Flash-induced enhancements in the proton NMR relaxation rate of Photosystem II particles: response to flash trains of 1–5 flashes

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Flash-induced enhancements of the proton NMR relaxation rate R_1 of the solvent in suspensions of PS II particles have been recorded for sequences of 1–5 saturating flashes. The one-flash relaxation transient is a positive R_1 enhancement of 0.0080 s^{-1}, which relaxes to the preflash baseline with a half-time of 25 s. The two-flash response is a positive relaxation transient of nearly identical amplitude to the one-flash response, but with a slower decay ($t_{1/2} \approx 40$ s). The appearance of a strongly relaxing paramagnetic center after one flash is consistent with the expected properties of an Mn(III) → Mn(IV) oxidation. In contrast, the absence of a further R_1 enhancement after the second flash shows that a strongly relaxing paramagnetic center is not formed by $S_2 \to S_3$ as is expected for manganese redox chemistry involving oxidation of Mn(III); the NMR experiment gives no indication that manganese redox chemistry occurs on the $S_2 \to S_3$ transition. The three-flash R_1 response is a positive R_1 transient of 0.0066 s^{-1}, which decays with a half-time of approx. 50 s to a stable value of 0.002 s^{-1} above the preflash baseline. Positive R_1 enhancements have been observed in the presence of carbonylcyanide *m*-chlorophenylhydrazone (CCCP), an agent known to accelerate the decay of S_2 and S_3, at concentrations which very effectively suppress the one-flash and two-flash decays. These enhancements indicate that the formation of the S_0 state from S_1 involves the production of a strongly relaxing center, and the sign of the R_1 response is consistent with the expected properties of an Mn(III) → Mn(II) reduction. The kinetic behavior of the three-flash R_1 transient is indicative of slow redox equilibria involving the strongly relaxing center of the S_0 state. Flash transients across a five-flash cycle exhibit oscillatory behavior with a local minimum on the fourth flash. Simulations of the flash profile indicate the presence of an R_1 contribution from a minor fraction of centers which are capable of transition to S_2 and S_3, but are inhibited at $S_3 \to S_0$.

Introduction

The catalytic cycle of photosynthetic water oxidation is known to involve a manganese cluster

which is driven by the photochemistry of PS II through a sequence of five oxidation states, the so-called S states (S_0, S_1, S_2, S_3, S_4). These oxidation states of the manganese center mediate the four-electron oxidation of water to molecular oxygen [1,2]. Absolute manganese requirement for the functioning of the water oxidation is well documented. Quantitation of manganese in PS II has indicated a stoichiometry of four tightly bound manganese ions per center [3–6], although a value of 2 [7] has also been reported.

Recently, spectroscopic probes of the S states

have been developed using ESR [8–10] and optical [11–16] spectroscopy. ESR signals apparently arise from two different forms of the S_2 state, one a multiline signal centered about $g = 2$ and the other a broad absorption at $g = 4.1$. These data suggest that the S_2 state consists of a mixed valence manganese cluster. An optical transient in the near infrared supports this interpretation [16]. Period-4 optical transients have also been observed in flash studies in the ultraviolet [12–14]. The analysis of these experiments has yielded four optical transients during the four S-state transitions. These transients, which are similar in nature, have been interpreted in terms of four sequential $Mn(III) \rightarrow Mn(IV)$ oxidations during the S-state cycle [14].

Recent work from this laboratory [17] has shown that the $S_1 \rightarrow S_2$ transition produces a detectable enhancement of the NMR R_1 relaxation rate of the solvent protons in suspensions of PS II particles. This NMR relaxation enhancement monitors the appearance of a strongly relaxing paramagnetic center which is produced during the $S_1 \rightarrow S_2$ transition. The sign of the proton NMR relaxation enhancement is consistent with the expected properties of a $Mn(III) \rightarrow Mn(IV)$ oxidation. Evidence linking the NMR relaxation transient to redox chemistry in the S states derives from a variety of observations. These include: (1) the decay kinetics of the transient, which correspond to known properties of S-state decay; (2) the suppression of the NMR transient by treatments which release manganese from PS II (washing by alkaline Tris buffer or 1 mM NH_2OH); (3) the sensitivity of the response to CCCP, an agent which accelerates S-state decay; (4) the observation that subextracting concentrations of NH_2OH, which are known to produce a two-flash retardation in the normal period-4 cycle of oxygen flash yields, also produce a two-flash delay in the appearance of the NMR relaxation transient; and (5) the ineffectiveness of atrazine-type inhibition of electron transport on the reducing side of PS II in suppressing the one-flash R_1 response.

In this communication we present a characterisation of the proton NMR relaxation enhancement transient in suspensions of PS II particles across a five-flash cycle. The purpose of these measurements is the development of an experimental probe of manganese redox chemistry that occurs during the $S_2 \rightarrow S_3$ and $S_0 \rightarrow S_1$ transitions.

Materials and Methods

PS-II-enriched membranes were prepared from market spinach by the procedure of Berthold et al. [18] as modified by Ghanotakis et al. [19]. The PS II membranes were suspended in a buffer containing 400 mM sucrose/15 mM NaCl/40 mM Na-Mes at pH 6.0, divided into 0.75 ml aliquots and stored at 190 K until use. Typical control rates for oxygen evolution activity were 400–450 μmol O_2 per mg Chl per h.

Samples were dark adapted for a minimum of 3 h on ice prior to start of measurements. Samples were kept in total darkness except when flash-illuminated. Addition of 0.5 mM EDTA, electron acceptor and other reagents (as detailed in figure legends) was made after the dark adaptation period.

NMR samples consisted of 30 μl of the PS II suspension at a concentration equivalent to 1.9 mg Chl per ml. The flash measurements utilized a cylindrical flat cell (8 mm diameter by 0.5 mm depth) milled into a lucite light pipe as described in Refs. 17 and 20. Samples were temperature-equilibrated in the NMR probe at 18–20°C for 10 min and then illuminated with a single actinic flash 6–6.5 min before beginning the measurement sequence. Details of the NMR instrumentation and the basic measurement sequence are described in Refs. 17 and 21

Flash illumination was provided by a Xenon Corporation model 457 power supply and a Novatron-722 flash lamp. Actinic flashes had a half-width of about 10 μs and were generated by dissipating electrical energy of 70 J per pulse. The flash output was collected by a parabolic mirror and focused into the sample chamber by means of a condensing lens, a right-angle prism, and a light pipe [17]. A saturation curve of the one flash proton NMR relaxation enhancement transient was recorded as a function of the voltage across the discharge capacitor. Flash energies higher than 50 J per pulse were found to be saturating.

Results

Enhancements of the NMR relaxation rates (R_1) of solvent water protons produced by actinic flash trains on PS II suspensions are shown in Figs. 1-3. In all experiments, samples were dark adapted for 3 h at $0°C$ and then illuminated with a single flash 6-6.5 min before commencement of the measurement sequence. This procedure maximizes the fraction of manganese centers in the S_1-state at the start of experiments.

Figs. 1a and 2a show the proton NMR relaxation enhancement transients produced by one flash and two flashes, repsectively. Amplitudes of the transients measured 150 ms after the final flash are 0.0080 and 0.0079 s^{-1}. These transients decay to the baseline with half-times of about 25 s (one flash) and 40 s (two flashes).

Fig. 1b and 2b show the effect of 1 μM CCCP on the one-flash and two-flash R_1 transients. CCCP is known to accelerate the deactivation of the higher S states (S_2 and S_3) back to the S_1 state

(ADRY effect). This effect is clearly evident in both the one-flash and two-flash R_1 transients. A CCCP titration [17] indicates that the R_1 response at 10 s is effectively suppressed by CCCP concentrations of at least 0.5 μM. In the presence of 1 μM CCCP, only the first point of each sequence, measured 150 ms after the flash, is unaffected. While the one flash response decays abruptly to the baseline, in the two-flash sequence a small, but observable, decay persists 10 s after the final flash. This is probably due to effects of double hits, which produce a small fraction of centers in the S_0 state.

Fig. 3 shows the R_1 response following three flashes in the absence (Fig. 3a) and presence (Fig. 3b) of ADRY reagent. Three flashes are expected to produce a maximum number of centers in the S_0 state, which in thylakoid membranes is relatively stable over periods of several tens of minutes. The observed proton NMR relaxation enhancement decays, following the third flash, from an initial amplitude of 0.0074 s^{-1} to a final, relatively stable enhancement 0.0020 s^{-1} above the preflash baseline. In order to distinguish the true, stable
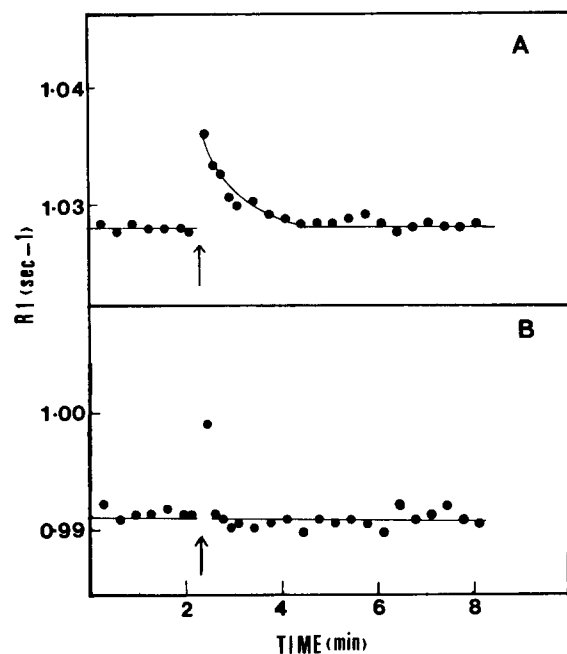

Fig. 1. Proton-solvent relaxation rate as a function of time in a suspension of PS II particles following a single actinic flash. Samples contained 1.5 mM $Fe(CN)_6^{-3}$ and 0.5 mM EDTA. (A) was without ADRY reagent, (B) contained 1 μM CCCP. Chlorophyll concentration was 1.87 $mg \cdot ml^{-1}$. Each trace is an average of 25 experiments.
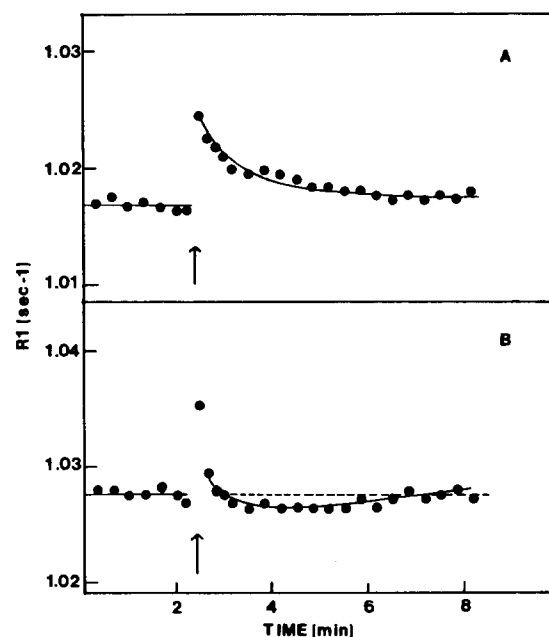

Fig. 2. Proton-solvent relaxation rate as a function of time in a suspension of PS II particles following two actinic flashes spaced 300 ms apart. Other experimental conditions are given in the legend of Fig. 1.
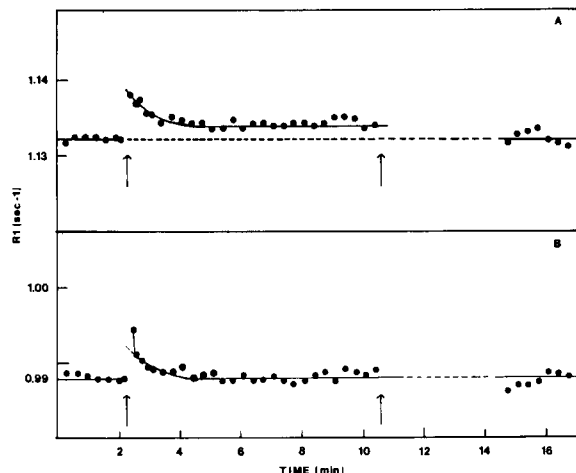
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Fig. 3. Proton-solvent relaxation rate as a function of time in a suspension of PS II particles following three actinic flashes spaced 300 ms apart. 8 min after the flash train, a single flash was applied followed by a 3.5 min dark delay to reset the S-state system to the S_1 state. Samples contained 1.5 mM $Fe(CN)_6^{-3}$ and 0.5 mM EDTA with (B) or without (A) 1 μM CCCP. Chlorophyll concentration is 1.87 mg·ml^{-1}. Each trace is an average of 24 experiments.

enhancement from effects of slow R_1 variation due to temperature drift, a single flash, followed by a 3.5 min dark delay, was applied 8 min after the three-flash sequence (indicated by the second arrow in Fig. 3). This second flash returns all centers to the S_1 state and thus defines the post-sequence baseline for the three-flash experiment. The S_1 baseline is shown by dashed lines in Figs. 3a and b.

The three-flash sequence produces a positive R_1 transient, which decays with a half-time of approx. 40 s to a stable ΔR_1 value of 0.002 s^{-1}. The decaying component of the transient was unexpected in terms of the presumed stability of S_0 state; it was initially interpreted as resulting from centers in the S_2 and S_3 states, due either to the use of non-saturating actinic flashes, or due to damaged centers unable to complete the $S_3 \rightarrow S_4 \rightarrow S_0$ transition. However, the addition of CCCP at a concentration sufficient to suppress entirely the S_2 and S_3 responses other than the first measured point, did not similarly suppress the three flash response. In the presence of CCCP, the R_1 measurements collected at times greater than 10 s after the three flash sequence reflect exclusively centers taken to the S_0 state. Thus the response
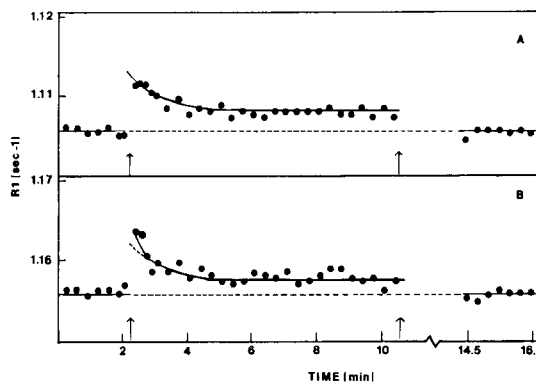

Fig. 4. Proton-solvent relaxation rate as a function of time in a suspension of PS II particles following three actinic flashes. Experimental conditions are the same as described in Fig. 3 except for the inclusion of 250 μM DCBQ in the suspension in place of $Fe(CN)_6^{-3}$.

due to $S_1 \rightarrow S_0$ is a positive R_1 transient, which exhibits both kinetic and stationary components. The kinetic component, for which $\tau_{1/2} \approx 50$ s, was observed both in the absence and presence of CCCP. In contrast, the stationary component, which reflects relaxation due to a stable para-
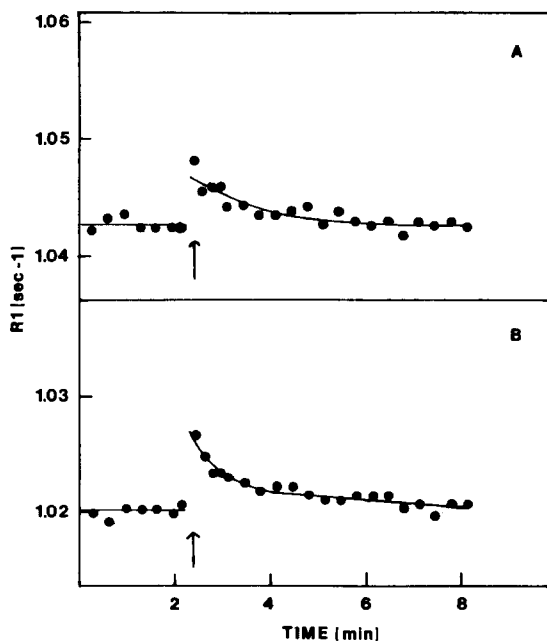

Fig. 5. Proton-solvent relaxation rate as a function of time in a suspension of PS II particles following four (A) and five (B) actinic flashes spaced 300 ms apart. Other experimental conditions are given in the legend of Fig. 1.

magnetic center in the S_0 state, was suppressed by CCCP.

This behavior suggested that the strongly relaxing center in S_0 might be oxidizable by $Fe(CN_6)^{-3}$ when CCCP is present as a redox mediator. To test this hypothesis, the 3-flash experiment was repeated using a weaker oxidant, DCBQ, as electron acceptor. Three flashes again produced a stable relaxation enhancement (Fig. 4A), which in this case persisted in the presence of CCCP (Fig. 4B). Thus the data of Figs. 3 and 4 demonstrate the presence of a strongly relaxing center in the S_0 state and suggest that this center may be oxidizable by $Fe(CN_6)^{-3}$, but not DCBQ, in the presence of CCCP.

Fig. 5 shows the proton NMR relaxation enhancement transient following the trains of four (Fig. 5a) and five (Fig. 5b) flashes. A positive, decaying R_1 enhancement is seen in both experiments. Fig. 6 contains a plot of the initial amplitude of the proton NMR relaxation enhancement transients as a function of flash number across the five flash cycle. In this cycle, the fourth flash represents a nominal return of the manganese center to the S_1 state and is expected to produce a local minimum in the flash response. This expectation is realized, although the amplitude of the oscillation is less than that normally encountered in measurements of O_2 flash yields. Effects leading to damping of the R_1 oscillation are considered further below.

Discussion

The efficiency with which membrane-bound paramagnetic ions are able to act as relaxation traps for solvent protons depends strongly on the oxidation state of the ion. Specifically, strongly relaxing centers are those which possess large paramagnetic moments and for which the electronic spin relaxation time is long, i.e., comparable to the inverse proton Larmor frequency (approx. 10^{-8} s at 20 MHz). Such long paramagnetic relaxation times are characteristic of ions in orbital singlet states, where the static zero-field splitting is small [22]. Among the various common manganese oxidation states, only Mn(II) and Mn(IV) ($^6A_1$ and $^4A_2$, respectively, in octahedral environments) possess orbital singlet electronic
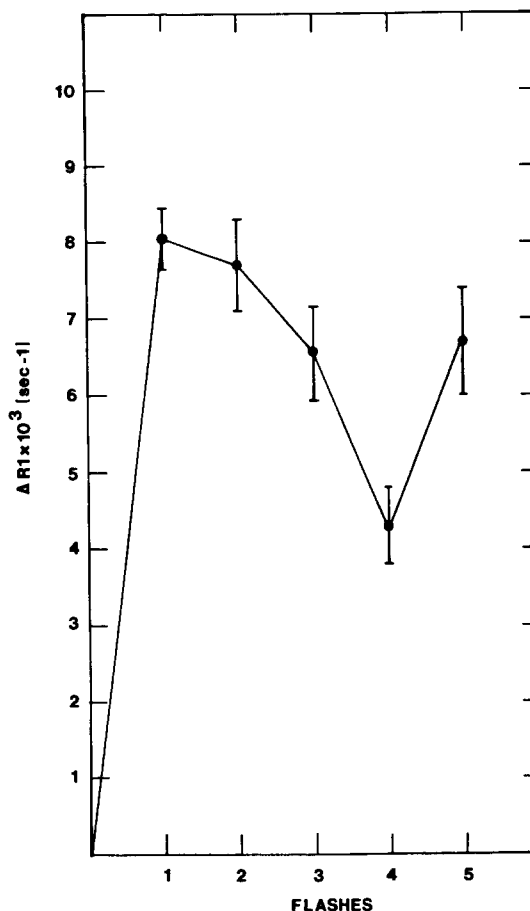

Fig. 6. Amplitude of the flash induced R_1 transient in suspensions of PS II particles as a function of flash number.

ground states. Thus the proton NMR relaxation enhancement experiment provides a reasonably selective probe of the appearance of disappearance of these two oxidation states during S-state transitions.

Fig. 6 summarizes the amplitudes of the observed proton NMR relaxation enhancement transients across a 5-flash cycle. In interpreting these transients in terms of the probable underlying manganese redox chemistry, we take the appearance of a strongly relaxing center on an oxidative S-state transition as evidence for a Mn(III) → Mn(IV) oxidation; the appearance of a strongly relaxing center on a reductive S-state transition is evidence for a Mn(III) → Mn(II) reduction. We believe that an interpretation based on individual ion oxidation states is probably ap-

propriate for the S-state system at room temperature. However, we cannot at present exclude the possible involvement of very strongly exchange-coupled manganese dimers or tetramers, in which case the observation of efficient NMR relaxation traps would be associated chemically with strongly spin-coupled spin-$\frac{1}{2}$ centers.

The $S_1 \to S_2$ transition is accompanied by the appearance of a strongly relaxing center, clearly indicative of a Mn(III) $\to$ Mn(IV) oxidation. The behavior of this transient has been described in detail previously [17]. The two-flash response is essentially identical in amplitude to the one-flash response although slower in its decay. Thus the second flash does not produce a second strongly relaxing center; the proton NMR relaxation enhancement experiment gives no indication that manganese redox chemistry takes place on this transition. The slowed decay after two flashes evidently reflects the altered kinetics of charge recombination between the S states and the quinone Q_B/Q_B^- on the acceptor side of PS II. Assuming that the acceptor side is largely oxidized during the dark-adaptation period, a single flash produces Q_B^-, and two flashes produce Q_B. Recombination from Q_B^- to the S states is a more efficient process than is recombination from Q_B.

The relaxation properties of the $S_1 \to S_0$ transition are described most clearly by the three flash experiments of Figs. 3 and 4. In summary, the $S_1 \to S_0$ transition produces a strongly relaxing center which exhibits both kinetic and stationary components. In the absence of ADRY reagent, three flashes produce a positive R_1 enhancement which persists over the time-course of the experiment. In the presence of ADRY reagent (CCCP, 1 μM) however, the three-flash R_1 enhancement relaxes to the S_1 baseline when the redox poise of the medium is strongly oxidizing (1.5 mM $Fe(CN)_6^{-3}$). Under less strongly oxidizing conditions (250 μM DCBQ), a stable R_1 enhancement persists both in the presence and absence of ADRY.

These results indicate that a strongly relaxing center is formed during the $S_1 \to S_0$ transition. The appearance of a strongly relaxing center on a reductive transition of the S states is suggestive of the formation of Mn(II) from Mn(III). The S_0 state can be oxidized by $Fe(CN)_6^{-3}$ in the dark to a state that is identical in its NMR relaxation properties to S_1. This oxidation reaction is accelerated by the ADRY reagent CCCP. When the redox posie is set by DCBQ, the strongly relaxing center associated with the S_0 state is stable both in the presence and absence of CCCP, evidently due to the lower midpoint potential of this acceptor system. It is interesting to note, however, that the R_1 response measured in the presence of DCBQ and CCCP (Fig. 4b) contains both kinetic and stationary components. The kinetic component, for which $t_{1/2} \approx 50$ s, evidently reflects a partial CCCP-catalysed oxidation of the S_0 state.

The conclusion from the NMR data that the $S_1 \to S_2$ transition involves a Mn(III) $\to$ Mn(IV) oxidation is in accord with other experimental observations. In particular, the formation at low temperature of a multiline EPR signal on this transition indicates that manganese redox chemistry is involved, as does the observed shift to higher energy of the mangnese K_α absorption edge. The S_2 state evidently comprises a mixed valence manganese cluster [8,16,23].

On the other hand, the apparent absence of manganese oxidation on the $S_2 \to S_3$ transition in the proton NMR relaxation enhancement experiment conflicts with the conclusion of Dekker et al. [14], who attributed a broad optical transient near 315 nm to a Mn(III) $\to$ Mn(IV) oxidation. However, other experimental observations appear consistent with the absence of manganese redox chemistry on this transition. In particular, the manganese K_α absorption edge is relatively little perturbed by the $S_1 \to S_3$ transition, in contrast to its behavior after one flash [24]. Moreover, the thermoluminescence band associated with the $S_2 \cdot Q_B^-$ recombination occurs at essentially the same temperature as that due to $S_3 \cdot Q_B^-$ recombination (B band) [25-30], suggesting that the holes associated with S_2 and S_3 may have similar potentials.

If the $S_2 \to S_3$ transition involves oxidation of Mn(III) as inferred from the ultraviolet transients, then the Mn(IV) formed does not provide an efficient relaxation trap for proton magnetisation. This situation could occur if the Mn(IV) produced were magnetically isolated from the pool of solvent protons. Mechanistic studies of the relaxation coupling in the proton NMR relaxation enhancement

experiment would be useful in this regard to assess whether magnetic coupling from manganese to the solvent protons involves a spin-diffusion mechanism [17]. If spin-diffusion coupling through the membrane dominates the relaxation pathway, magnetic isolation of manganese would be unlikely.

Our conclusion, based on the proton NMR relaxation enhancement data, that the $S_0 \to S_1$ transition is associated with a $Mn(II) \to Mn(III)$ oxidation also conflicts with the work of Dekker

et al. [14], who interpreted the 315 nm optical transient in terms of $Mn(III) \to Mn(IV)$. The chemical identity of the strongly relaxing center associated with S_0 can be further characterized through a study of the magnetic field dependence of the proton NMR relaxation enhancement. Such studies should clarify the proposed involvement of $Mn(IV)$.

The proton NMR relaxation enhancement transients across a five-flash cycle are compared to theoretical simulations in Fig. 7. The overall S-state transient following the jth flash is given by the expression

$$T_j = \sum_{i=0}^{3} P_i T_i$$

where P_i is the fractional population of the S_i state and T_i is the R_1 transient for the $S_1 \to S_i$ transition assuming 100% efficiency. S-state populations were calculated assuming a misses parameter of $\alpha = 0.1$ and a double-hits parameter of $\beta = 0.05$. The proton NMR relaxation enhancement experiment, unlike O_2 flash yields, is also sensitive to centers which are capable of oxidation to S_2 or S_3, but are incapable of completing the S-state cycle. For this reason, simulations were based on two different assumptions with respect to the presence of inactive centers. The first (- - - - - -) assumed that all centers were active in O_2 evolution, while the second ($\cdots\cdots$) assumed that 15% of the centers were incapable of S-state turnover beyond S_3. The amplitudes of the flash-induced R_1 transients were taken from the data of Figs. 1a, 2a, and 3b. T_1 is by definition zero. T_3 was taken equal to T_2, which is the ΔR_1 value following a single flash, normalized to 100% $S_2 + S_3$ population. T_0 was the ΔR_1 value measured 10 s after the 3-flash sequence in the presence of CCCP, also normalized to 100% S_0 population. R_1 data accumulated 10 s after the third flash were used to calculate T_0, since they contained contributions due only to S_0. Both simulations are normalized to the experimental one-flash transient.

The simulation which assumes that all centers are able to complete the S-state cycle was in only fair agreement with experiment on the fourth flash transient, where the calculated enhancement was
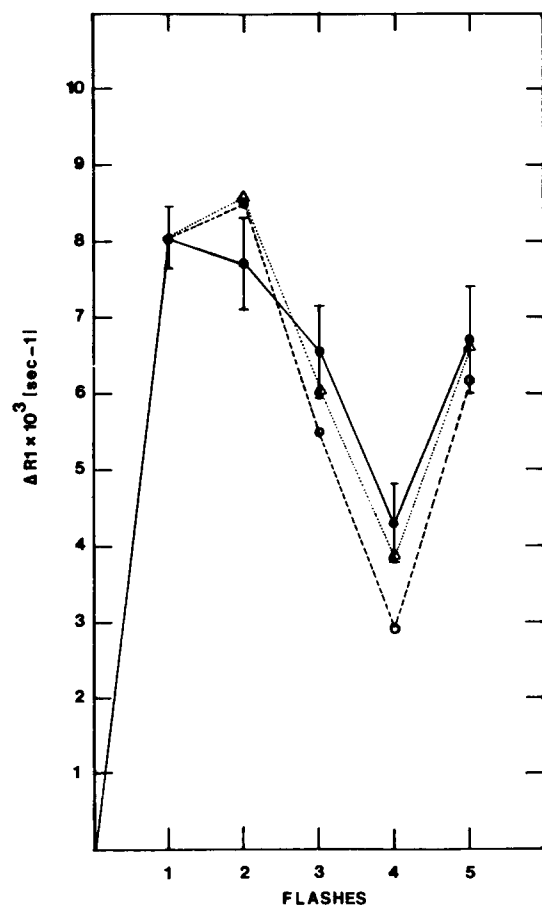

Fig. 7. Comparison of experimental and calculated proton NMR relaxation enhancement flash profiles. Solid line represents experimental points. (- - - - - -) is a simulation assuming a misses parameter $\alpha = 0.1$ and a double hit parameter $\beta = 0.05$. ($\cdots\cdots$) is a simulation assuming the same misses and double hit parameters, and also assumes a 0.15 fraction of damaged centers which are unable to cycle past the S_3 state. Both simulations are normalized to the experimental transient on the first flash.

substantially lower than the observed value. Inclusion of a small fraction of partially inactive centers improved the agreement at the third, fourth and fifth flashes. Measurements of the R_1 flash response were undertaken using several different PS II preparations, and the results shown in Fig. 6 were typical, i.e., the four-flash response was significantly greater than predicted assuming the absence of blocked centers. It is possible that partially blocked centers may result from loss of the 33 kDa peripheral polypeptide, which is known to be required for the $S_3 \rightarrow S_0$ transition.

Acknowledgement

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