# pH dependence of the multiline, manganese EPR signal for the '$S_2$' state in PS II particles

## Absence of proton release during the $S_1 \longrightarrow S_2$ electron transfer step of the oxygen evolving system

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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 $S_2$ Kok 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 occuring at pH 6.5–7.0. Both the rate and yield of the $S_2$ 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 $S_1 \rightarrow S_2$ 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* *$S_2$ 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:

$$2H_2O \longrightarrow O_2 + 4e^- + 4H^+$$

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 $S_0 \longrightarrow S_1$, those in [5] obtained 0, 1, 1, 2, while the results of [6] suggested 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 $S_1/S_0$ 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

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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 $S_1 \longrightarrow S_2$ 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 $\mu$M $O_2 \cdot$mg $Chl^{-1} \cdot h^{-1}$. 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 $K_3Fe(CN)_6$ 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 $\mu$M DCIP and 30 $\mu$M 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 ($\epsilon$) arises due to the lower $\epsilon$ value for the protonated form ($2.7 \times 10^3\ M^{-1} \cdot cm^{-1}$) when compared to the deprotonated form ($\epsilon = 22 \times 10^3\ M^{-1} \cdot cm^{-1}$). 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 × *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 0–4°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 dichromic 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 $O_2$-evolving activity during sample preparation.

## 3. RESULTS AND DISCUSSION

We have observed the $S_2$ 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 $S_1$ Kok state is the most populated one in the dark, the EPR spectrum corresponds predominantly to the $S_2$ Kok state.

Quantitative information regarding the $S_2$ 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 $S_2$ 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 $S_2$ EPR signal is also found to be independent of pH in this range, irrespective of whether $S_2$ multiline signal is generated by CW illumination at 200 K or by single laser flash at room temperature. Fig.2 shows the $S_2$ signal intensity, $O_2$ evolution rate and DCIP reduction rate as a function of pH. In contrast to the $S_2$ multiline signal, both the $O_2$ evolution and

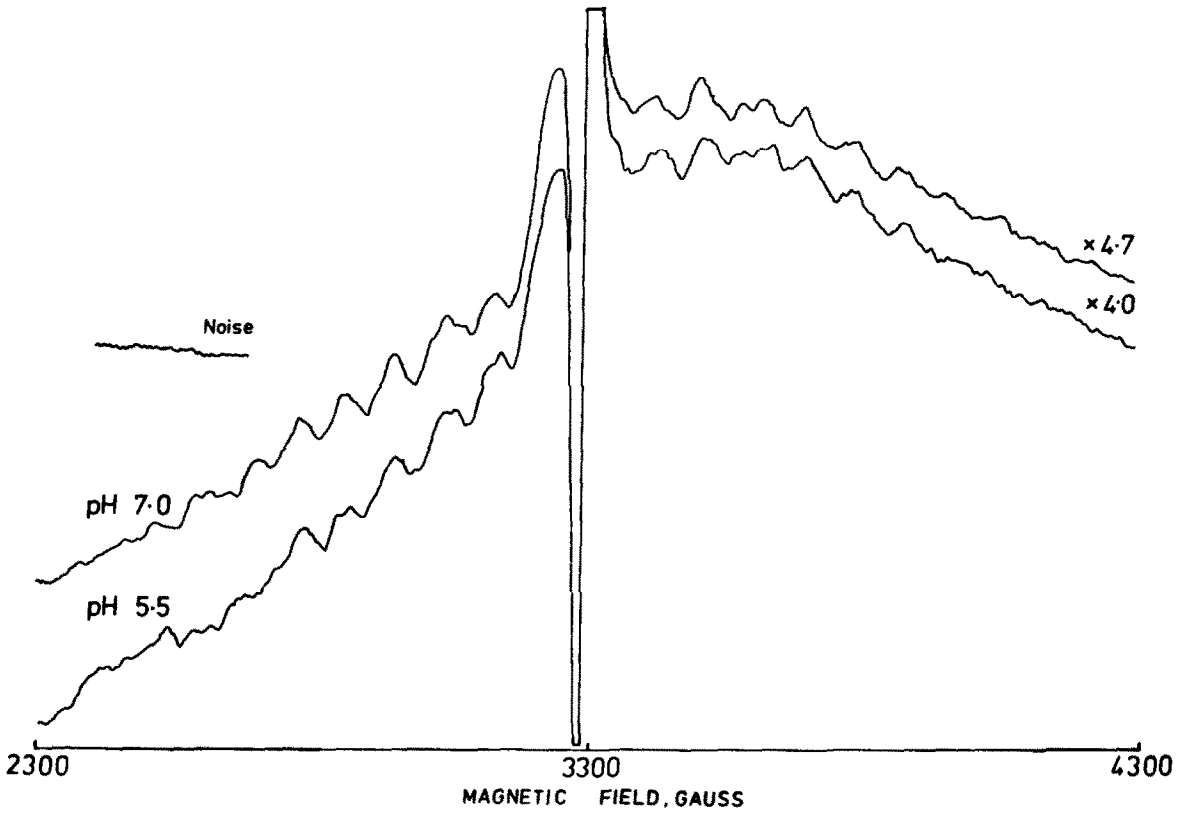


Fig.1. Light-induced (CW, 200 K) EPR spectrum (9.25 GHz) of the $S_2$ multiline signal at pH 5.5 and 7.0 in dark-adapted spinach PS II membranes. Spectra are recorded in the dark after illumination. Temperature, 10 K; microwave power, 50 mW; modulation amplitude, 32 G; scan rate, 500 G/min; time constant, 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.

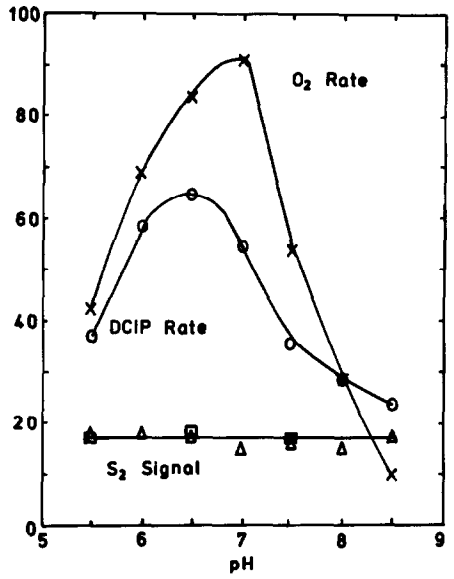


Fig.2. Plot of oxygen evolution rate (×—×), DCIP reduction rate (○—○) and the $S_2$ EPR signal intensity [(△—△), 5 min CW illumination at 200 K; or (□—□), single laser flash at 20°C] as a function of pH. The *y*-axis scale is in arbitrary units.

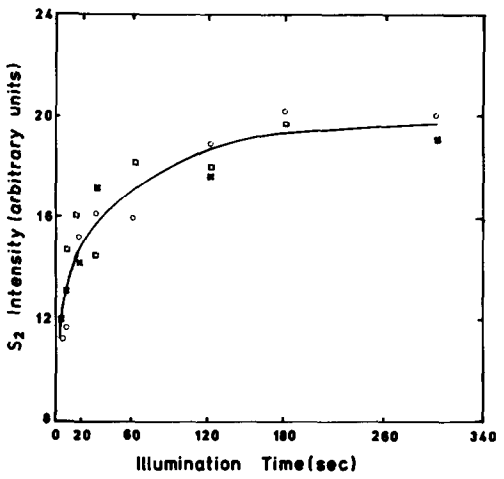


Fig.3. Plot of light-induced (CW, 200 K) $S_2$ multiline EPR signal intensity as a function of illumination time. (○) pH 5.5, (×) pH 6.5, (□) pH 7.5.

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 $\mu$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 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 $S_0 \longrightarrow S_1$, $S_2 \longrightarrow S_3$ and $S_3 \longrightarrow S_4$. 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 $S_2 \longrightarrow S_3 \longrightarrow S_4$ steps and not in the $S_0 \longrightarrow S_1 \longrightarrow S_2$ 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 $S_1 \longrightarrow S_2$ 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 $S_1 \longrightarrow S_2$ 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.

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