fundamental events of neurosecretion. Prior work has demonstrated that two subpopulations of adrenal cells which are significantly enhanced in their content of either epinephrine or norepinephrine can be isolated with their secretory machinery intact. This work has shown that adrenal cells isolated in this manner can be classified according to the catecholamine that they release upon stimulation because the catecholamines are released in the same relative proportions that they are stored in the cell. Verification of the voltammetric technique, which provides information on transient secretion, is possible with microscale chromatographic analysis. Because voltammetry is a nondestructive technique, any method of single-cell analysis may be subsequently performed to obtain complementary information. With this information, the nature of the differences between these subpopulations of adrenal cells may be more completely explored.

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Detection of a Paramagnetic Intermediate in the S_1 State of the Photosynthetic Oxygen-Evolving Complex†

S. L. Dexheimer[1a,b] and Melvin P. Klein*[,1b]

Contribution from the Lawrence Berkeley Laboratory and Department of Physics, University of California, Berkeley, California 94720. *Received October 22, 1990*

Abstract: We report the detection of a new electron paramagnetic resonance (EPR) signal that demonstrates the presence of a paramagnetic intermediate in the resting (S_1) state of the photosynthetic oxygen-evolving complex. The signal was detected using the method of parallel polarization EPR, which is sensitive to $\Delta m = 0$ transitions in high spin systems. The properties of the parallel polarization EPR signal in the S_1 state are consistent with an $S = 1$ spin state of an exchange-coupled manganese center that corresponds to the reduced form of the species giving rise to the multiline EPR signal in the light-induced S_2 state. The implications for the electronic structure of the oxygen-evolving complex are discussed.

Water is the terminal electron donor for the electron transfer processes that constitute the light reactions of plant photosynthesis. The splitting of water to produce molecular oxygen, four hydrogen ions, and four electrons takes place in photosystem (PS) II of green plants and cyanobacteria and is mediated by the oxygen-evolving complex, which contains redox-active manganese ions. Electron transfer in PS II begins with the photoexcitation of the primary donor followed by transfer of the photoexcited electron to the iron–quinone acceptor complex via an intermediate pheophytin species. Transfer of an electron from the oxygen-evolving complex via an intermediate tyrosine species reduces the photooxidized primary donor, allowing repetition of the photochemical cycle.[2] Since the oxidation of water to molecular oxygen is a four-electron process, while the reduction of the photooxidized primary donor is a single-electron process, the oxygen-evolving complex must couple the four-electron oxidation of water to the single-electron photochemistry of the rest of the reaction center. This function has been described in terms of an S-state model[3] in which the oxygen-evolving complex cycles through a series of states, S_0–S_4, as it transfers electrons to reduce the primary donor while accumulating oxidizing equivalents for water oxidation. When the complex reaches the state S_4, molecular oxygen is released and the complex reverts to the S_0 state. The resting state of the complex is the S_1 state.

The chemical identity and electronic structure of the species that constitute the S states and the relation of this structure to the function of the oxygen-evolving complex have been the subject of continued study and speculation.[4] Manganese is an essential part of the oxygen-evolving complex and is thought to form the binding site for water in the water oxidation process. Quantitation procedures have estimated a stoichiometry for four functional manganese ions per PS II unit,[5] but the structural organization and oxidation states of the manganese ions throughout the S-state cycle have not yet been established. Although the system has been thoroughly studied with conventional electron paramagnetic resonance (EPR) spectroscopy, only two signals attributed to manganese in the native enzyme have been reported, and both occur in the S_2 state. The multiline signal, comprised of approximately 19 hyperfine components centered near $g = 2$, is consistent with an $S = {}^1/_2$ spin state of an exchange-coupled mixed-valence manganese cluster.[6] A second signal appears at an effective g value of 4.1,[7-9] consistent with an $S = {}^3/_2$ species of nearly axial symmetry, and the correlation of the generation of this signal with an increase in the manganese X-ray absorption energy suggests that the signal originates from manganese.[10]

In this paper, we present a new EPR signal associated with the S_1 state of the oxygen-evolving complex and discuss its implications for the structure of the redox-active manganese centers. The presence of half-integral spin EPR signals in the S_2 state, together

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with the indication from X-ray edge studies[11] of a one-electron oxidation of manganese in the S_1 to S_2 transition, suggests the presence of a paramagnetic species of integral spin the S_1 state. We detected the integral-spin intermediate using the technique of parallel polarization EPR, a method that until recently has seen little application to systems of biological interest.[12-14]

Theory

In a conventional EPR experiment, the microwave magnetic field is oriented perpendicular to the applied magnetic field and induces magnetic dipole transitions with the selection rule $\Delta m = \pm 1$. A microwave magnetic field polarized parallel to the applied magnetic field may be used to detect magnetic dipole transitions corresponding to the selection rule $\Delta m = 0$, which can become partially allowed in spin systems with $S > {}^1/_2$ when zero-field Hamiltonian terms mix the Zeeman basis states.

The spin Hamiltonian for a spin $S > {}^1/_2$ in the molecular principal axis frame includes the terms:

$$H_{\text{Zeeman}} = \beta \mathbf{H}\cdot\mathbf{g}\cdot\mathbf{S} \quad (1)$$

$$H_{\text{zero-field}} = D[S_z^2 - {}^1/_3 S(S+1)] + E(S_x^2 - S_y^2) \quad (2)$$

where D is the axial zero-field splitting parameter and E is the rhombic zero-field parameter.[15] When only the Zeeman term is present, the eigenstates of the Hamiltonian are eigenstates of the z component of the spin angular momentum, where the axis of quantization is determined by the direction of the applied magnetic field. Mixing of the Zeeman states by the zero-field Hamiltonian leads to a splitting of the levels at zero field and produces eigenstates of the total spin Hamiltonian that are no longer eigenstates of the z component of the angular momentum. In general, the Hamiltonian parameters will not fall in a limit in which one of the terms may be treated as a perturbation on the other, and the resulting EPR properties can be determined only by exact diagonalization of the total Hamiltonian, as is done in the spectral simulations presented in this work. The properties of parallel polarization EPR transitions for the simplest case of an $S = 1$ spin state have been discussed previously,[14-16] and a perturbation treatment valid for non-Kramers spin states in which the zero-field Hamiltonian term is much larger than the Zeeman term has been presented.[13,17]

Experimental Methods

PS II-enriched thylakoid membranes were prepared from spinach using a Triton X-100 fractionation procedure.[18] Since adventitious $S = {}^5/_2$ Mn^{2+} ions can produce background signals in the parallel polarization, care was exercised during the sample preparation procedure to avoid manganese release, and the resulting PS II-enriched membranes were washed first in sucrose buffer (400 mM sucrose; 50 mM 4-morpholinoethanesulfonic acid (MES), pH 6; 15 mM NaCl; 5 mM $MgCl_2$) containing 1.5 mM EDTA and again in sucrose buffer containing 20 mM $CaCl_2$. The final pellet was suspended in either sucrose buffer or glycerol buffer (50 mM MES, pH 6; 15 mM NaCl; 5 mM $MgCl_2$; 50% (v/v) glycerol) to a final chlorophyll concentration of approximately 10 mg/mL. To avoid variable background features from paramagnetic condensed oxygen, the last washes, final resuspension, and sample tube loading were performed under an argon atmosphere using degassed buffers. The preparations used in this study had a high level of activity, as demonstrated by measured values of at least 600 μmol of O_2/mg of chlorophyll/h for the rate of oxygen evolution under continuous illumination at room temperature, using the method described previously.[10] Control samples depleted of the manganese ions associated with the oxygen-evolving complex were made from part of the preparation using a hydroxylamine treatment similar to that of Tamura and Cheniae:[19] PS II membranes at a chlorophyll concentration of 1 mg/mL were incubated in the dark for 30 min in sucrose buffer containing 2.5 mM hydroxylamine and 5 mM EDTA, and then washed in an EDTA-containing sucrose buffer.

Samples were poised in the S_1 state by dark adaptation at 4 °C for more than 1 h prior to freezing at 77 K and were later advanced to the S_2 state by illumination at low temperature.[20] Illuminations were performed using the methods described previously.[10] For the first set of measurements, initially dark-adapted samples suspended in either sucrose or glycerol buffer were advanced to the S_2 state by illumination for 5 min at 200 K. As a control, additional dark-adapted samples were illuminated at 77 K for 8 min to induce electron donation from cyt b_{559} rather than from the oxygen-evolving complex. To investigate the relation between the S_1 state signal and the EPR signals in the S_2 state, the changes in the S_1 state signal relative to the changes in the multiline and $g = 4.1$ signals were monitored in a series of measurements in which initially dark-adapted samples suspended in glycerol buffer were first illuminated at 140 K for 9 min, then warmed to 200 K in the dark for 90 s, and finally illuminated again at 200 K for 5 min. The reported values for the multiline and $g = 4.1$ signals are peak-to-peak amplitudes determined by the illuminated minus dark difference spectra. The multiline signal amplitude was taken as the average of the peak-to-peak amplitudes of two low-field hyperfine components that do not overlap cyt b_{559} features. These components were chosen because competitive donation from cyt b_{559} during the 140 K illumination results in cyt b_{559} signal intensity that precludes accurate measurement of the amplitude of a number of the hyperfine components of the induced multiline signal. The reported values for the S_1-state signal are peak-to-peak amplitudes determined from the parallel polarization spectra. All signal intensities are quoted in arbitrary units.

EPR spectra were collected using a Varian E-109 X-band spectrometer with a modified V-4536 bimodal cavity and an Air Products Helitran cryogenic system. The bimodal cavity could be excited in either of two TE_{102} modes: one having the conventional geometry in which H_1 is polarized perpendicular to the applied magnetic field, and the other with H_1 polarized parallel to the applied field. This arrangement allows conventional EPR signals to be monitored in the same experiment as the parallel polarization measurements. In the experiments described below, both parallel and perpendicular polarization EPR spectra were collected under each experimental condition. Spectrometer conditions for the parallel polarization spectra were: microwave frequency, 9.20 GHz; microwave power, 3 mW (and 20 mW for some samples in sucrose buffer); field modulation, 20 G at 100 kHz; scan time, 4 min; time constant, 2 s. Typically, 25 scans were averaged under each condition. When necessary, linear base lines were subtracted from the spectra to compensate for the monotonic background observed when the spectrometer was operated at high gain and high field modulation amplitude. Perpendicular polarization EPR spectra were collected under the same spectrometer conditions, except: microwave frequency, 9.34 GHz; microwave power, 0.5 mW; time constant, 0.5 s. The cryogenic system was operated as its stable base temperature of 4.2 K, and a GaAsFET microwave preamplifier was used to improve the spectrometer sensitivity.[21]

EPR spectral simulations were performed using an exact diagonalization of the spin Hamiltonian matrix. The Hamiltonian consisting of the Zeeman and zero-field terms given by eqs 1 and 2 was constructed in the molecular principal axis frame. The Hamiltonian was diagonalized at each of a series of magnetic field values, and since the samples consisted of randomly oriented centers, the Hamiltonian for each field value was diagonalized at each of a large number of orientations of the applied magnetic field with respect to the molecular axes, with the resulting contributions to the spectrum weighted by a solid-angle factor. Given values for the microwave transition frequency and the transition line width, the differences between the Hamiltonian eigenvalues were tested for the resonance condition. If an energy difference fell within a given number of line widths of the microwave frequency, the transition probabilities were computed and were weighted by the appropriate line-width factor, assuming either a Gaussian or Lorentzian frequency function. The parallel polarization transition probability was computed using a transition Hamiltonian $\beta\mathbf{H}_1\cdot\mathbf{g}\cdot\mathbf{S}$ with the components of $\mathbf{H}_1$ proportional to those of $\mathbf{H}_0$. Perpendicular polarization spectra could also be computed by averaging the transition probability over orientations of H_1 in the plane

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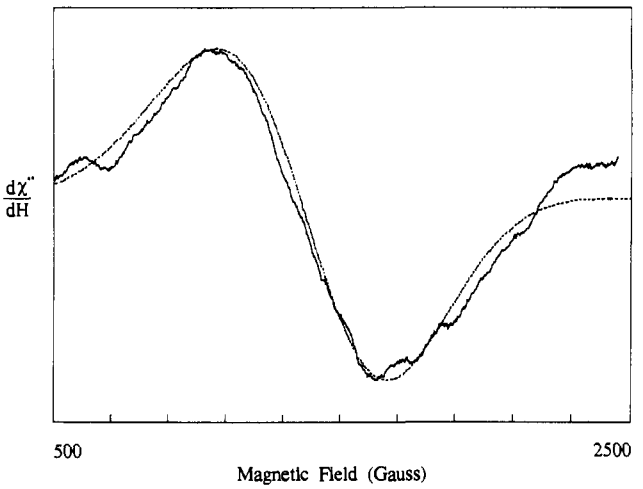

Figure 1. The parallel polarization EPR signal associated with the S_1 state of the oxygen-evolving complex, as observed in the dark-adapted minus 200 K illuminated difference spectrum of a photosystem II preparation. The signal appears at an effective g value of approximately 4.8 and has a peak-to-peak width of approximately 600 G. Irregular features apparent in the signal are not reproducible. Spectrometer conditions: microwave frequency, 9.2 GHz; microwave power, 3 mW; temperature, 4.2 K; field modulation, 20 G at 100 kHz; scan time, 4 min; time constant, 2 s. Twenty-five scans were averaged both before and after illumination. The broken line displays the result of a computer simulation described in the text. Simulation parameters: $S = 1$, $g = 2$, $D = -0.125$ cm^{-1}, $E = 0.025$ cm^{-1}.

perpendicular to $\mathbf{H}_0$. Derivatives of the simulated spectra were computed for comparison with experimental results.

Results

Detection of the Paramagnetic Intermediate. Figure 1 shows the dark-adapted minus 200 K illuminated difference spectrum in the parallel polarization. A weak, broad feature is present at low field in the S_1 state and diminishes upon advancement to the S_2 state by illumination at 200 K. The signal appears at an effective g value of approximately 4.8, and has a peak-to-peak width of roughly 600 G. Also included in the figure are the results of a computer simulation of the spectrum using an $S = 1$ spin Hamiltonian, the details of which are described below. Since the photosynthetic samples are relatively dilute in manganese and parallel polarization signals are expected to be weak, signal averaging was used to improve the sensitivity, as described in the previous section. In addition, since temperature fluctuations may distort the signal base line, the cryogenic system was operated at its stable base temperature of 4.2 K. Because of the presence of irregular background features, the parallel polarization S_1-state signal is most readily detected in a dark-adapted minus illuminated difference spectrum. However, the signal is evident in signal-averaged spectra of the dark-adapted state. No parallel polarization EPR response associated with the S_2 state was detected. This result is consistent with the previously proposed spin state and symmetry assignments for the multiline and $g = 4.1$ signals, since no parallel polarization transitions are expected for $S = {}^1/_2$ spin states or for axial $S = {}^3/_2$ states when the axial zero-field parameter D is sufficiently large relative to the microwave frequency to result in a resonance in the $g \approx 4$ region.

The spin relaxation properties of the S_1-state parallel polarization signal were found to be dependent on the cryoprotectants used in the samples. Similar effects have been observed for the multiline and $g = 4.1$ conventional EPR signals.[9] In the case of the S_1-state signal, the signal saturated more readily in the presence of glycerol than in the presence of sucrose: in sucrose-containing samples, the signal could be detected at a microwave power of 20 mW, whereas the signal was not observable at this power level in glycerol-containing samples. The signal could be detected with either cryoprotectant at a microwave power level of 3 mW.

Computer Simulation of the Parallel Polarization EPR Spectrum. Computer simulations of the parallel polarization spectrum were performed as described above, and the results of a simulation using an $S = 1$ spin Hamiltonian are displayed along with the signal in Figure 1. The simulation corresponds to a transition of Gaussian line shape between the $|-\rangle$ and $|+\rangle$ levels of an $S = 1$ spin state with zero-field parameters $D = -0.125$ cm^{-1} and $E = 0.025$ cm^{-1}. Since the simulation is relatively insensitive to a small degree of g anisotropy, an isotropic g value of 2 was assumed. Simulations assuming an $S = 2$ spin state did not accurately reproduce the line shape of the S_1-state signal. The $S = 1$ simulation also predicts allowed transitions in the conventional perpendicular polarization. However, the anisotropy introduced by the zero-field Hamiltonian results in very broad and, therefore, unobservably weak transitions between $|-\rangle$ and $|0\rangle$ and between $|0\rangle$ and $|+\rangle$ spread over a broad range about $g = 2$. A weak perpendicular polarization transition is also predicted between the $|-\rangle$ and $|+\rangle$ levels for molecular orientations in which the applied field is away from the molecular principal axes. However, this transition would fall in the region of two considerably more intense signals, specifically the $g = 4.3$ rhombic iron feature and the light-induced $g = 4.1$ signal, precluding its observation either in the dark-adapted state or in a dark minus illuminated difference spectrum.

Control Experiments. Illumination at 200 K is known to result in both the oxidation of the oxygen-evolving complex, as evidenced by the appearance of the multiline and $g = 4.1$ conventional EPR signals, and the reduction of the iron–quinone acceptor complex, as evidenced by the appearance of its conventional EPR signal[22] at $g = 1.9$. Three independent control experiments were performed to address the origin of the parallel polarization EPR signal in the S_1 state. First, the reversibility of the light-induced changes was tested: following illumination at 200 K, samples were thawed in the dark at 4 °C to allow relaxation back to the S_1 state. This resulted in the disappearance of the multiline, $g = 4.1$, and iron–quinone acceptor signals, as expected, and also resulted in the recovery of the S_1-state signal to its original amplitude, as evidenced by the magnitude of the dark minus illuminated difference spectrum following a second illumination at 200 K. The original amplitudes of the S_2-state signals were also recovered in the second illumination. As a second control, part of the preparation was depleted of the manganese ions associated with the oxygen-evolving complex using the hydroxylamine extraction and washing treatment described in the previous section. This treatment resulted in the loss of the parallel polarization signal in the S_1 state, in addition to the expected loss of the ability to generate the multiline and $g = 4.1$ signals by illumination at 200 K. In a third control experiment, illumination of intact preparations at 77 K instead of 200 K was used to induce electron donation to the iron–quinone acceptor complex by the alternate donor cyt b_{559} rather than the oxygen-evolving complex. This produced signals due to oxidized cyt b_{559} and the reduced iron–quinone acceptor complex in the conventional perpendicular polarization. No change was induced in the parallel polarization S_1-state signal following illumination at 77 K.

Variable Temperature Illumination. The relation of the parallel polarization signal in the S_1 state to the multiline and $g = 4.1$ conventional EPR signals in the S_2 state was investigated by illumination at different temperatures. For PS II membranes suspended in buffer containing glycerol, the relative yield of the two S_2-state signals depends on the illumination temperature:[9] illumination at 140 K produces mostly the $g = 4.1$ signal, while illumination at 200 K produces mostly the multiline signal. Moreover, when PS II membranes that have been illuminated at 140 K are warmed to 200 K in the dark, much of the $g = 4.1$ signal converts to the multiline signal. A second illumination at 200 K results in a further increase in the multiline signal amplitude, as well as a small increase in the $g = 4.1$ signal amplitude. The results of an experimental trial in which initially dark-adapted PS II membranes suspended in glycerol buffer were illuminated at 140 K, then warmed to 200 K in the dark, and finally illu-

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Table I. Amplitudes of the S_1 State, Multiline, and $g = 4.1$ Signals Measured under Each of a Series of Conditions[a]

	S_1 signal	multiline	$g = 4.1$
dark adapt	6.8	0	0
illuminate at 140 K	6.4	3	23
warm in dark at 200 K	4.8	12	5
illuminate at 200 K	2.3	29	13

[a] Signal amplitudes are in arbitrary units. Estimated uncertainties in the signal amplitudes are ±0.15 unit for the S_1-state signal, ±1 unit for the multiline signal, and ±1.5 units for the $g = 4.1$ signal.

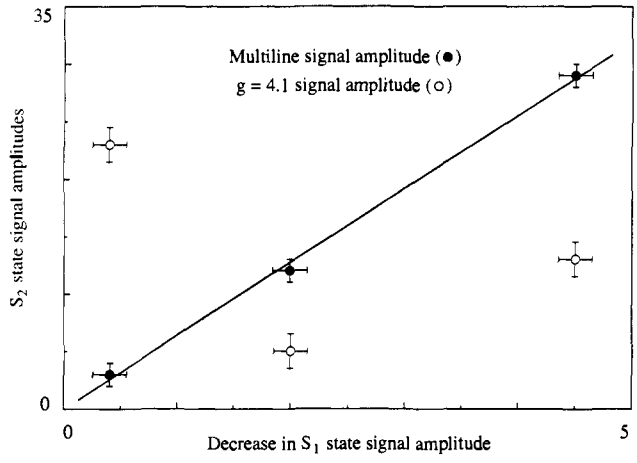

Figure 2. The amplitudes of the multiline (solid circles) and $g = 4.1$ (open circles) signals versus the decrease in the S_1-state signal from its initial value in the dark-adapted state measured under the conditions presented in Table I. The line represents a least-squares fit to the multiline signal data points.

minated again at 200 K are presented in Table I. The measured multiline and $g = 4.1$ signal amplitudes are plotted versus the decrease in the S_1-state signal from its initial value in the dark-adapted state in Figure 2. A clear proportionality is observed between the change in the S_1-state signal and the induced multiline signal, whereas the change in the S_1-state signal appears unrelated to the $g = 4.1$ signal.

Discussion

The observation of the parallel polarization EPR signal provides direct evidence for the presence of a paramagnetic intermediate in the S_1 state. The results of the control experiments and the variable temperature illumination measurements provide evidence for the assignment of the signal to manganese in the oxygen-evolving complex. First, the reversibility of the changes induced by illumination at 200 K confirms that the changes in the parallel polarization spectrum are associated with the transition between the S_1 and S_2 states and also confirms that the initial period of dark adaptation was sufficient to poise essentially all of the centers in the S_1 state.[23] The absence of the signal in preparations depleted of the manganese ions associated with the oxygen-evolving complex is consistent with manganese as the origin of the signal. In addition, the lack of any change in the parallel polarization spectrum upon illumination at 77 K, which induces electron donation from cyt b_{559} rather than the oxygen-evolving complex, implies that oxidation of manganese rather than reduction of the iron–quinone acceptor complex is responsible for the observed changes in the parallel polarization signal following advancement to the S_2 state by illumination at 200 K.

The results of the variable temperature illumination measurements show that the decrease in the S_1-state signal from its initial value in the dark-adapted state is always proportional to the amplitude of the multiline signal observed under each condition. In general, a decrease in the amplitude of an EPR signal may result either from a decrease in the population of the species that gives rise to the signal or from a change in spin relaxation rates. In this experiment, the decrease of the S_1 signal upon generation of the multiline signal would likely indicate either that the S_1-state signal corresponds to the reduced form of the exchange-coupled manganese center that gives rise to the multiline signal when oxidzed, or that the generation of the $S = {}^1/_2$ multiline signal substantially increases the spin relaxation rate of some other species giving rise to the S_1-state signal. However, we note that the relatively slow spin relaxation rates for the multiline signal, as evidenced by time-domain measurements[24] of $T_1 \approx 1$ ms at 4.2 K and the relative ease with which the multiline signal is saturated at low temperature in CW measurements,[9] would result in the multiline signal being a very poor relaxer for an $S = 1$ S_1-state species. In addition, this argument would necessitate hypothesizing the presence of an additional paramagnetic center in PS II, and no direct evidence for such a center exists. Since measurements of the change in the manganese X-ray K-edge absorption energy indicate a one-electron oxidation of manganese in the S_1 to S_2 transition, and since EXAFS measurements do not indicate a major structural change in this transition,[11] the proportionality of the decrease in the S_1-state signal to the amplitude of the multiline signal generated under different conditions suggests that the S_1-state signal arises from the one-electron reduced form of the species that gives rise to the multiline signal.

The field position and line shape of the parallel polarization signal are suggestive of a transition between the $|-\rangle$ and $|+\rangle$ levels of an $S = 1$ spin state with nonzero zero-field parameters, and the signal shows good agreement with an $S = 1$ computer simulation. This spin-state assignment is consistent with a species which, upon the loss of one electron, would produce the $S = {}^1/_2$ multiline signal in the S_2 state. Although $S = 2$ spin states can, in principle, give rise to low-field features in the parallel polarization, $S = 2$ simulations did not accurately reproduce the line shape of the S_1-state signal. The shape of the S_1-state parallel polarization signal is relatively symmetric, consistent with the weak orientation dependence of the splitting between the $S = 1|-\rangle$ and $|+\rangle$ levels when the zero-field interaction is less than or comparable to the Zeeman interaction.[14,15] In contrast, the intradoublet splittings of $S = 2$ states with zero-field parameters typical of transition metal ions are quite anisotropic, leading to asymmetric line shapes in powder spectra.[13,14,25] Parallel polarization spectra with line shapes and effective g values substantially different from those of the S_1-state signal were detected in $S = 2$ states of complexes of trivalent manganese,[14,25] while a low-field parallel polarization signal roughly similar to the S_1-state signal was detected in an $S = 1$ state of a multinuclear manganese complex.[26] The absence of any observable manganese hyperfine structure in the S_1 signal is most likely the result of the inhomogeneous broadening that often occurs in transitions within non-Kramers doublets.[13,14,25] The width of the S_1-state signal is comparable to the width of the manganese hyperfine structure observed for an exchange-coupled binuclear Mn_2(III,III) complex.[25] X-ray absorption edge studies[27] suggest that the S_1 state includes manganese ions in the Mn(III) oxidation state and that the S_1 to S_2 transition involves a change in the oxidation state of one of the ions from Mn(III) to Mn(IV). The simplest structure that could give rise to an $S = 1$ signal in the S_1 state and an $S = {}^1/_2$ signal upon advancement to the S_2 state is a weakly antiferromagnetically coupled binuclear Mn_2(III,III) complex with a thermally populated first excited state. Weak antiferromagnetic exchange interactions have been observed in synthetic binuclear Mn_2(III,III) complexes.[28] A low-lying spin state of an ex-

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change-coupled complex of higher nuclearity would also be consistent with the properties of the S_1-state signal.

The two S_2-state EPR signals show strikingly different behavior relative to the changes in the S_1-state signal in the variable temperature illumination measurements. Studies of the two S_2-state EPR signals[9,29] indicate that, upon advancement to the S_2 state, a given PS II unit will contribute either to the multiline signal or the the $g = 4.1$ signal, but the nature of this heterogeneity has not been clear. The comparison can be made more quantitative by using the signal amplitudes in Table I to estimate the percentage of oxygen-evolving complexes contributing to the $g = 4.1$ and multiline signals at each step of the experiment. Since the changes in the $g = 4.1$ and multiline signals induced upon warming to 200 K in the dark result from the conversion of oxidized oxygen-evolving complexes from the form that gives rise to the $g = 4.1$ signal to the form that gives rise to the multiline signal, rather than from the generation of any new oxidizing equivalents, the decrease in the $g = 4.1$ signal amplitude corresponds to the same number of oxidized oxygen-evolving complexes as the increase in the multiline signal amplitude at this step in the experiment. The resulting scaling factor may then be used to express each value for the multiline and $g = 4.1$ signal amplitude as a percentage of the total number of oxygen-evolving complexes oxidized during the course of the experiment, which is determined by the weighted sum of the amplitudes of the multiline and $g = 4.1$ signals following the final illumination at 200 K. This estimation neglects any donor–acceptor charge recombination that might occur during the short 200 K warming step. Since the $g = 4.1$ signal recombines more readily than the multiline signal,[9] this would lead to an underestimation of the fraction of centers contributing to the $g = 4.1$ signal in the first step of the experiment. However, since the conversion of the $g = 4.1$ signal to the multiline signal occurs much more rapidly than does recombination,[7] this error is expected to be small. The resulting estimates are as follows: in the first step of the experiment, illumination at 140 K generates a $g = 4.1$ signal intensity corresponding to at least 32% of the total number of PS II units in which the oxygen-evolving complex is oxidized during the course of the experiment, while the multiline signal amplitude corresponds to only 8% of these units. Upon warming in the dark at 200 K, most of those complexes initially giving rise to the $g = 4.1$ signal convert to produce additional multiline signal intensity, resulting in 34% of the centers giving rise to the multiline signal and 7% giving rise to the $g = 4.1$ signal. Following the final illumination at 200 K, 82% of the oxidized oxygen-evolving complexes give rise to the multiline signal and 18% to the $g = 4.1$ signal.

The preceding analysis suggests that the generation of the $g = 4.1$ signal is unrelated to the decrease in the S_1-state signal. This is shown most dramatically in the 140 K illumination step, in which a $g = 4.1$ signal amplitude corresponding to 32% of the PS II units is observed, while only a small decrease, proportional to the small multiline signal corresponding to 8% of the units, is seen in the S_1-state signal. This result, together with the proportionality of the changes in the S_1-state signal and the multiline signal shown in Figure 2, implies that upon advancement to the S_2 state, the species that gives rise to the S_1-state signal converts only to the multiline species and is independent of the $g = 4.1$ species. The results in Table I also indicate that upon warming in the dark at 200 K following illumination at 140 K, a substantial decrease is observed in the S_1-state signal that is proportional to the generated multiline signal, even though no new oxidizing equivalents are generated at this step of the experiment. These results can be explained in a straightforward manner if the multiline and $g = 4.1$ signals arise from magnetically distinct sites within the oxygen-evolving complex. In this case, we expect that generation of the $g = 4.1$ signal upon illumination at 140 K results from oxidation of one of the two sites in some fraction of the PS II units. We expect that the centers that would give rise to the multiline signal when oxidized and the S_1-state parallel polarization signal when reduced would be relatively unaffected by illumination at 140 K, consistent with the observation of a small multiline signal and a proportionally small decrease in the S_1-state signal. Warming in the dark to 200 K results in the conversion of oxidized PS II units giving rise to the $g = 4.1$ signal to the multiline form, presumably by electron transfer between the two sites, as originally proposed by Hansson et al.[29] Reduction of centers giving rise to the $g = 4.1$ signal would lead to a decrease in the amplitude of the $g = 4.1$ signal, and concurrent oxidation of the centers that give rise to the S_1-state signal would result in a decrease in the S_1-state signal proportional to the amount of induced multiline signal, as is observed. The second illumination, at 200 K, results in the oxidation of oxygen-evolving complexes that did not advance to the S_2 state in the first illumination. In a small fraction of these complexes, the $g = 4.1$ signal is induced, and in the remainder, the multiline signal is induced, leading again to a decrease in the S_1-state signal proportional to the induced multiline signal. The species that gives rise to the parallel polarization signal would be expected to remain reduced in those oxygen-evolving complexes that contribute to the $g = 4.1$ signal, and this would account for part of the parallel polarization signal amplitude remaining after the 200 K illumination. We expect that the remainder of the final parallel polarization signal would correspond to PS II units in which cyt b_{559} rather than the oxygen-evolving complex was oxidized during the initial illumination at 140 K.

The results are more difficult to reconcile with the proposal that the $g = 4.1$ and multiline signals originate from two thermally dependent conformations of a single strongly exchange-coupled cluster, as suggested by de Paula et al.[30] Since the $g = 4.1$ signal can be generated independently of any change in the S_1-state signal, it would be necessary to hypothesize that the cluster also exists in two forms in the S_1 state. The proportionality between the changes in the S_1-state parallel polarization signal and the generation of the multiline signal would imply that the conformation that would give rise to the multiline signal in the S_2 state would correspond to the parallel polarization signal in the S_1 state and that the form that would give rise to the $g = 4.1$ signal in the S_2 state would presumably be EPR silent in the S_1 state. However, such a model does not predict the observed behavior of the signals upon warming to 200 K in the dark. In the context of the single-cluster model, the observed changes in the S_2-state signals following warming in the dark result from a thermally induced conformational change. An analogous shift in the S_1 state from the form that would give rise to the $g = 4.1$ signal when oxidized to the form that would give rise to the multiline signal when oxidized would result in an increase in the S_1-state signal amplitude, rather than the observed decrease. The observed decrease in the S_1-state signal could be explained in terms of conformational changes only by hypothesizing that clusters in the S_1 and S_2 states somehow undergo opposite conformational shifts upon warming in the dark. Such a combination of conformational changes would in no way require that the relative amplitudes of the S_1-state signal and the multiline signal observed following warming in the dark show the same constant of proportionality as the relative amplitudes observed following the 140 K and 200 K illumination steps, yet such a proportionality is observed. The thermally induced shift in the S_1-state conformations that would be required to explain the observed decrease in the S_1-state signal upon warming the sample from 140 K to 200 K is also inconsistent with the known dependence of the relative yield of the $g = 4.1$ and multiline signals on the illumination temperature.[9]

The structure of the metal centers within the oxygen-evolving complex is not yet entirely clear. The presence of a bridged structure with a Mn–Mn distance of 2.7 Å was established by early EXAFS experiments.[31] Hansson et al.[29] proposed a model in which the multiline signal arises from a binuclear Mn_2(III,IV) species and the $g = 4.1$ signal arises from a mononuclear Mn(IV) species of nearly axial symmetry; however, these particular assignments may not be fully consistent with the observed EXAFS scattering amplitudes.[31,32] Recently, Kim et al.[33] detected hy-

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perfine structure indicative of an exchange-coupled manganese center in signals near $g = 4$ in ammonia-treated PS II preparations. In addition, Baumgarten et al.[34] measured light-induced changes in the magnetic susceptibility of fluoride-treated PS II preparations, which exhibit an altered EPR signal at $g = 4.2$ in the S_2 state,[7,35] that they interpreted as evidence of a magnetically coupled site. These chemically inhibited preparations, although giving rise to altered EPR properties, have been considered as possible analogues for the native $g = 4.1$ species, with the suggestion that both this signal and the multiline signal may originate from the same multinuclear cluster. However, we note that addition of exogenous ligands has been seen to significantly alter exchange couplings and bridging structure within multisite metalloprotein complexes, either by disrupting existing bridges or by leading to the formation of new bridging interactions,[36] suggesting that these chemically inhibited preparations may have exchange interactions different from those of the native enzyme.

In conclusion, the detection of the parallel polarization EPR signal in the S_1 state of the oxygen-evolving complex demonstrates the presence of a paramagnetic intermediate in the resting state of the enzyme and provides an additional spectroscopic probe for understanding the structure and mechanism of this complex. The behavior of the signal in the S_1 to S_2 transition suggests that the S_1-state signal arises from the reduced form of the species that produces the multiline signal in the S_2 state, and the properties of the S_1-state intermediate provide additional insight into the electronic structure of the manganese ions.

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Carbon-13 Chemical Shift Tensors in Aromatic Compounds. 3. Phenanthrene and Triphenylene

Arlen Soderquist, Craig D. Hughes, W. James Horton, Julio C. Facelli,† and David M. Grant*

Contribution from the Department of Chemistry, University of Utah, Salt Lake City, Utah 84112.
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Abstract: Measurements of the principal values of the ^{13}C chemical shift tensor are presented for the three carbons in triphenylene and for three different α-carbons in phenanthrene. The measurements in triphenylene were made in natural abundance samples at room temperature, while the phenanthrene tensors were obtained from selectively labeled compounds (99% ^{13}C) at low temperatures (~25 K). The principal values of the shift tensors were oriented in the molecular frame using ab initio LORG calculations. The steric compression at C_4 in phenanthrene and in corresponding positions in triphenylene is manifested in a sizable upfield shift in the σ_{33} component relative to the corresponding σ_{33} values at C_1 and C_9 in phenanthrene. The upfield shift in σ_{33} is mainly responsible for the well-known upfield shift of the isotropic chemical shifts of such sterically perturbed carbons. In phenanthrene C_9 exhibits a unique σ_{22} value reflecting the greater localization of π-electrons in the C_9–C_{10} bond. This localization of the π-electrons at the C_9–C_{10} bond in the central ring of phenanthrene also corresponds with the most likely ordering of electrons described by the various Kekulé structures in phenanthrene. The analysis of the ^{13}C chemical shieldings of the bridgehead carbons in triphenylene provides significant experimental information on bonding between rings in polycyclic aromatic compounds. The results confirm that the electronic structure of triphenylene is best described by three fairly isolated benzene rings linked by C–C bonds of essentially single bond character. Similarly in phenanthrene, the bonding structure which correlates the shielding information may be characterized by the dominance of two benzene rings comprising the biphenyl moiety. A strong C_9–C_{10} π-bond with only limited π-electron character in the C_{8a}–C_9 and C_{10}–C_{10a} bonds is indicated by both the experimental and theoretical results.

I. Introduction

Important information on carbon-13 chemical shift tensors may be obtained from solid state NMR spectra of either powders or single crystals. Single crystal data are required to determine completely all six elements of an experimental carbon-13 chemical shift tensor, but principal tensor values may be obtained from powdered solids whenever the spectral bands can be individually identified and assigned to a specific nucleus. The need for the powerful but more demanding single crystal methods is reduced in powders when molecular symmetry features and/or theoretical information are available to assist in specifying some or all of the principal axes associated with the measured principal shifts. The use of low-temperature NMR methods in powdered solids for measuring the principal components of the chemical shift tensor in small and medium size molecules is now well established,[1-9]

† Also with Utah Supercomputing Institute, University of Utah.

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