Larsson, S. (1988) *Chem. Scr. 28*, 15–23.
Mitchell, P. (1976) *J. Theor. Biol. 62*, 327–367.
Mitchell, P. (1987) in *Advances in Membrane Biochemistry and Bioenergetics* (Kim, C. H., Tedeschi, H., Diwan, J. J., & Salerno, J. C., Eds.) pp 25–52, Plenum Press, New York.
Moreadith, R. W., & Fiskum, G. (1984) *Anal. Biochem. 137*, 360–367.
Ohnishi, T., & Trumpower, B. L. (1980) *J. Biol. Chem. 255*, 3278–3284.
Ohnishi, T., Schagger, H., Meinhardt, S. W., LoBrutto, R., Link, T. A., & von Jagow, G. (1989) *J. Biol. Chem. 264*, 735–744.
Rich, P. R. (1984) *Biochim. Biophys. Acta 768*, 53–79.
Rieske, J. S. (1976) *Biochim. Biophys. Acta 456*, 195–247.
Robertson, D. E., & Dutton, P. L. (1988) *Biochim. Biophys. Acta 935*, 273–291.
Salerno, J. C., McCurley, J. P., Dong, J.-H., Doyle, M. F., Yu, L., & Yu, C.-A. (1986a) *Biochem. Biophys. Res. Commun. 136*, 616–621.
Salerno, J. C., Yoshida, S., & King, T. E. (1986b) *J. Biol. Chem. 261*, 5480–5486.
Schneider, H., Lemasters, J. J., & Hackenbrock, C. R. (1982) in *Function of Quinones in Energy Conserving Systems* (Trumpower, B. L., Ed.) pp 125–139, Academic Press, New York.
Tisdale, H. D. (1967) *Methods Enzymol. 10*, 213–215.
Trumpower, B. L., & Edwards, C. A. (1979) *J. Biol. Chem. 254*, 8697–8706.
Tsai, A. H., & Palmer, G. (1982) *Biochim. Biophys. Acta 681*, 484–495.
Vanneste, W. H. (1966) *Biochim. Biophys. Acta 113*, 175–178.
von Jagow, G., & Link, T. A. (1986) *Methods Enzymol. 126*, 253–271.
von Jagow, G., Ljungdahl, P. O., Graf, P., Ohnishi, T., & Trumpower, B. L. (1984) *J. Biol. Chem. 259*, 6318–6326.
Weber, S., & Wolf, K. (1988) *FEBS Lett. 237*, 31–34.
Yu, C.-A., & Yu, L. (1980) *Biochemistry 19*, 5715–5720.

Electron-Transfer Reactions in Manganese-Depleted Photosystem II†

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ABSTRACT: We have used flash-detection optical and electron paramagnetic resonance spectroscopy to measure the kinetics and yield per flash of the photooxidation of cytochrome b_{559} and the yield per flash of the photooxidation of the tyrosine residue Y_D in Mn-depleted photosystem II (PSII) membranes at room temperature. The initial charge separation forms $Y_Z^+ Q_A^-$. Following this, cytochrome b_{559} is oxidized on a time scale of the same order and with the same pH dependence as is observed for the decay of Y_Z^+; under the conditions of our experiments, the decay of Y_Z^+ is determined by the lifetime of $Y_Z^+ Q_A^-$. In order to explain this observation, we have constructed a model for electron donation in which Y_Z^+ and $P680^+$ are in redox equilibrium and cytochrome b_{559} and Y_D are oxidized via $P680^+$. Using our results, together with data from earlier investigations of the kinetics of electron transfer from Y_Z to $P680^+$ and charge recombination of $Y_Z^+ Q_A^-$, we have obtained the first global fit for electron donation in Mn-depleted PSII that accounts for the data over the pH range from 5 to 7.5. From these calculations, we have obtained the intrinsic rate constants of all the electron-donation reactions in Mn-depleted PSII. These rate constants allow us to calculate the free energy difference between Y_Z^+ P680 and Y_Z $P680^+$, which is found to increase by 47 ± 4 mV/pH from pH 5 to 6 and is observed to increase more slowly per pH unit for pH > 6. An important conclusion of our experimental work is that the rates of photooxidation of cytochrome b_{559} and Y_D are determined by the lifetime of the oxidizing equivalent on Y_Z/P680. Extension of our model to oxygen-evolving PSII samples leads to the prediction that the kinetics and yields of electron donation from cytochrome b_{559} and Y_D to $P680^+$ will depend on the S_2- or S_3-state lifetime.

The crystal structure of the reaction center from the purple nonsulfur bacterium *Rhodopseudomonas viridis* and its analogy to PSII[1] has significantly advanced our understanding of the electron-transfer pathway from the primary electron donor, P680, to plastoquinone along the electron-acceptor side of PSII (Michel & Deisenhofer, 1988, and references cited therein). However, the analogy between the bacterial reaction center and PSII does not extend to the electron-donor side. The ability of PSII to oxidize water and several components present only in the water oxidation system emphasize the structural and functional differences of electron donation in

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[1] Abbreviations: chl, chlorophyll; cyt b_{559}(ox, red), cytochrome b_{559}(oxidized, reduced); DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; E_m, reduction potential; EPR, electron paramagnetic resonance; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; kDa, kilodaltons; MES, 2-(*N*-morpholino)ethanesulfonic acid; PSII, photosystem II; Tris, tris(hydroxymethyl)aminomethane.

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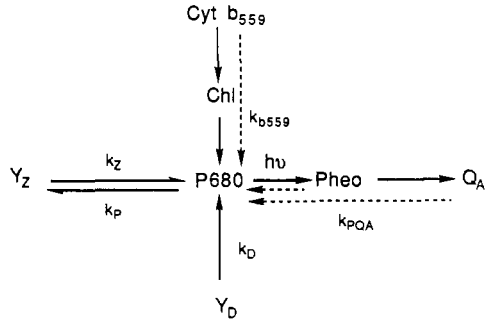
Scheme I: Pathways for Electron Donation to $P680^+$ in Mn-Depleted PSII



the bacterial and PSII reaction centers. In addition to the water oxidation pathway, there are at least two alternative electron donors to $P680^+$—cytochrome b_{559} and a redox-active tyrosine, Y_D (Y160 of the D2 polypeptide). Even the simplest PSII reaction center preparation, consisting of D1/D2/cytochrome b_{559}, contains these two alternative electron donors. Although the photooxidation of both cyt b_{559} and Y_D has been observed under certain conditions, their physiological functions remain unclear [reviewed by Cramer and Whitmarsh (1977)]. The aim of this study is to characterize, in detail, the involvement of cyt b_{559} and Y_D in the electron-donation reactions of PSII.

The present picture of electron donation in PSII includes three distinct electron donors—the Mn complex, cyt b_{559}, and Y_D, all of which ultimately result in the reduction of the oxidized primary electron donor, $P680^+$. Until now it has been difficult to model the electron-transfer reactions in the reaction center due to the lack of information concerning electron donation from cyt b_{559} and Y_D. The simplest model of electron transfer in PSII that is consistent with our kinetic and yield measurements of the photooxidation of cyt b_{559} and Y_D requires four pathways, each of which may reduce $P680^+$ (Scheme I): (1) the Mn path, in which the Mn complex mediates the oxidation of water and a tyrosine residue, Y_Z, acts as an intermediate for electron transfer from Mn to $P680^+$, (2) the cytochrome b_{559} path, in which chlorophyll mediates electron transfer from cytochrome b_{559} to $P680^+$ (Thompson & Brudvig, 1988), (3) the tyrosine Y_D path, in which a tyrosine residue, Y_D, donates electrons to $P680^+$, and (4) charge recombination with the electron acceptor Q_A, or Q_B (Yerkes et al., 1983; Robinson & Crofts, 1983). In this study, we have focused on PSII membranes with extracted or inhibited O_2-evolving complexes. In such centers, Y_Z is still the dominant electron donor to $P680^+$, but now Y_Z^+ is reduced primarily by recombination of $Y_Z^+ Q_A^-$ rather than by electron donation from the Mn complex.

In O_2-inactive PSII, the rate of electron transfer from Y_Z to $P680^+$ is significantly faster ($\approx$3 orders of magnitude; Reinman et al., 1981) than those of the two alternative electron donors, cyt b_{559} and Y_D (Floyd et al., 1971; Boussac & Etiene, 1982). Consequently, the quantum yield of oxidation of cyt b_{559} or Y_D by $P680^+$ is very low in comparison to the quantum yield for formation of Y_Z^+ per reaction center turnover. As a result, a detailed understanding of the electron-transfer events in PSII has been hampered in the past by the lack of sufficient data of the kinetics and yields of electron donation from the two alternative electron donors, cyt b_{559} and Y_D.

In earlier work, photooxidation of cyt b_{559} has only been reported in samples containing lipophilic anions, which are known to bypass or short circuit the physiological electron-transport mechanism in PSII (Ben-Hayyim, 1972; Heber et al., 1979; Velthuys, 1981; Yerkes & Crofts, 1984), in Tris-treated preparations, in which electron transport from Mn to Y_Z is inhibited (Knaff & Arnon, 1969b; Knaff & Arnon, 1970), or at low temperatures (Knaff & Arnon, 1969a). The fact that photooxidation of cyt b_{559} is only observed when electron donation from the Mn complex is prevented or circumvented supports the model to be presented in this paper, in which the cyt b_{559} pathway competes with the water oxidation path in electron donation to $P680^+$.

The photooxidation of Y_D has been studied in some detail in O_2-evolving PSII membranes (Babcock & Sauer, 1973). However, very little information is available in the literature about the room temperature photooxidation kinetics and yields of Y_D in O_2-inactive PSII membranes, even though O_2-inactive PSII can be prepared with most of Y_D reduced in the dark.

The primary objective of this study is to clarify the roles of cyt b_{559} and Y_D in PSII. Since cyt b_{559} is photooxidized by $P680^+$ and reduced by plastohydroquinone ($t_{1/2}$ = 100 ms in chloroplasts at room temperature; Whitmarsh & Cramer, 1978), it has been proposed that cyt b_{559} mediates cyclic electron transfer around PSII. We have suggested that such a cycle of electron transfer involving cyt b_{559} serves to protect PSII from damaging reactions caused by the extremely powerful oxidant $P680^+$ (Thompson & Brudvig, 1988). Y_D^+ has been proposed to function to maintain the Mn complex in a high-valent state and, thereby, prevent inactivation of O_2 evolution by release of Mn^{2+} (Styring & Rutherford, 1987). The first step in the further characterization of the functions of cyt b_{559} and Y_D is to determine the kinetics and yields of the reactions that lead to their photooxidation.

We have used hydroxylamine-treated PSII membranes that lack the Mn complex but retain all of the remaining electron-transfer species in order to determine the kinetics of electron donation of the cyt b_{559} pathway and the tyrosine Y_D path. In the work reported here, we have measured the room temperature rise kinetics and yields for the photooxidation of cyt b_{559} as well as the yield per flash for the photooxidation of Y_D as a function of pH using flash-detection optical and EPR spectroscopy. On the basis of our experimental results, together with kinetic measurements from past studies, we have constructed a kinetic model for the electron-donation reactions in O_2-inactive PSII and obtained a global solution that is consistent with the entire set of data.

Methods

Sample Preparation. PSII membranes were isolated from market spinach leaves by a modified version (Beck et al., 1985) of the isolation procedure described by Berthold et al. (1981). Treatment of PSII membranes with 5 mM NH_2OH (Miller & Brudvig, 1989) resulted in the inactivation of the O_2-evolving complex and partial loss of the 17- and 23-kDa polypeptides. These samples retained less than 10% O_2 evolution activity. The NH_2OH-treated PSII membranes were washed by dilution to 0.2–0.5 mg of chl/mL in a low-salt resuspension buffer (buffer A: 0.5 M sucrose, 50 mM MES, 15 mM NaCl, and 1 mM $CaCl_2$, pH 6.5) followed by centrifugation at 10000*g* for 10 min. The membranes were subsequently stored in the same resuspension buffer at 77 K. All manipulations were performed in dim green light at 4 °C.

Spectroscopic Measurement of the Photooxidation of Cyt b_{559}. The NH_2OH-treated PSII membranes were resuspended to a concentration of 0.5 mg of chl/mL in a buffer consisting of 15 mM NaCl, 2 mM ascorbate (to reduce cyt b_{559}), 20 mM MES, and 30% (v/v) ethylene glycol at pH 6.0, pelleted by centrifugation at 10000*g* for 10 min, and resuspended in the same buffer to a final concentration of 5.3 mg of chl/mL and stored at 77 K.

To measure the amount of dark-oxidized cyt b_{559}, EPR spectra of the NH_2OH-treated PSII membranes were collected before and after 10 min of illumination (700 W/m^2) at 77 K. A sample oxidized with 5 mM K_2IrCl_6 was used as an EPR intensity standard, corresponding to two ferricytochrome b_{559} per reaction center (de Paula et al., 1985). The spectrometer settings were as follows: microwave frequency, 9.1 GHz; microwave power, 0.08 mW; magnetic field modulation frequency, 100 kHz; magnetic field modulation amplitude, 20 G; and sample temperature, 7 K. Integration of the g_z turning point of the EPR signal was used to determine the amount of oxidized cyt b_{559}.

Optical spectroscopy measurements were performed at 25 °C on a flash-detection spectrophotometer similar to that described by Joliot et al. (1980). Continuous illumination was provided by a cluster of four light-emitting diodes (Toshiba TLRA 150C, λ_{max} = 650 nm). Actinic flashes were provided by either a xenon flash lamp (EG&G Model FX 199, 2-μs width at half-height) filtered by a red high band-pass filter (Schott RG 5, >660 nm) or a dye laser (Candela Co. Model SLL-250, 600-ns total duration, dye Oxazine 720, λ_{max} = 699 nm). The dye laser provided saturating actinic flashes, whereas the Xe flashes were ≈80% of saturation. No photooxidation of cyt b_{559} was detected in a test sequence consisting of only detecting flashes (EG&G Model FX-199U) filtered through the monochromator (HL, Jobin-Yvon).

For the determination of the yield of photooxidation per actinic flash as a function of pH, six buffer solutions consisting of 15 mM NaCl, 20 mM MES (for pH < 7), or 20 mM Hepes (for pH ≥ 7) and 30% (v/v) ethylene glycol were prepared and adjusted to pH 5.0, 5.6, 6.2, 6.6, 7.2, and 7.7. All samples were used within a 2-h interval of equilibration to 25 °C and dilution with buffer at any given pH to a concentration of 0.02–0.06 mg of chl/mL. Sample decay was monitored approximately every 20 min by a measurement of the final absorbance change at 560 nm observed during continuous illumination. The maximum absorbance change observed at 560 nm after 2 s of continuous illumination decreased to half its initial amplitude after 73 min at pH 5.0 and after 304 min at pH 6.2. All absorbance measurements were corrected for the amount of sample decay incurred in the time interval between the initial sample dilution and the absorbance measurement.

Chlorophyll concentrations were determined after each set of measurements as described by Vernon et al. (1966). All absorbance measurements were normalized to a chlorophyll concentration of 0.040 mg of chl/mL. An extinction coefficient of 17.5 mM^{-1} cm^{-1} for the oxidized-minus-reduced spectrum at 560 nm (Cramer et al., 1986) and a stoichiometry of 250 chl per PSII (Berthold et al., 1981) were assumed in the quantitation of photooxidized cyt b_{559}.

The kinetic data were analyzed by using a single-exponential curve-fitting routine of the KaleidaGraph program on a Macintosh computer. Each curve was fit to the equation $\Delta I/I = A - A\exp(-kt)$ by repetitive iterations until $R \geq 0.95$, where $\Delta I/I$ is the measured change in absorbance and A is a constant.

Spectroscopic Measurement of the Photooxidation of Tyrosine Y_D. At each of five values of pH (5.0, 6.2, 6.6, 6.7, and 7.7), six EPR samples were prepared. Each set consisted of NH_2OH-treated PSII membranes washed twice by dilution to 0.2–0.5 mg of chl/mL in resuspension buffer A (with adjusted pH), followed by centrifugation at 10000g for 10 min. The membranes were subsequently resuspended to 1.1–1.4 mg of chl/mL in buffer A. Chlorophyll concentrations were assayed by the method of Arnon (1949). After the addition of 0.38 mM ascorbate per mg of chl/mL of PSII, the samples were incubated for 1 h in the dark, on ice. Subsequently, the membranes were loaded into EPR tubes and dark adapted for 3–12 h, after which the samples were frozen to 77 K in complete darkness.

The following experimental procedure was repeated for each set of the samples at any given pH: (1) an EPR scan was taken of each sample in the dark-adapted state to determine the initial amount of Y_D^+; (2) after a 5-min dark equilibration period at 4 °C and a 1-min dark incubation at room temperature, each sample was illuminated by one, two, three, four, or five saturating flashes and immersed in liquid N_2 within 20 s after the last flash; (3) after flash excitation, a second EPR scan was taken to measure the yield per flash(es) of the photooxidation of Y_D; (4) following a second 5-min dark incubation period at 4 °C, each sample was illuminated (800 W/m^2) for 5 min in a transparent dewar filler with ice water, wiped dry, and cooled to 77 K; and (5), finally, a third EPR spectrum was collected to determine the total yield of oxidized Y_D after continuous illumination. Saturating flashes were obtained from a dye laser (Candela Co. Model SLL-66A, 600-ns total duration, dye Rhodamine 590 perchlorate).

The following spectrometer conditions were used for all measurements of the Y_D^+ signal: microwave frequency, 9.1 GHz; microwave power, 0.5 μW; magnetic field modulation frequency, 100 kHz; magnetic field modulation amplitude, 4 G; and sample temperature 15 K.

Results

EPR Characterization of Cytochrome b_{559} and Y_D in NH_2OH-Treated PSII Membranes. In order to quantitatively define the initial redox state of both cyt b_{559} and Y_D in our NH_2OH-treated PSII prep, we measured the amount of dark-oxidized cyt b_{559} and Y_D by low-temperature EPR. 34% of one cyt b_{559} heme equivalent was observed to be oxidized in dark-adapted, ascorbate-treated samples, which is consistent with the decrease in potential of cyt b_{559} caused by treatment with hydroxylamine (Larsson et al., 1984). Hydroxylamine-treated PSII membranes were found to contain a variable amount of Y_D^+ depending on the concentration of reductants and the length of time since the sample was exposed to light. Typically only ≈15% of Y_D remains oxidized after a >3-h dark-adaption period. Measurement of the photoinduced Y_D^+ EPR signal as a function of length of illumination at 273 K has shown that Y_D^+ has reached its maximum level after 5 min of continuous illumination. In addition, our EPR measurements indicate a half-time of ≈10 min for the dark reduction of photoinduced Y_D^+ at 273 K in NH_2OH-treated PSII (data not shown).

Photooxidation of Cytochrome b_{559} in NH_2OH-Treated PSII Membranes. Figure 1 shows the time course of the continuous light-induced absorbance change at 560 nm of NH_2OH-treated PSII membranes at pH 5.0. To ascertain that the absorbance change at 560 nm was due to cyt b_{559}, we stepped through the visible spectrum (390–580 nm) and measured the absorbance change at ≤5-nm intervals induced by continuous illumination. Figure 2 shows the absorbance difference spectrum obtained at room temperature in NH_2OH-treated PSII membranes after 0.8 s of continuous light. The isosbestic points, peaks, and troughs of the reduced minus photooxidized difference spectrum are in good agreement with those found in the photooxidized minus dark reduced spectrum of cyt b_{559} in the double-mutant S56 of the green alga *Chlorella sorokiniana* (Lavergne, 1987). The positions of the maxima and minima are also in accordance with the dithiothreitol-reduced minus ammonium persulfate

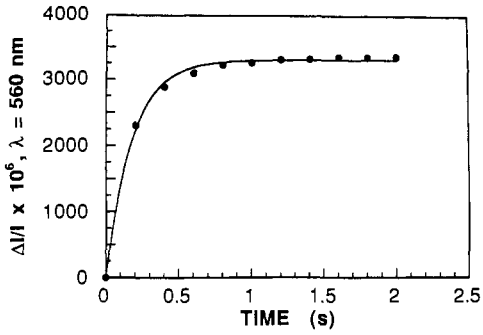


FIGURE 1: Time course of the light-induced absorbance change at 560 nm of dark-adapted NH_2OH-treated PSII membranes (pH 5.0) during 2 s of continuous illumination. The rate of photooxidation of cyt b_{559} obtained from a single-exponential fit to the experimental data is 4.3 ± 1.3 s^{-1}. The data are normalized to a chl concentration of 0.040 mg of chl/mL.

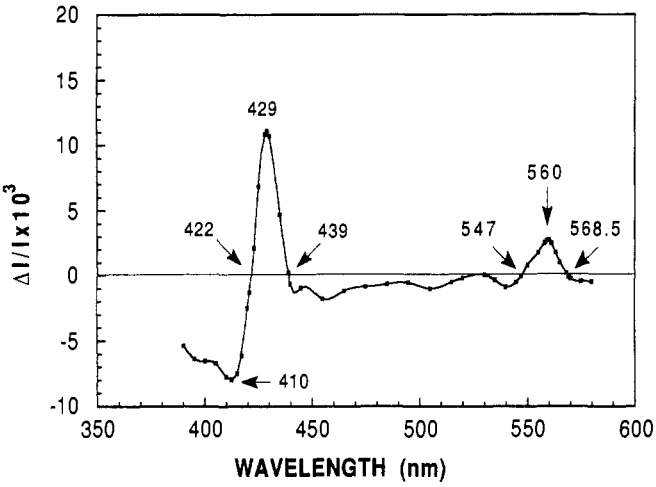


FIGURE 2: Spectrum of the light-induced absorbance change after 0.8 s of continuous illumination of dark-adapted NH_2OH-treated PSII membranes at pH 5.0. At 560 nm, the absorbance change after 0.8 s of continuous illumination is 93% of the maximum absorbance observed after 1.8 s of continuous light (see Figure 1). All data were normalized to a chl concentration of 0.040 mg of chl/mL.

oxidized spectrum of purified spinach cyt b_{559} (Garewal & Wasserman, 1974).

For further characterization of cyt b_{559} in NH_2OH-treated PSII membranes, we compared the light-induced absorbance changes at 560 nm, both in the absence and presence of 100 μM ferricyanide. Ferricyanide ($E_m \approx 415$ mV; Reilly, 1973) is known to readily oxidize cyt b_{559} ($E_m \approx 370$–0 mV, depending on treatment and conditions; Thompson et al., 1989). Addition of 100 μM ferricyanide, followed by a 15-min dark incubation period at room temperature, resulted in a 80% decrease in the yield of cyt b_{559} photooxidation from 5 s of continuous illumination (data not shown).

Two technical considerations of the flash kinetic measurements are the time interval necessary for the dark reoxidation of Q_A^- between flashes and the number of flashes during which the yield per flash of photooxidation of cyt b_{559} remains constant. Both questions are addressed in Figure 3, in which the absorbance change at 560 nm is plotted as a function of flash frequency, t. Within experimental error, the flash yield of photooxidation of cyt b_{559} remains constant during the first four actinic flashes. A spacing between flashes of ≥2 s was needed for the maximal flash yields of photooxidation of cyt b_{559}, due to the time required for reoxidation of Q_A^-. As a result of this measurment, we used a spacing of 5 s between flashes to obtain the highest flash yields in all subsequent repetitive flash experiments.

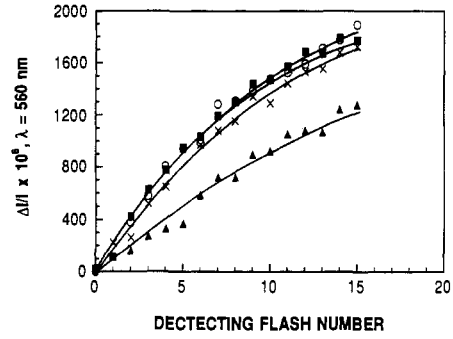


FIGURE 3: Flash-induced absorbance change at 560 nm of dark-adapted NH_2OH-treated PSII membranes at 0.04 mg of chl/mL as a function of flash frequency, t: t = 0.05 s (▲), t = 0.5 s (×), t = 2 s (■), and t = 5 s (○). The illumination sequence consisted of a set of 15 xenon actinic flashes spaced at time intervals of length t, during which $\Delta I/I$ was measured 49 ms after each actinic flash.

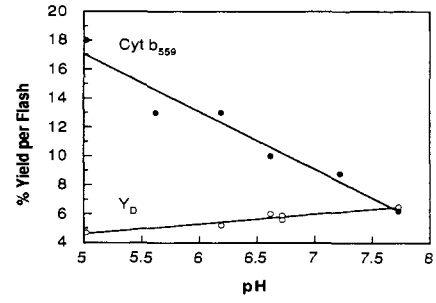


FIGURE 4: pH dependence of the yield per flash of the photooxidation of cyt b_{559} and Y_D. For the measurement of cyt b_{559}, an illumination sequence of four saturating laser flashes at 5-s intervals was employed; $\Delta I/I$ was measured 49 ms after each actinic flash. The average of three measurements was taken for the absorbance change per flash at each pH. Subsequently, the yield per flash was averaged over the first three actinic flashes (at each pH), since the absorbance change was observed to be linear during the first three to four flashes (see Figure 3). Details of the measurement of the yield per flash of the photooxidation of Y_D are provided in the text (see Methods).

Subsequently, we investigated the pH dependence of the yield of photooxidation of cyt b_{559} per saturating flash. The extent of photooxidation of cyt b_{559} was observed to decrease with increasing pH over the range 5.0–7.7, with a maximum yield per flash at pH 5.0 (Figure 4). Whereas the flash yield of photooxidation is clearly pH dependent, the final yield of cyt b_{559} photooxidation by continuous illumination was largely pH independent (data not shown).

The kinetics of cyt b_{559} oxidation following a saturating flash were determined by taking the absorbance difference between a dark adapted and a preilluminated sample. By this method, any absorbance changes not related to cyt b_{559} were eliminated. The illumination procedure for a single measurement included (1) an initial saturating actinic flash followed by a series of detection flashes to monitor the photooxidation of cyt b_{559}, (2) the application of 0.8 s of continuous illumination to completely photooxidize cyt b_{559}, followed by a dark adaption period of 1 min to allow for reoxidation of Q_A^-, and (3) a second saturating actinic flash, again followed by a series of detection flashes (equivalent to that of the first flash sequence) to correct for absorbance changes not related to cyt b_{559}. A possible complication to this procedure is the reduction of cyt b_{559} during the 1-min dark-adaption period. However, preliminary measurements had shown $\tau \gg 1$ min for the dark reduction of cyt b_{559}(ox).

The first actinic flash primarily produces the Y_Z^+ Q_A^- charge separation that, upon equilibration, results in the formation of a small fraction of cyt b_{559}(ox) Q_A^- (see Dis-

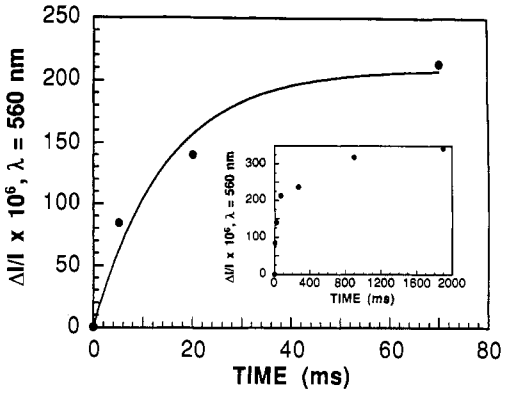


FIGURE 5: Time course of the flash-induced absorbance change at 560 nm in NH_2OH-treated PSII membranes (pH 5.0) by xenon flash excitation. As seen from the inset to Figure 4, the absorbance data are biphasic. However, we have characterized only the fast phase of photooxidation by fitting the data collected in the first 70 ms after the actinic flash to a single exponential curve (see Table I). A fresh, dark-adapted NH_2OH-treated PSII sample was used for each measurement. All data are normalized to a chl concentration of 0.040 mg of chl/mL.

Table I: Kinetic Parameters

reaction	pH	rate (s^{-1})	no. of expts
photooxidation of cyt b_{559}[a]	6.2	54 ± 40	3
	5.0	69	1
recombination of $Y_Z^+ Q_A^-$	5.0	≥19	2

[a] As seen in the inset to Figure 5, the absorbance data for the photooxidation of cyt b_{559} are biphasic. We have characterized only the fast phase of photooxidation by fitting the data collected in the first 70 ms after the actinic flash to a single-exponential curve.

cussion). Subsequently, the continuous illumination period induces the maximal photooxidation of cyt b_{559}. Since the lifetime of cyt b_{559}(ox) is much longer than 1 min, cyt b_{559} remains oxidized during the 1-min dark adaption period and for the remainder of the experiment. As a result, the only product formed by the actinic flash given after continuous illumination is $Y_Z^+ Q_A^-$. Therefore, the difference spectrum of the flash-induced absorbance before and after continuous illumination cancels the contribution at 560 nm of any species, other than cyt b_{559}, to the overall absorbance measurement. This subtraction procedure is only valid if the dark period following the continuous illumination is long enough to allow Q_A^- to be reoxidized to Q_A in all the reaction centers prior to the application of the second actinic flash. In a separate set of experiments, we determined the fractional amount of Q_A^- that did not undergo reoxidation to Q_A prior to the application of the second actinic flash (see below; discussion of Figure 6). Since reaction centers with Q_A reduced are photochemically inactive, the absorbance change as measured by the actinic flash subsequent to continuous illumination was corrected for the percent of nonfunctional reaction centers in all of our single-flash studies.

Figure 5 shows the absorbance change of NH_2OH-treated PSII membranes at pH 5.0 as measured by the three-step illumination procedure. The kinetic data for the single flash-induced photooxidation of cyt b_{559} at pH 5.0 and 6.2 are presented in Table I. To summarize, we have found the rate of photooxidation of cyt b_{559} to be independent of pH (for pH = 5.0–6.2) and of DCMU treatment (data not shown).

As mentioned above, an essential aspect of this measurement is that the dark period following the continuous illumination is of sufficient length for Q_A^- to undergo reoxidation to Q_A in all of the reaction centers prior to the application of the second actinic flash. However, the length of the dark-adaption

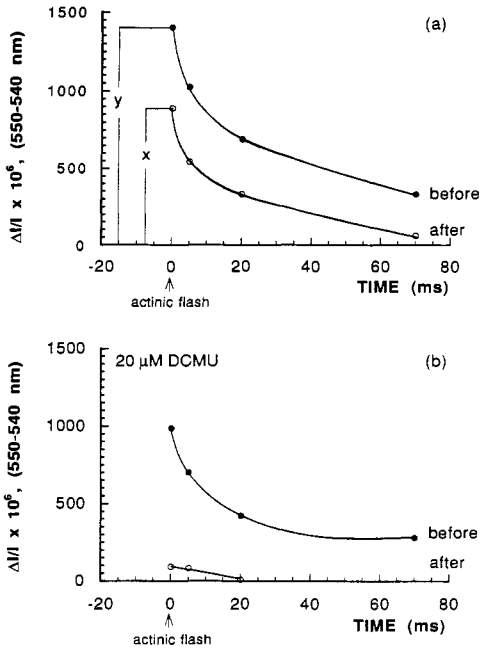


FIGURE 6: Time course of the 550–540-nm difference spectra of the flash-induced absorbance change before (labeled "before") and after (labeled "after") a period of 0.8 s of continuous illumination and 1 min of dark adaption of dark-adapted NH_2OH-treated PSII samples. The same detection flash sequence was used before and after continuous illumination; the first detection flash was given 100 μs after the xenon actinic flash. See Results for a complete description of the illumination procedure. The differences in the absorbance change represented by y and x in (a) represent the fraction of reduced Q_A at 100 μs after the actinic flash before and after a period of 0.8 s of continuous illumination and 1 min of dark adaption, respectively. All data are at pH 5.0 and normalized to a chl concentration of 0.040 mg of chl/mL.

period is limited by the kinetics of the dark rereduction of cyt b_{559} and the inherent rate of aggregation of our samples. The absorbance of C-550 is generally used as an assay for the photoreduction of Q_A [reviewed by Butler (1977)]. In our measurements, we used the absorbance change at 550 nm versus that at 540 nm to check the oxidation state of Q_A immediately after the 1-min dark period following continuous illumination.

A comparison of parts a and b of Figure 6 shows that the kinetics of the absorbance change following the first actinic flash are essentially the same in both the presence and the absence of 20 μM DCMU. Since DCMU is known to block electron transfer from Q_A^- to Q_B, these data suggest that the sharp increase and subsequent decay in the absorbance change is not due to Q_A^- to Q_B electron transfer but, instead, corresponds to the formation and subsequent recombination of the $Y_Z^+ Q_A^-$ primary charge separation. During continuous illumination and in the presence of DCMU, the primary charge separation ($Y_Z^+ Q_A^-$) continues to form and recombine until Q_A is entirely reduced by the formation of a secondary, stable charge separation, at which point no further stable photochemistry occurs. On the basis of the known rates for electron donation to $P680^+$, the only stable charge separations that can occur in the absence of exogenous electron donors are cyt b_{559}(ox) Q_A^- and $Y_D^+ Q_A^-$.

A single-exponential fit to the decay curve of Q_A^- following the first actinic flash on the dark-adapted sample indicates a half-time of 36 ms for the oxidation of Q_A^-. Thus, an upper limit for the rate of recombination of $Y_Z^+ Q_A^-$ in NH_2OH-treated PSII membranes is placed at $t_{1/2} \leq 36$ ms. The quantity $(1 - x/y)$ in Figure 6a is indicative of the number of PSII reaction centers in which Q_A^- is not reoxidized in the

dark period after continuous illumination. Our measurements at 550 and 540 nm indicate that Q_A^- remains reduced after continuous illumination and 1-min dark adaption in 37% of the reaction centers at pH 5.0 in the absence of DCMU (Figure 6a). The long lifetime of Q_A^- is most likely due to a largely reduced PQ_{pool} in our samples. When exposed to light (even dim green light), treatment of PSII with hydroxylamine results in the extensive reduction of the PQ_{pool}, which reoxidizes very slowly (Diner, unpublished results). Therefore, the hydroxylamine treatment, in addition to the presence of ascorbate and the absence of any exogenous or endogenous electron acceptors, is expected to significantly decrease the rate of Q_A^- to Q_B (or Q_B^-) electron transfer.

Figure 6a also shows the effectiveness of the 0.8 s of continuous illumination for the maximal photooxidation of cyt b_{559}. The single-flash, 550 - 540 nm absorbance difference measurement of the dark-adapted sample indicates that a small fraction of Q_A^- is not reoxidized within 70 ms after the first actinic flash given to the dark-adapted samples (curve labeled "before" in Figure 6a). We attribute the fraction of reduced Q_A still present at 70 ms after the actinic flash to the formation of the cyt b_{559}(ox) Q_A^- and Y_D^+ Q_A^- charge-separated states. In contrast, all of Q_A^- induced by the actinic flash given after 0.8 s of continuous light and 1-min dark adaption is entirely reoxidized within 70 ms by the Y_Z^+ Q_A^- recombination reaction (curve labeled "after" in Figure 6a). The result indicates that after preillumination and dark adaption, both secondary electron donors to $P680^+$, cyt b_{559}, and Y_D are oxidized and cannot undergo further photochemistry with Q_A on the time scale of this experiment.

For comparison, we repeated the same 550 - 540 nm measurement as described above in a DCMU-treated sample, shown in Figure 6b. In contrast to the measurement in the absence of DCMU, essentially all (90%) of Q_A remains reduced during the 1-min dark-adaption period in DCMU-treated samples and, subsequently, does not participate in any further photochemistry with Y_Z during the second flash sequence (compare the absorbance change at 100 μs in the two curves labeled "after" in Figure 6). Since only the reaction centers with reoxidized Q_A can turnover in the light, this result indicates that at most 10% of the reaction centers may form the charge separation Y_Z^+ Q_A^- during the second flash sequence. This result places a lower limit on the lifetime of reduced Q_A in DCMU-treated samples, where $\tau > 1$ min. This is consistent with the observation that the half-times for reduction of photooxidized cyt b_{559} and Y_D^+ by Q_A^- are both significantly longer than 1 min, where the lifetime of cyt b_{559}(ox) is significantly greater than 1 min and $t_{1/2}$ for reduction of photooxidized $Y_D^+ \approx 10$ min at 273 K (data not shown).

Finally, in order to quantitate the number of equivalents of cyt b_{559} contributing to the maximum absorbance change at 560 nm, we have measured the absorbance change at 560 nm during continuous illumination both in the absence (pH 5.0, Figure 1) and presence of 20 μM DCMU (pH 5.0, data not shown). Using an extinction coefficient of 17.5 mM^{-1} cm^{-1} for the oxidized minus reduced spectrum of cyt b_{559} at 560 nm and a stoichiometry of 250 chl/PSII (see Methods), we find that $\approx$1.1 equiv of cyt b_{559} per PSII are photooxidized after 1.8 s of illumination in the absence of DCMU.

The same quantitation for cyt b_{559} in reaction centers limited to one turnover by the addition of DCMU is complicated by the fact that the addition of DCMU to centers containing Q_B^- is known to cause Q_A to be reduced in the dark (Velthuys & Amesz, 1974). As a result, it is necessary to first determine the percentage of reaction centers that already have some Q_A reduced in the dark; only centers with oxidized Q_A are capable of turning over in the light and, thus, oxidizing cyt b_{559}. Assuming that all of Q_A is oxidized in the dark in samples not containing DCMU, the 550 - 540 nm difference spectra in the absence and presence of DCMU (Figures 6, parts a and b, respectively) indicate that 30% of Q_A is already reduced in the dark, prior to illumination, in centers containing DCMU. Therefore, in samples with 20 μM DCMU, only 70% of the Q_A is able to form a stable charge separation with cyt b_{559}, and, as a result, at most 70% of one heme equivalent is expected to be photooxidized in these samples. After 1.8 s of continuous illumination, we find that $\approx$58% of the PSII centers have photooxidized one heme in DCMU-treated samples (data not shown). These data indicate that, in the presence of DCMU, the majority of centers produce the stable charge separation, cyt b_{559}(ox) Q_A^-, and that $\approx$12% of Q_A is involved in a stable charge separation other than cyt b_{559}(ox) Q_A^-, most likely Y_D^+ Q_A^-.

Photooxidation of Y_D in NH_2OH-Treated PSII Membranes. To quantitate the yield of Y_D^+ per reaction center turnover as a function of pH, we used EPR to measure the increase in EPR signal II as a function of flash number n, where $n = 1$–5 (see Methods). A flash frequency of 0.2 Hz was used for flash numbers of $n > 1$. By measuring the amount of flash-induced S_2-state multiline EPR signal in O_2-evolving PSII preparations, we determined that the laser light was saturating for chlorophyll concentrations <1.4 mg of chl/mL (data not shown).

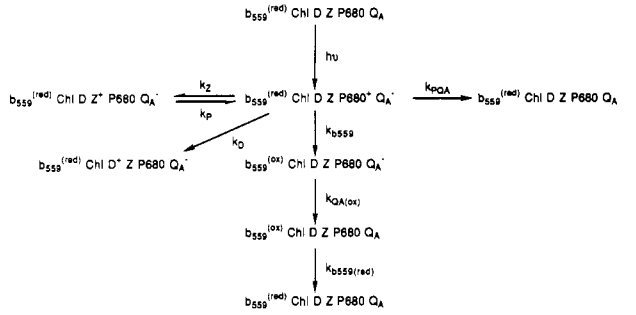
For the analysis, we subtracted the area of signal II present in the initial dark spectrum from the area of signal II observed after n flashes; the difference spectrum was normalized to the area of signal II measured after continuous illumination. Within experimental error, the yield per flash remained constant for at least five flashes (five was the maximum number of flashes applied to any given sample), so the data for the first five flashes were combined to obtain the yield per flash. The flash yield of photooxidation of Y_D increased with increasing pH over the range 5.0–7.7, with a maximum yield per flash at pH 7.7. As seen in Figure 4, the pH trend for the photooxidation of Y_D is opposite to that found for cyt b_{559}.

Discussion

The electron-donation pathways of O_2-inactive PSII are shown in Scheme I. Regardless of whether PSII contains functional O_2-evolving centers, electron donation from the tyrosine residue Y_Z of the Mn pathway to $P680^+$ is the dominant electron-donation reaction. Even in PSII membranes with inactive O_2-evolving centers, electron donation from Y_Z occurs on a microsecond time scale, whereas electron transfer from cyt b_{559} or Y_D proceeds on a millisecond to second time scale. Therefore, the initial charge separation in Mn-depleted PSII is Y_Z^+ Q_A^-. However, we find that photooxidation of cyt b_{559} and Y_D occurs on a much slower time scale than that for the formation of Y_Z^+ Q_A^-. In fact, the rise time for cyt b_{559} photooxidation is on the same time scale as Y_Z^+ Q_A^- recombination. To account for our measurements, we propose the reaction mechanism shown in Scheme II for the photooxidation of the two alternative electron donors, cyt b_{559} and Y_D.

One important issue in defining the kinetic mechanism for the photooxidation of both cyt b_{559} and Y_D (Scheme II) is the determination of the oxidant to each. From our measurements, we cannot easily distinguish between electron transfer from the alternative electron donor to $P680^+$ or to Y_Z^+. However, the slow rate of oxidation of cyt b_{559} and Y_D favors oxidation

Scheme II: Reaction Mechanism for the Photooxidation of Cytochrome b_{559}



by Y_Z^+ or by P680$^+$ in equilibrium with Y_Z^+. Consistent with the latter is the fact that cyt b_{559} is efficiently photooxidized in a mutant lacking Y_Z, which indicates that P680$^+$ can be a direct oxidant of cyt b_{559} (Diner, unpublished results). Moreover, the slow kinetics of cyt b_{559} (this work) and Y_D oxidation (Babcock & Sauer, 1973)[2] and the much more rapid kinetics for P680$^+$ reduction by Y_Z are incompatible with a model in which either alternative electron donor is oxidized prior to reduction of P680$^+$ by Y_Z. In order to account for these observations and our kinetic data, we have used a model in which P680$^+$ Q_A^- and Y_Z^+ Q_A^- exist in rapid equilibrium and, during the lifetime of the charge separation, P680$^+$ acts as an oxidant to cyt b_{559} and to Y_D. An equilibrium state between the two redox couples Y_Z/Y_Z^+ and P680/P680$^+$ has been suggested by several authors in the past (Bouges-Bocquet, 1980, and references cited therein; Yerkes et al., 1983).

Finally, with our measurements and data from previous studies, we now have sufficient information not only to propose a model (Schemes I and II), but, for the first time, the data set is complete enough to obtain a global fit of the kinetics of electron transfer in O_2-inactive PSII membranes.

Scheme I may be rewritten in terms of five rate equations:

$$d[P680^+]/dt = -(k_Z + k_D + k_{b559} + k_{PQA})[P680^+] + (k_P)[Y_Z^+] \quad (1)$$

$$d[Y_Z^+]/dt = (k_Z)[P680^+] - (k_P)[Y_Z^+] \quad (2)$$

$$d[\text{cyt } b_{559}(\text{red})]/dt = -(k_{b559})[P680^+] \quad (3)$$

$$d[Y_D(\text{red})]/dt = -(k_D)[P680^+] \quad (4)$$

$$d[Q_A^-]/dt = -(k_{PQA})[P680^+] \quad (5)$$

Solving the coupled set of differential equations yields the following solution for the concentration of P680$^+$ over time:

$$[P680^+] = C_1 \exp(-k_x t) + C_2 \exp(-k_y^* t) \quad (6)$$

where C_1 and C_2 are positive coefficients defined as

$$C_1 = (a - k_y)/(k_x - k_y) \quad (7)$$

$$C_2 = 1 - C_1 \quad (8)$$

and the rate constants k_x and k_y are given by the two solutions of the quadratic equation

$$k_x, k_y = \{(a + b) \pm [(a + b)^2 - 4(ab - bc)]^{1/2}\}/2 \quad (9)$$

The constants a, b, and c in eqs 7–9 represent the following collection of rate constants:

$$a = (k_Z + k_D + k_{b559} + k_{PQA}) \quad (10)$$

$$b = k_P \quad (11)$$

$$c = k_Z \quad (12)$$

The relative concentrations of the redox-active species are normalized on a scale from 0.0 to 1.0, where at $t = 0$, [P680$^+$] = 1.0, [Y_Z^+] = 0.0, [cyt b_{559}(red)] = 1.0, [Y_D(red)] = 1.0, and [Q_A^-] = 1.0. For the decay and rise times of P680$^+$ and Y_Z^+, respectively, and for the subsequent decay of Y_Z^+, we have used the data for Y_Z to P680$^+$ electron donation and Y_Z^+ Q_A^- charge recombination in Tris-treated PSII (Reinman et al., 1981; Dekker et al., 1984). For the final yields per flash for the photooxidation of cyt b_{559} and Y_D, we have used the information provided by this study (Figure 4).

The rate of P680$^+$ Q_A^- charge recombination has been determined previously (Conjeaud & Mathis, 1980; Reinman et al., 1981); however, these measurements are somewhat complicated due to competing electron donation from Y_Z. Commonly, two closely spaced actinic flashes are used to measure the rate of decay of P680$^+$ by recombination with Q_A^-; the time interval between the two flashes is set such that Y_Z^+ remains oxidized and Q_A^- is reoxidized during the time between flashes (generally, exogenous electron acceptors, such as ferricyanide, are present). With the proper time interval between the two flashes, the first flash results in the formation of the Y_Z^+ P680 Q_A^- charge separation and the second flash induces the state Y_Z^+ P680$^+$ Q_A^-. The rate of P680$^+$ Q_A^- recombination is obtained by measuring the time course of the absorbance change corresponding to the decay of P680$^+$ after the second flash. One problem that is not addressed in these measurements is the effect of the positive charge on Y_Z on the charge recombination of P680$^+$ Q_A^-; a positive charge in the vincinity of P680 may be expected to enhance the rate of recombination. In addition, it appears inevitable that at least in a small fraction of the reaction centers, Y_Z^+ is rereduced prior to the second flash; again, the presence of Y_Z would lead to an enhancement of the observed rate of decay of P680$^+$ after the second flash. To circumvent these potential problems, the rate of P680$^+$ Q_A^- charge recombination was measured in the Y_Z-deficient mutant of *Synechocystis* PC 6803 (D1-Y161F) by flash-detection optical spectroscopy (Diner, unpublished results). In agreement with previous results, the major phase of the recombination rate (85%) was observed to be independent of pH over the range 5.1–9.0. Measurement of the absorbance change at 432 nm following a saturating flash indicates $t_{1/2}$ = 810–930 μs for the major phase and $t_{1/2} \approx$ 10–130 ms for the minor phase of decay of P680$^+$ by charge recombination over the given pH range. In contrast, using the two-flash method, Reinman et al. (1981) have determined $t_{1/2}$ = 120 μs for the recombination of P680$^+$ Q_A^- in Tris-treated PSII membranes. We suggest that the presence of Y_Z^+ in the vicinity of P680$^+$ is the cause of the significantly enhanced the rate of P680$^+$ Q_A^- in these samples. On the basis of the measurements in the Y_Z-deficient PSII mutant, we have imposed the following limits on the intrinsic rate of P680$^+$ Q_A^- recombination: 7.4×10^{-4} μs^{-1} ≤ k_{PQA} ≤ 8.7×10^{-4} μs^{-1}.

By successive reiterations through five nested loops, we have obtained successful fits to the experimental data over the pH range 5.0–7.5. A more detailed account of the model calculations will be provided in a forthcoming publication.

At pH 5.0, 6.0, 7.0, and 7.5, we have calculated both the intrinsic rate constant for each electron-donation reaction in the reaction center and the relative concentration over time

[2] Babcock and Sauer (1973) report a half-time of ≈1 s for the room temperature photooxidation of Y_D after one actinic flash in oxygen-evolving thylakoid membranes. On the basis of this measurement and our model calculations, we infer that the rate of photooxidation of Y_D occurs on a similar time scale (millisecond to second) in O_2-inactive PSII membranes.

Table II: Summary of Model Calculations for the Intrinsic Rate Constants (Scheme I) as a Function of pH

rate (μs^{-1})[a]	pH 5.0	pH 6.0	pH 7.0	pH 7.5
k_Z	4.4×10^{-2}	$(7.7–8.4) \times 10^{-2}$	$(1.5–1.6) \times 10^{-1}$	$(2.0–2.1) \times 10^{-1}$
k_P	2.5×10^{-3}	$(6.5–9.0) \times 10^{-4}$	$(6.0–8.0) \times 10^{-4}$	$(3.0–4.0) \times 10^{-4}$
k_{PQA}	$(7.4–8.7) \times 10^{-4}$	$(7.4–8.7) \times 10^{-4}$	$(7.4–8.7) \times 10^{-4}$	$(7.4–8.7) \times 10^{-4}$
k_{b559}	2.0×10^{-4}	$(1.2–1.4) \times 10^{-4}$	$(0.70–1.0) \times 10^{-4}$	$(6.0–8.0) \times 10^{-5}$
k_D	6.5×10^{-5}	$(3.6–6.1) \times 10^{-5}$	$(5.0–7.0) \times 10^{-5}$	$(5.0–7.0) \times 10^{-5}$
K_{eq} ($=k_Z/k_P$)	18	93–120	190–270	500–700

[a] See Scheme I for definition of the rate constants.

of each redox-active species. If an effective rate constant had not been determined specifically at one of these pH values, it was obtained by interpolation. Table II summarizes the calculated intrinsic rate constants and the equilibrium constant ($K_{eq} = k_Z/k_P$).

Several important observations can be made at this point: the most significant of which is that these intrinsic rate constants, collectively, represent the first global solution consistent with the entire set of experimental parameters for the electron-donation reactions in O_2-inactive PSII reaction centers. Second, these calculations support a model in which the yields and kinetics of the two alternative electron donors are determined by the equilibrium between Y_Z P680$^+$ and Y_Z^+ P680. Knowledge of the intrinsic rate constants allows the calculation of the equilibrium constant between Y_Z^+ P680 and Y_Z P680$^+$, where $K_{eq} = k_Z/k_P$. By use of K_{eq} to calculate the free energy difference ($\Delta G°$) between Y_Z^+ P680 and Y_Z P680$^+$, $\Delta G°$ is observed to increase by $\approx 47 \pm 4$ mV/pH unit from pH 5 to 6 but is found to increase to a lesser extent at higher pH.

An additional observation from the calculations is that only the forward (k_Z) and the backward (k_P) rates of electron transfer in the equilibrium reaction between Y_Z^+ and P680$^+$ show significant pH dependence. In agreement with Yerkes et al. (1983), we also find that the increase in k_Z with pH is 1.7–1.9-fold rather than 10-fold (as would be expected for a single protonation event), suggesting that the pH dependence of this reaction is likely due to the protonation and deprotonation of groups not closely associated with Y_Z or P680. Yerkes et al. (1983) have also suggested an equilibrium between Y_Z and P680 to deconvolute their measurements of the decay of Y_Z^+ in Tris-treated PSII reaction centers. The rates for k_Z and k_P calculated by Yerkes et al. (1983) are on the same order of magnitude as determined in our model calculations. One point of discrepancy between our calculations and those of Yerkes et al. (1983) is the magnitude of K_{eq}. The reason for this disagreement is the value of k_{PQA} (the rate of recombination of P680$^+$ Q_A^-) used in each calculation; Yerkes et al. (1983) have used $k_{PQA} = 3.5 \times 10^{-3}$ μs^{-1}, whereas we have used $k_{PQA} = (7.4–8.7) \times 10^{-4}$ μs^{-1}. By substituting a rate of recombination (k_{PQA}) on the order of the one used by Yerkes et al. (1983) into our model calculations, we find a K_{eq} on the same order of magnitude as that found by Yerkes et al. (1983). In contrast to k_Z and k_P, our model calculations suggest that k_{b559} is only slightly pH dependent (k_{b559} increases by approximately a factor of 3 over the pH range 5.0–7.5) and k_D is pH independent over the given pH range.

With this understanding of electron donation in Mn-depleted PSII membranes, we can now proceed to characterize the kinetics of the cyt b_{559} and Y_D pathways in O_2-evolving samples. A significant result of our experimental work and a key aspect of our model of PSII is the redox equilibrium between Y_Z^+ and P680$^+$. By analogy, we suggest that the Mn-complex is also in redox equilibrium with Y_Z^+ as has been previously proposed by Bouges-Bocquet (1980). In O_2-evolving PSII membranes, Y_Z^+ is rapidly reduced by the Mn complex. Due to the short lifetimes of P680$^+$ and Y_Z^+, no fast photooxidation of cyt b_{559} or Y_D is expected immediately after one actinic flash. However, during the dark period after the actinic flash, we expect to observe oxidation of cyt b_{599} and Y_D with a rise time that corresponds to the decay of the S_2- and S_3-states [$t_{1/2}$(fast phase) = 1.4 and 1.6 s, respectively (Vermaas et al., 1984)]. In good agreement with this prediction is the observed rise time of Y_D^+ in O_2-evolving PSII, where $t_{1/2}$ of photooxidation of Y_D is $\approx$1 s in the S_2- and S_3-states (Babcock & Sauer, 1973; Velthuys & Visser, 1975). In contrast to the change in kinetics, we expect that the yield of cyt b_{559} and Y_D oxidation from a single flash in O_2-evolving PSII to be the same as in a Mn-depleted PSII preparation. Using a similar approach as presented in this study, we are currently investigating the single-flash kinetics and yields for the room temperature photooxidation of cyt b_{559} in O_2-evolving PSII samples.

Registry No. Cyt b_{559}, 9044-61-5; P680, 53808-91-6; Y, 60-18-4.

References

Arnon, D. I. (1949) *Plant Physiol. 24*, 1.

Babcock, G. T., & Sauer, K. (1973) *Biochim. Biophys. Acta 325*, 504.

Beck, W. F., de Paula, J. C., & Brudvig, G. W. (1985) *Biochemistry 24*, 3035.

Ben-Hayyim, G. (1972) *FEBS Lett. 28*, 145.

Berthold, D. A., Babcock, G. T., & Yocum, C. F. (1981) *FEBS Lett. 134*, 231.

Bouges-Bocquet, B. (1980) *Biochim. Biophys. Acta 594*, 85.

Boussac, A., & Etienne, A. L. (1982) *Biochem. Biophys. Res. Commun. 109*, 1200.

Butler, W. L. (1977) in *Encyclopedia of Plant Physiology, V* (Trebst, A., & Avron, M., Eds.) p 149, Springer-Verlag, Berlin.

Conjeaud, H., & Mathis, P. (1980) *Biochim. Biophys. Acta 590*, 353.

Cramer, W. A., & Whitmarsh, J. (1977) *Annu. Rev. Plant Physiol. 28*, 133.

Cramer, W. A., Theg, S. M., & Widger, W. R. (1986) *Photosynth. Res. 10*, 393.

Dekker, J. P., Van Gorkom, H. J., Brok, M., & Ouwehand, L. (1984) *Biochim. Biophys. Acta 764*, 301.

de Paula, J. C., Innes, J. B., & Brudvig, G. W. (1985) *Biochemistry 24*, 8114.

Floyd, R. A., Chance, B. & Devault, D. (1971) *Biochim. Biophys. Acta 226*, 103.

Garewal, H. S., & Wasserman, A. R. (1974) *Biochemistry 13*, 4063.

Heber, U., Kirk, M. R., & Boardman, N. K. (1979) *Biochim. Biophys. Acta 546*, 292.

Joliot, P., Béal, D., & Frilley (1980) *J. Chim. Phys. 77*, 209.

Knaff, D. B., & Arnon, D. I. (1969a) *Proc. Natl. Acad. Sci. U.S.A. 63*, 956.

Knaff, D. B., & Arnon, D. I. (1969b) *Proc. Natl. Acad. Sci. U.S.A. 64*, 715.

Knaff, D. B., & Arnon, D. I. (1970) *Biochim. Biophys. Acta 223*, 201.

Larsson, C., Jansson, C., Ljungberg, U., Åkerlund, H.-E., & Andersson, B. (1984) in *Advances in Photosynthesis Research, I* (Sybesma, C., Ed.) p 363, Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht, The Netherlands.
Lavergne, J. (1987) *Biochim. Biophys. Acta 894*, 91.
Michel, H., & Deisenhofer, J. (1988) *Biochemistry 27*, 1.
Miller, A.-F., & Brudvig, G. W. (1989) *Biochemistry 29*, 1385.
Reilly, J. E. (1973) *Biochim. Biophys. Acta 292*, 509.
Reinman, S., Mathis, P., Conjeaud, H., & Stewart, A. (1981) *Biochim. Biophys. Acta 635*, 429.
Robinson, H. H., & Crofts, A. R. (1983) *FEBS Lett. 153*, 221.
Styring, S., & Rutherford, A. W. (1987) *Biochemistry 26*, 2401.
Thompson, L. K., & Brudvig, G. W. (1988) *Biochemistry 27*, 6653.
Thompson, L. K., Miller, A.-F., Buser, C. A., de Paula, J. C., & Brudvig, G. W. (1989) *Biochemistry 28*, 8048.
Velthuys, B. R. (1981) *FEBS Lett. 126*, 272.
Velthuys, B. R., & Amesz, J. (1974) *Biochim. Biophys. Acta 333*, 85.
Velthuys, B. R., & Visser, J. W. M. (1975) *FEBS Lett. 55*, 109–112.
Vermaas, W. F. J., Renger, G., & Dohnt, G. (1984) *Biochim. Biophys. Acta 764*, 194.
Vernon, L. P., Shaw, E. R., & Ke, B. (1966) *J. Biol. Chem. 241*, 4104.
Whitmarsh, J., & Cramer, W. A. (1978) *Biochim. Biophys. Acta 501*, 83.
Yerkes, C. T., & Crofts, A. R. (1984) in *Advances in Photosynthesis Research, I* (Sybesma, C., Ed.) p 489, Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht, The Netherlands.
Yerkes, C. T., Babcock, G. T., & Crofts, A. R. (1983) *FEBS Lett. 158*, 359.

Structure of the Retinal Chromophore in 7,9-*dicis*-Rhodopsin†

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ABSTRACT: Bovine rhodopsin was bleached and regenerated with 7,9-*dicis*-retinal to form 7,9-*dicis*-rhodopsin, which was purified on a concanavalin A affinity column. The absorption maximum of the 7,9-dicis pigment is 453 nm, giving an opsin shift of 1600 cm^{-1} compared to 2500 cm^{-1} for 11-*cis*-rhodopsin and 2400 cm^{-1} for 9-*cis*-rhodopsin. Rapid-flow resonance Raman spectra have been obtained of 7,9-*dicis*-rhodopsin in H_2O and D_2O at room temperature. The shift of the 1654-cm^{-1} C=N stretch to 1627 cm^{-1} in D_2O demonstrates that the Schiff base nitrogen is protonated. The absence of any shift in the 1201-cm^{-1} mode, which is assigned as the C_{14}–C_{15} stretch, or of any other C–C stretching modes in D_2O indicates that the Schiff base C=N configuration is trans (anti). Assuming that the cyclohexenyl ring binds with the same orientation in 7,9-*dicis*-, 9-*cis*-, and 11-*cis*-rhodopsins, the presence of two cis bonds requires that the N–H bond of the 7,9-dicis chromophore points in the opposite direction from that in the 9-cis or 11-cis pigment. However, the Schiff base $C=NH^+$ stretching frequency and its D_2O shift in 7,9-*dicis*-rhodopsin are very similar to those in 11-*cis*- and 9-*cis*-rhodopsin, indicating that the Schiff base electrostatic/hydrogen-bonding environments are effectively the same. The C=N trans (anti) Schiff base geometry of 7,9-*dicis*-rhodopsin and the insensitivity of its Schiff base vibrational properties to orientation are rationalized by examining the binding site specificity with molecular modeling.

Vertebrate visual pigments contain an 11-*cis*-retinal chromophore bound via a protonated Schiff base linkage to a specific lysine residue of the ~41 000-dalton apoprotein opsin (Birge, 1981). The absorption maxima of these pigments range from 440 to 580 nm (Lythgoe, 1972). The amino acid sequences of a number of opsins (Hargrave et al., 1983; Nathans et al., 1986; Ovchinnikov, 1982) have made it possible to identify protein perturbations that may be responsible for this broad range of absorption maxima (Kosower, 1988; Loppnow et al., 1989; Nathans et al., 1986). The primary event in vision is an isomerization around the C_{11}=C_{12} bond of the chromophore to form a twisted all-trans photoproduct (Eyring et al., 1980; Hubbard & Kropf, 1958; Kandori et al., 1989b; Yoshizawa & Wald, 1963). Although bathorhodopsin had been thought to be the first intermediate, several reports have indicated that there is an intermediate prior to bathorhodopsin (Kobayashi, 1980; Peters et al., 1977; Shichida et al., 1984), and Kandori et al. (1989a) and Shichida et al. (1984) have identified photorhodopsin as the first one-photon photoproduct. The specific protein–chromophore interactions which dictate

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