluorescence quencher in PSII, that is during the photoassembly process of the OEC. Mn-depleted PSII is more susceptible to photoinhibition than O_2-evolving PSII (Aro et al., 1993). This would imply that PSII is more susceptible to photodamage before the OEC becomes fully functional during photoassembly. The absence of a fully functional OEC to reduce rapidly $P680^+$ would increase electron donation from Chl_Z. If a small amount of Chl_Z^+ were generated under steady-state high-light conditions, it could help protect the system from photoinhibition by quenching some of the excitation energy before it produces a charge separation. In this way, the rate of charge separation might be reduced to match the slower photoassembly process.

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REFERENCES

Arnon, D. I. (1949) *Plant Physiol. 24*, 1−15.
Aro, E.-M., Virgin, I., & Andersson, B. (1993) *Biochim. Biophys. Acta 1143*, 113−134.
Babcock, G. T., Ghanotakis, D. F., Ke, B., & Diner, B. A. (1983) *Biochim. Biophys. Acta 723*, 276−286.
Beck, W. F., de Paula, J. C., & Brudvig, G. W. (1985) *Biochemistry 24*, 3035−3043.
Berthold, D. A., Babcock, G. T., & Yocum, C. F. (1981) *FEBS Lett. 134*, 231−234.
Bruce, D., Samson, G., & Carpenter, C. (1997) *Biochemistry 36*, 749−755.
Buser, C. A., Thompson, L. K., Diner, B. A., & Brudvig, G. W. (1990) *Biochemistry 29*, 8977−8985.
Buser, C. A., Diner, B. A., & Brudvig, G. W. (1992) *Biochemistry 31*, 11449−11459.
Butler, W. L. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3420−3422.
Butler, W. L., Visser, J. W. M., & Simons, H. L. (1973) *Biochim. Biophys. Acta 292*, 140−151.
Chen, G.-X., Blubaugh, D. J., Homann, P. H., Golbeck, J. H., & Cheniae, G. M. (1995) *Biochemistry 34*, 2317−2332.
Crofts, J., & Horton, P. (1991) *Biochim. Biophys. Acta 1058*, 187−193.
Demmig-Adams, B. (1990) *Biochim. Biophys. Acta 1020*, 1−24.
de Paula, J. C., Innes, J. B., & Brudvig, G. W. (1985) *Biochemistry 24*, 8114−8120.
Duysens, L. N. M., & Sweers, H. E. (1963) in *Studies on Microalgae and Photosynthetic Bacteria* (Japanese Society of Plant Physiologists, Ed.) pp 353−372, University of Tokyo Press, Tokyo.
Hillier, W., Lukins, P., Seibert, M., & Wydrzynski, T. (1997) *Biochemistry 36*, 76−85.
Horton, P., Ruban, A. V., & Walters, R. G. (1996) *Annu. Rev. Plant Physiol. Plant Mol. Biol. 47*, 655−684.
Hoshino, M., Ikehara, K., Imamura, M., Seki, H., & Hama, Y. (1981) *Photochem. Photobiol. 34*, 75−81.
Krieger, A., Moya, I., & Weis, E. (1992) *Biochim. Biophys. Acta 1102*, 167−176.
Kyle, D., Arntzen, C., Franck, F., & Inoue, Y. (1983) *Photochem. Photobiol. 38*, 609−614.
Long, S. P., Humphries, S., & Falkowski, P. G. (1994) *Annu. Rev. Plant Physiol. Plant Mol. Biol. 45*, 633−662.
Miller, A.-F., & Brudvig, G. W. (1989) *Biochemistry 28*, 8181−8190.
Miller, A.-F., & Brudvig, G. W. (1991) *Biochim. Biophys. Acta 1056*, 1−18.
Mullineaux, C., Pascal, A., Horton, P., & Holzwarth, A. (1993) *Biochim. Biophys. Acta 1141*, 23−28.
Okayama, S., & Butler, W. L. (1972) *Biochim. Biophys. Acta 267*, 523−529.

Renger, G., & Schreiber, U. (1986) in *Light Emission by Plants and Bacteria* (Govindjee, Amesz, J., & Fork, D. C., Eds.) pp 587−619, Academic Press, Inc., New York.

Schatz, G. H., Brock, H., & Holzwarth, A. R. (1988) *Biophys. J. 54*, 397−405.

Schreiber, U., & Neubauer, C. (1990) *Photosynth. Res. 25*, 279−293.

Schweitzer, R. H., & Brudvig, G. W. (1995) in *Photosynthesis: from Light to Biosphere* (Mathis, P., Ed.) pp 807−810, Kluwer Academic Publishers, Dordrecht, The Netherlands.

Schweitzer, R. H., & Brudvig, G. W. (1996) *Biospectroscopy 2*, 167−171.

Tamura, N., & Cheniae, G. (1987) *Biochim. Biophys. Acta 890*, 179−194.

Tang, X.-S., Zheng, M., Chisholm, D. A., Dismukes, G. C., & Diner, B. A. (1996) *Biochemistry 35*, 1475−1484.

van Gorkom, H. J. (1986) in *Light Emission by Plants and Bacteria* (Govindjee, Amesz, J., & Fork, D. C., Eds.) pp 267−289, Academic Press, Inc., New York.

van Noort, P. I., Zhu, Y., LoBrutto, R., & Blankenship, R. E. (1997) *Biophys. J. 72*, 316−325.

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