Time-Resolved Fluorescence Measurements of Photosystem II: The Effect of Quenching by Oxidized Chlorophyll Z

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Chlorophyll Z (Chl_Z) is a unique redox-active chlorophyll bound to His118 in the D1 subunit of photosystem II (PSII). Previous work has shown that Chl_Z^+ is a potent quencher of fluorescence. In this study, we have used time-resolved fluorescence spectroscopy to determine the effect of Chl_Z^+ on the decay components in PSII at low temperature. Both EPR and fluorescence measurements were made to ascertain the redox state of the sample under the conditions of the fluorescence measurements. We have compared spinach PSII membranes, which contain both the core antenna and the peripheral LHCII antennae, with PSII core complexes from *Synechocystis* PCC 6803, which contain only the core antenna. The presence of Chl_Z^+ decreases the steady-state fluorescence intensity by about 10-fold, and this decrease is associated with a large decrease in the yield of the nanosecond decay components. In samples containing a fractional amount of Chl_Z^+, there is a good correlation between the fluorescence decay time and the amount of Chl_Z^+. Chl_Z^+ is an effective quencher of emission from all of the core antenna chlorophylls. This result is consistent with "rapid exciton equilibration" within the PSII core antenna. However, emission from the peripheral LHCII antenna was less effectively quenched by Chl_Z^+, indicating that exciton equilibration is incomplete among the core and peripheral antennas, at least at 77 K.

Introduction

The photosystem II (PSII) complex of higher plants and green algae contains about 200 chlorophyll molecules that function to absorb light and transfer the excitation energy into the reaction center where a photoinduced charge separation occurs. About 40 of these chlorophylls are bound to the PSII core, which consists of the CP47 and CP43 chlorophyll-binding proteins and the D1/D2 proteins that form the reaction center (RC). The remaining chlorophylls are bound to the light-harvesting complex II (LHCII) proteins, which comprise the peripheral antennae. The PSII complex in cyanobacteria lacks the peripheral LHCII antennae, but the PSII core is very similar to the higher plant system.

The D1/D2 proteins of PSII are homologous to the L/M heterodimer of the reaction center from purple nonsulfur bacteria.[1] One difference, however, is that the D1/D2 complex has two extra accessory chlorophylls.[2] We have recently shown that these two accessory chlorophylls are ligated to the symmetrically positioned residues D1-His118 and D2-His117.[3] Chlorophyll Z (Chl_Z) is a unique redox-active accessory chlorophyll ligated to His118 in the D1 subunit. Chl_D is the accessory chlorophyll coordinated to D2-His117 and is not redox-active. Chl_Z and Chl_D may play an important role as a bridge for energy transfer between the core antenna chlorophylls and the reaction center.[4]

Fluorescence has been widely used to probe the energy and electron-transfer processes in a variety of PSII preparations (for review see ref 5). The absorption and emission spectra of PSII are heterogeneous due to the presence of a series of spectral forms of chlorophyll.[5,6] In the pebble-mosaic model, K. Sauer[7] suggested that very fast excitation energy transfer occurs between the pigments within one pigment−protein complex, but clearly slower transfer steps may be involved in the energy transfer between the pigment−protein complexes. Recent data show that the absorption of light in the PSII core antenna is followed by ultrafast excitation energy transfer among all pigments of an individual pigment pool (cluster) with lifetimes from 500 fs to several picoseconds. In PSII membrane particles with an open RC, the time-resolved fluorescence study of Dau and Sauer[8] revealed a 15 ps energy-transfer component and trapping with a mean lifetime of 200 ps. These data were interpreted in terms of the model of rapid exciton equilibrium between the radical pair and the excited state of the Chl in the PSII core[9] (see also refs 8 and 10). According to the "rapid exciton equilibrium model", the exciton equilibrium distribution is a result of energy-transfer processes between chlorophylls in PSII on a time scale of 5−30 ps followed by trapping in the open RC. Trapping occurs with a mean lifetime of 200 ps determined by the charge-separation reactions (trap-limited kinetics). The energy-transfer processes in PSII at 77 K were reported to occur in the range of 30−40 ps[11] and recently in the range of 5−30 ps.[12] The variability of the rate of the energy-transfer component was recently explained by inhomogeneous broadening in a pigment complex with a pronounced dispersion in the rate constants for energy transfer.[12] The heterogeneity of the PSII core fluorescence decay processes is under debate. In PSII with open RCs, the fluorescence decays show three groups of components:[5] one or more fast phases (50−200 ps), one or more middle phases (0.4−1.0 ns), and one or more slow

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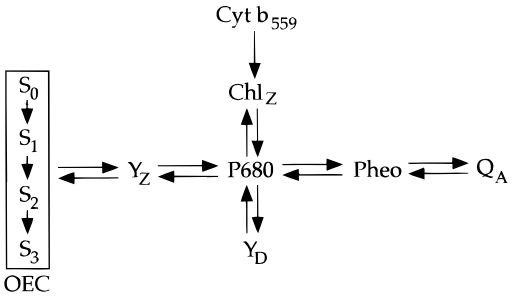
SCHEME 1: Electron-Transfer Pathways in PSII Where P680 Is the Primary Electron Donor, Pheo Is a Pheophytin Molecule, Q_A Is a Bound Quinone Molecule, Y_D Is a Redox-Active Tyrosine, Chl_Z Is a Redox-Active Chlorophyll Molecule, Cyt b_{559} Is Cytochrome b_{559}, Y_Z Is a Redox-Active Tyrosine on the Main Electron-transfer Pathway, and S_i Are the S-States of the O_2-Evolving Complex[a]



[a] The double arrows indicate the reversibility of electron-transfer pathways.

phases (1.2−2.2 ns). If the RCs in PSII are closed ($P680^+Pheo\text{-}Q_A^-$), the fluorescence decays slow giving rise to nanosecond fluorescence.[13] Although the differences in sample, time resolution, and analysis procedures may be a source for the variety of fluorescence decay components, structural heterogeneity of PSII (α/β heterogeneity)[14] resulting in a multiphasic trapping process and protein-coupled radical-pair relaxation[12,15] seem to contribute to the fluorescence decay processes with lifetimes in the range of 0.5−10 ns in PSII. In closed PSII, formation of reduced Q_A was suggested to modulate the nanosecond fluorescence of the PSII core due to the electric field dependent slowing of the primary charge separation in the RC.[16,17]

Low-temperature fluorescence emission spectra of PSII preparations are better resolved and exhibit more structure. Three emission bands have been characterized, called F680, F685, and F695.[18] The changes in fluorescence emission at low temperature could be due to incomplete equilibration among pigment pools or trapping of excitation within a given pigment pool. It is of note with respect to this special volume in honor of Ken Sauer and Mel Klein that the first picosecond fluorescence study of spinach chloroplasts at low temperature was reported by Sauer and co-workers.[19]

Low-temperature illumination of PSII produces a stable charge separation. Therefore, low-temperature fluorescence studies reflect closed PSII centers in which Q_A is reduced. It has been known for a long time that the redox state of Q_A affects the chlorophyll fluorescence intensity.[20] When Q_A is reduced, photochemical quenching of fluorescence is blocked and an increase in the fluorescence yield is observed owing to the primary charge separation between P680 and Pheo with subsequent delayed fluorescence. Although Q_A is normally the electron acceptor when a stable charge separation is generated at low temperature, the electron donor depends on the illumination temperature and chemical treatment of the sample.[21] When the O_2-evolving complex (OEC) is intact and for illumination temperatures greater than or equal to 200 K, electron donation from the OEC predominates; at lower temperature or in Mn-depleted PSII, the alternate electron-donation pathway consisting of cytochrome b_{559} (cyt b_{559}) and Chl_Z predominates (Scheme 1). As a result, the specific redox state of PSII present during low-temperature fluorescence measurements will vary depending on the pretreatment of the sample and the illumination conditions. However, the redox state of the sample has typically not been measured in previous low-temperature fluorescence studies of PSII.

Recently, we have shown that Chl_Z^+ is a potent quencher of fluorescence.[22] In samples treated with a chemical oxidant such as ferricyanide to oxidize cyt b_{559}, Chl_Z is stably photooxidized in high yield by illumination at temperatures below 100 K.[21] This allows a unique quencher to be reversibly formed in PSII. Therefore, Chl_Z^+ provides a well-defined and controllable quencher within the PSII reaction center that can be used to probe energy-transfer processes in PSII. In this study, we have used time-resolved fluorescence spectroscopy to determine the effect of Chl_Z^+ on the decay components in PSII at low temperature. Both EPR and fluorescence measurements were made to ascertain the redox state of the sample under the conditions of the fluorescence measurements. We have compared spinach PSII membranes, which contain both the core antenna and the peripheral LHCII antennae, with PSII core complexes from *Synechocystis* PCC 6803, which contain only the core antenna. We find that the fluorescence lifetime is highly dependent on the redox state of Chl_Z.

Experimental Section

Sample Preparation. O_2-evolving core complexes of PSII were isolated from *Synechocystis* PCC 6803 using the method of Tang and Diner.[23] The polypeptide composition of this preparation includes CP47, CP43, D1, D2, cyt b_{559}, and the 33 kDa (manganese stabilizing) polypeptide; these core complexes are free of PSI, cytochrome b_6/f complex, and cyanobacterial ATPase.[23] The O_2-evolution activities of the preparations used in this work were typically 1800−2600 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1} after purification on the DEAE−Toyopearl anion exchange column (650S resin, TosoHaas). Chlorophyll concentrations were determined optically after methanol extraction using an extinction coefficient of 79.24 mL (mg of Chl)$^{-1}$ cm^{-1} at 665 nm.[24] PSII samples used for spectroscopic measurements were diluted to 20 μg of Chl/mL in buffer containing 50 mM MES−NaOH at pH 6.0, 20 mM $CaCl_2$, 10 mM NaCl, 30 mM $MgSO_4$, 0.03% (w/w) dodecyl maltoside, and 40−50% (v/v) glycerol. *Synechocystis* PSII samples were reduced by adding sodium ascorbate and sodium dithionite to final concentrations of 15 and 1 mM, respectively, and oxidized by adding potassium hexachloroiridate to a final concentration of 2 mM. After these additions, the samples were incubated in the dark on ice for 5−60 min before freezing in liquid nitrogen in the dark.

PSII membranes were prepared from market spinach and stored in the dark at 77 K in buffer containing 20 mM MES−NaOH at pH 6.0, 15 mM NaCl, and 30% v/v ethylene glycol at a chlorophyll concentration of 2−5 mg/mL.[25,26] PSII membrane samples used for spectroscopic measurements were diluted to a concentration of 20 μg of Chl/mL in the same buffer. O_2-evolution rates were typically 330−420 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}. Spinach PSII samples were reduced by adding sodium ascorbate to a final concentration of 15 mM and oxidized by adding potassium hexachloroiridate to a final concentration of 2 mM. After these additions, the samples were incubated in the dark on ice for 5−60 min before freezing in liquid nitrogen in the dark.

Fluorescence Measurements. Fluorescence spectra at 85 K were taken with a Perkin-Elmer fluorescence spectrophotometer (model 650-40) equipped with a home-built nitrogen-flow glass cryostat. The time-dependent fluorescence changes during continuous illumination were recorded with a program written with LabView (National Instruments) on a Macintosh

SE. After the beginning of the measurement, the excitation shutter was opened and the fluorescence intensity was recorded versus time. Only the excitation beam illuminated the sample during the measurements ($\lambda_{ex} = 468$ nm, $\lambda_{em} = 685$ nm). The excitation and emission slit widths were 10 and 2 nm, respectively, for both types of measurement. The light intensity was sufficient to form Q_A^- within a few seconds, and the subsequent fluorescence intensities represent measurements of F_{max}.

Single-Photon Counting Measurements. The time-resolved fluorescence kinetics were measured using single-photon counting (SPC) as was previously described.[27] The sample was frozen to 77 K in the dark and placed into an optical Dewar in liquid nitrogen. The samples were excited at 600 nm using a cavity-dumped dye laser (Spectra Physics) synchronously pumped by a mode-locked Nd:YAG laser at a repetition rate of 3.8 MHz. The full width at half-maximum (fwhm) of the excitation laser pulses was 5 ps. The instrument response function measured at 600 nm from excitation light scattered off the frozen sample was 30 ps fwhm. Prior to collection of time-resolved data, the samples were illuminated with the excitation laser for a minimum of 10 min at 77 K in order to form a stable charge-separated state. The decay kinetics were collected in files of 2048 channels at 10.3 ps/channel, giving a dynamic range of about 16 ns for each decay. These decay curves were deconvolved from the instrument response function by fitting to a sum of exponentials, $\sum_i A_i \exp(-t/\tau_i)$, where A_i and τ_i are the amplitudes and lifetimes of the ith component, respectively. The results of three-, four-, or five-component fits were evaluated by comparing the χ^2 values and residuals of each fit, and four components were deemed sufficient to represent the data accurately. The kinetics in the 670−740 nm region were globally fit and decay associated spectra (DAS) of the fit amplitudes versus the emission wavelength were constructed. Positive and negative amplitudes in the DAS represent fluorescence decay and rise components, respectively. The excitation pulses were less than 1 nJ/pulse, thus avoiding singlet−singlet annihilation. The fit amplitudes were scaled to the steady-state fluorescence intensity by setting the sum of the products of the amplitudes and the lifetimes equal to the intensity, $I = \sum_i A_i \tau_i$, at each detection wavelength.

EPR Measurements. EPR measurements of PSII samples at 20 μg/mL 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.[28] All EPR spectra were taken at 15 K, 1.4 μW, 9.07 GHz, 3280 G field center, 100 G sweep width, and a 4 G modulation amplitude. The EPR signal of the dark-stable tyrosine radical in PSII, $Y_D^{\bullet}$, induced by 0 °C illumination for 6 min was taken to be one electron spin per PSII and was used as a spin standard.[29,30] 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 ±20% was typical for these EPR measurements.

Results

Figures 1 and 2 show steady-state fluorescence data for *Synechocystis* and spinach PSII, respectively, which illustrate the quenching properties of Chl_Z^+. The oxidized samples were pretreated with a chemical oxidant to oxidize cyt b_{559}. Previous EPR measurements on spinach PSII membranes have shown that illumination of such chemically oxidized samples at temperatures less than or equal to 100 K results in formation

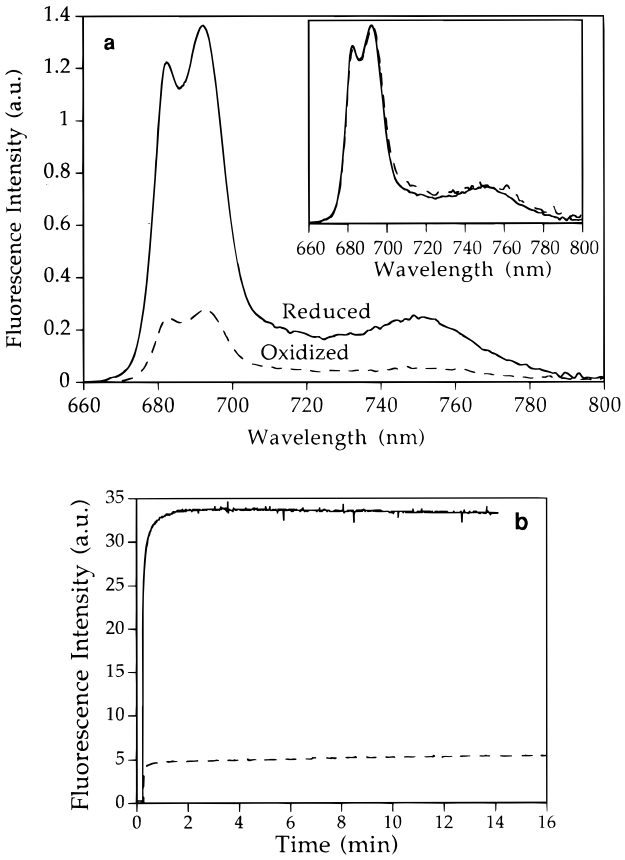


Figure 1. Fluorescence of oxidized (dashed lines) and reduced (solid lines) *Synechocystis* PSII at 85 K. (a) Steady-state spectra excited at 468 nm. Inset shows steady-state spectra scaled to the peak intensity at 740 nm. (b) Fluorescence intensity measured at 685 nm versus time of illumination with 468 nm light at 85 K.

of the charge-separated state $Chl_Z^+Q_A^-$.[21] EPR measurements confirm that this charge-separated state was also formed in high yield in *Synechocystis* PSII (Table 1). The reduced samples were pretreated with a chemical reductant to reduce cyt b_{559}. Previous EPR measurements on spinach PSII membranes have shown that illumination of such chemically reduced samples at temperatures less than or equal to 100 K results in formation of the charge-separated state cyt $b_{559}{}^{ox}Q_A^-$.[21] EPR measurements also confirm that the yield of Chl_Z photooxidation at 77 K was much smaller in chemically reduced *Synechocystis* PSII (Table 1).

The time course of the fluorescence intensity shows that upon illumination at 85 K, *Synechocystis* PSII reaches a stable charge-separated state after a few minutes of illumination (Figure 1b), and continued illumination does not alter the redox state of the sample. This demonstrates that the *Synechocystis* PSII samples were not changing during the SPC measurements (see below). EPR measurements of similar samples show that a small amount of Chl_Z^+ was formed in the reduced sample and roughly 80% of the centers formed Chl_Z^+ in the oxidized sample upon illumination at 77 K; see Table 1. Identical samples were used during the SPC measurements and it is expected that similar amounts of oxidized Chl_Z were present during these experiments. If no Chl_Z^+ had been formed in the reduced sample and 100% of Chl_Z^+ had been formed in the oxidized sample, the difference in the fluorescence intensity of the oxidized and reduced samples would have been even greater.

The fluorescence intensity of the reduced spinach PSII membrane sample decreased slowly during illumination. Previous work has shown that this slow decrease in fluorescence

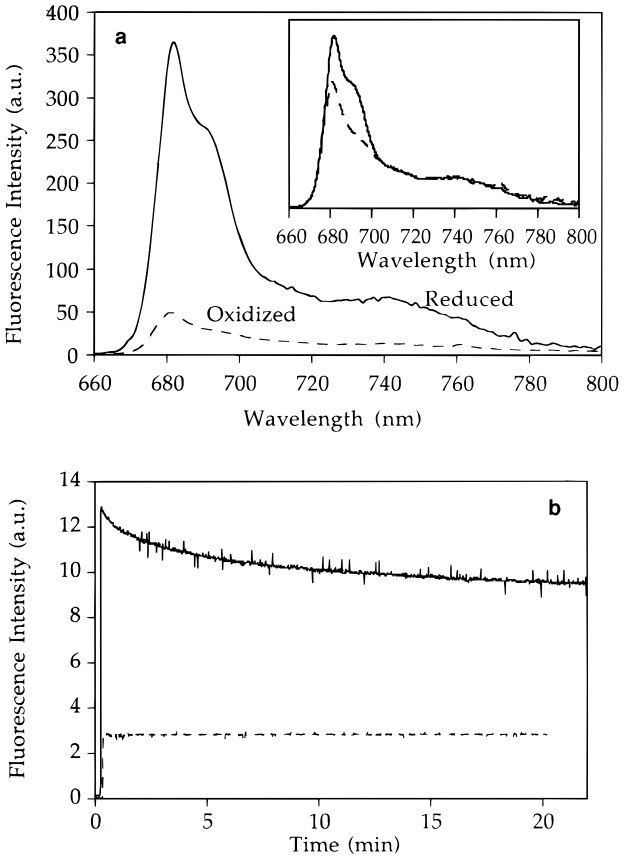


Figure 2. Fluorescence of oxidized (dashed lines) and reduced (solid lines) spinach PSII membranes at 85 K. (a) Steady-state spectra excited at 468 nm. Inset shows steady-state spectra scaled to the peak intensity at 740 nm. (b) Fluorescence intensity measured at 685 nm versus time of illumination with 468 nm light at 85 K.

TABLE 1: EPR Quantitation of $Y_D^{\bullet}$ and Chl_Z^+ in *Synechocystis* PSII

sample	illumination sequence	$Y_D^{\bullet}$	Chl_Z^{+a}
reduced	dark	0.20	0.00
	10 min at 77 K	0.20	0.20
oxidized	dark	0.70	0.00
	10 min at 77 K	0.70	0.80
	6 min at 0 °C	1.00	0.00

[a] Chl_Z^+ amounts were estimated by integrating the organic radical region of the spectrum and subtracting the area of $Y_D^{\bullet}$ measured in the same sample prior to illumination.

during prolonged illumination is due to slow electron transfer from Q_A^- to O_2, which allows multiple turnovers of the photosystem and further oxidation of Chl_Z^+ with a concomitant increase in quenching.[22] Because the reduced spinach PSII sample did not reach a stable redox state until after a much longer continuous illumination, the steady-state fluorescence measurements of the reduced sample (Figure 2a) were made after 15 min of preillumination at 85 K in order to minimize the distortion of the spectral shape due to quenching induced during the measurement. The SPC measurements of the reduced PSII membrane sample were made as quickly as possible to avoid significant formation of Chl_Z^+. (The data in Figure 3b were collected after about 10 min of illumination at 77 K with the excitation beam.) During the SPC measurements of the spinach PSII membrane samples, the oxidized sample was in a highly quenching state with near quantitative oxidation of Chl_Z and the reduced sample was in an intermediate quenching state with a fraction of the centers containing oxidized Chl_Z.

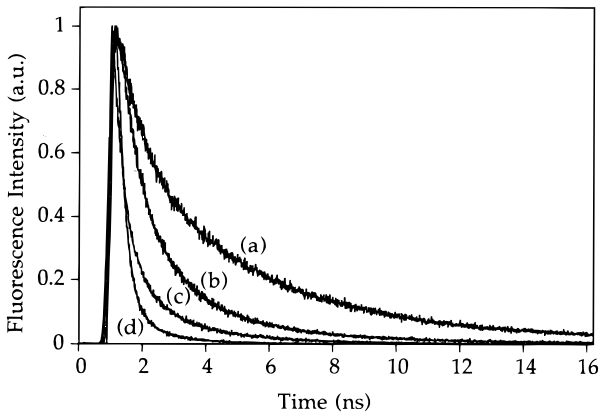


Figure 3. Time-resolved fluorescence at 77 K monitored at 695 nm and excited at 600 nm for (a) reduced *Synechocystis* PSII, (b) reduced spinach PSII membranes, (c) oxidized *Synechocystis* PSII, and (d) oxidized spinach PSII membranes.

Although the intensity of the steady-state fluorescence was significantly decreased in oxidized *Synechocystis* PSII relative to that of the reduced sample, the normalized emission spectra were not significantly different (Figure 1a, inset). This indicates that Chl_Z^+ effectively quenches excitation energy in the entire core antenna and that the core antenna system is energetically well connected in these samples even at cryogenic temperatures.

In spinach PSII membranes, the fluorescence intensity of the oxidized samples was also significantly quenched relative to that of the reduced samples (Figure 2a) but the fluorescence intensity at 695 nm was quenched to a greater degree than that at 680 nm. This indicates that the antenna chromophores giving rise to the 680 nm band are slightly less well connected than those giving rise to the 695 nm band. If the 680 nm emission is predominately from the peripheral antenna[18] and the 695 nm emission is predominately from the core antenna, these results would indicate that the peripheral antenna is less well connected than the core antenna at 85 K.

SPC measurements of oxidized and reduced PSII samples show that the oxidized samples have shortened decay kinetics compared with the reduced samples; see Figure 3. The reduced *Synechocystis* PSII sample, in which about 20% of the centers contained Chl_Z^+, had the slowest decay time with a 1/e time of approximately 3 nanosecond (Figure 3a). In the oxidized *Synechocystis* PSII sample, in which about 80% of the centers contained Chl_Z^+, the 1/e time was approximately 1 ns (Figure 3c). The reduced spinach PSII membrane sample (Figure 3b) had decay kinetics between those of the reduced and oxidized *Synechocystis* PSII samples, with a 1/e time of roughly 2 nanosecond. This intermediate decay time is attributed to the partial formation of oxidized Chl_Z during prolonged illumination of the reduced spinach PSII membrane sample,[22] which results in an intermediate shortening of the decay kinetics (Figure 2b). The oxidized spinach PSII membrane sample decay had the shortest decay time, which is attributed to the stoichiometric formation of Chl_Z^+ in this sample.[21] Overall, there is an excellent correlation between the amount of Chl_Z^+ induced in each sample and the shortening of the fluorescence decay kinetics.

DAS spectra were generated from fits of the fluorescence decays of reduced and oxidized *Synechocystis* PSII; see Figure 4a. The reduced sample decays were globally fit to a sum of four exponentials with lifetimes of 0.06, 0.45, 2.5, and 6.3 ns. The short lifetime component shows a positive amplitude at short wavelengths and a negative amplitude at longer wavelengths indicative of energy transfer. The SPC measurements

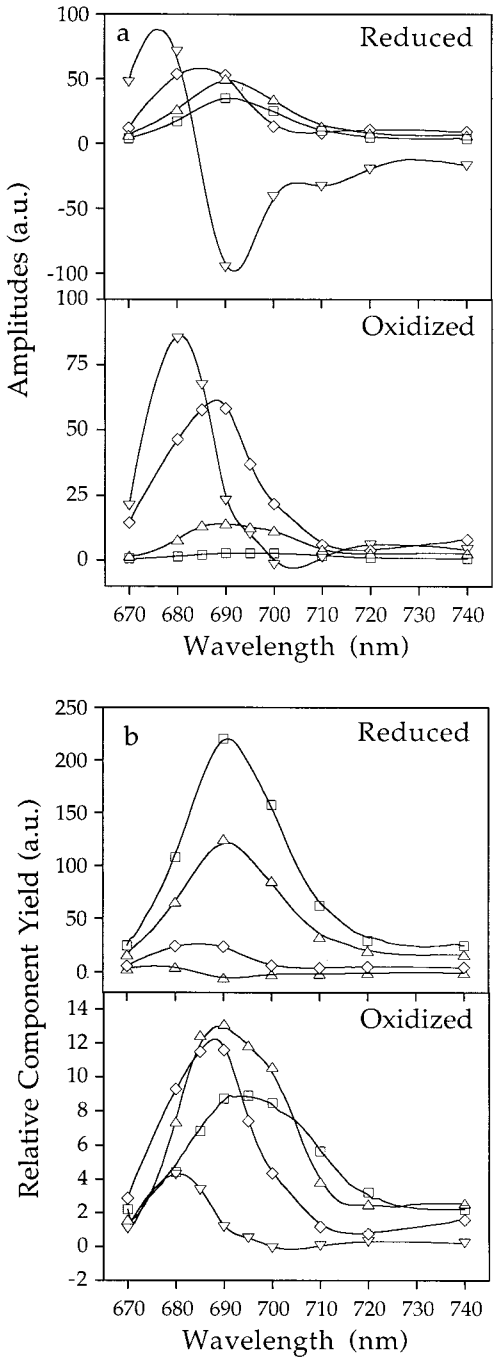


Figure 4. (a) Decay associated spectra of *Synechocystis* PSII for the reduced sample at (▽) 0.06, (◇) 0.45, (△) 2.5, and (□) 6.3 ns and the oxidized sample at (▽) 0.05, (◇) 0.20, (△) 0.93, and (□) 3.2 ns. (b) Decay associated relative fluorescence yield. Symbols are the same as those in part a.

were carried out with a 16 ns window to get an accurate measure of the nanosecond lifetimes. Under these conditions quantitation of the shortest lifetime was not as precise, though a short lifetime of a few tens of picoseconds was necessary for a good fit to the data. The 0.45 ns component band was centered at 685 nm, while the two nanosecond components were centered at 690 nm. Global fits to the oxidized sample decays resulted in four lifetimes of 0.05, 0.2, 0.93, and 3.2 ns. The shortest component shows little negative amplitude at long wavelengths; because the time-resolution was not optimized for fast energy-transfer measurements, this result is probably due to the conditions under which the experiments were made rather than a lack of energy transfer in the oxidized sample. The amplitude of the shortest component was similar to that of the reduced sample at short wavelengths. The amplitude of the few hundred picosecond component was similar to that of the reduced sample, though the lifetime was shortened from 0.45 to 020 ns. The two nanosecond lifetime components were shortened from 2.5 and 6.3 ns in the reduced sample to 0.9 and 3.2 ns in the oxidized sample with a corresponding decrease in the amplitude of these components. A plot of the fluorescence yields associated with each component reveals that the drop in the steady-state fluorescence of the oxidized samples is due to a drop in the yield associated with the two nanosecond components. The relative yields of the three positive components (0.45, 2.5, and 6.3 ns) in the reduced sample are 4.5, 16.2, and 79.3%, respectively, and for the three components of the oxidized sample (0.20, 0.93, and 3.2 ns) 2.8, 3.5, and 2.9%, respectively, taking the integrated fluorescence of the reduced sample to be 100%. The fluorescence yield of all three components combined is about 11 times lower in the oxidized sample compared with that in the reduced sample. This degree of quenching is comparable to what was obtained in the steady-state fluorescence measurements; see Figure 1a.

Discussion

We have presented low-temperature steady-state and time-resolved fluorescence emission data of PSII samples containing a varied amount of Chl_Z^+. The presence of Chl_Z^+ decreases the steady-state fluorescence intensity, and this decrease is associated with a large decrease in the yield of the nanosecond decay components. In samples containing a fractional amount of Chl_Z^+, there is a good correlation between the fluorescence decay time and the amount of Chl_Z^+. These results demonstrate that Chl_Z^+ is a potent fluorescence quencher.

As shown in Table 1, it is difficult to avoid generating some Chl_Z^+ when PSII samples are illuminated at 77 K. Even when the samples were treated with chemical reductants to prereduce cyt b_{559}, a small amount of Chl_Z was photooxidized. This is due to two factors. First, the low-potential forms of cyt b_{559} are difficult to reduce, and unless cyt b_{559} is prereduced, illumination at low temperatures will yield Chl_Z photooxidation (Scheme 1). Second, O_2 can act as an electron acceptor at 77 K,[22] and this leads to multiple turnovers of PSII and some photooxidation of Chl_Z, even in samples that are treated with chemical reductants to prereduce cyt b_{559} (Figure 2b). Therefore, it is likely that a significant and probably variable amount of Chl_Z^+ has been present in all previous low-temperature fluorescence studies of PSII samples. Owing to its potent quenching, the amount of Chl_Z^+ needs to be evaluated before interpreting low-temperature fluorescence data.

We have compared the steady-state fluorescence emission spectra of *Synechocystis* PSII core complexes, which contain only the core antenna, with spinach PSII membranes, which contain both the core antenna and the peripheral LHCII antenna. Because the normalized emission spectra of *Synechocystis* PSII core complexes are the same for the oxidized and reduced samples, we conclude that Chl_Z^+ is an effective quencher of emission from all of the core antenna chlorophylls (Figure 1a, inset). This result is consistent with recent experimental results, which indicate that the PSII fluorescence properties are determined by "rapid exciton equilibration".[8] However, emission from the peripheral LHCII antenna was less effectively quenched by Chl_Z^+ (Figure 2a, inset), indicating that exciton equilibration is incomplete among the core and peripheral antenna, at least at 77 K.

In our time-resolved fluorescence study of the *Synechocystis* PSII core without and with Chl_Z^+ (Figure 4), the time window

(16 ns) was not optimized for the measurements of the fast fluorescence components. However, an energy-transfer component (40−60 ps) was required to fit the data. This component is likely to represent the fast energy-transfer process, which in the PS II core antenna showed a variability of lifetimes (from 5 to 30 ps).[12] This heterogeneity was recently explained by the inhomogeneously broadened dispersion of rates of energy transfer.[12] The presence of this phase at low-temperature implies that the steady-state fluorescence properties of the PSII core are determined by the thermal equilibrium distribution of excited states of the PSII core pigments (Figure 1a).

Our data show that both reduced and oxidized samples at 77 K exhibit kinetic heterogeneity of the fluorescence decaying components with lifetimes in the range of 0.2−6 nanosecond (Figure 4a, upper panel). The *Synechocystis* PSII core with reduced Q_A and without Chl_Z^+ is in the closed state. This preparation does not contain the LHCII; therefore, all fluorescence presumably originates from the CP43/CP47 core antenna and the D1/D2/cyt-b_{559} RC. In the absence of the RC, the CP43/CP47 fluorescence would decay within nanoseconds. The exciton/radical pair equilibrium model[8−10] predicts that the excitation in the PSII core antenna (CP43/CP47) rapidly equilibrates with the RC. This process is followed by trapping of the excitation and formation of the radical pair $P680^+Pheo^-$. In closed RCs, the radical pair tends to recombine and repopulate the $P680^*$ excited state, which is in equilibrium with the excitation states of the core antenna. This model suggests that the emission due to both the trapping process in the PSII core and the repopulation of P680* would be characterized by similar spectral profiles. In Figure 4a (upper panel), the 2.5 nanosecond and the 6.3 nanosecond DAS of the reduced sample have similar spectral shapes. On the basis of the exciton/radical pair equilibrium model, these decaying components could represent an equilibrated excited state of the PSII core antenna. Thus, the faster 60 ps DAS and the 450 ps DAS could reflect the equilibration process slowed at 77 K as compared to room temperature. In the oxidized state (Figure 4a, lower panel), the decaying components with lifetimes of 930 ps and 3.2 ns largely resemble the shape of the nanosecond spectra of the reduced sample and may represent the equilibrated state.

However, we cannot rule out an alternative interpretation of the data. The kinetic and structural heterogeneity of the PSII seem to contribute to the fluorescence decay processes with lifetimes in the range of 0.5−10 ns and result in a multiphasic trapping process and protein-coupled radical-pair relaxation.[12−15] The 450 ps DAS could represent the trapping of the excitation in the PSII core antenna by the primary donor as opposed to the 100 ps trapping phase and 500 ps charge-stabilization phase in open PSII at room temperature.[31] The RC may also contribute to the heterogeneity of the fluorescence decays. In a recent 77 K time-resolved fluorescence study of D1/D2/cyt-b_{559} PSII complexes, Konermann et al.[12] ascribed 0.1, 0.6, 2, and 8 ns components to a series of sequentially relaxing radical-pair states. In closed PSII, the nanosecond fluorescence decays were reported to be modulated by the electric fields imposed by a negative charge on the reduced Q_A.[17] Therefore, the 2.5 and 6.3 ns DAS in the reduced sample in Figure 4a could be ascribed to a repopulation of the excited states in the PSII core due to charge recombination in the RC. The possibility of the contribution to the nanosecond fluorescence decays of energy-uncoupled pigment clusters of the core also could not be ruled out.

Formation of Chl_Z^+ in relatively close proximity to the primary donor P680 results in a shortening of the lifetimes of all fluorescence-decaying components and amplitudes of the long-lived components (Figure 4 parts a and b, lower panels). Our previous study[22] and the data of this study show that Chl_Z^+ is a very effective quencher of the fluorescence in the PSII core antenna. The other effective quenchers of the antenna fluorescence may be the oxidized primary donor, $P680^+$, and free chlorophyll in solution. Earlier, the 5−6 nanosecond phase at low temperatures was attributed to free chlorophyll in solution.[11] It is unlikely that free chlorophyll is present in amounts sufficient to quench the fluorescence in our preparations. The shape of the steady-state emission spectra of the PSII core particles at 77 K is unchanged both at reducing and oxidizing conditions (Chl_Z^+ content is about 20 and 80%, respectively). The Chl_Z^+ content is easily monitored by EPR measurements, and its accumulation is reversible. There is also no indication of the emission at 677 nm ascribed to the fluorescence of free Chl at 77 K.

This work represents the initial characterization of the effect of Chl_Z^+ on the fluorescence emission of PSII. The ability to induce a potent quencher in a controllable way and at a well-defined location in the PSII reaction center presents a new opportunity to study the excited-state dynamics in PSII. In the future, more detailed studies as a function of temperature and in natural membranes may provide information on the exciton hopping and trapping processes within a single PSII complex and also between neighboring PSII complexes.

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