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Reversible and irreversible effects of alkaline pH on Photosystem II electron-transfer reactions

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Incubation of highly active, O_2-evolving PS II preparations at alkaline pH inhibits donor side electron-transfer reactions in two distinct fashions, one reversible the other irreversible. In both cases, O_2 evolution is inhibited, with concomitant loss of the light-induced multiline and $g = 4.1$ EPR signals and an increased steady-state level of EPR Signal II induced by continuous illumination. However, the inhibition that is observed between pH 7.0 and 8.0 is readily reversible by resuspension at low pH, while above pH 8.0 the effect is irreversible. In addition, under repetitive flash conditions the ms decay kinetics remains largely unchanged at pH $\leq$ 8.0 but shows about a 2-fold increase in amplitude and is slowed at pH above 8.0. The irreversible component of inhibition most likely can be attributed to the loss of Mn and the 16, 24 and 33 kDa proteins. The reversible component may be mediated by displacement of Cl^- from an anion-binding site by OH^- or by titration of ionizable groups on the protein(s) associated with water-splitting. We propose that the reversible inhibition blocks electron transfer between the O_2-evolving complex and an intermediate which serves as the direct donor to Signal II, while the irreversible inhibition blocks the reduction of Signal II by this intermediate donor species.

Introduction

The loss of the water-splitting activity of chloroplast membrane preparations at alkaline pH in the presence of uncouplers has been observed by a number of investigators [1–4]. From these experiments it has been concluded that it is the internal thylakoid pH which controls water-splitting activity and photosynthetic electron flow through PS II by its effect on the O_2-evolving apparatus located on the lumenal side of the membrane [1]. The flash illumination study of Briantais et al. [3] indicates that the target for alkaline inactivation is the S_2 state of the oxygen-evolving complex.

Highly active PS II preparations [5–7] offer an excellent means of examining the effect of pH on the water-splitting reactions. Because the lumenal surface of the membrane is exposed to the external medium in these preparations [7], the direct influence of pH on the water-splitting apparatus can be investigated without interference from an intervening membrane barrier. Kuwabara and Murata [6,8] found that suspension of PS II preparations in Tricine buffers at pH $\geq$ 8.0 produced a loss of O_2 evolution [6], the release of the 33, 24 and 16 kDa proteins [8] and a significant decrease in the Mn content [8], indicating a substantial perturba-

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Abbreviations: Mes, 4-morpholineethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Chl, chlorophyll; DCBQ, 2,5-dichlorobenzoquinone; PS, Photosystem.

tion of the O_2-evolving apparatus. In those studies [6,8], the O_2-evolution activity of the preparations was assayed at low pH (6.5) subsequent to the treatment at high pH; using this inhibition assay, only 10–15% of the activity was lost from samples treated at pH 8.0 as compared to those maintained at pH 6.5. These results can be contrasted to those of Sandusky et al. [9], who observed that PS II preparations assayed directly in pH 8.0 buffer exhibited only 10% of the activity of a sample assayed at pH 6.0. This difference in pH-dependence of the O_2-evolution activities obtained via the two assay procedures implies that alkaline pH affects the water-splitting complex in more than one fashion.

More recently, Damoder and Dismukes [10] presented a profile of O_2-evolution activity versus assay pH which differs from the results of Sandusky et al. [9]. Additionally, Damoder and Dismukes [10] found that the light-induced amplitude of the low-temperature multiline EPR signal [11,12] is independent of pH in the range 5.5–8.5. Because the multiline EPR signal is assigned to the S_2 state [12,14,17,18], they concluded that no protons are released from the water-splitting complex at the $S_1 \rightarrow S_2$ transition. However, using the assay procedure of Kuwabara and Murata [Refs. 6 and 8, vide supra], we observed a substantial decrease in the amplitude of multiline signal and O_2-evolution above pH 8.0 [19], consistent with the loss of Mn and essential peptides reported for this pH regime by Kuwabara and Murata.

The differences and discrepancies among the results cited above indicated to us that additional studies of the effect of pH on the water-splitting complex and associated electron transport components were warranted. Thus we have measured the O_2-evolution activity, the amplitudes of the low-temperature multiline EPR signal [11,12] and the photoinduced $g = 4.1$ EPR signal [13,14], and EPR Signal II decay kinetics [15,16], both for PS II preparations suspended in alkaline pH buffers and for preparations treated at alkaline pH and subsequently returned to low pH (6.0). We find that there are two modes of alkaline inactivation of the water-splitting complex: a reversible component of inactivation between pH 7.0–8.0 and an irreversible component above pH 8.0. Contrary to the results of Damoder and Dismukes [10], we find that the amplitude of the multiline EPR signal associated with the S_2 state of the O_2-evolving complex [12,14,17,18] is not independent of pH. Instead, we observe that for both modes of inhibition, the loss of the multiline EPR signal parallels the loss of O_2-evolution activity. Evidence is provided which indicates that the mechanism of inhibition is different for the two modes of inactivation, and a model for the sites of inactivaiton is presented.

Materials and Methods

O_2-evolving PS II preparations were obtained as described previously [19]. High pH treatment was accomplished by suspending samples in an incubation buffer containing 15 mM NaCl and either 50 mM Mes (pH 6.0 and pH 6.5), 50 mM Hepes (pH 7.0–pH 8.0) or 50 mM Tricine (pH 8.25–pH 9.0) at a chlorophyll concentration of 1–3 mg/ml. Samples were incubated on ice for 1 h under ambient light. Aliquots were withdrawn for assay of O_2-evolution, and the suspensions were centrifuged (10 min, $34\,000 \times g$). The irreversible effects of high pH treatment were investigated by additionally washing the pellets once in pH 6.0 incubation buffer. Pellets were resuspended in the appropriate incubation buffer containing approx. 50% glycerol to a final chlorophyll concentration of 3–5 mg/ml. Samples were placed in quartz EPR tubes and dark-adapted for 1 h before freezing in liquid N_2. Continuous illumination of the EPR samples was performed with a 400 W tungsten source filtered through 10 cm of water. Sample temperature was maintained with a Varian model 906790-03 temperature controller or a solid CO_2/CH_3OH bath. Low-temperature EPR spectra were recorded with a Varian E109 spectrometer as described previously [13]. Typically, spectra were obtained by averaging four scans of the magnetic field. The ms decay kinetics of EPR Signal II in a flowing sample were measured with the same spectrometer utilizing a Phase R DL-1400 dye laser for sample excitation as described previously [15,16]. Steady-state Signal II measurements were performed in the dark and under saturating, continuous illumination with a tungsten lamp using the same experimental set-up as that used for the kinetics experiments. Instrument settings are listed

in the figure legends. Steady-state O_2-evolution rates were measured as described previously [16]. Typically, control rates at pH 6.0 of 250–400 μmol O_2/(mg Chl · h) were obtained.

Results

Fig. 1A shows the low-temperature multiline EPR signal produced by continuous illumination at 190 K of samples incubated at various pH values. The amplitude of the EPR signal decreases monotonically in the range pH 6.0–8.0. At pH 8.0 there remains about 20% of the pH 6.0 signal amplitude. No difference in the line shapes or line positions is observed in this pH range. For samples at higher values of pH the third line downfield from $g = 2$ appears anomalously large. This is probably a result of the overlapping g_y feature from a small amount of photoxidized cytochrome *b*-559 (high potential). Also, the portions of the spectra upfield from $g = 2$ may be modified by signals from the photoreduced acceptor Q_A. Fig. 1B shows that returning samples to pH 6.0 after incubation at pH $\leq$ 8.0 results in significant restoration of multiline signal amplitude. Although this restoration is nearly complete in samples incubated at pH 7.5 or pH 7.75, the sample at pH 8.0 returns to only about 80% of the control level. At pH greater than 8.0 the decreases are largely irreversible (Fig. 2). Again, there is no evidence for alteration of line shape or position.

As reported previously in similar PS II preparations, steady-state O_2-evolution activity is also inhibited when assayed at alkaline pH (Fig. 2, closed symbols). The activity is half-maximal at pH 7.25–7.5 and is not detectable by pH 8.5. On the same graph, we have plotted multiline signal amplitudes produced under conditions identical to those in Fig. 1. Within experimental error, the steady-state O_2-evolution activity and multiline signal amplitude decrease in parallel as the pH increases.

Both O_2-evolution activity and multiline signal amplitude are inhibited by incubation at pH $\geq$ 8.0, even when samples are subsequently assayed at pH 6.0 (Fig. 2, open symbols). However, this irreversible effect occurs at higher pH values; half-maximal inhibition is not achieved until about pH 8.25–8.5. In addition, the inhibitory effect occurs within a narrower range of pH.

We have also investigated the effects of alkaline pH on the light-induced $g = 4.1$ EPR signal as-

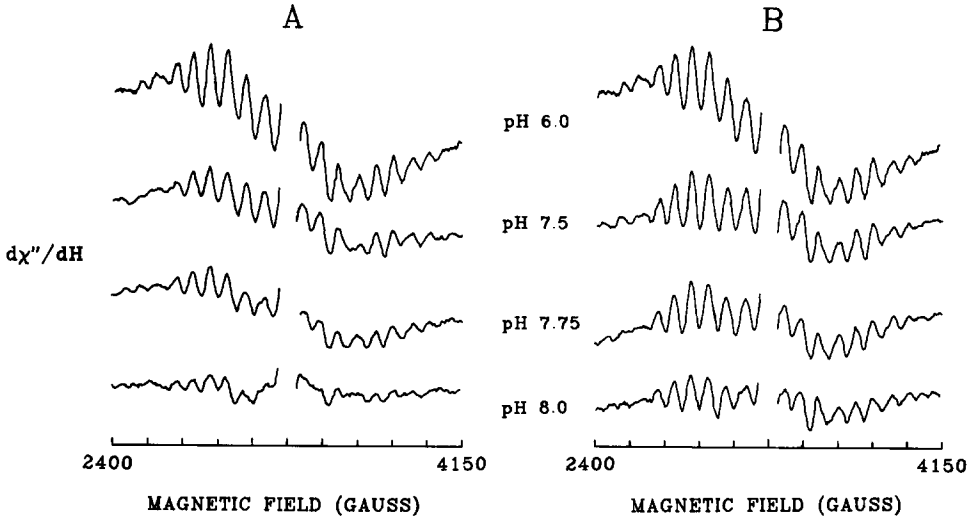

Fig. 1. The effect of pH on the formation of the multiline EPR signal. Each PS II sample was illuminated at 190 K for 3 min. Spectra of the dark-adapted samples have been subtracted. Amplitudes have been normalized for differences in chlorophyll concentration. (A) From top to bottom: pH 6.0, pH 7.5, pH 7.75, pH 8.0. (B) Same as (A) except samples were resuspended at pH 6.0 after alkaline pH treatment. Spectrometer conditions: temperature, 10 K; microwave power, 20 mW; microwave frequency, 9.19 GHz; field modulation, 32 G; modulation frequency, 100 kHz; scan rate, 2000 G/min; time constant, 64 ms.

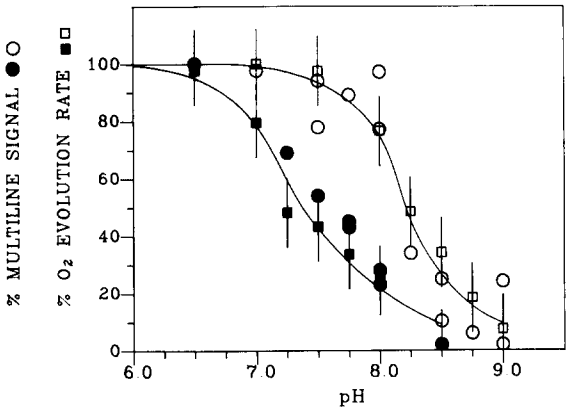

Fig. 2. The reversible and irreversible effects of pH on the steady rate of O_2-evolution and the amplitude of the multiline EPR signal. Closed symbols: O_2-evolution rates and multiline signal amplitudes measured at the indicated pH. Open symbols: O_2-evolution rates and multiline signal amplitudes measured at pH 6.0 following incubation at the indicated pH. O_2-evolution rates and multiline signal amplitudes are normalized relative to control samples incubated at pH 6.0. O_2-evolution rates represent the average of 2 or 3 separate experiments. Error bars indicate a deviation of $\pm 12\%$ about the mean, which represents the largest S.D. observed at any pH. EPR samples were illuminated and spectra were recorded as in Fig. 1. Multiline signal amplitudes were taken as the peak-to-peak height of five lines downfield from $g = 2$. Each point represents a single determination.

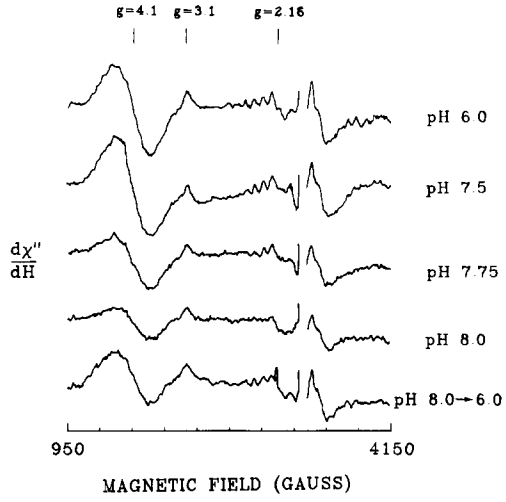

Fig. 3. The effect of pH on the formation of the $g = 4.1$ EPR signal. Samples were illuminated at 140 K for 1 min. Spectra of the dark-adapted samples have been subtracted. From top to bottom: pH 6.0, pH 7.5, pH 7.75, pH 8.0, pH 8.0 taken back to pH 6.0. Spectrometer conditions were as in Fig. 1.

signed to an intermediate donor between the O_2-evolution complex and the PS II reaction center [13,14]. Fig. 3 shows difference spectra obtained by subtraction of the dark-adapted spectrum from the spectrum after continuous illumination at 140 K. As reported previously [13], the prominent features produced by the illumination were the broad derivative-shaped signal at $g = 4.1$, signals at $g = 3.1$ and $g = 2.16$ assigned to the oxidation of cyt *b*-559 HP and a broad negative peak upfield from $g = 2$, assigned to the reduction of Q_A. In addition, a very small multiline signal was induced in a small fraction of centers which were advanced to the S_2 state during illumination or sample handling. Compared to the amplitude at pH 6.0, the $g = 4.1$ signal is perhaps somewhat larger at pH 7.5, but is decreased to about one third at pH 8.0. The linewidth, however, remains constant. As seen for the O_2-evolution activity and multiline signal, the pH effect on the $g = 4.1$ signal is reversible at pH < 8.0; returning the pH 8.0 sample to pH 6.0 resulted in restoration of the signal to about 60% of the control amplitude. At pH ≥ 8.0 this signal is irreversibly lost. More precise quantitation of the $g = 4.1$ signal is hampered by the presence of a 5-fold larger background signal centered at $g = 4.3$, which can lead to artifacts in the difference spectra due to small microwave frequency shifts.

Fig. 4 shows the effect of alkaline pH on the ms decay kinetics of EPR Signal II under repetitive flash conditions (8–12 flashes per turnover). The kinetic traces at both pH 6.0 and pH 7.75 contain a dominant phase with a half-time of 3–4 ms and a minor slow phase with a half-time of approx. 200 ms. The amplitude of the decay trace at pH 7.75 is about 25% larger than at pH 6.0 when normalized for differences in Chl concentration. In contrast, the decay amplitude is more than 2-fold larger for the sample incubated at pH 8.75 and subsequently assayed at pH 6.0. This indicates that there is a substantial loss of the μs component of the Signal II reduction kinetics [16]. Moreover, the kinetics in the ms regime are altered after treatment at pH 8.75; the dominant phase is slower, with a half-time of about 20 ms.

Under continuous illumination a substantial steady-state increase in EPR Signal II is expected

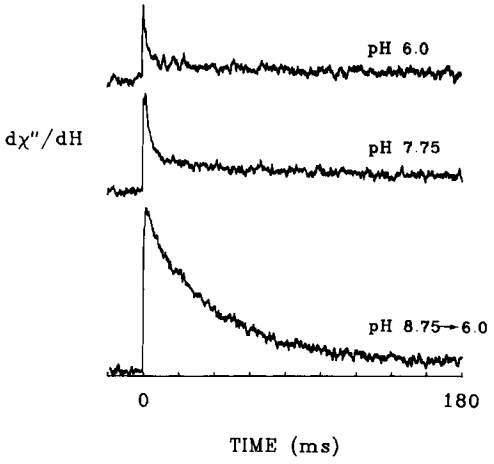

Fig. 4. The effect of pH on the decay kinetics of EPR Signal II. From top to bottom: pH 6.0, pH 7.75, pH 8.75 taken back to pH 6.0. The kinetics were measured at $g = 2.010$, the low-field maximum of EPR Signal II, using a Varian E-109 spectrometer. Instrument settings: field modulation, 100 kHz; modulation amplitude, 5 G; microwave power, 20 mW; half-time of instrument rise, 0.25 ms (time constant, out). Flowing sample of 3 ml (2.5 mg Chl/ml) contained 2.5 mM potassium ferricyanide, 2.5 mM potassium ferrocyanide, and 500 μM DCBQ. Each trace represents the sum of 5000 flashes. Sample temperature was maintained at 17 ± 1°C. Amplitudes are normalized for Chl concentration. Laser excitation: 5–10 mJ/pulse at the sample; laser flash frequency, 1 Hz; 8–12 flashes/sample turnover while flowing.

in preparations where the rereduction of Signal II is blocked or slowed significantly. Table I shows the effect of alkaline pH on the steady-state level of Signal II induced by continuous, saturating illumination. These measurements were performed in the presence and absence of DCBQ, which acts as a more efficient acceptor than ferricyanide alone. As a reference, we have included a sample treated with 0.8 M $CaCl_2$, which, as we have shown previously, blocks or greatly slows the rereduction of Signal II [16]. At pH 6.0, in the presence of DCBQ, a small increase in Signal II amplitude is observed upon illumination, possibly due to centers inactivated during purification. This represents about a 10% increase over the dark level of Signal II (data not shown). At pH 7.75, in the absence of DCBQ, about half of the maximal Signal II increase is seen, whereas in the presence of DCBQ a maximal increase is observed. The effect of incubation at pH 7.75 is partially reversed on returning to pH 6.0. In contrast, the sample incubated at pH 8.75 and assayed at pH 6.0 displays a nearly maximal increase in Signal II independent of the presence of DCBQ.

TABLE I

THE EFFECT OF pH ON STEADY-STATE EPR SIGNAL II AMPLITUDE INDUCED BY CONTINUOUS ILLUMINATION

Steady-state EPR Signal II amplitudes were measured at $g = 2.010$ using the same instrument and flow system as that used for the time resolved experiments. Instrument settings: field modulation, 100 kHz; modulation amplitude, 4 G; microwave power, 5 mW; time constant, 0.5 s. Flowing sample of 2 ml (2.5 mg Chl/ml) contained 1 mM potassium ferricyanide, 0.1 mM potassium ferrocyanide $\pm$ 500 μM DCBQ. The amplitudes represent the increase in signal intensity at the low-field maximum of EPR Signal II produced by saturating, continuous illumination and are normalized for differences in Chl concentration.

Sample	Light-induced amplitude of EPR signal II [a]	
	−DCBQ	+DCBQ
pH 6.0	0.17	0.25
pH 7.75	0.56	0.96
pH 7.75–6.0	0.20	0.40
pH 8.75–6.0	0.71	0.79
0.8 M $CaCl_2$, pH 6.0	1.1	1.0

[a] Arbitrary units. Estimated uncertainty, ± 0.05.

Discussion

Our evidence indicates that alkaline pH inhibits the O_2-evolution activity and the normal electron transfer reactions of the donor side of PS II in two distinct fashions. Both types of inhibition lead to a loss of light-induced amplitude of the multiline and $g = 4.1$ EPR signals and to an increase in the steady-state level of EPR Signal II produced by continuous illumination. However, the inhibition that is observed between pH 7.0 and 8.0 can be reversed readily by resuspension at low pH, while above pH 8.0 an irreversible inactivation is observed.

The irreversible component of inhibition can be ascribed to the depletion of Mn and the 16, 24 and 33 kDa proteins, which are known to be involved in the water-splitting process [19]. The loss of these peptides and Mn at alkaline pH (Ref. 8 and N.V.

Blough and K. Sauer, unpublished observations) parallels reasonably closely the pH profile of the irreversible inhibition. Moreover, we have previously shown that the depletion of all three of these proteins by $MgCl_2$ (or $CaCl_2$) extraction produces a similar loss of multiline signal amplitude and the μs phase of Signal II decay (Refs. 16 and 19; Table I and Fig. 3).

In contrast, the reversible component of inhibition cannot be assigned to the depletion either of these three proteins or of Mn, because under the conditions of these experiments, these species are not lost appreciably at pH < 8.0 (Ref. 8 and N.V. Blough and K. Sauer, unpublished observations). Although the precise mechanism of the inhibition is not yet apparent, we can suggest two possible explanations for the effect. First, the reversible inactivation may be mediated by the interaction of OH^- with an anion-binding site. Based on the dependence of O_2-evolution on Cl^- concentration in halophyte thylakoids, Critchley et al. [20] proposed that reversible, ionic binding of Cl^- at or near the O_2-evolving complex facilitates PS-II electron transport. These authors also suggested a mechanism for the inhibitory effect of alkaline pH on O_2-evolution involving displacement of bound Cl^- by OH^- [20,21]. In a PS II preparation from spinach, Yocum and co-workers [9] found that the displacement of bound Cl^- from the water-splitting site by SO_4^{2-} occurs at pH 7.5 but not at pH 6.0, and it was suggested that chloride binding also is pH dependent in this system. Alternatively, the reversible inactivation may result from titration of ionizable groups on the protein(s) involved in O_2 evolution, causing the complex to adopt a nonfunctional conformation.

Based on our results, we can assign two different sites for the reversible and irreversible components of inhibition. In both cases, steady-state buildup of EPR Signal II under saturating, continuous illumination is indicative of a decrease in the rate of electron transfer from the O_2-evolving complex to Z, the species giving rise to Signal II. However, the buildup is not maximal in the reversible inhibition unless DCBQ, a more efficient acceptor than ferricyanide, is present. This implies that under steady-state conditions reduction of Z is slowed but not completely blocked by this treatment. At the same time, reversible inhibition leaves the transient decay kinetics of Signal II under repetitive flash conditions largely unchanged, indicating that a substantial portion of the Signal II decay is in the sub-ms regime. This suggests that the donor to Z is still present and is rereduced between flashes. This donor is not the species giving rise to the multiline or $g = 4.1$ EPR signals, because these signals are not generated upon alkaline pH inactivation. The results presented here are consistent with our model in which we proposed an intermediate electron carrier Y between the water-splitting site and Z [16]. Thus, the effect of reversible inhibition is to block electron transfer from the O_2-evolving complex to the intermediate donor Y. In agreement with our model, the ms decay kinetics in dark-adapted samples at pH 7.75 subjected to a series of flashes show no evidence of accumulation of oxidizing equivalents at the water-splitting site (James Cole and Michael Boska, unpublished observations). The 2-fold increase in the amplitude of the ms decay phases of Signal II under repetitive flash conditions seen upon irreversible inactivation is very similar to what we previously observed with $CaCl_2$-washed samples [16]. This increase represents the loss of a sub-ms component, which is not observable with 100 kHz field modulation. The loss of this decay component, which was assigned to rapid equilibration between Y and Z, indicates that the site of irreversible inactivation is the electron transfer from Y to Z.

Below is a model for the donor side of PS II showing the different sites for the reversible component of alkaline inactivation at pH < 8.0 and the irreversible component at pH > 8.0.

Below pH 8.0:

$$S_1\text{-}S_4 \cdots g = 4.1 \overset{/}{\not\rightarrow} Y \rightarrow Z \rightarrow \text{P-680}$$

Above pH 8.0:

$$S_1\text{-}S_1 \cdots g = 4.1 \rightarrow Y \overset{/}{\not\rightarrow} Z \rightarrow \text{P-680}$$

The species giving rise to the $g = 4.1$ EPR signal, has been implicated as an intermediate electron donor in PS II, functioning when the O_2-evolving complex is poised in the S_1 [13] or S_2 [14] states. It is uncertain whether this species also

functions in higher S-state transitions. Our results indicate that the $g = 4.1$ species is labile to reversible alkaline inactivation, but Y, the species responsible for the sub-ms reduction of signal II, is not, suggesting that the $g = 4.1$ species is more closely associated with the manganese-containing water-splitting complex.

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