Electron Paramagnetic Resonance Kinetic Studies of the S States in Spinach Thylakoids†

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ABSTRACT: The Tyr_z^+ decay kinetics have been analyzed by using time-resolved EPR to determine the half-time of each $S_i \rightarrow S_{(i+1)}$ transition in the O_2-evolving complex of spinach thylakoids under physiological conditions. Using dark-adapted thylakoids and appropriate single-turnover flash sequences, we were able to detect the signal II_{vf} kinetics of the $Tyr_z^+ S_0 \rightarrow Tyr_z S_1$, $Tyr_z^+ S_1 \rightarrow Tyr_z S_2$, $Tyr_z^+ S_2 \rightarrow Tyr_z S_3$, and $Tyr_z^+ S_3 \rightarrow (S_4) \rightarrow Tyr_z S_0$ transitions. To correct for damping of the S state synchronization during the flash sequence, the Kok parameters were estimated by measuring the oxygen flash pattern in situ using nitroxide-based EPR oximetry. Following deconvolution of the individual S state contributions, the signal II_{vf} decay kinetics yield the following half-times for the S state transitions: $S_0 \rightarrow S_1$ in 40−60 μs, $S_1 \rightarrow S_2$ in 85 μs, $S_2 \rightarrow S_3$ in 140 μs, and $S_3 \rightarrow (S_4) \rightarrow S_0$ in 750 μs. Preliminary results with detergent-solubilized PSII membranes suggest that the $S_3 \rightarrow S_0$ transition at least is slowed by a factor of ~2 in this system. Ramifications of these half-times in terms of electron transfer events on the donor site of PSII are discussed.

In photosystem II (PSII of chlorophyll *a*-containing plants), water is oxidized to oxygen by a tetranuclear manganese cluster in which the four required oxidizing equivalents are sequentially accumulated from the primary electron donor (P680). Since one oxidizing equivalent is stored after each photoreaction, an oscillation pattern with a periodicity of four is observed in oxygen evolution using short single-turnover saturating flashes. The formal redox states of the manganese cluster are known as the S states, labeled S_0−S_4, where the index represents the number of oxidizing equivalents stored (Kok et al., 1970). S_0 and S_1 are dark stable states, while S_2 and S_3 deactivate to S_1 upon dark incubation (Kok et al., 1970). The S_4 state is metastable and spontaneously converts to S_0, accompanied by the release of an oxygen molecule. A tyrosine residue, Tyr_z, is an intermediate electron carrier which transfers the oxidizing equivalents from $P680^+$ to the Mn cluster (Debus et al., 1988; Metz et al., 1989). Thus, the reaction may be written as $S_i + Tyr_z^+ \rightarrow S_{(i+1)} + Tyr_z$ (Hoganson & Babcock, 1988).[2] Since the rate of Tyr_z^+ re-reduction is S state-dependent, the half-time of each $S_i \rightarrow S_{(i+1)}$ transition can therefore be obtained by measuring the Tyr_z^+ re-reduction kinetics.

Previous measurements of the S state transition times have been made using EPR (Babcock et al., 1976; Cole & Sauer, 1987; Hoganson & Babcock, 1988) and absorption spectroscopy techniques (Velthuys, 1981; Dekker et al., 1984; Lavergne, 1984; Renger & Weiss, 1986; Saygin & Witt, 1987; Van Leeuwen et al., 1993; Rappaport et al., 1994). However, there is considerable disagreement between groups concerning the turnover kinetics in the various S states, and there is as yet no general agreement (Table 1). In particular, the early transitions ($S_0 \rightarrow S_1$ and $S_1 \rightarrow S_2$) have not been well resolved by EPR, due to instrument response time limitations and the difficulty of preparing a sample with a sufficiently high initial S_0 population (Hoganson & Babcock, 1988).

Examination of Table 1 shows that there is reasonable consensus on the slowest, most easily measured transition, i.e. $S_3 \rightarrow S_0$. The kinetics of this transition appear to become progressively slower, from <1 to over 4 ms, as the biochemical preparation becomes enriched in PSII (from chloroplasts through to core particles). There is also a clear trend of the transition kinetics becoming monotonically faster in the earlier S states, although the spread of the estimated half-times also increases. The largest uncertainty is found for the $S_0 \rightarrow S_1$ transition. This transition has not previously been resolved by EPR, and this failure has been rationalized on the basis of its expected speed (~50 μs), as inferred from optical measurements. These latter are challenging, as there is no unique S state optical absorption signal, only broad probably Mn-derived bands in the UV range below 300 nm. These must be kinetically deconvoluted from the period two oscillation in UV absorbance due to acceptor side processes, as well as from the inevitable S state mixing which occurs through imperfect flash advancement due to double turnovers (β) and misses (α) (Kok et al., 1970; Joliot & Kok, 1975). Despite these difficulties, there was until recently a rough consensus from (inferred) EPR results and the optical data of several groups that the $S_0 \rightarrow S_1$ transition was the fastest, with a $t_{1/2}$ of <100 μs. However, Rappaport et al. (1994) have recently presented a very detailed optical study which suggests that the $S_0 \rightarrow S_1$ transition is in fact unexpectedly

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[1] Abbreviations: EPR, electron paramagnetic resonance; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; OEC, oxygen-evolving complex; PBQ, *p*-benzoquinone; PPBQ, phenyl-*p*-benzoquinone; PDT, ^{15}N-perdeuterated tempone; PSI, photosystem I; PSII, photosystem II; Tris, tris(hydroxymethyl)aminomethane.

[2] Although the oxidized form of Tyr_z is usually written Tyr_z^+, the radical is in fact the neutral, deprotonated species (Hoganson et al., 1995).

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Table 1: Previously Reported Half-Times for PSII S State Advancement following Single-Turnover Flash[a]

$t_{1/2}$ (μs) $S_0 \rightarrow S_1$	$t_{1/2}$ (μs) $S_1 \rightarrow S_2$	$t_{1/2}$ (μs) $S_2 \rightarrow S_3$	$t_{1/2}$ (μs) $S_3 \rightarrow (S_4) \rightarrow S_0$	ref
≤100	≤100	≈400	≈1000	Babcock et al., 1975 chloroplasts (EPR)
	~70		~1000	Velthuys, 1981 chloroplasts
30 ± 10	110 ± 20	350 ± 50	1300	Dekker et al., 1984 PSII preparations
	30−40			Lavergne, 1984 *Chlorella sorokiniana*
40 ± 20	110 ± 30	220 ± 40	1200 ± 200	Renger & Weiss, 1986 PSII membrane fragments
		600	1300	Cole & Sauer, 1987 PSII preparations (EPR)
	60	60	800	Koike et al., 1987 PSII particles of thermophilic cyanobacterium (50 °C)
50	40	110 or 220	1500	Saygin & Witt, 1987 cyanobacteria PSII particles
	100	325		Hoganson & Babcock, 1988 PSII membranes (EPR)
<3	95	380	4500	Van Leeuwen et al., 1993 PSII core particles
250	55	290	1200	Rappaport et al., 1994 PSII membranes (pH 6.5)

[a] Includes results for higher plants and photosynthetic bacteria. Data from optical or EPR kinetic measurements.

slow, with a $t_{1/2}$ of 250 μs, although their kinetic estimates for the other three transitions were in general accord with earlier results. This is potentially a very significant observation, because a 250 μs $S_0 \rightarrow S_1$ transition should have been resolvable in earlier EPR studies of Tyr_z^+ reduction. The failure to observe such a slow kinetic could mean that the Tyr_z^+ EPR signal is for some reason broadened or absent in the S_0 state, which would have important implications for recent models of Tyr_z function in the water oxidation chemistry (Hoganson et al., 1995).

Compared to optical absorbance techniques, the measurement of Tyr_z^+ decay kinetics by EPR provides unambiguously the S state half-times, as the Tyr_z^+ EPR signal can be detected at room temperature without interference from other photosystem signals (Blankenship et al., 1975; Warden et al., 1976). In intact systems, the signal is conventionally called signal II_{vf} (very fast). In manganese-depleted PSII samples, the Tyr_z^+ EPR signal is designated signal II_f (fast). There is another photooxidizable, spectroscopically similar tyrosine in PSII, Tyr_d. This does not participate in normal turnover and rapidly attains a state of ~100% oxidation during illumination. Its EPR signal is called signal II_s (slow).

In this study, the S state half-times have been determined by measuring the decay rates, at high (10 μs) time resolution, of signal II_{vf} in different S states using intact thylakoids as the physiologically active material. For deconvolution of the individual S state contributions, nitroxide-based EPR oximetry (Strzalka et al., 1990) was used to measure the flash-induced O_2 release pattern. This allows determination of both the initial S_0/S_1 distribution and Kok parameters (α and β) under exactly the same experimental conditions as used for the signal II_{vf} decay kinetic determinations.

We report the first detection of the $S_0 \rightarrow S_1$ transition by EPR and show that Tyr_z^+ is indeed visible, with the same apparent intensity in this transition as in the other three transitions. The kinetics of the $S_0 \rightarrow S_1$ transition are not anomalously slow but are indeed the fastest of all the S state transitions.

MATERIALS AND METHODS

Thylakoid membranes were isolated from market spinach. About 400 g of leaves was deveined and homogenized in 50 mM HEPES-NaOH (pH 7.5), 5 mM $MgCl_2$, 50 mM NaCl, and 0.3 M mannitol. The homogenate was strained through eight layers of cheesecloth and one layer of nylon mesh (35 μm) and centrifuged at 1000*g* for 10 min. The pellet was resuspended in 50 mM HEPES-NaOH (pH 7.5), 5 mM $MgCl_2$, and 50 mM NaCl to give an osmotic shock and centrifuged at 1500*g* for 10 min. The pellet was finally resuspended in the homogenizing buffer and the chlorophyll concentration adjusted to 1.5 mg of Chl/mL. For Tris-washed samples, the thylakoids were incubated in 200 mL of 0.8 M Tris (pH 8.0) for 20 min instead of the osmotic shock stage. All preparation steps were performed at 4 °C, and the chlorophyll concentration was determined by the method of Porra et al. (1989).

The O_2 evolution activity was measured with a Clark type electrode in 50 mM HEPES-NaOH (pH 7.5), 5 mM $MgCl_2$, 50 mM NaCl, 0.3 M mannitol, 0.25 mM PPBQ, 0.5 mM $K_3Fe(CN)_6$, and 5 mM NH_4Cl as the assay mixture. The chlorophyll concentration during the assay was about 20 μg/mL. The steady state rates of oxygen evolution were typically between 220 and 260 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}. No change in the oxygen activity was observed during storage of thylakoids at 4 °C for the duration of signal II_{vf} measurements (several hours).

PSII membranes were prepared by the method of Ghanotakis et al. (1984), but the Triton X-100 level was decreased by 50%. We found that, with the reduction of the Triton level, the amount of uncoupled inactive centers was substantially decreased, with not more than 10% PSI contamination.

All EPR measurements were carried out at 8−10 °C on a Bruker ESP 300E spectrometer equipped with a TM011 cavity. A Gilson minipulse 3 pump was used to control the flow of the sample through the suprasil quartz flat cell from a long tube acting as a reservoir and kept in a water bath at 4 °C in the dark. Saturating 10 μs xenon flashes from an EG&G electro-optics flash lamp were used to excite the sample. Optical fiber was used as a light guide to illuminate the sample directly in the EPR cavity.

^{15}N-perdeutrated tempone (PDT) (from C/D/N isotopes, Quebec, Canada) was used as an oxygen probe to measure the flash-induced O_2 release [see Strzalka et al. (1990)].

The signal II_{vf} kinetic measurements were performed using a 100 kHz modulation frequency, a 100 mW microwave power, a 4 G modulation amplitude, and a 10 μs time constant (unless otherwise stated), at the low-field peak of signal II_s ($g = 2.010$). PBQ (1 mM) and $K_3Fe(CN)_6$ (1 mM) were included as the electron acceptors. A field-independent flash artifact signal was measured at a position 50 mT offset from $g = 2$ and subtracted from all of the signal II_{vf} kinetic signals.

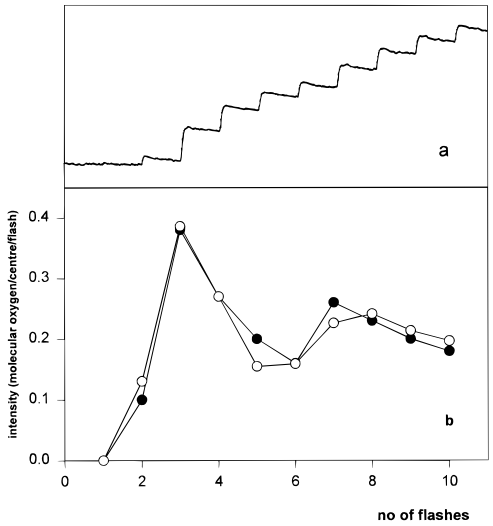

FIGURE 1: (a) Flash-induced oxygen release detected by monitoring the amplitude increase of the ^{15}N-perdeutrated tempone (PDT) signal at $g = 2.0132$ from an S_1-rich sample. Instrumental parameters were as follows: microwave power 100 mW; time constant, 10 ms; microwave frequency, 9.7926 GHz; modulation amplitude, 2 G; modulation frequency, 100 kHz; and an average of 90 events. (b) Relative flash pattern of the PDT signal versus the number of flashes. The filled circles are the experimental data points. Using the Kok model as described in the text, the open circles are the calculated fit with 23% misses, 10% double hits, and an initial S state distribution of 90% S_1 and 10% S_0.

Timing and control of the EPR acquisition, flash lamp, and pump were performed by the ESP 300E spectrometer computer. The flash lamp was triggered 200 μs after signal recording commenced when acquiring a kinetic signal. The pump flow rate was constant and chosen so that the residence time in the EPR cuvette was sufficiently long to ensure near uniform advancement of the S state by a given flash sequence for the aliquot within the microwave-exposed region of the cavity.

Oxygen yield flash patterns were measured on a home-built unmodulated Joliot type electrode (Research School of Biological Sciences) at 25 °C. For flash excitation, the flash lamp was triggered by a computer. The isolated and amplified signals were then digitized and stored in the computer.

RESULTS AND DISCUSSION

Figure 1a shows the flash-induced oxygen release measured within the EPR cuvette, as detected by the increase the in the ^{15}N-perdeutrated tempone EPR signal magnitude. The results were obtained using a 250 ms flash interval in the presence of 1 mM PBQ and 1 mM ferricyanide. The flash pattern was measured on an S_1-rich sample (described below) with the same optical arrangements, temperature, and sample flow conditions used for signal II_{vf} measurements. At the high microwave power levels used in these experiments (100 mW), an increase in the oxygen concentration causes a proportional increase in the peak height of the PDT signal. This is because the measurement is performed in the high-power saturation regime and the presence of oxygen shortens the relaxation time, partially relieves the signal saturation, and so increases the signal magnitude.

A fitting program based on the following Kok type model was used to deconvolute the measured oxygen-induced EPR changes in the PDT signal amplitude, to obtain the initial

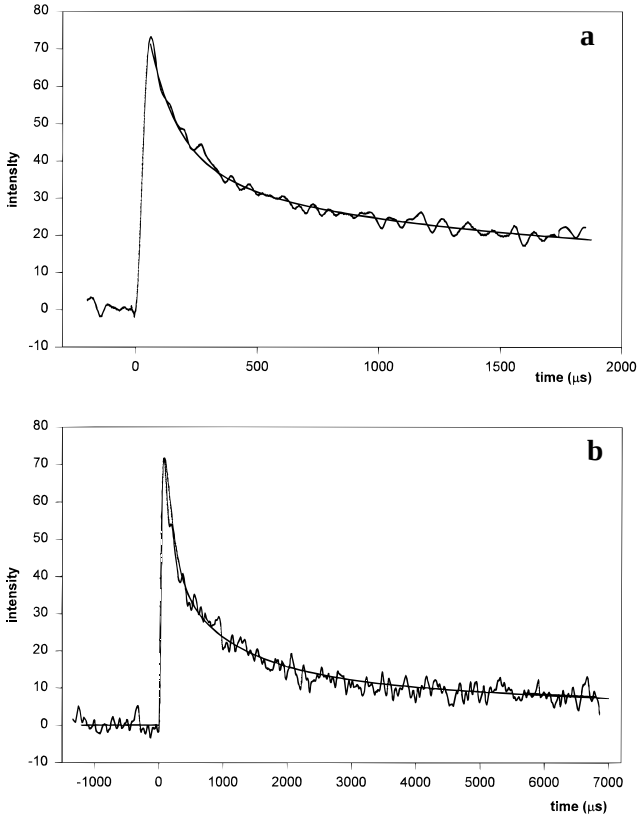

FIGURE 2: (a) Signal II_{vf} decay kinetics and fit of the summed signals from 12 flashes on a dark-adapted sample with a 10 μs time constant. (b) Signal II_{vf} decay kinetics and fit of the same flash sequence on a dark-adapted sample with a 40 μs time constant. The signal (in arbitrary units) is divided by the number of averaged events in both cases [(a) 73 210 and (b) 18 000]. Spectrometer conditions were as described in Materials and Methods.

S_0/S_1 distribution and the α and β parameters.

$$I_n = (1 - \alpha)[S_3]_{n-1} + \beta[S_2]_{n-1} \quad (1)$$

$$[\mathbf{S}]_n = \mathbf{K}\cdot[\mathbf{S}]_{n-1} \quad (2)$$

$$\mathbf{[S]}_n = \begin{bmatrix}[S_0]\\ [S_1]\\ [S_2]\\ [S_3]\end{bmatrix}_n \text{ and}$$

$$\mathbf{K} = \begin{bmatrix}\alpha & 0 & \beta & 1-\alpha\\ 1-\alpha-\beta & \alpha & 0 & 0\\ \beta & 1-\alpha-\beta & \alpha & 0\\ 0 & \beta & 1-\alpha-\beta & \alpha\end{bmatrix} \quad (3)$$

I_n is the fractional oxygen yield on the nth flash, determined by the corresponding change in nitroxide peak intensity relative to the steady state change per flash. $[S_i]$ is the fractional population of centers in the S_i state. $\mathbf{[S]}_n$ is the vector defining the fractional S state distribution in the sample after n flashes. **K** is the "turnover matrix", incorporating the probabilities for double turnovers (β) and misses (α) per flash[3] (Messinger et al., 1991).

Figure 2 shows the signal II_{vf} kinetics obtained by repeated flashing of a thylakoid sample aliquot, summed over many

[3] This assumes that double hits do not occur in the S_3 transition (Messinger et al., 1991).

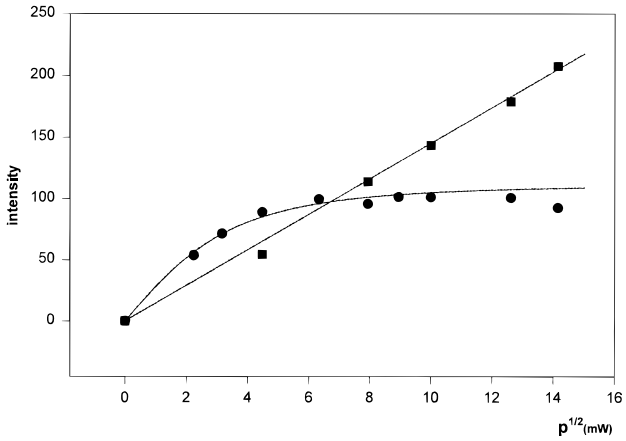

FIGURE 3: Microwave power dependence of the total turnover amplitude (from eq 4) for signal II_{vf} (■) from functional thylakoids and signal II_f (●) from Tris-washed thylakoids, normalized by the number of averaged events. Spectrometer conditions were as for Figure 2 with a 40 μs time constant for signal II_{vf} and a 1.2 ms time constant for signal II_f. The initial slopes of the signal amplitude versus root power are proportional to the intrinsic signal amplitudes in both cases. For the Tris-washed sample, the signal amplitude was fitted to the expression signal amplitude = (initial slope) $\sqrt{P}/\sqrt{(1 + P/P_{1/2})}$ (Pace et al., 1991), where $P_{1/2}$ is the half-saturation power.

aliquots. In each case, the dark-adapted aliquot was subjected to a train of 12 flashes and the kinetic signals acquired on each flash. The response should correspond closely to that from a uniform mixture of the four S states. Panels a and b of Figure 2 show the observed time courses with instrument response times of 10 and 40 μs, respectively. The smooth curves are model fits as discussed below. In addition to components with sub-millisecond kinetics, corresponding to turnover of functionally intact centres, there is a slowly relaxing component ($t_{1/2} \sim 7$ ms) corresponding to about 10% of the total signal intensity and presumably arising from damaged/uncoupled centers. A similar proportion of uncoupled centers was seen in PSII particles (Hoganson & Babcock, 1988). In our hands, this proportion was somewhat variable but was not observed to exceed 15%.

Babcock et al. (1989) compared the relaxation behavior of Tyr_z^+ and Tyr_D^+ in intact PSII membranes at room temperature and showed that Tyr_z^+ gave little or no evidence of saturation at microwave powers up to 200 mW. Tyr_D^+ saturated at ~10 mW. Figure 3 shows a comparison of the microwave power saturation behavior of Tyr_z^+ in intact (signal II_{vf}) and Tris-washed (signal II_f) Mn-depleted thylakoids. In both cases, the signals were generated by repetitive flashing, as in Figure 2, but with only a single flash per aliquot for the Tris-washed material. Up to 200 mW, the Tyr_z^+ signal from intact material shows no detectable saturation effects, whereas the signal from Tris-washed material saturates at ~20 mW. All kinetic measurements of signal II_{vf} were made using a 100 mW microwave power. The first step in performing the kinetic measurements on signal II_{vf} was to generate an S_1 rich sample (~90%). To achieve this, a sample with a total volume of 80−140 mL and a Chl concentration of 1.5 mg/mL was subjected to a preflash procedure. The sample was circulated continuously, and light, other than flash exposure in the EPR cavity, was rigorously excluded. A flow rate of ~10 mL/min produced a dark adaptation time of ~10 min, which ensured essentially complete relaxation of higher S states to the S_1 state during

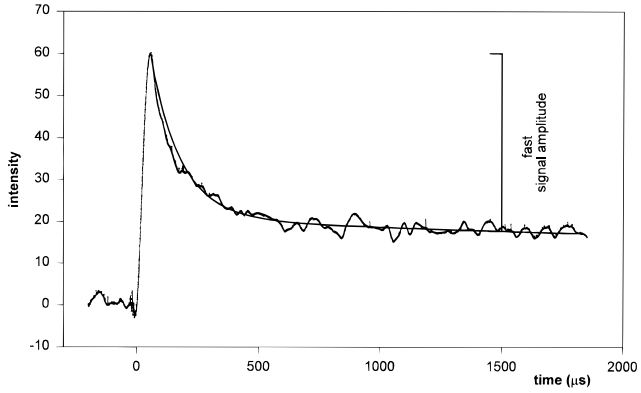

FIGURE 4: Signal II_{vf} decay kinetics and fit of one flash on an S_1-rich sample. The signal amplitude is divided by the number of averaged events (36 930). Illumination procedure cycle: one flash (signal collected) and dark adaptation. The signal amplitude of the fast-decaying component (≤100 μs) at $t = 50$ μs is shown.

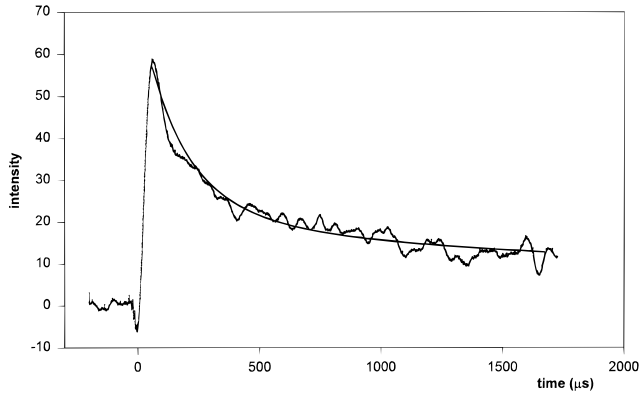

FIGURE 5: Signal II_{vf} decay kinetics and fit of the second flash of four serial flashes on an S_1-rich sample. The signal amplitude is divided by the number of averaged events (25 671). Illumination procedure cycle: one flash, dark adaptation, four flashes (the signal of the second flash was collected), and dark adaptation.

this period (Messinger et al., 1993). The continuously flowing sample was flashed at a rate of 0.5 Hz, for a total time corresponding to two passages through the EPR cavity.

To obtain the Tyr_z^+ kinetics for the $S_1 \rightarrow S_2$, $S_2 \rightarrow S_3$, and $S_3 \rightarrow S_0$ transitions, the procedure was as follows. An S_1-rich sample, prepared as above, was subjected to three flashes (250 ms interval), and the three kinetic traces accumulated in separate files on the spectrometer computer. The aliquot in the EPR cuvette was then displaced by the pump and the sequence repeated. After one complete passage of the sample, it was reprepared in the S_1 state and the procedure repeated (up to 25 times for one sample). Figures 4−6 show the results of extensive averaging for the first through third flash Tyr_z^+ kinetic responses, respectively. Already evident, in the un-deconvoluted data, is a progressive slowing of the Tyr_z^+ re-reduction kinetics with advancement from S_1 to S_3.

Because of the relatively large misses factor involved with flash turnover in the EPR cavity, loss of S state coherence after three flashes becomes significant. This makes it very difficult to reliably resolve the $S_0 \rightarrow S_1$ kinetics in a four-flash experiment. To address this, the sample preparation protocol was modified to produce a system with defined the S_0/S_1 composition only. Following the initial preparation of the S_1-rich sample, the sample was again cycled through the cuvette and each aliquot exposed to a three-flash sequence. During dark relaxation, all states other than S_0

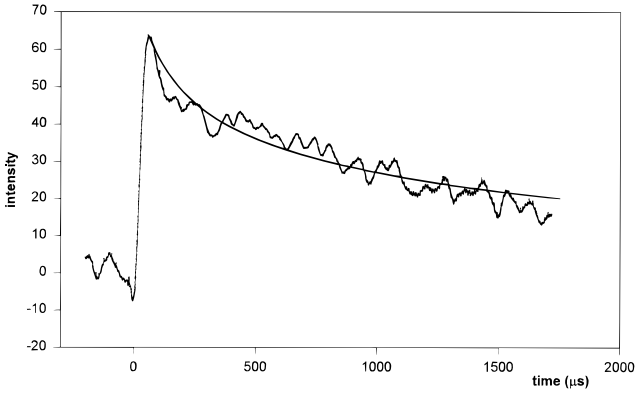

FIGURE 6: Signal II_{vf} decay kinetics and fit of the third flash of four serial flashes on an S_1-rich sample. The signal amplitude is divided by the number of averaged events (26 562). Illumination procedure cycle: one flash, dark adaptation, four flashes (the signal of the third flash was collected), and dark adaptation.

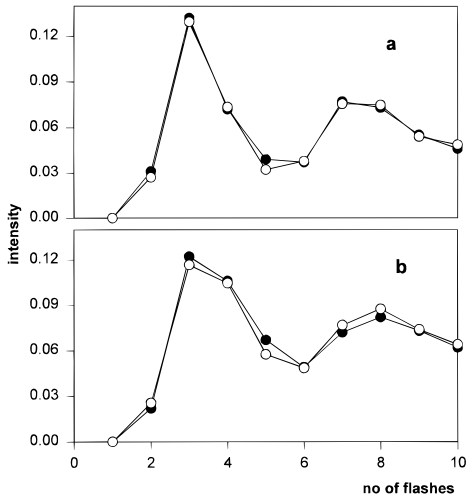

FIGURE 7: Oxygen flash patterns obtained on aliquots of the S_1-rich sample (a) and the S_0-enriched samples (b) using Joliot type electrode. The filled circles are the experimental data points, and the open circles are the calculated fits with 18% misses, 8% double hits, and an initial S state distribution of 90% S_1 and 10% S_0 for the S_1-rich sample and 60% S_1 and 40% S_0 for the S_0-enriched sample.

revert to S_1. The resulting proportion of S_0/S_1 may be calculated from the Kok parameters determined for turnover within the EPR cavity (Figure 1), but was confirmed by flash pattern measurement of a sample aliquot on a Joliot type electrode (Figure 7). The S_0 content was 40%. The resulting "S_0-enriched" sample was then passed again through the cuvette and each aliquot subjected to a single flash with data acquisition. Figure 8 shows the resulting averaged response. Again, even without deconvolution, the kinetics of Tyr_z^+ re-reduction in Figure 8 are evidently faster than those in Figures 4−6.

To deconvolute the pure, individual S state kinetic contributions, the following assumptions were made.

(a) The rise and fall kinetics of the active centers in each S state are monoexponential, with the rise time governed by the spectrometer response time (10 μs). A fixed (for a given sample) fraction (∼10%) of "nonfunctional" centers turn over with a slow, S state-independent, decay time constant of ∼10 ms (as in Figure 2).

(b) The total amplitude of signal turnover is the same in all S states for a given sample.

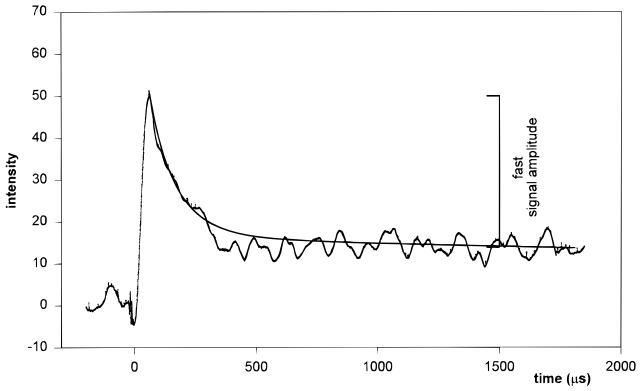

FIGURE 8: Signal II_{vf} decay kinetics and fit of one flash on an S_0-enriched sample. The signal amplitude is divided by the number of averaged events (20 780). Illumination procedure cycle: one flash, dark adaptation, three flashes, dark adaptation, one flash (the signal was collected), and dark adaptation. The signal amplitude of the fast-decaying component (≤100 μs) at $t = 50$ μs is shown.

Table 2: Kinetics of S State Advancement Found in This Work Monitoring Tyr_z^+ Re-Reduction by EPR

$t_{1/2}$ (μs) $S_0 \rightarrow S_1$	$t_{1/2}$ (μs) $S_1 \rightarrow S_2$	$t_{1/2}$ (μs) $S_2 \rightarrow S_3$	$t_{1/2}$ (μs) $S_3 \rightarrow (S_4) \rightarrow S_0$
40−60	85	145	750

Then for the ith S state, the Tyr_z^+ signal, $I_i(t)$, is assumed to have the form

$$I_i(t) = (1 - e^{-t/\tau_r})(I_a e^{-t/\tau_i} + I_{slow} e^{-t/\tau_{slow}}) \qquad (4)$$

where τ_r is the signal rise time constant, τ_i the decay time constant for active centers, and τ_{slow} the decay time constant for inactive centers. I_a and I_{slow} are the signal amplitude for active and inactive centers, respectively.

Despite the fast τ_r value used (10 μs), the Tyr_z^+ signal rise at this time resolution is not observed to be monoexponential due to interference from the tail of the chemically induced dynamic electron polarization signal from PSI (Blankenship et al., 1975, 1977; Boska et al., 1983). The signal appears as an emission spike at the field used to monitor Tyr_z^+ but is essentially gone after ∼50 μs following the flash. For this reason, fitting of eq 4 to the data was restricted to times of ≥50 μs following the flash.

The smooth curves in Figures 4−6 and 8 then represent the least-squares fits of eq 4 to the data, assuming the Kok parameters described from Figure 1. The resulting decay half-times for the individual S states are listed in Table 2. The smooth curves in Figure 2 give the fits, using the above parameters, for steady state turnover with a uniform S state distribution. From the fitting, the uncertainty in the $t_{1/2}$ values given in Table 2 is estimated at 5% for all transitions except the $S_0 \rightarrow S_1$ transition. The uncertainty on the latter is greater, and we provide limits within which the fits are insensitive to the value chosen.

Several conclusions may be drawn from our results. Firstly, Tyr_z^+ turnover is visible by EPR in all S states, with essentially the same amplitude. Secondly, the $S_0 \rightarrow S_1$ transition is indeed the fastest, with a time scale comparable to the original estimates derived from UV optical measurements (Velthuys, 1981; Dekker et al., 1984; Renger & Weiss, 1986; Saygin & Witt, 1987). If the $S_0 \rightarrow S_1$ transition was faster than several tens of microseconds, a significant decrease in the signal amplitude would be observed in Figure

8. This is not the case; in fact, the fast phase (<100 μs) signal amplitudes in Figures 4 and 8 are respectively about 40 and 35 units. If the half-time of the $S_0 \rightarrow S_1$ transition was in the range of several hundreds of microseconds [as per Rappaport et al. (1994)], however, a slow-decaying signal would be resolved in Figure 8. It should be noted that, in earlier EPR studies of signal II_{vf}, estimates concerning the $S_0 \rightarrow S_1$ transition kinetics were obtained from the fourth flash in a multiple-turnover series. However, even though in these experiments the amount of $S_0 \rightarrow S_1$ transition on the fourth flash was probably substantial, the transition could not be reliably resolved due to the contribution of the slow $S_3 \rightarrow S_0$ component. Therefore, in the earlier work, because of the $S_3 \rightarrow S_0$ contamination, the possibility that the half-time of the $S_0 \rightarrow S_1$ transition was in the range of several hundreds of microseconds could not be absolutely excluded. Some inference could be drawn from the amplitudes of the third and fourth flash signals, but of course, the validity of these estimates would be highly dependent on the Kok model and parameters chosen. In such a case, the contribution of the fast transitions ($S_0 \rightarrow S_1$ and $S_1 \rightarrow S_2$) would be important and the assumption that the $S_0 \rightarrow S_1$ transition is EPR silent could probably not then have been excluded. Thirdly, although we confirm the earlier consensus position that the turnover kinetics become monotonically slower as one proceeds through the Kok cycle, this does not in fact occur in a steady progression. Rather, the first three transitions, up to the S_3 state, all occur within approximately the same time scale ($\sim$100 μs), while the final $S_3 \rightarrow S_0$ step is almost 1 order of magnitude slower ($\sim$1000 μs). Since experimental errors and artifacts in our system are much more likely to obscure rather than reinforce such a time scale distribution, we regard the above conclusion as sound.

Although our intention here was to examine Tyr_z turnover in physiologically intact material, we note from the data in Tables 1 and 2 that the kinetics we observe are generally somewhat faster than those which have been previously reported in PSII-enriched systems. This is certainly true for the $S_3 \rightarrow S_0$ transition, which is the most reliably determined under normal circumstances. To examine if this effect is real, we have undertaken a preliminary study of Tyr_z^+ turnover in PSII membranes, using the same experimental arrangements employed for the thylakoid work. Figure 9 shows the observed Tyr_z^+ decay kinetics in PSII membranes, using a summed 12-flash turnover series as in Figure 2b. The $S_3 \rightarrow S_0$ transition may be readily deconvoluted from the rest (which appear to exhibit the same pattern as seen in thylakoids, i.e. all being at least 5 times faster than $S_3 \rightarrow S_0$). The resulting $t_{1/2}$ estimated for $S_3 \rightarrow S_0$ in PSII membranes is $\sim$1.2 ms, notably slower than that observed under comparable conditions in thylakoids. However, we cannot presently exclude that this is simply a pH effect or a detergent treatment effect. For reasons of sample stability, the thylakoid measurements were performed at pH 7.5, while those on the detergent-solubilized PSII membranes were made at pH 6.0. This matter is currently under examination.

The approximate constancy of the S state turnover kinetics, up to the S_3 state, is quite remarkable, given that oxidative equivalents are being progressively stored in the oxygen-evolving complex during this process. This suggests that charge balancing must occur rigorously on each S state advancement, presumably by proton release. Although the S state-associated pattern of proton release into the aqueous

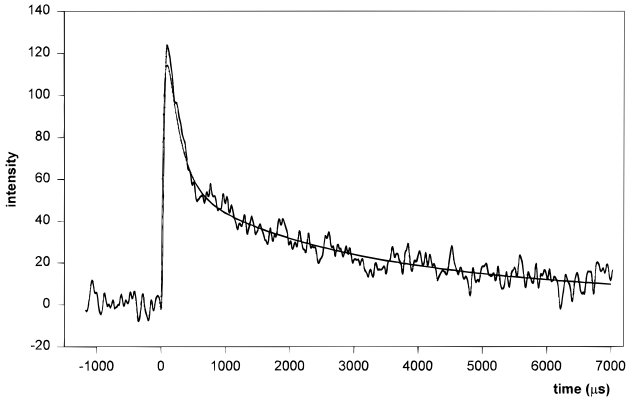

FIGURE 9: Signal II_{vf} decay kinetics and three-parameter fit of the summed signals from 12 flashes on a dark-adapted PSII membrane at a chlorophyll concentration of 2 mg/mL in a buffer that contained 50 mM MES (pH 6.0), 10 mM $MgCl_2$, 10 mM NaCl, 10 mM $CaCl_2$, and 0.4 M sucrose. The experimental conditions are exactly as those of Figure 2b. The signal amplitude is divided by the number of averaged events (10 000). The fitting assumes a single average turnover for the S_0, S_1, and S_2 states ($t_{1/2} \sim 150$ μs) and a separate kinetic for the $S_3 \rightarrow S_0$ transition.

phase has been the subject of debate for some time, our results would support the recent conclusion of Bögershausen and Junge (1995) that the stoichiometry of H^+ release is essentially the same on each step of the S cycle.

In contrast to the early turnover events, the $S_3 \rightarrow (S_4) \rightarrow S_0$ transition is comparatively slow and close to the dioxygen release time (600 μs; Strzalka et al., 1990). This, coupled with the recent water isotope exchange results of Messinger et al. (1995), showing facile substrate water exchange with the OEC up to the S_3 state, strongly suggests that no water oxidation "chemistry" occurs in the early S states. Thus, dioxygen bond formation must occur as a concerted four-electron process. We are currently engaged in a study to determine if there is any discernible difference between the $S_3 \rightarrow S_0$ turnover, by Tyr_z^+ re-reduction, and oxygen release kinetics, when all components are measured in the same system.

Finally, there is one interesting observation which emerges from the data in Figure 3; that is, the apparent spin concentration of the Try_z^+ kinetic signal is only $\sim$0.5 per PSII (assuming 1.0 per PSII in Tris-washed samples). Such an effect has been observed previously (Hoganson & Babcock, 1988; Warden et al., 1976), where the estimates were 0.3−0.4 on the basis of Tyr_D signal quantitation. These earlier values were believed to have been underestimated, due to spectrometer response time and turnover efficiency limitations. Such effects are essentially absent here, with fast spectrometer response and flash efficiency affecting both the active and Tris-treated samples equally. Moreover, Hoganson and Babcock (1988) have shown that the Tyr_z^+ signal in functional PSII centers is not significantly different in overall shape (particularly width) from the signal in Tris-washed PSII (or indeed from the Tyr_D^+ signal). Thus, the observation of a reduced apparent amplitude of the functional Tyr_z^+ signal per center *turning over* has no simple experimental or instrumental explanation. Moreover, Andréasson et al. (1995) have shown that a maximum ratio of Tyr_z^+/Tyr_D^+ equal to $\sim$0.45 is generated by continuous illumination in PSII reversibly blocked by Ca^{2+} depletion. The above raises the possibility that about half of the Tyr_z^+ signal intensity is "lost", because centers are EPR silent for some

reason, or greatly broadened. In this regard, it has recently been reported that Tyr_z^+ is in close association with Mn in samples inhibited by Ca^{2+} depletion (Gilchrist et al., 1995), leading to massive signal broadening, and that the phenoxide oxygen of Tyr_z may be actively involved in proton abstraction chemistry (Hoganson et al., 1995). Whether either of these factors plays a role in reducing the room-temperature visible Tyr_z^+ EPR signal intensity we cannot judge at this point, but our data strongly indicate that, whatever the origin of the reduction, it operates essentially to the same extent in each S state of the intact system.

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