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Assignment of the $g = 4.1$ EPR signal to manganese in the S_2 state of the photosynthetic oxygen-evolving complex: an X-ray absorption edge spectroscopy study *

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X-ray absorption spectroscopy at the Mn K-edge has been utilized to study the origin of the $g = 4.1$ EPR signal associated with the Mn-containing photosynthetic O_2-evolving complex. Formation of the $g = 4.1$ signal by illumination of Photosystem II preparations at 140 K is associated with a shift of the Mn edge inflection point to higher energy. This shift is similar to that observed upon formation of the S_2 multiline EPR signal by 190 K illumination. The $g = 4.1$ signal is assigned to the Mn complex in the S_2 state.

The O_2-evolving complex cycles through five intermediate oxidation states, labeled S_0–S_4. The S_4 state spontaneously decays to produce O_2 and the S_0 state. Recent research has sought to define the structural nature of these intermediates and the mechanism of electron transfer from the O_2-evolving complex to the photochemical reaction center. Manganese is an essential cofactor of the O_2-evolving complex [1]. EXAFS studies are consistent with a bridged binuclear Mn structure for the S_1 [2] and S_2 [3] states. A multiline EPR signal is associated with the S_2 state [4,5] and has been assigned to a mixed-valence Mn cluster [4], with recent work favoring a tetrameric Mn structure [6]. The S_2 multiline EPR signal may be trapped by rapid cooling following a single flash at room temperature [4] or by continuous illumination at 190 K [5]. Recently, Casey and Sauer reported that illumination at 140 K generated a broad EPR signal centered at $g = 4.1$, which relaxed on warming to 190 K in the dark to produce the multiline signal [7]. The $g = 4.1$ signal was assigned to a species functioning as an electron transfer intermediate between S_1 and the reaction center. In contrast, Zimmerman and Rutherford cogenerated the $g = 4.1$ and multiline signals on illumination at 200 K, and assigned the $g = 4.1$ signal to an intermediate between the states S_2 and S_3 [8]. More recently, these authors demonstrated that several common cryoprotectants or ethanol inhibit the formation of the $g = 4.1$ signal above 140 K [9]. In the absence of cryoprotec-

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Abbreviations: Chl, chlorophyll; Mes, 4-morpholineethanesulfonic acid; cytochrome b-559 HP, cytochrome *b*-559 (high-potential form); EXAFS, extended X-ray absorption fine structure.

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tants, the $g = 4.1$ signal exhibits period four oscillations following a train of room temperature flashes, with maxima on the first and fifth flashes. These observations have provided strong evidence that the $g = 4.1$ signal is actually associated with the S_2 state [9]. De Paula et al. [10] have proposed that both of these signals arise from the same Mn site. Based on studies of the temperature dependence of the signals, these authors have assigned the multiline signal to an $S = 1/2$ excited state and the $g = 4.1$ signal to an $S = 3/2$ ground state of two different configurations of the Mn complex [6]. In this scheme, on warming from 140 K to 200 K a change in the Mn magnetic exchange couplings causes a decrease in the spacing between these two levels, which results in population of the $S = 1/2$ excited state. However, thermal depopulation of the $S = 1/2$ state on cooling to 4 K is not accompanied by formation of a $g = 4.1$ feature associated with the $S = 3/2$ spin state; conversely, no multiline signal is detected if samples illuminated at 140 K are examined at 20–30 K in the spectrometer [6]. Thus, the mechanism by which the $g = 4.1$ signal is converted to the multiline signal by the effect of warming or action of cryoprotectants is not yet clear, but it must involve some conformational changes of the Mn complex.

While the association of the $g = 4.1$ signal with the S_2 state is reasonably certain, the absence of resolved ^{55}Mn hyperfine structure precludes making a definite assignment to the Mn complex. We have addressed this question utilizing X-ray absorption spectroscopy at the Mn K-edge. This technique probes the oxidation state and site symmetry of the Mn complex and is element specific. Observations of a light-induced positive shift in the manganese X-ray absorption edge energy, correlated with the production of the S_2 multiline EPR signal, provided direct evidence for the oxidation of Mn at the $S_1 \rightarrow S_2$ transition [11,12]. In this paper we examine the changes in the Mn edge which accompany formation of the $g = 4.1$ EPR signal.

O_2-evolving Photosystem II preparations were prepared by Triton X-100 extraction of spinach chloroplasts as previously described [2]. Samples were suspended at 20–30 mg Chl/ml in 50 mM Mes/15 mM NaCl/5 mM $MgCl_2$ (pH 6.0) with about 30% glycerol added as a cryoprotectant and were loaded into lucite sample holders. Following 1 h of dark adaptation at 4°C, the samples were frozen to 77 K until use. Prior to illumination, samples were equilibrated at 140 K or 190 K in an unsilvered nitrogen gas-flow dewar. At these temperatures reoxidation of Q_A^- is blocked [13], thus limiting the donor side to a single turnover. Samples were illuminated for 2 min in this dewar by a 400 W tungsten source filtered through a 5 cm water bath. X-ray absorption spectra were obtained at the Stanford Synchrotron Radiation Laboratory, Stanford, CA, on beam line IV-1 during dedicated operation of the SPEAR storage ring. Energy resolution was 1 eV using an Si⟨111⟩ double crystal monochromator. The uncertainty in the measurement of the edge inflection energy is ±0.1 eV. Edge spectra were taken in the fluorescence mode using a plastic scintillator array as previously described [2]. Energy calibration was provided by simultaneous measurement of the narrow 'white-line' feature of $KMnO_4$ with each scan. During X-ray measurements, the sample temperature was maintained at about 150 K in a nitrogen gas-flow cryostat. EPR spectra were recorded before and after X-ray exposure with a Varian E-109 spectrometer equipped with an Air Products Model LTR liquid helium cryostat.

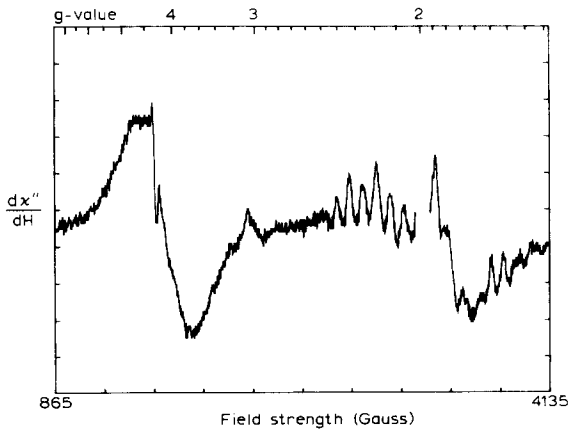

Fig. 1. The $g = 4.1$ EPR signal induced upon illumination at 140 K. The spectrum of the dark-adapted sample has been subtracted. The sharp feature at $g = 4.3$ is a subtraction artifact. Spectrometer conditions: temperature, 8 K; microwave power, 50 mW; microwave frequency, 9.2 GHz; modulation amplitude, 32 G; modulation frequency, 100 kHz; scan time, 4 min; time constant, 128 ms.

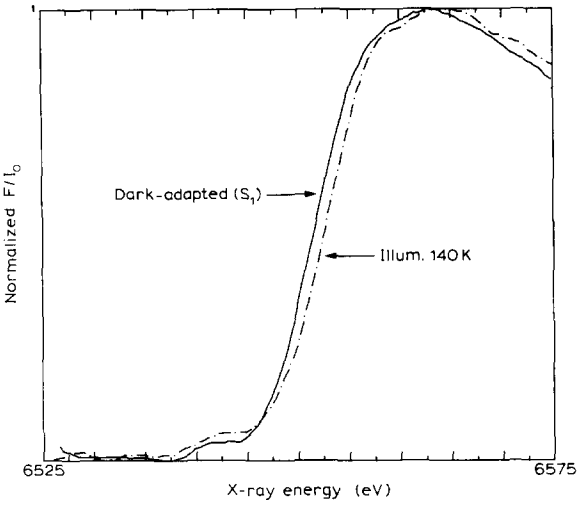

Fig. 2. Effect of 140 K illumination on the X-ray absorption K-edge spectra of Mn. Spectra are of a dark-adapted (S_1) sample and the same 140 K illuminated sample as in Fig. 1. The spectra were collected at 150 K in darkness. A linear pre-edge background was subtracted from the edge spectra and the data were smoothed for presentation by linear regression over fixed 2.7 eV regions about each point.

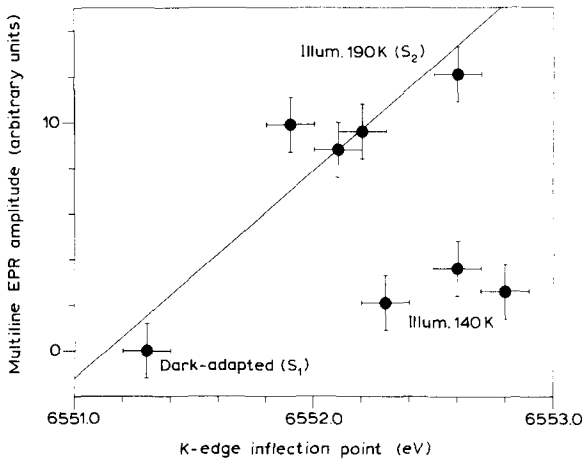

Fig. 3. Mn K-edge inflection energy plotted against multiline EPR signal amplitude for samples which were dark-adapted and illuminated at 190 K or 140 K. The edge inflection energies were determined by fitting the spectra to quadratic polynomials over 2.7–6.8 eV regions about each point and taking the first derivative of the smoothed data. The edge inflection energies did not vary systematically with the energy width of the smoothing function. Multiline EPR amplitudes were taken as the sum of peak-to-peak amplitudes for five lines downfield from $g = 2$. A linear least-squares line is drawn through the dark-adapted (S_1) and 190 K illuminated (S_2) data points.

Fig. 1 shows the EPR difference spectrum induced upon illumination of a Photosystem II sample at 140 K. Several light-induced features are apparent: the broad $g = 4.1$ signal, a low amplitude multiline signal centered about $g = 2$, a peak near $g = 3.1$ representing the g_z feature of the spectrum of oxidized cytochrome *b*-559 HP and the broad Q_A^- signal at $g = 1.9$. The $g = 4.1$ signal contains a narrow subtraction artifact owing to the presence of a sharp feature at $g = 4.3$ in the dark spectrum. The multiline amplitude is about 25% of that induced on illumination at 190 K (see Fig. 3). A decrease of up to 20% in the amplitude of the light-induced EPR signals was sometimes observed following X-ray spectroscopy. Because the O_2-evolution activity is not significantly decreased following exposure to the X-ray beam [11], this loss in signal amplitude is probably due to charge recombination and not X-ray damage.

The Mn K-edge absorption spectra of the same sample exhibiting the $g = 4.1$ EPR signal and another sample poised in the S_1 state are compared in Fig. 2. A shift in the absorption edge to higher energy is apparent in the 140 K illuminated sample. Because Mn is not associated with the acceptor Q_A or the competitive donor cytochrome *b*-559, this shift is correlated with the $g = 4.1$ signal. No significant changes in the edge structure are apparent. Both spectra contain a pre-edge $1s \rightarrow 3d$ transition at about 6543 eV which becomes allowed in a noncentrosymmetric coordination environment. Because we have observed no significant changes in the Mn EXAFS features upon formation of the S_2 state by illumination at 190 K [3], it is unlikely that substantial changes in the structure of the Mn complex are induced at 140 K. Thus, the edge shift most likely reflects a change in Mn oxidation state and not a redistribution of electron density at Mn due to ligand rearrangement or exchange. The absence of a change in the edge shape is also consistent with a simple oxidation state change. The edge inflection energy was 6551.3 eV for the S_1 sample and 6552.8 eV for the sample exhibiting the $g = 4.1$ signal. This 1.5 eV shift is of similar magnitude to what is observed upon formation of the S_2 multiline EPR signal at 190 K (see Fig. 3 and Ref. 3).

Thus, formation of either the multiline or the $g = 4.1$ signal results from a similar Mn oxidation state change.

Because some multiline signal is induced in the samples illuminated at 140 K, it is possible that the edge shift is associated not only with the $g = 4.1$ signal but also with the multiline signal. To test this possibility, we have constructed a correlation plot of the edge shifts observed in several samples illuminated at 190 K or 140 K versus the amplitude of the multiline signal (Fig. 3). A best fit line is drawn through the dark-adapted and 190 K illuminated sample data points. As we previously reported [11,12], there is a good correlation between the amplitude of multiline signal and the magnitude of the edge inflection energy shift for the samples illuminated at 190 K, and the data for the illuminated samples cluster along the upper portion of the correlation line. However, data points for samples illuminated at 140 K fall in a separate domain to the lower right portion of the plot and are quite distant from the correlation line. The distribution in the edge inflection energies and multiline signal amplitudes within each set of illuminated samples reflects some variation in S-state composition. The maximum edge shift for the samples illuminated at 140 K is about the same as that induced at 190 K, but it is much larger than expected on the basis of correlation with multiline signal amplitude. Thus, formation of the $g = 4.1$ signal is directly associated with the observed edge shift.

Our results provide direct evidence that the formation of the $g = 4.1$ signal is correlated with the oxidation of Mn and thus arises from the Mn-containing O_2-evolving complex. The similarity of the Mn edges from samples poised in the S_2 state by illumination at 190 K and from samples exhibiting the $g = 4.1$ signal is strong evidence that the $g = 4.1$ signal is another S_2-state EPR signal, in agreement with the assignment of Zimmerman and Rutherford [9]. Our data also support the model that the $g = 4.1$ and multiline signals both arise from the Mn complex [10]. However, any structural differences between the centers which may give rise to the $g = 4.1$ and multiline signals are not apparent in the Mn edge spectra and remain unclear.

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