

pH dependence of the multiline, manganese EPR signal for the 'S₂' state in PS II particles

Absence of proton release during the S₁ → S₂ electron transfer step of the oxygen evolving system

Reddy Damoder and G. Charles Dismukes

Department of Chemistry, Princeton University, Princeton, NJ 08544, USA

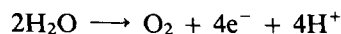
Received 14 June 1984

The pH dependence of oxygen evolution rates, 2,6-dichlorophenolindophenol (DCIP) reduction rates and the intensity of the multiline manganese EPR signal associated with the S₂Kok state has been studied using oxygen-evolving spinach (PS) II particles. The oxygen evolution and DCIP reduction rates are found to be very sensitive to pH, with the maximal rates occurring at pH 6.5–7.0. Both the rate and yield of the S₂ multiline manganese EPR signal intensity, produced by single flash excitation at room temperature or by continuous illumination at 200 K, are found to be independent of pH, indicating that no proton is released from this manganese site during the S₁→S₂ electron transfer. These results agree with those from other laboratories showing no proton release on this transition, but using techniques monitoring other species.

Photosystem II Manganese EPR Photosynthetic oxygen evolution Photosynthetic water oxidation
Electron transfer S₂ multiline EPR signal

1. INTRODUCTION

Photosynthetic oxygen evolution occurs in a 4-step process [1] by the oxidation of water catalyzed by a manganese (Mn)-containing enzyme (review [2,3]). Each step involves a light-driven reaction between two of the 5 intermediate oxidation states of the enzyme, identified as S_n states (*n* = 0–4). It is generally believed that one electron is transferred during each of these 4 steps. It has been established that 4 protons are also released in this 4-electron oxidation, thus establishing the electroneutrality of the overall reaction:



There has been considerable debate in the literature regarding the number of protons released during each step. Authors in [4] found a stoichiometry of 1, 0, 1, 2 for proton release with the reactions starting with S₀ → S₁, those in [5] obtained 0, 1, 1, 2, while the results of [6] sug-

gested the release of 0.75, 0, 1.25, 2 protons during the 4 steps. This non-integral stoichiometry was explained by assuming two parallel mechanisms of 1, 0, 1, 2 and 0, 0, 2, 2 with 75 and 25% distribution, respectively. This distribution corresponds to the steady-state population ratio for S₁/S₀ in the initially dark-adapted chloroplasts. Authors in [7] found a proton pattern of 1, 1, 1, 1. More recent results [8–12] all support the 1, 0, 1, 2 stoichiometry, which is the generally accepted result.

The two main experimental techniques used to measure pH changes occurring inside the thylakoids, as a result of proton release after each of a train of short flashes on dark-adapted chloroplasts are (i) use of fast and sensitive glass electrodes [6] and (ii) use of a dye such as cresol red or neutral red to monitor color changes of the dye occurring with changes of pH [5,8,10]. More recently, some experimentalists have sought the use of different techniques, other than those based

on pH changes, to obtain supporting evidence for the proton release mechanism. Authors in [9] used delayed fluorescence from chlorophyll, others [11] used thermoluminescence from chlorophyll, and those in [12] used ESR to detect the pH-sensitive spin-label tempamine.

In the measurements noted above it is not possible to distinguish between protons released from the membrane upon flash-induced electron transfer from those protons which are specifically released via water oxidation. A direct measurement of the proton equilibrium of the water oxidizing enzyme has not been previously reported owing to the absence of spectroscopic techniques for the detection of the enzyme. However, authors in [13,14] have reported a flash-induced, multiline EPR signal in spinach thylakoids arising from an apparent binuclear manganese center in a mixed-valence oxidation state identified with the S_2 Kok-state of the water oxidizing enzyme. This signal has also been characterized in [15,16] with general agreement on its association with the S_2 state. Since it is easy to obtain quantitative information from this EPR signal, we have examined the pH dependence of the $S_1 \rightarrow S_2$ transition as revealed by the S_2 multiline signal intensity in PS II particles. This also provides a direct measure of the proton release at the binuclear manganese site. It can therefore distinguish between protons released at this presumed active site, vs ionization of other groups which may contribute to pH changes.

2. EXPERIMENTAL

Spinach thylakoid membranes are obtained as in [17]. PS II particles are obtained from grana thylakoid membranes by a slight modification of the method of [18]. Details of this preparation will be described elsewhere (unpublished). The PS II particles obtained are highly active, showing oxygen evolution rates of 500–600 $\mu\text{M O}_2 \cdot \text{mg Chl}^{-1} \cdot \text{h}^{-1}$. The oxygen rates are assayed using a YSI model 53 oxygen monitor and a Clark-type oxygen electrode at 20°C. The electron acceptors used were 1.7 mM $\text{K}_3\text{Fe}(\text{CN})_6$ and 1.3 mM phenyl-*p*-benzoquinone (PPBQ). DCIP reduction is measured by following the absorption changes at 590 nm on a HP 8450 A spectrophotometer. Samples containing 40 μM DCIP and 30 μM chlorophyll in appropriate buffers are used for all O_2 rate measurements. The kinetics of reduction

are measured for at least 30 s, with a time resolution of 1 s. The decay curve is linear for about 10 s. The initial rates of DCIP reduction were obtained from the linear portion of the curve. The DCIP absorption at 590 nm is pH dependent. It was noted in [19] that DCIP is a weak acid with pK_a of 5.9. The observed pH dependence of the extinction coefficient (ϵ) arises due to the lower ϵ value for the protonated form ($2.7 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$) when compared to the deprotonated form ($\epsilon = 22 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$). These differences were taken into account in calculating the DCIP reduction rates at each pH value.

The suspension medium in all samples contains 0.33 M sorbitol, 15 mM NaCl and 4 mM MgCl_2 . To this is added one of the following buffers: 20 mM Mes (for pH 5.5, 6.0 and 6.5), 20 mM Hepes (for pH 7.0 and 7.5) or 20 mM Tricine (for pH 8.0 and 8.5).

The PS II particles stored at -78°C in 50% glycerol at pH 6.5 were thawed and diluted 10 times with the suspension medium of required pH and spun down at $40000 \times g$ for 20 min. The pellet is resuspended in suspension medium and then diluted to 50% with glycerol to give a final chlorophyll concentration of 4–6 mg/ml. This suspension is transferred into 4-mm quartz tubes for EPR. All steps are carried out at $0\text{--}4^\circ\text{C}$.

The EPR samples are dark adapted for 15 min, frozen to 200 K (methanol and dry ice mixture) and then illuminated at 200 K for 5 min with the visible light from a GE ELH 300 W quartz dichromatic lamp filtered by a 0.04 M CuSO_4 solution (16 cm). The light intensity at the sample is 0.9 W/cm^2 . Another set of dark-adapted samples at room temperature were flashed once with a Nd-YAG laser pulse (25 ns) and immediately quench cooled to 200 K. The samples are then stored in the dark in liquid nitrogen until EPR spectra are measured within 24 h. Non-illuminated dark-adapted samples are used as controls. EPR measurements are performed as in [17].

The O_2 -evolution and DCIP reduction rates are measured using the same samples used for obtaining EPR spectra. Similar rates are also obtained when freshly thawed PS II particles are used to measure the pH dependence of these rates in the absence of glycerol. Thus the PS II membranes used to obtain EPR spectra and rate data did not

lose any of their O₂-evolving activity during sample preparation.

3. RESULTS AND DISCUSSION

We have observed the S₂ EPR spectrum reproducibly in several PS II preparations and with a much better signal-to-noise ratio than observed with thylakoids. Because only one electron can be transferred by CW illumination at 200 K or by single laser flash at room temperature, and the S₁ Kok state is the most populated one in the dark, the EPR spectrum corresponds predominantly to the S₂ Kok state.

Quantitative information regarding the S₂ EPR signal intensity could be obtained from the peak heights and widths of the 5 well-defined low field lines ($g > 2.0$). We estimate the area under each of these transitions as (peak height) $\times$ (peak width)².

Linewidths vary only weakly with pH. The area under the 5 peaks is summed and normalized for chlorophyll concentration, which was usually nearly the same in all samples. Fig.1 shows the S₂ multiline signal at two different pH values, 5.5 and 7.0. It can be clearly seen that, when the differences in the chlorophyll concentrations are taken into account, the signal intensities are the same in the two spectra. No differences in either the line positions or line shapes are observed at different pH values between pH 5.5 and 8.5 for the major peaks. The signal intensity of the S₂ EPR signal is also found to be independent of pH in this range, irrespective of whether S₂ multiline signal is generated by CW illumination at 200 K or by single laser flash at room temperature. Fig.2 shows the S₂ signal intensity, O₂ evolution rate and DCIP reduction rate as a function of pH. In contrast to the S₂ multiline signal, both the O₂ evolution and

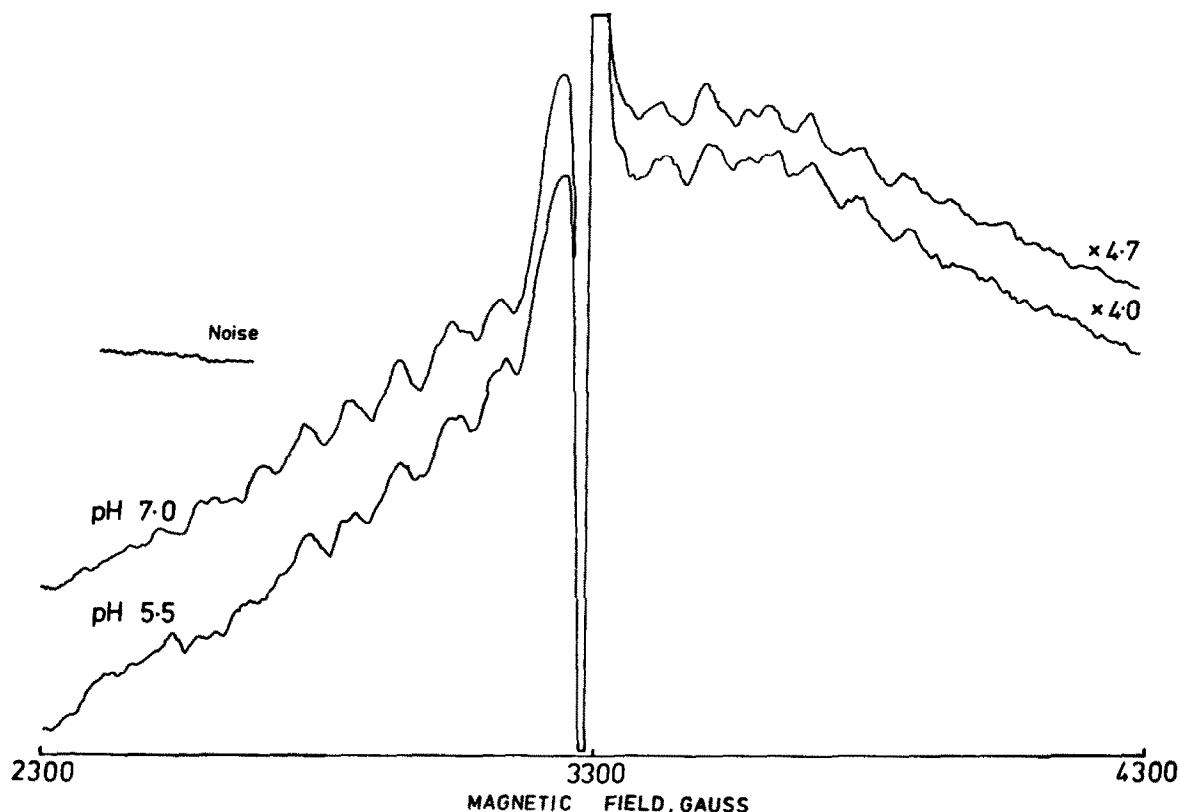


Fig.1. Light-induced (CW, 200 K) EPR spectrum (9.25 GHz) of the S₂ multiline signal at pH 5.5 and 7.0 in dark-adapted spinach PS II membranes. Spectra are recorded in the dark after illumination. Temperature, 10 K; microwave power, 50 mW; modulation amplitude, 32 G; scan rate, 500 G/min; time constant, 0.3 s. The chlorophyll concentrations in the two samples of pH 5.5 and 7.0 are 4.0 and 4.7 mg/ml, respectively.

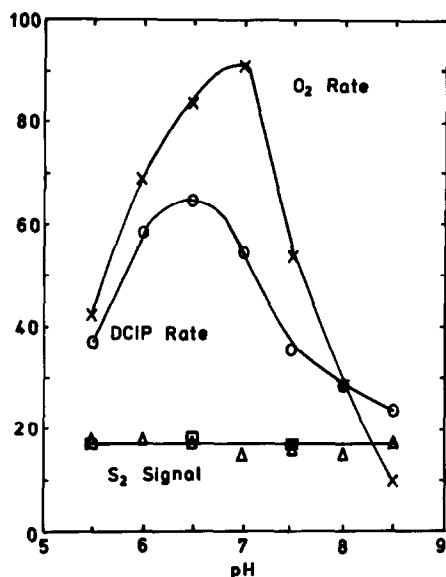


Fig. 2. Plot of oxygen evolution rate ($\times-\times$), DCIP reduction rate ($\circ-\circ$) and the S_2 EPR signal intensity [$(\Delta-\Delta)$, 5 min CW illumination at 200 K; or $(\square-\square)$, single laser flash at 20°C] as a function of pH. The y-axis scale is in arbitrary units.

DCIP reduction rates show a marked pH dependence, with the maximum rates occurring in the pH range 6.5–7.0. It is also observed that the Signal II EPR spectrum is also independent of pH in this interval.

The kinetics of S_2 multiline signal appearance at pH 5.5, 6.5 and 7.5 during illumination at 200 K are also independent of pH as shown in fig. 3. More than 50% of the multiline signal is generated by illumination for 1 s under the conditions used here. Maximum S_2 signal intensity is formed after 3 min illumination and does not change with further illumination.

All the acceptors used here ($K_3Fe(CN)_6$, PPBQ and DCIP) accept electrons between the intrinsic PS II acceptor, Q_B , and the plastoquinone pool. The observed rates reflect purely PS II electron transfer, as indicated by the complete loss of rate upon addition of 30 μ M dichlorophenyldimethylurea (DCMU), a potent inhibitor of PS II electron transport.

It is generally believed that two protons are released, on average, per one electron transferred through the entire chain; one proton being liberated from the donor side of PS II due to water

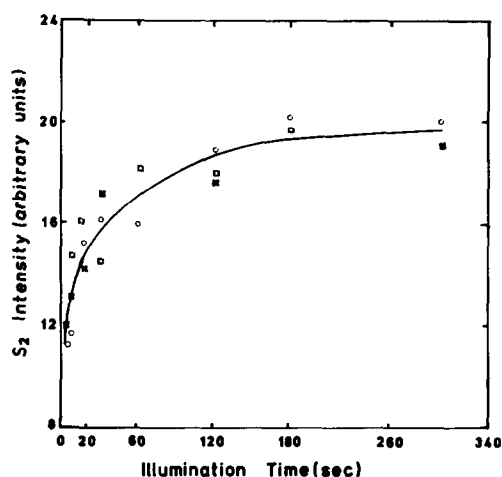


Fig. 3. Plot of light-induced (CW, 200 K) S_2 multiline EPR signal intensity as a function of illumination time. ($\circ$) pH 5.5, ($\times$) pH 6.5, ($\square$) pH 7.5.

oxidation, and the other from the donor side of PS I at the plastoquinone reoxidation step [4,21]. These two proton-releasing steps have been differentiated kinetically, with the former step having much faster half-rise time (microsecond range), compared to that of the latter step (millisecond range). Authors in [10] have correlated the proton release rates and electron abstraction rates during each electron transfer step with the conclusion that the proton is released after the electron is transferred in steps $S_0 \rightarrow S_1$, $S_2 \rightarrow S_3$ and $S_3 \rightarrow S_4$. In view of these correlations our results on the pH dependence of the steady-state V_{O_2} rate and DCIP reduction rate indicate that these overall processes are inhibited primarily in the $S_2 \rightarrow S_3 \rightarrow S_4$ steps and not in the $S_0 \rightarrow S_1 \rightarrow S_2$ steps. The lack of inhibition of the S_2 multiline EPR signal indicates that reactions leading to its formation by one electron transfer are unaffected by pH between 5.5 and 8.5. Consequently O_2 rate inhibition must take place in the higher S_n state transitions. The results of the experiment do not allow us to deduce the site where this rate limitation is imposed and, indeed may not even be located within the O_2 evolving complex itself. The average pK_a of the functional groups involved in this inhibition is 6.5–7.0 under our sample conditions. The lack of proton release for $S_1 \rightarrow S_2$ is consistent with the EPR description of the S_2 multiline signal arising from a mixed-valence Mn dimer having an $S = 1/2$ ground state

with no paramagnetic states for oxidized water present. The S_2 state appears to precede water oxidation. It should be mentioned that the pH effects observed on the rates are reversible, hence the water-oxidizing machinery is not destroyed at the extreme pH values studied.

The lack of dependency on pH (5.5–7.5) for the rate of S_2 multiline formation (fig.3) indicates that the efficiency of formation of the S_2 state is unaffected in this pH interval, even though O_2 evolution changes by more than a factor of two (fig.2). We therefore conclude that the rate-limiting reactions leading to formation of the S_2 state at 200 K, which include as candidates electron transfer steps leading up to oxidation of the O_2 evolving complex and reduction of the primary acceptor Q_A , do not exhibit an appreciable pH dependency.

The major controversy in the proton release mechanism has been for the $S_1 \rightarrow S_2$ electron transfer step. The observed pH independence of the S_2 multiline EPR signal intensity and its rate of appearance indicates directly that no protons are released from the binuclear Mn center during this transfer.

ACKNOWLEDGEMENT

This work is supported by a grant from the National Institutes of Health – General Medical Sciences GM 28789-03.

REFERENCES

- [1] Kok, B., Forbush, B. and McGloin, M. (1970) *Photochem. Photobiol.* 11, 457–475.
- [2] Sauer, K. (1980) *Acc. Chem. Res.* 8, 249–256.
- [3] Ames, J. (1983) *Biochim. Biophys. Acta* 726, 1–12.
- [4] Saphon, S. and Crofts, A. (1977) *Z. Naturforsch.* 32C, 617–626.
- [5] Junge, W., Renger, G. and Auslander, W. (1977) *FEBS Lett.* 79, 155–159.
- [6] Fowler, C.F. (1977) *Biochim. Biophys. Acta* 462, 414–421.
- [7] Hope, A.B. and Morland, A. (1979) *Aust. J. Plant Physiol.* 6, 289–304.
- [8] Velthuys, B.R. (1980) *FEBS Lett.* 115, 167–170.
- [9] Bowes, M.H. and Crofts, A.R. (1981) *Biochim. Biophys. Acta* 637, 464–472.
- [10] Forster, V., Hong, Y.-Q. and Junge, W. (1981) *Biochim. Biophys. Acta* 638, 141–152.
- [11] Inoue, Y. (1981) *Biochim. Biophys. Acta* 634, 309–320.
- [12] Willic, B. and Lavergne, J. (1982) *Photobiochem. Photobiophys.* 4, 131–144.
- [13] Dismukes, G.C. and Siderer, Y. (1980) *FEBS Lett.* 121, 78–80.
- [14] Dismukes, G.C. and Siderer, Y. (1981) *Proc. Natl. Acad. Sci. USA* 78, 274–278.
- [15] Brudvig, G.W., Casey, J.L. and Sauer, K. (1983) *Biochim. Biophys. Acta* 723, 366–371.
- [16] Hansson, O. and Andreasson, L.-E. (1982) *Biochim. Biophys. Acta* 679, 261–268.
- [17] Abramowicz, D.A. and Dismukes, G.C. (1984) *Biochim. Biophys. Acta*, in press.
- [18] Yamamoto, Y., Doi, M., Tamura, N. and Nishimura, M. (1981) *FEBS Lett.* 133, 265–268.
- [19] Armstrong, J.McD. (1964) *Biochim. Biophys. Acta* 86, 194–197.
- [20] Rutherford, A.W., Crofts, A.R. and Inoue, I. (1982) *Biochim. Biophys. Acta* 682, 457–465.
- [21] Teimann, R., Renger, G., Graber, P. and Witt, H.T. (1979) *Biochim. Biophys. Acta* 546, 498–519.