The Origins of Nonphotochemical Quenching of Chlorophyll Fluorescence in Photosynthesis. Direct Quenching by $P680^+$ in Photosystem II Enriched Membranes at Low pH†

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ABSTRACT: In most plants and algae, a down-regulation of photosynthesis under "excess" light conditions occurs which is associated with a quenching of chlorophyll *a* fluorescence. This nonphotochemical quenching of chlorophyll *a* fluorescence most likely arises from a mechanism which protects photosystem II from excessive excitation and resulting photoinhibition. In this report, nonphotochemical quenching of variable chlorophyll *a* fluorescence was induced by low pH in photosystem II enriched spinach thylakoid membranes. The origin of quenching was investigated with picosecond fluorescence decay spectroscopy in samples suspended in buffers ranging from pH 6.5 to pH 4.0. The yield of a relatively slow (approximately 1.5 ns) fluorescence decay process associated with the photosystem II reaction center decreased with decreasing pH. There were no significant changes in the yield of faster decay components associated with photosystem II antenna chlorophyll *a* processes. These results suggest a reaction center based rather than antenna chlorophyll based mechanism for nonphotochemical quenching in these preparations. Measurements of the photosystem II absorption cross section revealed no decrease in the functional antenna size at low pH which also supports a reaction center quenching mechanism. The kinetics of electron transfer in photosystem II were investigated using a pump probe spectrometer which measured simultaneously the flash-induced absorbance change at 820 nm (formation of oxidized photosystem II reaction center pigment, $P680^+$) and the variable fluorescence yield (formation of reduced photosystem II, electron acceptor, Q_A^-). A large increase in the lifetime of $P680^+$ at low pH was correlated with fluorescence quenching. After flash excitation of photosystem II the loss of fluorescence quenching occurred with the same kinetics as the reduction of $P680^+$. In conflict with reaction center based quenching mechanisms based on charge recombination between $P680^+$ and Q_A^- , the oxidation rate of Q_A^- was unaffected by low pH and under all conditions occurred at a slower rate than the reduction of $P680^+$. Our data are discussed in terms of a model for low pH dependent nonphotochemical quenching in photosystem II based on direct quenching by $P680^+$.

The light harvesting capability (antenna size) of photosystem II (PSII)[1] is a compromise between maximizing electron transport under low light conditions and minimizing photoinhibition under excess light. The effective antenna size of PSII is believed to be actively regulated by several related processes which are cumulatively termed nonphotochemical quenching (*q*N), the dissipation of absorbed light energy as heat in competition with photochemical utilization in the reaction centers.

Three processes contributing to *q*N have been described. These are photoinhibition, light state transitions, and high-energy or ΔpH-induced quenching. These processes can be separated both kinetically and by their differing sensitivity to uncouplers, electron transport, and enzyme inhibitors and by light quality and quantity (Quick & Stitt, 1989). Photoinhibitory quenching (*q*I) is induced by exposure to excess light, has been correlated with damage to PSII, and has the slowest kinetics (hours) of onset and recovery (Aro et al., 1993). Quenching by state transitions (*q*T) is induced by preferential excitation of PSII, involves the phosphorylation of the light harvesting complex of PSII (LHCII) and its subsequent dissociation from PSII, and occurs on a time scale of many minutes (Allen, 1995). High-energy or ΔpH-induced quenching (*q*E) is correlated with the buildup of ΔpH across the thylakoid membrane (Briantais et al., 1979) and the formation of the carotenoid zeaxanthin (Demmig-Adams, 1990) and occurs on a time scale of many seconds to a few minutes.

Much of the total fluorescence quenching observed during induction of photosynthesis at moderate light intensities is due to *q*E (Quick & Horton, 1984), and the inhibition of *q*E formation has been correlated with an increased susceptibility of PSII to photoinhibition (Krause & Behrend, 1986). *q*E has thus been proposed to be an indicator of an excitation

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[1] Abbreviations: Chl, chlorophyll; ΔA_{820}, flash-induced absorbance increase at 820 nm; F_o, minimal fluorescence yield; F_m, maximal fluorescence yield; F_v, $F_m - F_o$; Q_A, primary quinone electron acceptor of photosystem II; *q*N, nonphotochemical quenching of chlorophyll *a* fluorescence; *q*E, energy-dependent quenching of chlorophyll *a* fluorescence; LHCII, light harvesting complex associated with photosystem II; P680, reaction center of photosystem II; Phe, pheophytin; PS, photosystem.

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energy quenching mechanism which safely releases absorbed energy as heat to serve a photoprotective role for PSII (Horton & Ruban, 1992).

Numerous mechanisms have been proposed for high-energy fluorescence quenching. They localize quenching either in the antenna pigment bed (Crofts & Yerkes, 1994; Demmig-Adams, 1990; Genty et al., 1990; Ruban et al., 1992) or in the reaction center (Krieger et al., 1992; Noctor & Horton, 1990; Schreiber & Neubauer, 1990; Weis & Berry, 1987). Quenching in the antenna could involve LHCII and/or the PSII core Chl antenna. Reaction center quenching could involve excitation trapping by a nonphotochemical quenching state of the reaction center (Weis & Berry, 1987), an energy-dissipating recombination reaction (Krieger et al., 1992; Schreiber & Neubauer, 1990), or a cyclic electron transport pathway (Noctor & Horton, 1990). Distinguishing between these possibilities has been the focus of much recent work and the origin of considerable controversy. The two proposed sites of *q*E quenching (antenna and reaction center) are not mutually exclusive; it is possible that quenching occurs at both sites to different extents depending on environmental conditions and the organism under study.

In vitro systems of PSII -enriched membranes and thylakoid membranes show an electron donor side inhibition of PSII at low pH which is associated with *q*E (Crofts & Horton, 1991; Krieger et al., 1992; Krieger & Weis, 1990). Inhibition of the donor side of PSII has been suggested by Schreiber and Neubauer (1990) to be a prerequisite for either recombination or cyclic electron transport quenching mechanisms. Alternatively, the inhibition of electron donation to PSII may allow the special pair Chl $P680^+$ or the auxilliary Chl Z^+ to act as a quencher (Horton & Ruban, 1992; Thompson & Brudvig, 1987).

In this report, we investigate the origins of the low pH induced qN observed in PSII-enriched membranes. Our picosecond fluorescence spectroscopy measurements and absorption cross-section measurements support a reaction center based quenching mechanism, and our electron transport measurements strongly support direct quenching by $P680^+$ rather than recombination or cyclic electron transport mechanisms.

EXPERIMENTAL PROCEDURES

Isolation of PSII-Enriched Membranes. PSII enriched thylakoid membranes were isolated from market spinach as described in Ghanotakis et al. (1984). The membranes were frozen in liquid nitrogen and stored at −80 °C at a concentration of about 2 mg of Chl/mL until use. Chl *a* and *b* concentrations were measured as in Zeigler and Egle (1965). The Chl *a*/*b* ratio of the membranes was 1.65, and 77 K fluorescence emission spectra were dominated by PSII peaks at 685 (F685) and 695 nm (F695) as compared to PSI emission at 735 nm (F735). The F685/F735 ratio at 77 K of the membranes was typically 4.0 (not shown).

For all experiments, the PSII-enriched membranes were resuspended in buffer containing 0.3 M sorbitol, 50 mM KCl, 5 mM $MgCl_2$ and either 30 mM MES for pH 6.5−5.5 or 30 mM succinic acid for pH 5.5−4.0. There were no significant differences in any results at pH 5.5 between samples resuspended in MES or succinic acid buffers.

Picosecond Fluorescence Spectroscopy. Picosecond time-resolved fluorescence decay kinetics were determined with the single photon timing apparatus described in Bruce and Miners (1993). Excitation pulses of approximately 60 ps duration at 665 nm were provided by a pulsed laser diode (Hammamatsu PLP-01 with an LDH067 laser head) operated at 10 MHz. The photon timing circuitry received reference pulses from a fast avalanche photodiode (Hammamatsu S2381), and fluorescence was detected by a proximity-focused microchannel plate photomultiplier (Hammamatsu R2809U-11). The instrument response function had a half-width of 100 ps, and the time resolution limit after deconvolution was approximately 20 ps.

Instrument response functions were determined by collecting excitation scattered from a suspension of 0.5 μm latex beads in distilled water before and after the determination of every fluorescence decay. Individual fluorescence decays were collected until at least 5×10^4 counts in the peak channel were attained.

In each experiment, 50 mL of sample at a concentration of 2 μg of Chl *a*+*b*/mL was circulated through a flow-through measuring cuvette with a 200 μL volume at a rate of about 50 mL/min. For measurements at the minimal fluorescence level (F_o), the sample was kept completely in the dark except for the brief exposure (about 240 ms) to the laser beam. For measurements at F_m, the sample flowed through a 2 mL glass bulb placed just before (approximately 200 ms) the measuring cuvette which was illuminated with white light at an intensity of 2000 μEm^{-2} s^{-1}. The F_m samples were thus exposed to a 1 min dark incubation followed by a 2 s saturating light treatment (to close the reaction centers) and a 200 ms dark interval just before measurement.

Room temperature steady-state fluorescence emission spectra were collected with a diode array based fluorometer described previously (Brimble & Bruce, 1989). A spectrum was obtained for each sample at both F_o and F_m using the same circulating system used for collection of the picosecond fluorescence decay data.

For each experiment, fluorescence decay curves were collected at 675, 680, 685, 690, 695, and 700 nm. Model curve fits were generated by a convolution of the instrument response function with a sum of exponential decay components. The decay curves from all six emission wavelengths were modeled simultaneously using a global fitting routine, to determine the spectral shape of each decay component. The fitting program used an optimized Levenberg/Marquat algorithm and was developed by Warren Zipfel (ICS, Ithaca, NY).

Simultaneous Detection of Pump Probe Fluorescence Yield and the Flash Induced Absorbance Change at 820 nm Associated with $P680^+$. Fluorescence was detected with the pump probe flash fluorometer described in Falk et al. (1994). The pump flash (250 ns half-width, 620 nm) was generated by a Phase-R DL-32 flash lamp pumped dye laser. The probe flash (normally timed 60 μs after the pump flash) was a weak xenon flash covered with a Corning 4−96 blue filter.

Simultaneous detection of $P680^+$ by measurement of the flash induced absorbance change at 820 nm (ΔA_{820}) was accomplished with the single beam spectrometer described in Samson and Bruce (1995). The measuring beam was generated by an 820 nm diode laser (Spindler and Hoya) and the actinic flash by the flash lamp pumped dye laser described above.

Measurements of Chl *a* fluorescence yield and ΔA_{820} were done on the first flash of dark-adapted samples, or, alternatively, the data were averaged from 16 pump/probe flash

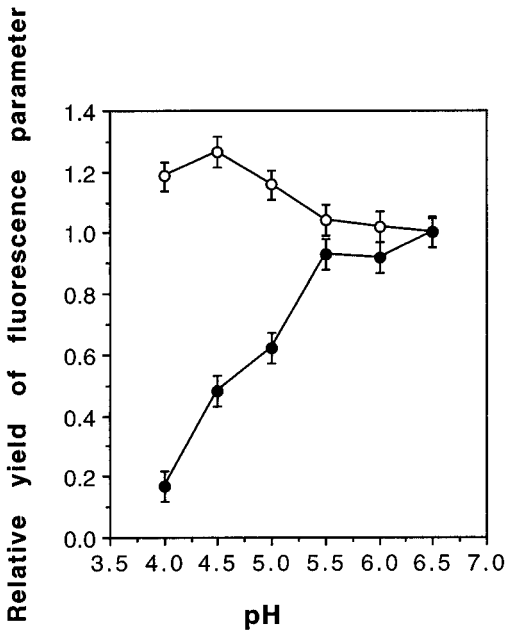

FIGURE 1: Relative yield of F_o (open circles) and F_v (filled circles) in PSII-enriched membranes suspended in buffers of varying pH. Values are the mean of three repeats; standard deviations are shown.

pairs given at a frequency of 0.3 Hz following a series of 25 saturating Xe flashes (at a frequency of 2 Hz). The suspensions of PSII-enriched membranes used for those measurements were dark-incubated for 10 min at a Chl*a*+*b* concentration of 10 μg/mL. One min before measurements, 200 μM FeCN was added to the samples to avoid accumulation of reduced Q_A during the repetitive flash treatment. Effective absorption cross sections were determined from flash intensity saturation curves for both variable fluorescence yield (F_v) and ΔA_{820} which were generated by changing the intensity of the pump flash with a neutral density filter wedge. Each dark-adapted and preflashed sample was exposed to only one pump flash, requiring the use of multiple samples to construct each saturation curve. Cross sections were calculated by fitting the saturation curves to a Poisson probability distribution as in Falk et al. (1994).

The lifetime of Q_A^- was determined by measuring fluorescence yield with the probe flash at various times after a saturating pump laser flash. The time interval between pump and probe was varied from 20 μs to 10 ms.

RESULTS AND DISCUSSION

Steady-State and Time-Resolved Fluorescence Emission. Steady-state fluorescence emission spectra (not shown) were determined at F_m and F_o for PSII-enriched membranes suspended in buffer of varying pH from 4 to 6.5 as described under Experimental Procedures. As reported by Kreiger et al. results showed F_v ($F_m - F_o$) to be strongly quenched at low pH while F_o was not (Figure 1). This selective quenching of F_v is usually interpreted to be consistent with a reaction center based mechanism for nonphotochemical quenching as competition between an antenna-based quenching mechanism and energy trapping by the reaction center is expected to decrease the F_o level.

Although measurements of relative changes in F_m and F_o are often used as primary evidence for the site of nonphotochemical quenching, they are indirect. Accurate measures of F_o are often difficult, and many factors can influence fluorescence yield (Samson & Bruce, 1995, 1996). The kinetic models (bipartite, tripartite) most often used to relate F_m and F_o to absorbance cross sections and rates of photochemistry (Butler & Strasser, 1977; Butler, 1978) were developed prior to knowledge of the primary acceptor (Phe) and that PSII is a shallow trap (multiple visits of an exciton to a reaction center before stable charge separation) (Dau, 1994). More data than F_m and F_o levels alone are required to determine a molecular mechanism for nonphotochemical quenching.

The exciton/radical pair equilibrium model (Shatz et al., 1988) is currently the most successful at explaining excited state dynamics of PSII and the origins of F_o and F_m (Dau, 1994). The model was developed to explain the picosecond fluorescence decay kinetics of PSII and incorporates both the concept of a shallow trap and the presence of Phe as primary acceptor. The exciton/radical pair equilibrium model separates the process of light capture and charge separation leading to $P680^+/Q_A^-$ into two kinetic components and thus predicts biexponential fluorescence decay kinetics in time-resolved fluorescence investigations. At both F_o and F_m, the first component of fluorescence emission (τ_A) reflects an equilibrium between energy transfer in the antenna, creation of P680*, and reversible radical pair ($P680^+/Phe^-$) formation. The second kinetic component (τ_B) is not in equilibrium with these primary processes and at F_o (open reaction centers) results from "charge stabilization" or electron transfer from Phe^- to Q_A. At F_m, the reaction center already contains Q_A^-, and the second component arises from the relaxation of the primary radical pair to ground state. This relaxation to ground is a distinct and much slower process than the rapid recombination of $P680^+/Phe^-$ which leads to P680*.

Identification of PSII-Associated Fluorescence Decay Components. To gain more insight into the origins of fluorescence quenching by low pH, we applied picosecond fluorescence spectroscopy to resolve the fluorescence emission at F_o and F_m into a sum of kinetic components which could be analyzed in terms of the exciton/radical pair equilibration model. We found the decay kinetics of PSII-enriched membranes to be modeled well with a sum of four exponential decay components at both F_o and F_m (Figure 2). At pH 6.5, the fastest decay component (C1) had very similar lifetimes (approximately 90 ps) and amplitudes at both F_o and F_m. Although this decay component was not significantly red shifted compared to the other decay components, its lifetime, relatively low amplitude, and the similarity in both lifetime and amplitude at F_o and F_m suggest an origin in PSI. The second fastest decay component (C2) had a lifetime of approximately 350 ps at F_o which increased to 500 ps at F_m. The relative amplitude of this component decreased by a factor of 4 upon PSII trap closure (transition from F_o to F_m), suggesting an origin in PSII. The third decay component (C3) had a lifetime of 730 ps in open centers and 1.3 ns in closed centers. The relative amplitude of this component increased by approximately 3 times upon PSII trap closure. The amplitude of the fourth component was smaller in open than closed centers, and the lifetime was approximately 1.7 ns in open centers and 2.4 ns in closed centers. The major changes in decay components observed upon trap closure were the concomitant decrease in amplitude of C2 and the increase in amplitude and lifetime of C3.

Previous fluorescence decay experiments with pea thylakoids showed four exponential decay components associated with PSII and were analyzed in terms of the exciton radical

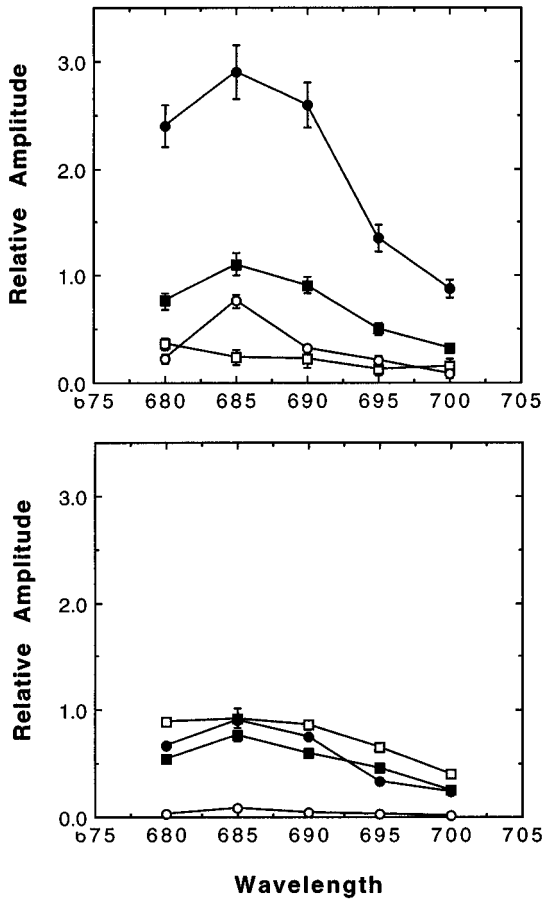

FIGURE 2: Decay-associated spectra for the four components used in the global fitting analysis of picosecond fluorescence decays obtained at 680, 685, 690, 695, and 700 nm from PSII-enriched membranes at pH 6.5. Data in the top panel are for membranes with closed reaction centers (F_m), and data in the lower panel are for membranes with open centers (F_o). The filled squares show the spectra for the fastest decay component, "C1", which had a lifetime of 95 ps in closed centers and 85 ps in open centers. Open squares show the spectra for the "C2" decay component, which had a lifetime of 500 ps in closed centers and 360 ps in open centers. Filled circles show the "C3" component, which had a lifetime of 1.3 ns in closed centers and 730 ps in open centers. Open circles show the "C4" component, which had a lifetime of 2.4 ns in closed centers and 1.7 ns in open centers. The data presented are the means of at least three independent experiments, and standard deviations are shown. The uncertainty in the lifetimes is approximately 10%. See Experimental Procedures for details of the measurement technique and global decay analysis procedure.

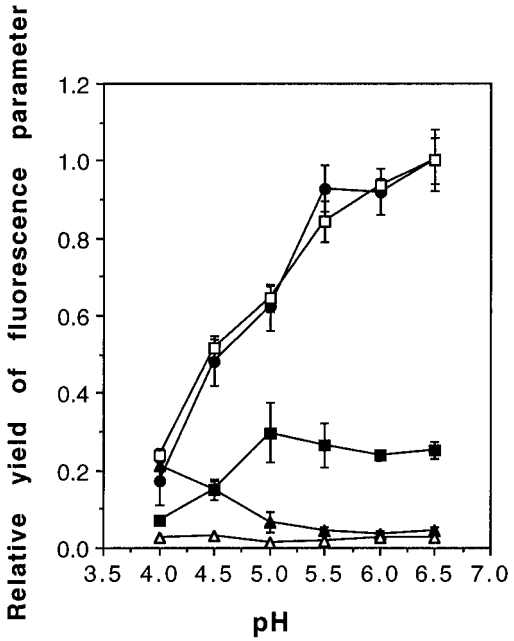

FIGURE 3: Relative yield (lifetime times amplitude) of each of the decay components arising from the global analysis of picosecond fluorescence decays of PSII-enriched membranes at F_m is compared to the yield of F_v (filled circles) as a function of pH. Open triangles show the yield of "C1", filled triangles the yield of "C2", open squares the yield of "C3" and filled squares the yield of "C4". The data presented are the means of at least three independent experiments, and standard deviations are shown. See text for further details.

pair model with the assumption that two forms of PSII (PSII α and PSII β) contributed to the observed kinetics (Roelofs et al., 1992). The authors assigned values of 250 ps (PSII α) and 320 ps (PSII β) for τ_A, 520 ps (PSII α) and 670 ps (PSII β) for τ_B at F_o and 380 ps (PSII α) and 650 ps (PSII β) for τ_A, 1.8 ns (PSII α) and 2.9 ns (PSII β) for τ_B at F_m. These values for τ_A and τ_B are reasonably close to the values of C2 (360 ps) and C3 (730 ps) at F_o and C2 (500 ps) and C3 (1.3 ns) at F_m found in the present study. There are, however, large variations found in the relative lifetimes and amplitudes of decay components found in two previous studies of PSII-enriched membranes (Krieger et al., 1992; Vass et al., 1993). This most likely results from differences in the PSII-enriched preparations. For example, the data of Krieger et al. (1992) were similar to the current study in that the amplitudes and lifetimes of the fastest decay component (C1) were reasonably constant at F_o and F_m. Athough not stated by Krieger et al., this behavior of C1 coupled to its very short lifetime in their study (20−30 ps) indicates an origin in PSI similar to the conclusion we have reached regarding C1 in the current study. In contrast, Vass et al. (1993) found the lifetime of their "C1" to be much longer and to increase dramatically at F_m indicative of an origin in PSII. These results suggest a lower amount of contaminant PSI in the PSII preparation used by Vass et al. (1993). In addition to preparation differences, variations in the degree of overlap between unresolved decay components may also contribute to differences seen in the three studies. However, in all three studies, the lifetime of a component similar to C2 in this study increased and its amplitude decreased upon transition from F_o to F_m, and both the lifetime and amplitude of a component similar to C3 of this study increased upon transition from F_o to F_m. In terms of the exiton radical pair model, it is likely that much of "C2" in all three studies was related to energy transfer, trapping, and primary radical pair formation at F_o and F_m (τ_A) and most of "C3" to charge stabilization at F_o and primary radical pair relaxation at F_m (τ_B).

Effect of Low pH on PSII-Associated Fluorescence Decay Components. Kreiger et al. (1992) showed that most of the F_m quenching induced by low pH in isolated PSII particles was associated with changes in only one of the decay components, C3, whose amplitude decreased by a factor of approximately 2 and whose lifetime decreased from 1.7 ns to 650 ps as pH decreased from 6.5 to 4.0. However, the results of their study depended upon the kinetic analysis of individual fluorescence decay curves taken at only one emission wavelength (681 nm) for each condition and pH. In the present study, we expanded upon the experiments of Krieger et al. (1992) by applying global decay analysis to a set of fluorescence decays collected at five emission wavelengths for each condition and pH (Figure 2 and Figure 3).

Our data support the work done by Krieger et al. (1992) and showed that most of the F_m quenching induced by low pH arose from quenching of only one of the decay components, C3, whose amplitude decreased by a factor of approximately 2 and whose lifetime decreased from 1.3 ns to 700 ps as pH decreased from 6.5 to 4.0. The relative contribution of each decay component (lifetime times amplitude) to F_m is plotted as a function of pH in Figure 3 and is seen to closely follow the F_v quenching. The yield of the C1 component is unaffected by pH, and that of C4 decreases somewhat at low pH. The relative yield of the C2 component is shown to increase at low pH which was also observed by Krieger et al. (1992).

Krieger et al. (1992) proposed that recombination between Q_A^- and $P680^+$ was responsible for the quenching of F_m and of their C3 component at low pH. They suggested that the probability of recombination was increased due to the increased lifetime of $P680^+$ at low pH (Meyer et al., 1989). In order for the recombination to result in decreased fluorescence yield, Krieger et al. (1992) suggested the following: "For some reasons, in the low pH state the free energy change related to recombination of $P680^+ \cdot Q_A^-$ may not be sufficient to create excited states of the reaction center and, thus, energy would be lost as heat." Implicit to this model is the idea that the origin of F_m fluorescence is recombination between Q_A^- and $P680^+$ which leads to excitation of the reaction center. As well described in a recent review by Dau (1994), recombination between Q_A^- and $P680^+$ is an unlikely source of variable fluorescence. More consistent with recent data (Dau, 1994) is the exciton radical pair model which proposes the origin of variable fluorescence to result from a relatively long-lived radical pair in equilibrium with energy transfer and trapping in closed centers (Schatz et al., 1988).

In terms of this model, the pH-sensitive C3 component at F_m arises from the slow relaxation of the radical pair ($P680^+$, Phe^-) to the ground state when charge stabilization (electron transfer from Phe^- to Q_A) no longer occurs in the presence of Q_A^-. In both the current study and that of Krieger et al. (1992), the selective loss of amplitude of this "C3" component indicates that the amount of radical pair formation and relaxation is dramatically reduced at low pH. Furthermore, as the faster decay components were unaffected in both studies by low pH, it appears that excitation energy transfer to and trapping by the reaction center are relatively unaffected by low pH.

These results strongly support a reaction center based mechanism for low pH induced quenching of PSII-enriched membranes. The loss of the "C3" decay component could result from an alteration of the reaction center by low pH which leads to a much faster relaxation of the primary radical pair to the ground state or from an alternative electron acceptor that oxidizes Phe^-. Either of these mechansims would have to occur on a time scale roughly equivalant to that of electron transfer from Phe to Q_A in open centers in order to explain the observed decay kinetics.

Another possibility is direct quenching by $P680^+$. Previous studies have shown the lifetime of $P680^+$ to be increased dramatically at low pH (Meyer et al., 1989) . Under conditions of saturating illumination, such as that used to measure F_m, the probability of an exciton reaching a reaction center containing $P680^+$ is greatly increased. If reaction centers containing $P680^+$ trap excitons with the same efficiency as open centers but then quench the excitation energy, their fluorescence decay kinetics could be similar to those of open centers. Under F_m measurement conditions, the "quenched" centers containing $P680^+$ would appear as "open centers", and the amplitude of decay components associated with "closed" centers would be decreased. A model invoking $P680^+$ as quencher would thus assume that the trapping efficiency of $P680^+$ is very similar to that of P680 and that the subsequent loss of excitation by relaxation to the ground state occurs on a similar time scale as charge stabilization in open reaction centers.

Table 1: Absorbance Cross Section of PSII Determined from Flash Saturation Curves for F_v and ΔA_{820} at pH 6.5 and 5.0[a]

		absorbance cross section of PSII (Å^2)	
sample		pH 6.5	pH 5.0
dark-adapted	F_v	27 ± 5.5	29 ± 3
	ΔA_{820}	37 ± 2.7	35 ± 5
preflashed	F_v	38 ± 8	35 ± 4
	ΔA_{820}	37 ± 2.3	37 ± 2.6

[a] Cross sections were determined for dark adapted samples exposed to only 1 flash and to samples exposed to 16 preflashes (0.5 Hz). Values are the means and standard deviations of three independent measures. The excitation wavelength was 620 nm.

To verify both the idea of reaction center based quenching and the hypothesis that $P680^+$ acts as the quencher, we used more direct techniques to study the absorbance cross section of PSII and the lifetime of $P680^+$ as a function of pH.

Absorbance Cross Sections, Lifetime of P680$^+$, and Origin of Fluorescence Quenching at Low pH in PSII-Enriched Membranes. To differentiate between antenna and reaction center quenching mechanisms and to avoid the assumptions made when inferring cross-section changes from F_m and F_o changes alone, we measured the absorption cross section of PSII directly. To decrease the effective absorbance cross section of PSII, a quenching mechanism must compete with energy transfer, trapping, and/or primary radical pair production. Antenna quenching mechanisms which introduce additional competitive deexcitation pathways in the antenna are of this type. In contrast, quenching mechanisms which create completely quenched reaction centers in a non competitive way will not affect the absorbance cross section of the remaining centers. Mechanisms which lose energy via alternative electron transport after primary radical pair production (PSII cyclic) or recombination of the reduced secondary electron acceptor (Q_A^-) and $P680^+$ would be of this type. The creation of a quenching center containing P680+ as direct irreversible quencher would also not decrease the cross section of the remaining unquenched PSII centers. See Mauzerall and Greenbaum (1989) for a review of absorbance cross-section determinations and theory.

We determined PSII absorbance cross sections from light saturation curves of both pump probe fluorescence yield and the formation of $P680^+$ as measured by the flash-induced absorbance increase at 820 nm (ΔA_{820}) as described under Experimental Procedures (Table 1). Cross sections were determined from dark-adapted samples exposed to only one flash and from preflashed samples. As shown in Table 1, the absorbance cross section of PSII in preflashed samples was the same when measured by either technique and was not decreased at low pH. For single- flash samples, the absorbance cross section was slightly smaller when measured by the variable fluorescence technique as compared to the ΔA_{820} technique. However, suspension at low pH did not decrease the absorbance cross section as measured by either

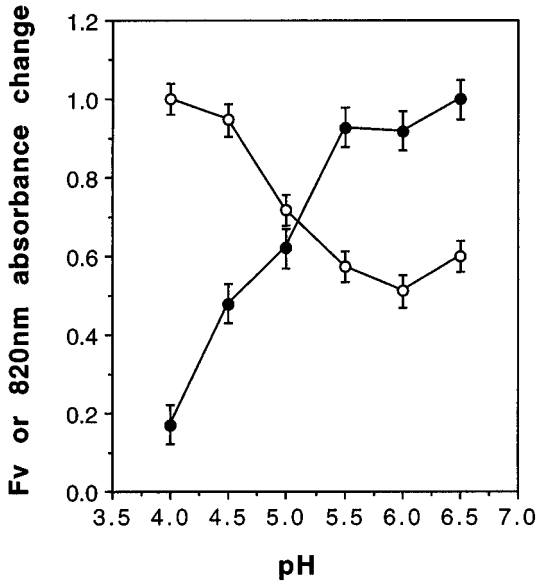

FIGURE 4: Maximum yield of the flash-induced absorbance change at 820 nm (ΔA_{820}) (open circles) is compared to the yield of F_v (filled circles) in PSII-enriched membranes as a function of pH. The data presented are the means of at least three independent experiments, and standard deviations are shown. See text for further details.

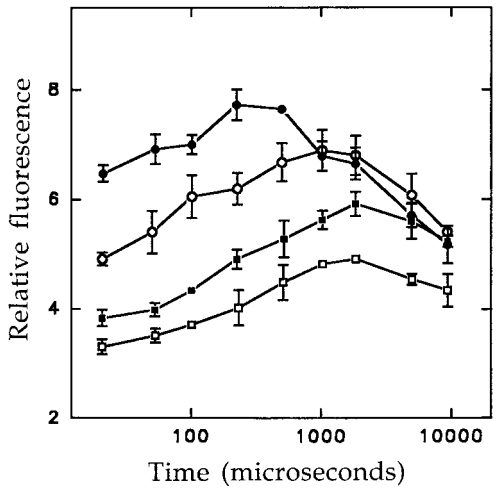

FIGURE 5: Relative yield of fluorescence elicited by a weak single-turnover probe flash at various times after a saturating single-turnover flash (see Experimental Procedures for details of pump probe fluorescence protocol). Filled circles, pH 6.5; open circles, pH 5.0; filled squares, pH 4.5; and open squares, pH 4.0. Samples were PSII-enriched membranes exposed to a a series of 25 preflashes and then signal-averaged for 16 pulse pairs. All data points show the mean and standard deviation of four independent measures.

technique. These data strongly support a reaction center based quenching mechanism for the nonphotochemical quenching of PSII-enriched membranes at low pH.

The amplitude of the ΔA_{820} signal used to determine the absorption cross sections increased at low pH (Figure 4). This was due to the longer decay time of the signal at low pH (impaired electron donation to $P680^+$) which allows more of the transient absorbance change to be detected by our limited time resolution (approximately 1 μs) kinetic spectrophotometer. At lower pH, the number of impaired PSII centers increased, and the resulting increase in ΔA_{820} amplitude was correlated with the quenching of F_v as shown in Figure 4. At least part of the microsecond ΔA_{820} signal still present at pH 6.5 is likely due to contaminant PSI. This results in an underestimation of the relative change in amplitude of ΔA_{820} and complicates direct comparisons to the relative change in amplitude of F_v. The ΔA_{820} data confirm previous reports (Meyer et al., 1989) of inhibition of $P680^+$ reduction at low pH and show a correlation between the quenching of F_v and the number of reaction centers containing $P680^+$. Kreiger and Weis (1990) previously showed that preillumination increased the degree of F_v quenching in low-pH samples. We found that a few single-turnover flashes were sufficient to stimulate the F_v quenching at low pH and furthermore that this increased quenching was correlated with an increase in the ΔA_{820} amplitude (data not shown). The preflash treatment did not modify the origin of the low pH induced F_v quenching as judged by the similar effective PSII absorbance cross sections in dark-adapted and preflashed samples (Table 1). These data strongly support the tight correlation observed between F_v quenching and number of PSII centers containing $P680^+$.

Schrieber and Neubauer (1990) have previously proposed that either PSII cyclic electron transport or recombination of $Q_A^-/P680^+$ would be stimulated by an increase in $P680^+$ lifetime and that these are possible mechanisms of non-photochemical quenching at low pH. To differentiate between direct quenching by $P680^+$ and either of these mechanisms, we determined the time course of fluorescence quenching after a single-turnover flash to compare to the kinetics of Q_A^- oxidation.

Kinetics of the Quenching of F_v at Low pH. The time course of fluorescence quenching was measured with the pump probe fluorometer described under Experimental Procedures. Figure 5 compares the amplitude of fluorescence elicited by the probe flash at different time intervals (from 30 μs to 10 ms) after the saturating pump flash at pH 4.0, 4.5, 5.0, and 6.5. In Figure 5, the samples were exposed to a series of 25 Xe preflashes, and the data are the average of the following 16 pump/probe flash pairs. Data collected from dark-adapted samples exposed to only one pump/probe flash pair were similar to those of the preflashed samples (not shown).

The fluoresence is observed to first rise to a maximal level and then decrease as the time interval between pump and probe flash increases. The increase in fluorescence yield on a microsecond time scale reflects the loss of quenching states produced by the actinic flash as previously described by Mauzerall (1972). The fluorescence decay is on a millisecond time scale and follows the kinetics of oxidation of Q_A^-. Due to the impaired acceptor side associated with PSII enriched membrane preparations, this decay is considerably slower than that found in intact thylakoids (Renger et al., 1993). It is clear that most of the quenching of variable fluorescence observed at low pH is observed in the microsecond rise component rather than the millisecond decay component. At pH 5 and 4.5, quenching is dramatic at time intervals less than 1 ms but is completely relieved by 5 ms after the pump flash. The rise and decay kinetics at pH 4.0 are very similar to those at pH 4.5 although there appears to be an additional quenching at all time intervals. The origin of this extra quenching at very low pH is thus not related to long-lived quenching states produced by the actinic flash and may represent a fast (nanosecond) recombination reaction at pH 4. The millisecond fluorescence decay kinetics (which reflect the rate of oxidation of Q_A^-) are unaffected by low pH. These data clearly show that most of the quenching of variable fluorescence at low pH (down to pH 4.5) in PSII-

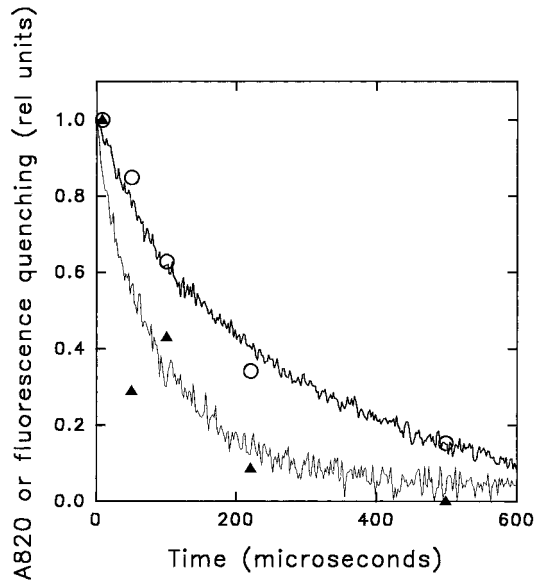

FIGURE 6: Decay kinetics of ΔA_{820} (reduction of $P680^+$) after a single-turnover flash in dark-adapted samples at pH 6.5 (light trace) and pH 4.5 (heavy trace) are compared to the decay of fluorescence quenching after a single-turnover flash in identical samples at pH 6.5 (filled triangles) and pH 4.5 (open circles). Data were normalized to facilitate comparison of decay kinetics. The initial amplitudes of both ΔA_{820} and fluorescence quenching were approximately 2 times higher at pH 4.5 than at pH 6.5.

enriched membranes results from an increase in the lifetime of quenching states produced by the pump flash. The data show that quenching by low pH is not related to an increase in the rate of oxidation of Q_A^-. Models for quenching which depend upon either microsecond or longer recombination between Q_A^- and $P680^+$ or cyclic electron transport around PSII involving Q_A^- predict an acceleration of Q_A^- oxidation at low pH and are therefore not supported by these data. Our results do support the idea of $P680^+$ as quencher at low pH.

In Figure 6, the decay kinetics of ΔA_{820} ($P680^+$) are compared to the decay kinetics of fluorescence quenching at both pH 6.5 and pH 4.5. As expected, the ΔA_{820} decay kinetics were slower at pH 4.5 than pH 6.5, reflecting the inhibition of $P680^+$ reduction at low pH. The remarkable similarity between the decay kinetics of the fluorescence quenching and the decay of ΔA_{820} at both pH values strongly supports the correlation between quenching and the presence of $P680^+$. A tight correlation between the fluorescence quenching and ΔA_{820} decay kinetics was also observed at pH 6.0, 5.5, 5.0, and 4.0 (data not shown).

In summary, our results with fluorescence yield, fluorescence lifetime, absorbance cross sections, and the decay kinetics of fluorescence quenching and $P680^+$ after PSII-turnover strongly support the idea that $P680^+$ quenches fluorescence directly at low pH in PSII enriched thylakoids. Our work confirms earlier suggestions that the inhibition of electron donation to PSII may allow the special pair Chl $P680^+$ or the auxilliary Chl Z^+ to act as a quencher (Horton & Ruban, 1992; Thompson & Brudvig, 1987). We do not suggest that $P680^+$ is responsible for all *q*E quenching but propose that under conditions of low lumenal pH significant quenching by $P680^+$ does occur and is the basis of the "reaction center" based quenching phenomena associated with ΔpH-induced *q*E.

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REFERENCES

Allen, J. F. (1995) *Physiol. Plant. 93*, 196−205.
Aro, E. M., Virgin, I., & Andersson, B. (1993) *Biochim. Biophys. Acta 1143*, 113−134.
Briantais, J.-M., Vernotte, C., Picaud, M., & Krause, G. H. (1979) *Biochim. Biophys. Acta 548*, 128−138.
Brimble, S., & Bruce, D. (1989) *Biochim. Biophys. Acta 973*, 315−323.
Bruce, D., & Miners, J. (1993) *Photochem. Photobiol. 58*, 464−468.
Butler, W. L. (1978) *Annu. Rev. Plant Physiol. 29*, 345−378.
Butler, W. L., & Strasser, R. J. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 3382−3385.
Crofts, A. R., & Yerkes, C. T. (1994) *FEBS Lett. 352*, 265−270.
Crofts, J., & Horton, P. (1991) *Biochim. Biophys. Acta. 1058*, 187−193.
Dau, H. (1994) *Photochem. Photobiol. 60*, 1−23.
Demmig-Adams, B. (1990) *Biochim. Biophys. Acta 1020*, 1−24.
Falk, S., Bruce, D., & Huner, N. P. A. (1994) *Photosynth. Res. 42*, 145−155.
Genty, B., Harginson, J., Briantais, J. M., & Baker, N. R. (1990) *Photosynth. Res. 25*, 249−257.
Ghanotakis, D. F., Babcock, G. T., & Yokum, C. F. (1984) *Biochim. Biophys. Acta 765*, 388−398.
Horton, P., & Ruban, A. V. (1992) *Photosynth. Res. 34*, 375−385.
Krause, G. H., & Behrend, U. (1986) *FEBS Lett. 200*, 298−302.
Krieger, A., Moya, I., & Weis, E. (1992) *Biochim. Biophys. Acta 1102*, 167−176.
Krieger, W., & Weis, E. (1990) in *Current Research in Photosynthesis* (Baltscheffsky, M., Ed.) pp 563−566, Kluwer, Dordrecht, The Netherlands.
Mauzerall, D. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1358−1362.
Mauzerall, D., & Greenbaum, N. L. (1989) *Biochim. Biophys. Acta 974*, 119−140.
Meyer, B., Schlodder, E., Dekker, J. P., & Witt, H. T. (1989) *Biochim. Biophys. Acta 974*, 36−43.
Noctor, G., & Horton, P. (1990) *Biochim. Biophys. Acta 1016*, 228−234.
Quick, W. P., & Horton, P. (1984) *Proc. R. Soc. London, Ser. B 220*, 371−382.
Quick, W. P., & Stitt, M. (1989) *Biochim. Biophys. Acta 977*, 287−296.
Renger, G., Gleiter, H., Haag, E., & Reifarth, C. (1993) *Z. Naturforsch. 48C*, 234−240.
Roelofs, T. A., Lee, C. H., & Holzwarth, A. R. (1992) *Biophys. J. 61*, 1147−1163.
Ruban, A. V., Rees, D., Pascal, A. A., & Horton, P. (1992) *Biochim. Biophys. Acta 1102*, 39−44.
Samson, G., & Bruce, D. (1995) *Biochim. Biophys. Acta 1232*, 21−26.
Samson, G., & Bruce, D. (1996) *Biochim. Biophys. Acta 1276*, 147−153.
Schatz, G. H., Brock, H., & Holzwarth, A. R. (1988) *Biophys. J. 54*, 397−405.
Schreiber, U., & Neubauer, C. (1990) *Photosynth. Res. 25*, 279−293.
Shatz, G., Brock, H., & Holzwarth, A. R. (1988) *Biophys. J. 54*, 397−405.
Thompson, L. K., & Brudvig, G. W. (1988) *Biochemistry 27*, 6653−6658.
Vass, I., Gatzen, G., & Holzwarth, A. R. (1993) *Biochim. Biophys. Acta 1183*, 388−396.
Weis, E., & Berry, J. A. (1987) *Biochim. Biophys. Acta 894*, 198−208.
Zeigler, R., & Egle, K. (1965) *Beitr. Biol. Pflanz. 41*, 11−37.

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