Oxygen from water is coordinated to manganese in the S_2 state of photosystem II

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Received 15 October 1985

Oxygen ligands from water are bound to the manganese cluster giving rise to the 'multiline' EPR signal from the S_2 state of photosystem II, as shown by the observation of hyperfine broadening of the EPR signal in the presence of ^{17}O-enriched water. The binding must occur in the S_0, S_1 or S_2 state. The estimated upper limit for the ^{17}O hyperfine coupling constant, 0.5 mT, excludes superoxide and hydroxyl radicals in the S_2 state, but not peroxide.

EPR *$H_2{}^{17}O$* *Manganese* *Oxygen evolution* *Photosynthesis* *Photosystem II*

1. INTRODUCTION

In the photosynthetic process of higher plants and algae, water is oxidized to dioxygen in 4 consecutive photoreactions of PS II. The intermediate oxidation states of the PS II electron donor side are usually designated with S_0, S_1 (predominant in the dark), S_2 and S_3. Each photoreaction involves a one-electron abstraction from the donor side. O_2 is formed in the $S_3 \longrightarrow S_0$ transition, which completes the cycle.

The absolute requirement for manganese in photosynthetic oxygen evolution suggested early that this element was involved in the stabilization of the different S states, but direct evidence for changes in the oxidation state of manganese was only recently achieved through the application of EPR [1–5], X-ray [6] and optical absorption spectroscopy [7,8]. However, questions concerning the number of manganese ions involved and their ligands, the S state at which water binds, the coordination of water to manganese and the oxidation states of oxygen and manganese in the different S states remain to be answered (review [9]).

We have addressed some of these issues by studying the effect of ^{17}O-labelled water on the EPR signal discovered by Dismukes and Siderer [2] and ascribed by them to interacting manganese ions in the S_2 state, an assignment that has been confirmed by others [3–5]. The line broadening induced by hyperfine coupling to ^{17}O nuclei ($I = 5/2$) shows that oxygen ligands from water are bound to the manganese cluster when it gives rise to the S_2 state EPR signal. The estimated upper limit for the hyperfine coupling constant, 0.5 mT, excludes superoxide and hydroxyl radicals in the S_2 state. Some possible binding geometries are discussed.

2. MATERIALS AND METHODS

Oxygen-evolving (600 μmol O_2/mg Chl per h) PS II particles were prepared from spinach as described [10]. $H_2{}^{17}O$ buffers were made by adding water with 50.7% enrichment (Merck, Sharp and Dohme) or water diluted to 32% enrichment to a freeze-dried Mes buffer (20 mM Mes-NaOH (pH

Abbreviations: $A(^{17}O)$, ^{17}O hyperfine coupling constant; Chl, chlorophyll; Mes, 4-morpholineethanesulfonic acid; PPBQ, phenyl-*p*-benzoquinone; PS II, photosystem II

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00145793/86/$3.50

6.3), 400 mM sucrose, 15 mM NaCl, 5 mM $MgCl_2$).

The PS II particles were centrifuged at 40000 × *g* for 35 min in Eppendorf tubes, and the pellet dispersed in the 32% buffer. The particles were deliberately exposed to strong room light (with the purpose of exchanging the water bound in the active site) while keeping the sample at 0°C. The washing procedure was repeated once with the 50.7% buffer. A final ^{17}O enrichment of 42% was estimated from the original buffer content of the sample and its weight at different steps of the preparation. The particles (0.3 ml at 14 mg Chl/ml) were transferred to an EPR tube and allowed to dark adapt for 15 min at 0°C. After addition of the electron acceptor PPBQ (4 mM), the sample was frozen in the dark at 200 K and illuminated [4] for 10 min with continuous white light (3 kW/m^2). A control sample with normal $H_2{}^{16}O$ buffer was made in parallel with the $H_2{}^{17}O$ sample.

The EPR measurements were made with a Bruker ER 200D-SRC spectrometer, interfaced to an Aspect 2000 minicomputer and equipped with an Oxford Instruments ESR 900 helium flow cryostat. For spectra in figs 2 and 4 the rapid scan accessory was used with a sweep amplitude of 20 mT and a sweep time of 5 s.

In the simulations of the ^{17}O broadenings, the $A(^{17}O)$ was assumed to be isotropic. The simulations could then be made by superposition of ^{16}O spectra offset from the original position by amounts corresponding to the $A(^{17}O)$ and with weights given by the ^{17}O nuclear spin (5/2), the number of equivalent nuclei and the estimated enrichment.

3. RESULTS AND DISCUSSION

PS II particles illuminated at 200 K in the presence of ^{17}O-enriched water show an S_2 state EPR signal with a small but significant line broadening compared with the control in normal water (fig.1A,B). The broadening does not manifest itself as an increased peak-to-peak width but is seen as a decrease in the spectral resolution in all parts of the signal. This is usually the case when small ligand hyperfine effects are being observed on complex EPR signals with intrinsic linewidths comparable with the splittings. Parts of the signal were selected for a more extensive signal averaging (fig.2A–D). The spectral regions were chosen outside the 0.31–0.36 T range to avoid interference from extraneous monomeric Mn(II) (0.05/250 Chl) that also showed a ^{17}O broadening. This signal, which is almost always present in the absence of EDTA (as in this case), is more easily seen at a higher temperature (fig.1C, see below).

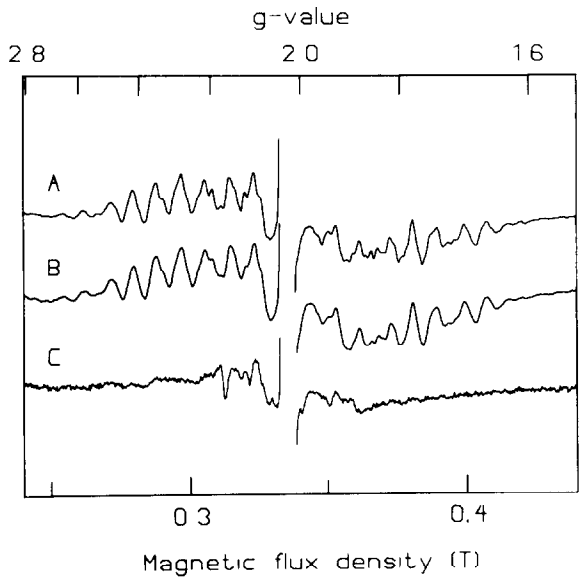

Fig.1. Effect of $H_2{}^{17}O$ on the S_2 EPR signal from PS II particles. The S_2 signal was produced by 200 K illumination of PS II particles (14 mg Chl/ml) in the absence (A) or presence (B) of $H_2{}^{17}O$ (42%). Microwave frequency, 9.47 GHz; power, 20 mW; modulation amplitude, 1.25 mT; time constant, 50 ms; temperature, 11 K. The spectra are the average of 8 scans. C, same as A, but microwave power, 0.2 mW, and temperature, 30 K.

The broadening induced by ^{17}O conclusively shows that water (or a higher oxidation state of oxygen) is directly coordinated to the manganese cluster when it gives rise to the S_2 EPR signal. It should be noted that the exchange of $H_2{}^{17}O$ for $H_2{}^{16}O$ was made under conditions of turnover of the S states, followed by 15 min of dark adaptation before freezing the samples. Therefore, with the plausible assumptions that the water binds reversibly to a specific S state and that the ^{17}O effect is due to oxygen ligands that will be released as O_2 in the $S_3 \longrightarrow S_0$ transition, it follows that the water binding occurs in the S_0 or S_1 state, or

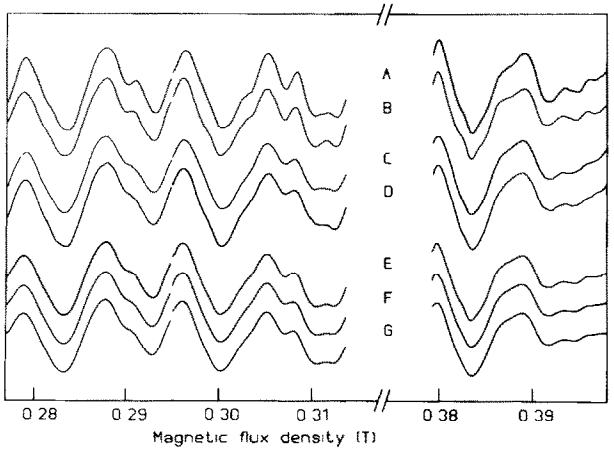

Fig.2. Effect of $H_2{}^{17}O$ on the S_2 EPR signal. B,D, same samples as in fig.1A,B, respectively. A,C, duplicate samples in $H_2{}^{16}O$ and $H_2{}^{17}O$, respectively, to show the reproducibility of the ^{17}O effect. Spectrometer conditions as in fig.1A,B, but 64 scans. E–G, simulations of the ^{17}O broadening using spectrum B with 42% abundance of ^{17}O and the following combinations of oxygen ligands and $A(^{17}O)$: one O, 0.45 mT (E); two O, 0.36 mT (F); three O, 0.27 mT (G).

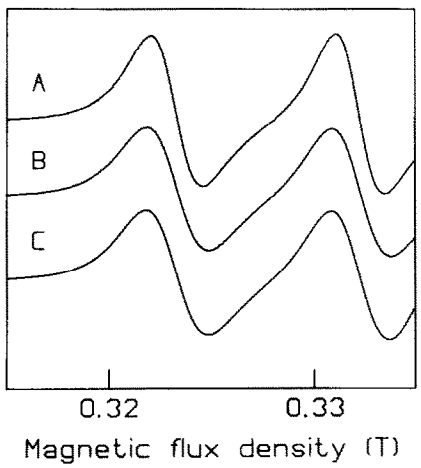

Fig.3. Room-temperature EPR spectra of 1 mM $MnCl_2$ in $H_2{}^{16}O$ (A) and $H_2{}^{17}O$ (45.6%) (B). C, simulation using spectrum A with 6 equivalent oxygen ligands, $A(^{17}O)$ = 0.23 mT and a ^{17}O abundance of 45.6%.

possibly in the S_2 state. S_2 is considered less likely because of the restricted mobility of water at 200 K.

Due to its relatively rapid exchange of oxygen with water, bicarbonate cannot be ruled out as responsible for the broadening. However, the ion seems to be important mainly on the acceptor side of PS II and with no major role in oxygen evolution [9]. The exchange of oxygen with other possible ligands (carboxyl-, carbonyl- and hydroxyl groups) is expected to be negligible [11].

To extract quantitative information from the observed broadening, a more thorough investigation was made. The ^{17}O enrichment in the samples was determined independently from the broadening of the monomeric Mn(II) signal. Since, in this case, water is the only likely ligand that can exchange oxygen and since the $A(^{17}O)$ of water does not seem to depend on other ligands present in an Mn(II) complex [12], $A(^{17}O)$ was assumed to be equal to $A(^{17}O)$ in hexaquo Mn(II). The latter was determined from simulations of the broadening at room temperature (fig.3) and low temperature (not shown) at a known ^{17}O enrichment. The value obtained, 0.23 mT, is close to the value, 0.21 mT, estimated from NMR measurements [12]. Then, the broadening of the monomeric Mn(II) signal in the PS II samples was analysed using $A(^{17}O)$ = 0.23 mT. Simulations with less than 6 water ligands resulted in good agreement only for unrealistically high enrichments ($\geq$50%). However, with the assumption of 6 water ligands, good correspondence with the experiments could be obtained with ^{17}O enrichments of 40–45% (fig.4), in agreement with the 42% estimated from the dilutions. Thus, the monomeric Mn(II) signal must be ascribed to hexaquo Mn(II) and indeed the spectrum in fig.1C is very similar to that obtained from frozen solutions of magnetically dilute hexaquo Mn(II) (not shown). Finally, the broadening of the S_2 EPR signal was simulated with an ^{17}O enrichment of 42%. Simulations with 1, 2 and 3 oxygen ligands resulted in equally good fits if the $A(^{17}O)$ was properly adjusted (fig.2E–G). Four or more ligands, with a correspondingly lower $A(^{17}O)$, were also possible (not shown). Thus, because of the lack of resolution in the experimental spectra, the number of oxygen ligands could not be determined unambiguously. However, 2 oxygen ligands would be consistent with the results obtained from experiments with chemical analogues of water [13].

The uncertainty in the hyperfine coupling constants derived in fig.2E–G is estimated to be $\pm$ 0.04 mT. Then the simulations yield an upper limit of 0.5 mT for $A(^{17}O)$. This excludes such oxidation states as superoxide or hydroxyl radicals,

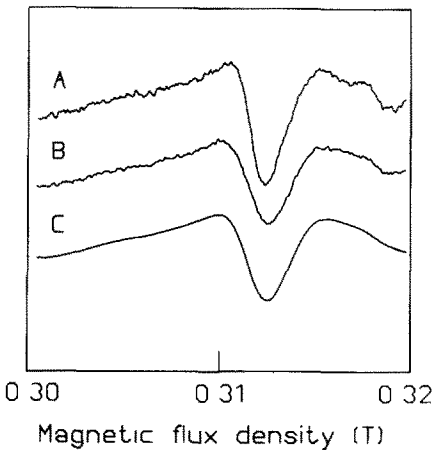

Fig.4. ^{17}O broadening of the EPR signal from extraneous $Mn(H_2O)_6^{2+}$ in the PS II samples. A,B, same samples as in fig.1A,B, respectively. Spectrometer conditions as in fig.1C, but 128 scans. C, simulation using spectrum A with the same assumptions as in fig.3C, but 42% ^{17}O abundance.

since the complete transfer of an electron spin to the ligands would be expected to give rise to a much larger $A(^{17}O)$ [14].

It is tempting to discuss the above findings in terms of Mn_2 models for S_2 where the oxygen ligands bridge the metal ions, in analogy with what is known about the dioxygen binding site in cytochrome-*c* oxidase [15]. Since results from X-ray [6] and optical absorption spectroscopy [8] seem to exclude the Mn(II) oxidation state in S_2, only Mn_2(III,IV) models are considered. A structure similar to the di-μ-oxo Mn_2(III,IV) core of certain model compounds is supported by EXAFS [16] and near infrared absorption spectroscopy [17]. Such a structure would give 2 equivalent $A(^{17}O)$, but would probably also be accompanied by a large exchange coupling constant, contrary to what has been derived from EPR studies [18,19]. However, structures like, for example, μ-peroxo Mn_2(III,IV) or $Mn(III)OH^-\ldots O^{2-}Mn(IV)$ [20], with a few more intervening bonds, can account for the moderate exchange coupling as well as for the $A(^{17}O)$ observed in the present work.

ACKNOWLEDGEMENTS

We wish to thank Mr Bruno Källebring for writing the computer display program and Drs Roland Aasa, Jörgen Bergström, Lars-Gunnar Franzén, Thomas Nilsson and Stenbjörn Styring for valuable discussions. This work was supported by the Swedish Natural Science Research Council.

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