The Microwave Power Saturation of SII_{slow} Varies with the Redox State of the Oxygen-Evolving Complex in Photosystem II†

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ABSTRACT: The microwave power for half-saturation ($P_{1/2}$) for the radical in photosystem II giving rise to signal II_{slow} (SII_s) has been measured by EPR in samples illuminated by a series of flashes. The charge storage state of the oxygen-evolving complex (S_0–S_4) was monitored by measuring the multiline EPR signal arising from the S_2 state. The following results were obtained: (1) SII_s becomes easier to saturate after tris(hydroxymethyl)aminomethane (Tris) washing, a treatment that partially removes the Mn cluster. (2) $P_{1/2}$ for SII_s oscillates with the flash number. $P_{1/2}$ is lower in S_1 (in dark-adapted material and after four flashes) than in S_2, S_3, or S_0. (3) $P_{1/2}(S_2) = P_{1/2}(S_3)$. (4) At 8 K $P_{1/2}(S_0) > P_{1/2}(S_2)$, but at 20 K $P_{1/2}(S_0) < P_{1/2}(S_2)$. (5) $P_{1/2}$ for SII_s increases with temperature (8–70 K) in the S_1 state. SII_s is more difficult to saturate in S_2, S_3, and S_0 than in S_1 over the investigated temperature range. In addition, the increase in $P_{1/2}$ is complex around 20–30 K in S_2, S_3, and S_0. (6) In S_0, $P_{1/2}$ for SII_s decreases with time (decay half-time 30–60 s) to a stable level significantly above the dark level. The data are explained in terms of cross relaxation between the radical giving rise to SII_s and an efficient relaxer, which is suggested to be the Mn cluster. This relaxes more slowly in S_1 than in the other S states. Since it is known that a mixed-valence Mn cluster is present in S_2, and because $P_{1/2}$ of SII_s in S_3 and S_0 is comparable to that in S_2, it is suggested that mixed-valence Mn clusters are present in the S_3 and S_0 states also. Different models with these features can be proposed, the simplest of which is the following: S_0 [Mn(II)–Mn(III)], S_1 [Mn(III)–Mn(III)], S_2 [Mn(III)–Mn(IV)], and S_3 [Mn(III)–Mn(IV)].

The water-splitting cycle of the oxygen-evolving system of photosystem II (PSII)[1] involves five intermediates, S_0–S_4 (Kok et al., 1970). S_0 is the most reduced state, and the more oxidized states are formed by successive electron transfers to the photooxidized primary electron donor P_{680}^+. Four consecutive charge separations are needed for the formation of one oxygen molecule. S_0 and S_1 were observed to be the stable states in the dark, and in most materials the dark population of the S states has been found to be 75% S_1 and 25% S_0 or, after very long dark incubation, 100% S_1 (Vermaas et al., 1984; Styring & Rutherford, 1987). The oxygen molecule is formed in the S_3–S_4–S_0 transition in which S_4 is assumed to be very short-lived.

The requirement of Mn atoms in oxygen evolution has been known for several years, and many different spectroscopic techniques have been applied in order to probe the chemical nature of the Mn cluster and to determine the redox changes that accompany each S-state transition. These methods include EPR, optical, X-ray absorption edge, and EXAFS spectroscopies [for recent reviews, see Dismukes (1986) and Babcock (1987)]. In addition, S-state-dependent changes in the water proton relaxation rate have been observed in NMR measurements (Srinivasan & Sharp, 1986a,b). Although the results are sometimes contradictory, it is generally agreed that the water-splitting process involves manganese redox chemistry and the current models involve a bi- or tetranuclear Mn cluster as the active site in the water splitting process.

Electrons from the Mn cluster (ultimately from water) are transferred to P_{680}^+ by an intermediate electron carrier Z. The nature of Z is still not clear [for a discussion, see Rutherford and Styring (1988) and Babcock (1987)], but in its oxidized form, it gives rise to a radical EPR spectrum called signal $II_{very\ fast}$ (SII_{vf}) with fast reduction kinetics (Blankenship et al., 1975). In cases when the Mn cluster is destroyed or decoupled from Z, its oxidized form gives rise to a similar EPR spectrum with slower reduction kinetics called SII_{fast} (SII_f) (Babcock & Sauer, 1975).

Also on the donor side of PSII is another redox component D, which, when oxidized, gives rise to an EPR signal that was observed by Commoner (Commoner et al., 1956). This signal is similar to SII_f but has much slower decay kinetics and is consequently called SII_{slow} (SII_s) (Babcock & Sauer, 1973). D^+ does not change its oxidation state during catalysis and is thought not to participate in the steady-state transfer of electrons from water to P_{680}^+. However, D reacts with the S states under different circumstances. The oxidized form, D^+, was recently shown to oxidize S_0 to S_1 in a slow dark reaction (Styring & Rutherford, 1987). The reduced form, D, reduced S_2 and S_3 rapidly in chloroplasts and thylakoids ($t_{1/2} \approx 1$ s) (Babcock & Sauer, 1973; Velthuys & Visser, 1975) and more slowly in PSII-enriched membranes ($t_{1/2} \approx 30$ s) (Styring & Rutherford, 1987).

In a study of the microwave power saturation of SII_{vf} Warden et al (1976) observed that the signal was more difficult

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[1] Abbreviations: D^+, component giving rise to EPR signal II_{slow}; EPR, electron paramagnetic resonance; ESE, electron spin echo; EXAFS, extended X-ray absorption fine structure; $P_{1/2}$, microwave power at half-saturation; P_{680}, primary electron donor chlorophyll(s) of PSII; PPBQ, phenyl-*p*-benzoquinone; PRE, proton relaxation enhancement; PSII, photosystem II; S_0–S_4, charge storage states of the oxygen-evolving enzyme; SII_f, SII_s, and SII_{vf}, signal II fast, slow, and very fast, respectively; T_1, spin–lattice relaxation time; Tris, tris(hydroxymethyl)aminomethane; XAES, X-ray Absorption edge; Z, electron carrier functioning between P_{680} and the Mn cluster. Z^+ gives rise to EPR signal $II_{very\ fast}$.

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to saturate at room temperature than was SII_f (after Tris washing) or SII_s. SII_f and SII_s showed similar saturation properties. From similar studies of the microwave power saturation of SII_f in the presence of variable amounts of Mn, it was concluded that the Mn cluster and Z^+ interacted magnetically (Yocum & Babcock, 1981; Yocum et al., 1981). Recently, electron spin-echo spectroscopy has been used to measure the spin-lattice relaxation time (T_1) of SII_s at 4 K in different S states (de Groot et al., 1986; Britt et al., 1987). Both groups found that the S_1 to S_2 transition was accompanied by a large increase in the spin–lattice relaxation rate (T_1^{-1}) of SII_s. This was interpreted as indicative of an oxidation of a Mn(III) to a Mn(IV), where Mn(IV) was expected to be a more efficient relaxer of SII_s than Mn(III) (de Groot et al., 1986). This group also extended their studies to the other S states and interpreted their data in accordance with a model (Dekker et al., 1984) in which each transition S_0–S_1, S_1–S_2, and S_2–S_3 is accompanied by a Mn(III) to Mn(IV) change.

Despite the considerable effort that has been put into investigations of the problem of Mn redox chemistry during the water-splitting process, it is not clear what changes occur, and in particular, the S_2–S_3 and S_0–S_1 transitions are poorly characterized. In this work the question of Mn redox chemistry is addressed with another method in which the microwave power saturation of SII_s at low temperatures is measured as a function of S state in samples of well-defined S-state composition.

A preliminary report of some of these data has been presented elsewhere (Rutherford & Styring, 1988).

Experimental Procedures

Materials. Thylakoid membranes were prepared from market spinach as described by Ford and Evans (1983). Before use in the EPR experiments the membranes were suspended to the desired chlorophyll (Chl) concentration in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.5) containing 15 mM NaCl, 5 mM NaCl, 5 mM $MgCl_2$, and 30% (v/v) ethylene glycol. PSII-enriched membranes were prepared according to Berthold et al. (1981) with the modifications of Ford and Evans (1983). They were a kind gift from Dr. L. G. Franzén (University of Göteborg, Göteborg, Sweden). The membranes were stored at 77 K at 10 mg of Chl/mL and were resuspended in 20 mM 4-morpholinoethanesulfonic acid (MES) buffer (pH 6.3) containing 0.4 M sucrose, 1 mM $CaCl_2$, 10 mM NaCl, and 30% (v/v) ethylene glycol before use. Tris washing was performed in 0.8 M Tris-HCl at pH 8.3. PSII-enriched membranes were diluted to 0.1 mg of Chl/mL in the Tris-HCl buffer, and the treatment was carried out under gentle stirring with a paintbrush for 20 min in room light on ice. The Tris-HCl was removed by spinning down the membranes and resuspending them in the appropriate buffer. This procedure was repeated twice. When used, PPBQ was added from a 20 mM solution in dimethyl sulfoxide.

Sample Preparation. Thylakoid membranes or PSII-enriched membranes at approximately 2 mg of Chl/mL were transferred to calibrated EPR tubes and incubated on ice in the dark for 2 h and at room temperature for 1 min. Thereafter, a saturating preflash was given, and the samples were allowed to equilibrate in total darkness at 20 °C for 10 min. Then PPBQ was added as an exogenous acceptor in the case of PSII-enriched membranes, while the thylakoid membranes were used without further additions. One minute after the addition of acceptor the appropriate number of flashes (0–6) was given, and the samples were frozen within 2 s in an ethanol–solid CO_2 bath (200 K). The samples were immediately transferred to liquid nitrogen. The preflash treatment was considered necessary in these experiments since it synchronizes the centers in the D^+S_1 state (Styring & Rutherford, 1987). Centers present as D^+S_1 from the start (≈75%) were converted to S_2 with the preflash. In the absence of an exogenous acceptor S_2 decays to S_1 during the subsequent dark interval. In some experiments a waiting time at 20 °C in absolute darkness was allowed after the flash sequence and prior to freezing.

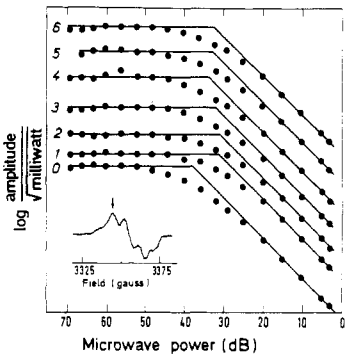

FIGURE 1: Microwave power saturation for SII_s in thylakoid membranes. The samples were given one preflash and 10 min thereafter zero to six flashes. On the abscissa the microwave power is given as dB, which with our spectrometer is defined as $P_{dB} = \log (200/P_{mW}) \times 10$. For clarity, the curves are displaced vertically and only the asymptotes at high and low microwave power are drawn. The inset shows a spectrum of SII_s recorded at 0.5μW microwave power. The arrow shows the field position chosen for the saturation measurements throughout the study. Spectrometer conditions: modulation amplitude 2.8 mT, temperature 20 K, microwave frequency 9.44 GHz, and modulating frequency 100 kHz. The concentration of thylakoid membranes was 2 mg of Chl/mL.

EPR Spectroscopy. EPR measurements were made on a Bruker ESR200D spectrometer equipped with a 90-dB microwave bridge that permitted the use of low microwave powers (with this setup measurements can be performed as low as 5 nW). The instrument was equipped with an Oxford Instruments cryostat and temperature controller. The temperature was controlled from this unit at a constant flow rate of helium during all measurements, which provided comparable temperatures at the sample level from sample to sample with a given meter reading.

The amplitude of the S_2-state multiline signal was estimated from the added sum of low-field peaks as earlier (Styring & Rutherford, 1987). In the power saturation studies the amplitude of SII_s was measured at the field position for the low-field peak marked in Figure 1.

Treatment of Power Saturation Data. The amplitude (I) of an EPR absorption is given by

$$I = kCP^{0.5}/(1 + P/P_{1/2})^{0.5b} \quad (1)$$

where P is the microwave power in milliwatts, $P_{1/2}$ is the power for half-saturation in milliwatts, C is the concentration of spins, k is an apparatus-dependent constant, and b is the inhomogeneity parameter (Sahlin et al., 1986; Rupp et al., 1978; de Paula & Brudvig, 1985). In flashed samples, SII_s is in different environments in the different S states, and in this case the amplitude of the signal is the sum of the amplitudes for each population ($i = S_0$–S_3), i.e.

$$I = k \sum_{i=S_0}^{S_3} [C_i P^{0.5}/(1+ P/P_{1/2,i})^{0.5b}] \quad (2)$$

The concentration of each S state (C_i) was calculated from the miss factor in each flash, which was determined from the variation with flash number of the amplitude of the S_2-state

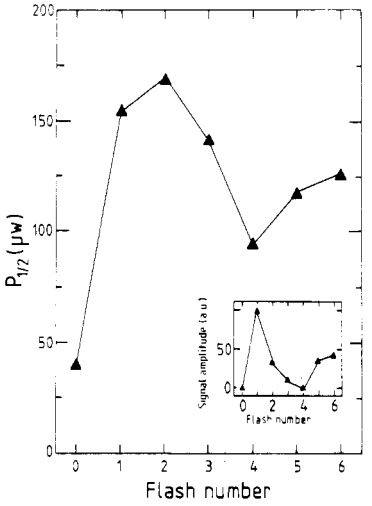

FIGURE 2: $P_{1/2}$ for SII_s thylakoid membranes measured at 20 K. The data are derived from Figure 1. The inset shows the variation of the amplitude of the S_2-state multiline signal in the same samples. The oscillation sequence can be satisfactorily simulated assuming 20% misses. The multiline signal was detected at 8 K, with a microwave power of 32 mW and a modulation amplitude of 22 mT.

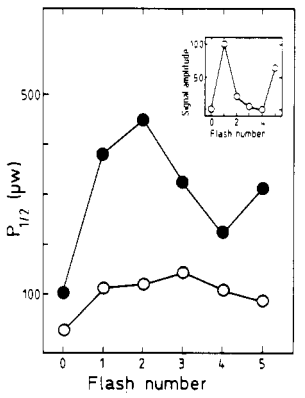

FIGURE 3: Flash-dependent variation of measured $P_{1/2}$ for SII_s in PSII-enriched membranes. The measurements were performed according to the method presented in Figure 1 in the same samples at either 20 K (filled circles) or 8 K (open circles). The membranes, at 2 mg of Chl/mL, were given the desired number of flashes after the preflash treatment described under Experimental Procedures. The inset shows the oscillation of the S_2-state multiline signal. This pattern can be satisfactorily fitted assuming 8% misses. The spectroscopic conditions were as in Figures 1 and 2.

multiline signal (insets in Figures 2, 3, and 4b).

$P_{1/2}$ is, in the case of a homogeneous population of spins, easily determined graphically from a plot of log $(I/P^{0.5})$ vs log P. In this case $P_{1/2}$ is found by extrapolating the straight parts of the curves (see Figure 1) to their intersecting point and reading the $P_{1/2}$ value on the abscissa. In the dark sample, which is homogeneous (100% S_1), the read $P_{1/2}$ value is $P_{1/2}$ for SII_s in S_1 centers. In the flashed samples the plot results in an apparent $P_{1/2}$ value (see Figure 1). This value gives an indication of the real $P_{1/2}$ in each of the different S states, since the flashed samples are dominated by only one S state; for example, the first flash results in a sample dominated by S_2. However, the true $P_{1/2}$ for SII_s in the different S states can be obtained as follows.

$P_{1/2}$ for SII_s in S_1 centers was determined graphically in the dark sample. This permitted the calculation of the intensity due to S_1 centers in the flashed samples according to eq 2. Then, for example, in the one-flash sample, a new set of intensities for SII_s in S_2 centers, I_{S_2}, was calculated (eq 2) by subtracting the calculated intensity in the S_1 centers from the experimentally measured intensities. $P_{1/2}$ for SII_s in S_2 centers

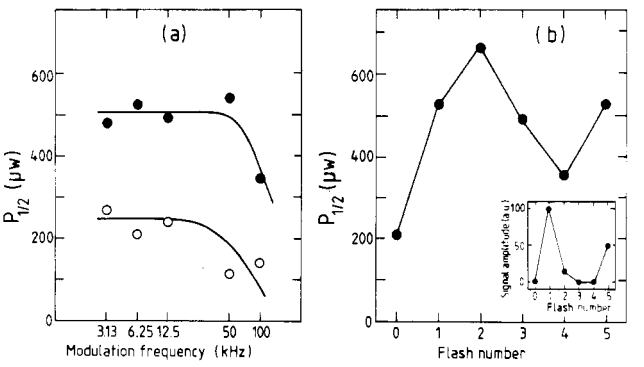

FIGURE 4: (a) Effect of modulation frequency on the measured $P_{1/2}$ for SII_s in PSII-enriched membranes that were dark-adapted or given one flash (after preflash treatment as in Figure 3). (b) Effect of flash number on $P_{1/2}$ for SII_s in PSII-enriched membranes when the saturation study was performed at 6.25-KHz modulation frequency. The measurements in (a) and (b) were performed at 20 K with the spectroscopic conditions from Figure 1. The inset in (b) shows the oscillation of the S_2-state multiline signal in this experiment. It can be fitted assuming 13% misses. The multiline signal was measured as in Figure 2.

was determined graphically from a plot of log $(I_{S_2}/P^{0.5})$ vs log P. $P_{1/2}$ for SII_s in S_3 and S_0 centers were determined in the same manner by using the two- and three-flash samples, respectively.

The inhomogeneity parameter b was determined directly from the power-saturation curves (see Figure 1) in which the slope in the saturating limit is $0.5b$. b was determined as 1.06 in thylakoids and 1.25 in PSII-enriched membranes.

RESULTS

(1) Continuous-Wave Power-Saturation Studies of SII_s in Different S States. The microwave power saturation of SII_s was measured in EPR samples of thylakoids or PSII-enriched membranes after different numbers of saturating flashes. The intensity of the signal measured under nonsaturating conditions was independent of the number of flashes given. In Figure 1 the power-saturation data are presented for SII_s measured at 20 K in samples of thylakoid membranes. The data are presented as a plot of log (amplitude/power$^{0.5}$) vs microwave power (in dB). The plot gives a line parallel to the abscissa in the nonsaturating part of the curve but deviates from this line as saturation occurs. Finally, it becomes a straight line with a slope that is dependent on the inhomogeneity parameter (Sahlin et al., 1986; de Paula & Brudvig, 1985). The slope is constant if the line width of the signal remains unchanged between the samples and over the temperature range studied. The line width of SII_s is difficult to determine since the different parts of the signal saturate differently (Hales & Das Gupta, 1981). This results in a different shape of the signal at high and low powers [the well-known SII_s line shape (Figure 1, inset) is obtained at low microwave power]. However, the line width remains unchanged between the different samples when spectra recorded with high power are compared (data not shown). Therefore, the slope is set similar in the curves in Figure 1. The power at half-saturation ($P_{1/2}$) is directly read on the abscissa at the point of intersection of the linear parts of the curve. It should be noted that this $P_{1/2}$ value is an apparent value, which is largely determined by the dominant S population in each sample (see Experimental Procedures).

From Figure 1 it is clear that the apparent $P_{1/2}$ changes with the flash number, i.e., with the S state. It is also seen that the precision in the measurements is considerable and the error in $P_{1/2}$ is estimated to be less than ±0.5 dB. The powers at

Table I: Calculated Values for $P_{1/2}$ for Each S State Separately[a]

sample	$P_{1/2}$ (μW)			
	S_0	S_1	S_2	S_3
thylakoids, 20 K	89	32	191	178
PSII-enriched membranes, 20 K	310	105	458	490
PSII-enriched membranes, 8 K	155	46	105	107
PSII-enriched membranes, 20 K, mod freq 6.25 KHz	448	210	790	726

[a] Values are calculated from the microwave power dependent variation in the intensity of SII_s after each flash in the experiments in Figures 2–4. The intensities are corrected for the S-state composition and $P_{1/2}$ is then determined as described under Experimental Procedures.

half-saturation extracted from Figure 1 have been plotted vs flash number in Figure 2. Similar data measured at 20 K in PSII-enriched membranes are shown in Figure 3 (closed symbols). The first flash induces a large increase in $P_{1/2}$ for SII_s. In both materials $P_{1/2}$ increases with a factor of nearly 4, which shows that D^+ relaxes faster in S_2 than in dark-adapted material. SII_s remains difficult to saturate, i.e., $P_{1/2}$ remains high, also after the second and third flashes (dominated by S_3 and S_0, respectively), while $P_{1/2}$ is again lower after the fourth flash, reflecting the return of a majority of the centers to the S_1 state. After five flashes $P_{1/2}$ increases again, corresponding to the re-formation of S_2.

In PSII-enriched membranes, power saturation of SII_s was also measured at 8 K (Figure 3, open symbols). Independent of flash number, SII_s was easier to saturate than at 20 K. However, the change follows the same pattern except that SII_s becomes more difficult to saturate after three flashes than after one or two. Also, at 8 K SII_s becomes easier to saturate after four flashes than after three flashes, but the $P_{1/2}$ value after four flashes appears anomalously high as compared with $P_{1/2}$ in the dark sample. The explanation for this high value is the presence of a fraction of centers in the S_0 state ($\approx$25%) in which SII_s relaxes fastest at 8 K.

In the experiments presented in Figures 2 and 3, the S_2-state multiline EPR signal was used to monitor the S-state changes in the flash series. The amplitude of the signal oscillates with the flash number, as shown in the insets in Figure 2 (thylakoid membranes) and Figure 3 (PSII-enriched membranes). From these patterns the miss parameter was estimated to be approximately 20% (Figure 2) and 8% (Figure 3), respectively. The lower amount of misses in PSII-enriched membranes was due to the use of an exogenous acceptor, which efficiently oxidizes the Q_A–Q_B acceptor complex. In this way recombination between the reduced acceptor and the higher S states is minimized. From the data in Figures 2 and 3, using these miss parameters and assuming that all centers were in the S_1 state from the start (achieved with the preflash treatment), it was possible to calculate (see Experimental Procedures) $P_{1/2}$ for each S state separately (Table I).

For unknown reasons SII_s is easier to saturate in thylakoids than in PSII-enriched membranes. However, a difference might be expected since the kinetics for the redox reaction between the S states and D/D^+ differ in the two kinds of preparation (Styring & Rutherford, 1987), indicating slight modifications in the environment of the components. Nevertheless, the general observations from the values in Table I are qualitatively similar in the two materials. $P_{1/2}$ for SII_s is lower in the S_1 state than in the other S states. The formation of the higher S states, S_2 and S_3, generates a fast-relaxing species that increases the relaxation rate of SII_s, resulting in an increase in $P_{1/2}$. $P_{1/2}$ is increased to the same level in S_2 and S_3 measured at either 8 or 20 K (the differences in Table I are within the experimental error). Thus, the power-saturation studies give no indication for a change of the fast relaxer between S_2 and S_3.

The generation of S_0 also results in an increase of $P_{1/2}$ for SII_s as compared with the dark state. However, in S_0, the fast relaxer that interacts with SII_s is different from that in the S_2 and S_3 states. This is clear when the $P_{1/2}$ values for SII_s measured at different temperatures in PSII-enriched membranes are compared. At 8 K the formation of S_0 results in an increase in $P_{1/2}$ for SII_s as compared with the value in the S_2 and S_3 states, while at 20 K, $P_{1/2}$ is lower in S_0 than in S_2 or S_3. Thus, the characteristics of the fast relaxer in the S_0 state are clearly distinguished from those in the other S states.

The estimation of the S-state composition is less accurate the higher the flash number. Nevertheless, it was possible, using the numbers from Table I and the known S-state composition, to stimulate with reasonable accuracy (not shown) the measured $P_{1/2}$ values after four, five, and six flashes in thylakoid membranes. In PSII-enriched membranes the simulation of $P_{1/2}$ was accurate for the five-flash sample, while the simulation resulted in a somewhat lower value than the measured $P_{1/2}$ in the four-flash sample.

It is of note that when S_2 is formed by illumination at 200 K, a large increase in the $P_{1/2}$ value of SII_s also occurs. Furthermore, thawing for 10 s and refreezing of the sample, a treatment that hardly affected the amplitude of the S_2 multiline signal, had no effect on the $P_{1/2}$ value of SII_s.

(2) Effect of Modulation Frequency on $P_{1/2}$ for SII_s. During the measurements of $P_{1/2}$ for SII_s at low temperatures it was pointed out to us that the signal might be measured under so-called rapid-passage conditions (this was suggested by Prof. Tore Vänngard, Department for Biochemistry and Biophysics, Chalmers Institute for Technology, Göteborg, Sweden). Rapid passage is an EPR phenomenon that occurs if the spin–lattice relaxation rate (T_1^{-1}) is slow compared with the modulation frequency used. Under such circumstances the magnetic field changes too rapidly for the spin system to follow it, and the result is a decrease in the amplitude of the detected signal. The decrease is larger when the signal is run at high microwave power conditions, i.e., under saturating conditions, and results in an artifically decreased amplitude. Thus, if a measurement is performed under rapid-passage conditions, the measured $P_{1/2}$ would become lower than the real value. However, rapid-passage phenomena can be avoided if the measurements can be performed with a modulation frequency that is low compared with T_1^{-1}. This was tested in an experiment in which $P_{1/2}$ for SII_s was measured with different modulation frequencies in a dark-incubated sample and in a sample that was given one flash (Figure 4a). It is seen that measurements performed with 100-kHz modulation frequency (used in the other experiments in this study) resulted in $P_{1/2}$ values that were lower than those measured with the lower modulation frequencies (3.13–12.5 kHz). The increase in $P_{1/2}$ measured when the modulation frequency was lowered indicates that measurements performed at 100 kHz indeed were carried out under rapid-passage conditions. The use of lower modulation frequencies then avoids this phenomenon. The observation (Figure 4a) that the measured $P_{1/2}$ of SII_s is independent of the modulation frequency below 12.5 kHz suggests that these measurements are performed under slow-passage conditions. SII_s relaxes slower in a dark-incubated sample (is easier to saturate) than in a sample that was given one flash (Figures 1–3). This is in agreement with the observation (Figure 4a) that SII_s in the dark-incubated sample still seems to be detected under rapid-passage conditions with 50-kHz modulation frequency, while in the sample that was

given one flash, this measuring frequency resulted in slow-passage conditions, i.e., the $P_{1/2}$ value similar to those measured at lower modulation frequencies.

The measurements in Figures 1–3 were performed under rapid-passage conditions (the modulation used was 100-kHz frequency). Therefore, it was necessary to investigate whether the variation of $P_{1/2}$ for SII_s with the flash number remained similar under slow-passage conditions. The measurements were performed with a modulation frequency of 6.25 kHz, which is in the modulation frequency independent limit for $P_{1/2}$ (Figure 4a). The data (Figure 4b) show an oscillatory pattern similar to the data obtained with 100-kHz modulation frequency (Figure 3). The inset in Figure 4b shows the oscillation of the S_2-state multiline signal. This pattern could be satisfactorily simulated assuming 13% misses. This allowed calculation of $P_{1/2}$ for SII_s in each S state (see Table I) and showed that the relaxation of SII_s was approximately similar in the S_2 and S_3 states. The relaxation in S_0 was slower than in S_2 and S_3, as was also the case when the measurements were performed with 100-kHz modulation frequency at this temperature. Thus, the $P_{1/2}$ values are qualitatively similar when SII_s is detected under slow-passage conditions as well as under rapid-passage conditions.

(3) Temperature Dependence between 8 and 70 K of $P_{1/2}$ for SII_s. The results presented in Figure 3 indicated that the change in $P_{1/2}$ for SII_s with flash number was different when the measurements were performed at different temperatures. Therefore, a study was performed in which the $P_{1/2}$ was measured between 8 and 70 K in a dark-adapted sample and in a sample given one flash. These two samples were chosen since they were expected to show the largest differences (compare Figures 2 and 3). Figure 5A shows how the power for half-saturation of SII_s varies with temperature in PSII-enriched membranes when the measurement is performed with 100-kHz modulation frequency. In the dark-adapted sample, $P_{1/2}$ increases with temperature in a regular manner, except for a slight deviation around 20 K (see below). In the sample given one flash $P_{1/2}$ is higher at all temperatures and seems to increase in parallel with $P_{1/2}$ in the dark-adapted sample above 40 K. However, around 20 K $P_{1/2}$ deviates dramatically from the behavior in the dark-adapted sample and increases very rapidly with temperature between 8 and 20 K to reach a maximum near 20 K. It thereafter decreases between 20 and 30 K to a level that is significantly higher than $P_{1/2}$ in the dark-adapted sample. This decrease results in the half-saturation power being lower at 30 K than at 20 K. At about 35 K the curve levels off and begins to increase again. In samples that had been given two or three flashes the variation of $P_{1/2}$ with temperature was similar to that in the sample that was given one flash (data not shown). Also, in thylakoid membranes the temperature influence on $P_{1/2}$ was similar (data not shown).

The decrease in $P_{1/2}$ between 20 and 30 K in S_2 centers (Figure 5A) is anomalous, and the temperature dependence of $P_{1/2}$ was therefore measured under slow-passage conditions. In Figure 5B the results obtained with 6.25-kHz modulation frequency are shown. SII_s is more difficult to saturate in S_2 centers (Figure 5B, triangles) (these samples contained 90% S_2 and 10% S_1) than in S_1 centers (Figure 5B, squares) over the temperature interval studied. The temperature dependence of S_2 centers is different from that in the dark sample with a fast increase in $P_{1/2}$ between 5 and 20 K. Thus, also at low modulation frequency the anomalous temperature behavior is observed. The curve then levels off and increases more slowly, seemingly in parallel with the curve in the dark sample.

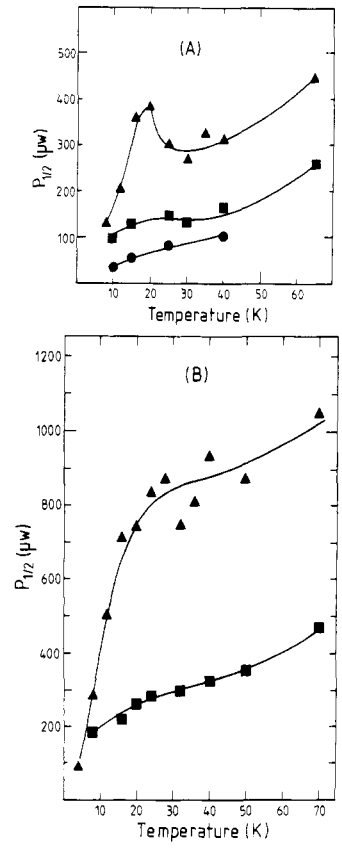

FIGURE 5: Temperature dependence for SII_s saturation in PSII-enriched membranes measured with (a) 100-kHz and (b) 6.25-kHz modulation frequency in dark-adapted material (■) or a sample given one flash (▲) after a preflash and acceptor addition as in Figure 3. (●) Effect of Tris washing on $P_{1/2}$ for SII_s. Spectroscopic conditions except for the modulation frequency as in Figure 1.

Despite the simpler behavior of the curve in the dark sample as compared with the flashed samples, the present precision does not permit a clear assignment of the relaxation to an Orbach or a Raman mechanism even in the dark sample (not shown).

The observation of the peak of $P_{1/2}$ around 20 K in Figure 5A also explains the large difference in $P_{1/2}$ for SII_s observed between 8 and 20 K (Figure 3). A slightly different behavior in the temperature dependence between the S states of $P_{1/2}$ for SII_s (although of the same general appearance) could also explain the difference of SII_s saturation in the S_0 state as compared with the S_2 and S_3 states (Table I).

The precision in the data does not permit unambiguous distinction in the temperature dependence of $P_{1/2}$ between the S_2, S_3, and S_0 states. However, the relaxation of SII_s in S_1 is clearly different and less complex. It is possible that the small deviation around 20 K from a regular increase in $P_{1/2}$ for SII_s in the dark-incubated sample (Figure 5A) is due to a minor fraction of centers remaining in the S_2 state after the preflash.

(4) Effect of Tris Washing on $P_{1/2}$ for SII_s. The results above show that illumination of PSII with one, two, or three flashes introduces a species that increases the relaxation rate of SII_s. The flash illumination does not change SII_s (D^+) itself, since no change in the nonsaturated amplitude could be observed after the flashes. However, it is likely that magnetic interactions between SII_s and the Mn cluster can explain the changes in $P_{1/2}$ for SII_s with flash number. Therefore, the effect of Tris washing on SII_s relaxation was tested. Tris washing is known to remove some of the Mn atoms in the oxygen-evolving complex (Yocum et al., 1981). The power-

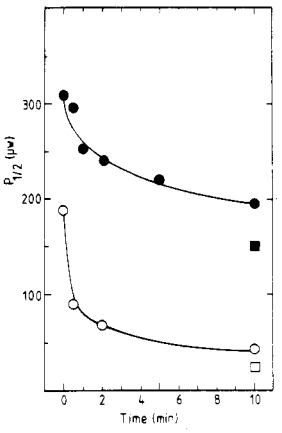

FIGURE 6: Decay of $P_{1/2}$ for SII_s with the dark incubation time after three flashes in thylakoid membranes at 2 mg of Chl/mL (open circles) and PSII-enriched membranes at 2 mg of Chl/mL (closed circles). The experimental procedures were as in Figures 1 and 3, respectively, except that the samples were kept in the dark at 20 °C for the indicated times before freezing. The squares indicate the $P_{1/2}$ measured in a parallel dark-adapted sample. The temperatures for the measurements were 25 K in thylakoids and 20 K in PSII-enriched membranes, and the spectroscopic conditions were as in Figure 1.

saturation data for SII_s in a Tris-washed sample are shown in Figure 5A (circles), and it is clear that SII_s is easier to saturate (between 8 and 40 K) than in the dark-incubated sample. This experiment indicates that the mere presence of the Mn cluster increases the relaxation rate of SII_s.

(*5*) *Kinetic Component in $P_{1/2}$ for SII_s in the S_0 State.* Recent proton relaxation enhancement (PRE) measurements in the different S states (Srinivasan & Sharp, 1986b) showed that the fast proton relaxer in S_0 underwent a change in the minutes time scale. Immediately after three flashes (in S_0) the proton relaxation rate was high but the fast-relaxing species disappeared rapidly ($t_{1/2} \approx 50$ s). The stable PRE after the decay remained higher than the dark base-line level.

It was therefore of interest to investigate whether the fast-relaxing species interacting with SII_s in S_0 showed an analogous kinetic component. Thylakoid membranes of PSII-enriched membranes were given three flashes to obtain a maximal fraction of the centers in the S_0 state. The samples were then incubated at different times in the dark at 20 °C before they were frozen. Figure 6 shows the changes in $P_{1/2}$ for SII_s with time in such an experiment performed with thylakoid membranes (open circles) and PSII-enriched membranes (closed circles). $P_{1/2}$ decreased to a level that was significantly higher than that in the dark-adapted sample. The decay was fast in thylakoid membranes ($t_{1/2} < 30$ s), while in PSII-enriched membranes, $P_{1/2}$ for SII_s in S_0 decreased more slowly ($t_{1/2} \approx 1$ min).

In these experiments a decrease in the $P_{1/2}$ of SII_s after three flashes would be expected from deactivation back to the S_1 state of any centers where S_2 or S_3 were present. The contribution to the decrease in $P_{1/2}$ from such deactivation can be estimated from the misses calculated from the oscillation of the multiline signal. Thus, for thylakoids after three flashes 51% S_0, 39% S_3, and 10% S_2 were present (20% misses in the experiment in Figure 6). With the $P_{1/2}$ values for S_2 and S_3 at this temperature (25 K), their contribution to the decrease in $P_{1/2}$ can be estimated. If the decrease in $P_{1/2}$ was due to S_2 and S_3 decay alone, the expected $P_{1/2}$ value would be approximately 100 μW. In fact, the measured $P_{1/2}$ value was 40 μW. The extra decrease in $P_{1/2}$ seems to be due to a slowing down of the relaxation rate for SII_s in S_0.

In the same way the proportion of the decrease in $P_{1/2}$ attributable to S_2 and S_3 deactivation can be estimated for PSII-enriched membranes. In the experiment in Figure 6 the misses were estimated as 13%, which resulted in 66% S_0, 29% S_3, and 5% S_2 after three flashes. If S_2 and S_3 deactivation were solely responsible for the $P_{1/2}$ decrease, a value of 250 μW would be predicted after 10 min. In fact, the measured $P_{1/2}$ was 195 μW. The discrepancy is too big to be explained by experimental data scatter and is probably due to a change in S_0.

In the experiments with PSII-enriched membranes an exogenous electron acceptor was used, which prevents the usual recombination–deactivation pathway of S_2 and S_3 decay (Lavergne & Etienne, 1981; Rutherford et al., 1982). Under these conditions, the $t_{1/2}$ for S_2 and S_3 deactivation has been measured to be 4 and 5 min, respectively (Styring & Rutherford, 1988). The phase of the decrease in $P_{1/2}$ occurring with a $t_{1/2}$ of 30–60 s is therefore not expected from S_2 and S_3 decay and can probably be attributed to a change in the relaxation properties of SII_s in S_0.

The end point for this change is a $P_{1/2}$ value that is significantly higher than that in the S_1 state. However, it should be noted that S_0 was present in only a fraction of the centers (51% S_0 and 49% S_1 and 66% S_0 and 25% S_1 in Figure 6 for thylakoids and PSII-enriched membranes, respectively). Thus, the true and value for $P_{1/2}$ in S_0 centers is higher than that measured directly in Figure 6, and it can be roughly estimated to be 60 μW in thylakoids and 245 μW in PSII-enriched membranes. These values are significantly lower than the calculated values for $P_{1/2}$ in S_0 centers immediately after the flashes, which are about 130 μW in thylakoid membranes and 300 μW in PSII-enriched membranes.

Qualitatively these data are similar to the PRE data in that the relaxation rate is decreased with a decay half-time of $^1/_2$–1 min. In addition, the final level reached is higher than the dark level.

(*6*) *Rapid Probe to Changes in Relaxation of SII_s.* The results presented in the previous sections show that SII_s is easier to saturate when the oxygen-evolving complex is in the S_1 state than in the other S states. Thus, power-saturation measurements of SII_s provide an EPR probe for investigations of S-state transitions and redox chemistry in the oxygen-evolving complex. However, measurements of $P_{1/2}$ values are tedious, and a more rapid assay would be useful. This possibility has been investigated, and the use of the ratio in the amplitude of SII_s at 126 μW (32 dB) and a nonsaturating power (0.2 μW, 60 dB) is proposed as a probe. The results show essentially the same behavior as the plot of $P_{1/2}$ vs flash number (see Figures 2 and 3). Thus, this ratio seems useful for rapid investigations of the saturation properties of SII_s.

DISCUSSION

In this paper, the power saturation of SII_s measured at low temperatures is used as a probe of the changes in the Mn cluster in the oxygen-evolving complex during the S-state transitions. The results can be summarized in the following points: (1) When the Mn cluster is partially removed by Tris washing, SII_s becomes easier to saturate. (2) $P_{1/2}$ for SII_s oscillates with the flash number. It is lowest in dark-adapted material (S_1) and after four flashes when S_1 is re-formed in a majority of the centers. (3) $P_{1/2}$ for SII_s varies with the S states. $P_{1/2}(S_1) < P_{1/2}(S_0)$, $P_{1/2}(S_2)$, $P_{1/2}(S_3)$; $P_{1/2}(S_2) = P_{1/2}(S_3)$; $P_{1/2}(S_0)$ is higher than $P_{1/2}(S_2)$ at 8 K and lower at 20 K. (4) In S_1, $P_{1/2}$ for SII_s increases with the temperature in a almost linear manner, while $P_{1/2}$ in S_2, S_3, and S_0 has a complex temperature dependence around 20–30 K. (5) In S_0, $P_{1/2}$ for SII_s seems to decrease to a stable value above the dark level. The half-decay time is ≈30 s in thylakoid membranes

and ≈60 s in PSII-enriched membranes. (6) At low temperatures, SII_s relaxes slowly compared with the modulation frequency normally used. However, this can be avoided by use of lower modulation frequencies and does not interfere with the essence of the data.

Tris washing partially extracts Mn from the Mn cluster and inhibits its function (Babcock, 1987). In earlier work it was observed that Tris washing resulted in slower relaxation of Z^+ (SII_f), which was taken as evidence that Z^+ was situated close to the Mn cluster (Yocum et al., 1981). In addition, D^+ relaxes more slowly in a mutant that lacks the Mn cluster (Rutherford et al., 1988) and after Tris washing [Figure 4; see also Yamada et al. 1987)], suggesting that Z^+ and D^+ are more equivalent with respect to the Mn cluster than was previously thought.

The observation here that $P_{1/2}$ for SII_s varies with the S states is in agreement with the earlier ESE spectroscopy work (de Groot et al., 1986; Britt et al., 1987) in that SII_s and the Mn cluster interact magnetically. T_1 for SII_s was found to be shorter in S_2 than in S_1, which was interpreted as being due to cross relaxation between SII_s and a fast relaxer formed in the S_1–S_2 transition. This fast relaxer was suggested to be the Mn cluster.

In the present work it was observed that $P_{1/2}$ varied with flash number (Figures 1–3). The samples in this study have a S-state composition that can be accurately deduced from the oscillation of the S_2-state multiline signal. This permitted an estimation of $P_{1/2}$ for SII_s in each S state (Table I) and at 8 K:

$$P_{1/2}(S_1) < P_{1/2}(S_2) = P_{1/2}(S_3) < P_{1/2}(S_0)$$

Since $P_{1/2}$ is proportional to T_1^{-1}, these values are in marked contrast to the earlier electron spin-echo results. De Groot et al. (1986) found T_1 to be much shorter in S_3 than in S_2 and longer in S_0 than in S_1. The reason for these discrepancies is probably the sample preparation. De Groot et al. (1986) used low-temperature illumination (250 K) to form S_3 and an increase to pH 8.3 to form S_0. In fact, a period of continuous illumination at 250 K results in the formation of not only S_3 but also a large amount of S_0 (results not shown). The formation of S_0 by an increase of the pH was verified by measurements in the UV (de Groot et al., 1986). However, it is known that high pH results in loss of Mn from PSII (Cole et al., 1986), which would transform SII_s to a slowly relaxing species (compare Tris washing in Figure 4). In fact, recent ESE measurements of T_1 for SII_s in flashed samples (Hoff et al., 1987) are entirely consistent with the power-saturation data.

The question now arises as to what information on the nature of the Mn cluster can be obtained from the $P_{1/2}$ data. In the earlier ESE work the stepwise acceleration of the T_1^{-1} of SII_s, which was observed upon advancing through the S states, was attributed to oxidation of Mn(III) to Mn(IV), on the assumption that higher valences states of Mn would show faster T_1^{-1} values (de Groot et al., 1986). This interpretation fitted with the model of Mn oxidation derived from UV spectroscopy, in which a Mn(III) to Mn(IV) transition was proposed for each of the S_0 to S_1, S_1 to S_2, and S_2 to S_3 transitions (Dekker et al., 1984). The $P_{1/2}$ measurements reported here cast serious doubt on the T_1 values measured by ESE for the S_0 to S_1 and S_2 to S_3 transitions. Hence, a reinterpretation of the S-state-dependent SII_s saturation characteristics is necessary. Clearly, the $P_{1/2}$ data cannot be simply explained in terms of an increasing relaxation rate arising from increasing Mn valence state since the least oxidized S state, S_0, is the fastest relaxing species (at least at 8 K). As an alternative explanation, the flash-dependent relaxation effects could reflect the magnetic properties of the Mn cluster as a whole. This seems reasonable since no broadening of SII_s was observed under any of the conditions studied, indicating that the distance between D^+ and the Mn cluster is large relative to the distances between the Mn atoms [i.e., the Mn–Mn distance is 2.7 Å (Yachandra et al., 1986) and perhaps 3.3 Å (Guiles et al., 1987)].

An alternative explanation of the S-state-dependent changes in $P_{1/2}$ for SII_s could involve changes in the environment of D^+ caused by conformational changes in or around the Mn cluster during the S transitions. Conformational changes during turnover have been suggested in a recent model for the Mn cluster (Brudvig & Crabtree, 1986), but these are small compared with the assumed distance between D^+ and the Mn cluster and thus unlikely to interfere drastically with the environment of D^+. Furthermore, there is evidence against conformation changes in the Mn cluster in the S_1 to S_2 transition (Yachandra et al., 1987) although this transition results in the largest change in $P_{1/2}$ for SII_s. Also, the observation (reported above) that the power-saturation characteristics of SII_s remained unchanged when a sample in which S_2 was formed by illumination at 200 K was thawed to room temperature argues against conformation changes as the origin for the changes in $P_{1/2}$. Therefore, the following discussion will be limited to a model in which the $P_{1/2}$ changes are caused by changing magnetic interactions between D^+ and the Mn cluster.

In the S_2 state an EPR signal is observed that arises from a spin = $^1/_2$ mixed-valence Mn cluster (Dismukes, 1986). Formation of S_2 by one or five flashes results in a marked increase in the $P_{1/2}$ of SII_s. This effect seems likely to be due to an interaction between D^+ and the mixed-valence Mn cluster. The formation of S_3 after two flashes gives no detectable change in the $P_{1/2}$ of SII_s relative to that measured in the presence of S_2. In addition, the unusual temperature dependence of the $P_{1/2}$ of SII_s seen in the presence of S_2 is unchanged in S_3 (data not shown). The most straightforward interpretation for this is that the redox state of the Mn does not change on the S_2 to S_3 transition, since it seems unlikely that the relaxation properties of the Mn cluster would be unchanged upon oxidation. This interpretation finds support from earlier measurements [e.g., XAES (Guiles et al., 1987), PRE (Srinivasan & Sharp, 1986b), and near-infrared spectroscopy (Dismukes & Mathis, 1984)] [see also Saygin and Witt (1984)]. On the other hand, although it can be easily imagined that the loss of the multiline signal upon S_3 formation could occur without Mn oxidation,[2] it is difficult to visualize a magnetic change in the Mn cluster that would result in loss of the multiline signal but that would not influence the relaxation properties. Nevertheless, such a situation could occur if, in S_3, an extra paramagnet is present that interacts weakly with the Mn cluster causing a small splitting of the sublevels of each spin multiplet (leading to loss of the multiline signal) but not significantly affecting the energy differences *between* the spin multiplets (leaving the relaxation properties largely unchanged) (this explanation was suggested to us by Dr Örjan

[2] The observation that the S_2-state multiline EPR signal disappears upon the formation of S_3 should not be taken as strong evidence for the oxidation of Mn. In S_2, a minor change in the environment of the Mn, such as that induced by replacement of Cl^- by SO_4^{2-}, results in loss of the multiline signal even though the oxidation state of the Mn cluster is proposed to be the same (Ono et al., 1986). Readdition of Cl^- to such samples in the S_2 state results in formation of the multiline signal. Thus, neither the disappearance nor the formation of the multiline signal is necessarily associated with Mn redox chemistry.

Hansson). In fact is is not unreasonable to propose that such a paramagnet exists in S_3 since, if the Mn cluster does not undergo oxidation, an unknown species must store the positive charge equivalent. The S_3 to S_0 change, which involves the transient formation of S_4 and the release of O_2, almost certainly results in a reduction of the Mn cluster. Although the temperature dependence of the $P_{1/2}$ of SII_s in S_0 is markedly different from that in the S_2 and S_3 states, the $P_{1/2}$ value in S_0 is nevertheless high. Indeed, at low temperature S_0 is the fastest relaxing state. Since the $P_{1/2}$ for SII_s in S_0 is at a level comparable to that measured for S_2, a reasonable explanation for these observations is that S_0 is also a mixed-valence Mn cluster (spin = $^1/_2$). A cluster containing (or made up of) Mn(II) and Mn(III) ions is an obvious candidate. The involvement of Mn(II) in the S_0 state has been proposed earlier [e.g., Radmer and Cheniae (1977), Srinivasan & Sharp (1986b), and Styring and Rutherford (1987)]. The S_1 state present in the dark or formed by four flashes has the lowest $P_{1/2}$ values for SII_s. This can be accounted for if the Mn cluster in S_1 does not contain strongly interacting mixed-valence Mn. A Mn cluster made up from interacting Mn(III) ions (ground-state spin = 0) is a good candidate. The low $P_{1/2}$ value found in S_1 compared with S_0 and S_2 can be taken as evidence that Mn is oxidized during both the S_0 to S_1 and S_1 to S_2 transitions.

The above discussion is based on the assumption that $P_{1/2}$ of SII_s is modulated by the magnetic properties of the whole Mn cluster. If the Mn cluster sensed by D^+ is dimeric [i.e., the simplest current model for the Mn cluster (Hansson et al., 1987)], then a simple explanation for the power-saturation data is provided if mixed-valence dimers (spin = $^1/_2$) of Mn are responsible for the high $P_{1/2}$ of D^+ in S_0, S_2, and S_3, while in S_1 the Mn is more diamagnetic. A reasonable model would be the following S_0 [Mn(II)–Mn(III)], S_1 [Mn(III)–Mn(III)], S_2 [Mn(III)–Mn(IV)], and S_3 [Mn(III)–Mn(IV)]. More complex models can be proposed if a tetrameric cluster is involved.

The coupling of D^+ with the Mn cluster in S_2 results in not only a faster relaxation rate but also a different temperature dependence of the $P_{1/2}$ than that found in S_1. In the range 8–20 K the $P_{1/2}$ increases much more rapidly with temperature in S_2 than in S_1 (Figure 5). This is expected due to the population of higher spin, and hence faster relaxing, excited states of the Mn cluster at higher temperatures [see Sahlin et al. (1987)]. The anomalous flattening off of the $P_{1/2}$ vs temperature curve at higher temperatures in S_2 (Figure 5) could be due to a population of excited states of the Mn cluster that have relaxation rates so rapid that the enhancement of D^+ relaxation becomes less efficient [see Brudvig et al. (1984), Sahlin (1986), and Sahlin et al. (1987) for recent discussions of relaxation phenomena]. Alternatively, such an anomalous temperature curve is predicted if the Mn cluster has its spin states in an unusual order; a model of the Mn cluster in the S_2 state with such characteristics (i.e., spin = $^3/_2$ ground state, spin = $^1/_2$ first excited state) has been recently proposed (de Paula et al., 1986). Further characterization of the temperature-dependent relaxation phenomena is in progress.

The $P_{1/2}$ measurements of SII_s reported here are very similar to the PRE NMR data reported earlier (Srinivasan & Sharp, 1986a,b). This is a surprise since a good relaxer of a radical at low temperature is expected to be a very poor relaxer of protons from water at room temperature. However, very little is known about the relaxation properties of the system at higher temperatures. It is possible that the similarities between the power-saturation data and the PRE data are more than coincidences in that the PRE may also reflect magnetic properties of the whole Mn cluster and thus be dominated by the Mn interactions, which could be present at both room and low temperatures. The change in relaxation properties of S_0, which was observed in the PRE measurements and seems to be reflected in the $P_{1/2}$ measurements reported here, could be due to conformational changes and/or to charge redistribution within the Mn cluster.

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Spectroscopic Studies of Quinonoid Species from Pyridoxal 5′-Phosphate†

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ABSTRACT: To establish the state of protonation of quinonoid species formed nonenzymically from pyridoxal phosphate (PLP) and diethyl aminomalonate, we have studied absorption spectra of the rapidly established steady-state mixture of species. We have evaluated the formation constant and the spectrum of the mixture of Schiff base and quinonoid species. For *N*-methyl-PLP a singly protonated species with a peak at 464 nm is formed from the unprotonated aldehyde and the conjugate acid of diethyl aminomalonate with a formation constant K_f of 240 M^{-1}. The very intense absorption band with characteristic vibrational structure (most evident as a shoulder at 435 nm) is accompanied by a weaker, structured band at about 380 nm and a weak, broad band at 330 nm. We suggest that the 380-nm band may represent a tautomeric form of the quinonoid compound. Protonation of the phosphate group appears to affect the spectrum only slightly. The corresponding mixture of Schiff base and quinonoid species formed from PLP has a very similar spectrum at pH 6–7. It has a formation constant K_f of 230 M^{-1} and a pK_a of 7.8, which must be attributed to the ring nitrogen atom. The dissociated species, which may be largely carbanionic, has a strong structured absorption band at 430 nm and a weaker one, again possibly a tautomer, in the 330-nm region. The analysis establishes that in all species a proton remains on either the phenolic oxygen or the imine nitrogen. Proton NMR spectroscopy, under some conditions, reveals only two components: free PLP and what appears to be Schiff base. However, we suggest that the latter may, in fact, be a quinonoid form, either alone or in rapid equilibrium with the Schiff base. Absorption spectra of quinonoid species formed in enzymes are analyzed and compared with the spectra of the nonenzymic species.

A generally accepted mechanism of catalysis by pyridoxal 5′-phosphate (PLP)[1] dependent enzymes postulates formation of a Schiff base (II; Scheme I) between the PLP (I) and an amino acid substrate. This is followed by withdrawal of an electron pair from a suitably oriented bond around the α carbon atom into the conjugated system represented by the imine double bond and the pyridine ring (Braunstein &

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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; SB, Schiff base; Q, quinonoid species.

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