Volume 221, number 2, 245–248 FEB 05081 September 1987

A comparison between the multiline EPR signals of spinach and *Anacystis nidulans* and their temperature dependence

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Received 6 July 1987

The multiline EPR signals arising from manganese in the S_2 state of the oxygen-evolving system of spinach and the cyanobacterium *Anacystis nidulans* have very similar properties and are affected identically by NH_3, suggesting that the system is highly conserved. The temperature dependence of the signal amplitude follows Curie behavior down to sub-helium temperatures. This is in contrast to previous reports, which were taken as evidence for a tetrameric manganese cluster. Thus, it seems that it is not yet possible from EPR data alone to distinguish between this model and a dimeric structure.

Oxygen evolution; EPR; Manganese; Temperature dependence; *(Anacystis nidulans)*

1. INTRODUCTION

Manganese plays a key role in the photosynthetic production of oxygen in higher plants and cyanobacteria. Each reaction center is associated with four manganese ions. The arrangement of these ions is not yet known in detail, but the so-called multiline EPR signal [1] from the S_2 state provides proof that the metal is present in a cluster containing at least two ions. In fact, the anomalous temperature dependence of the EPR signal has been taken as evidence that all four ions form the cluster [2]. Modifications in the signal on removal of the two extrinsic 16 and 24 kDa polypeptides have then been suggested to arise from changes in the interaction within this cluster [3].

The present results show that the multiline signals from spinach and *Anacystis nidulans* are very similar in spite of the lack of the two extrinsic polypeptides in the latter species. Also, unsuccessful efforts to reproduce the anomalous temperature dependence are reported. Thus, in our view, EPR data alone do not permit unambiguous conclusions concerning the arrangement of the manganese ions.

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2. MATERIALS AND METHODS

2.1. *Photosynthetic material*

O_2-evolving PS II particles were prepared from *A. nidulans* photosynthetic membranes as described in [4] and modified in [5], except that the final sucrose gradient centrifugation step was omitted. The PS II particles, about 2 mg chlorophyll (Chl)/ml, were suspended in 16 mM Mes/NaOH, pH 6.0, containing 20% (v/v) glycerol, 0.4 M mannitol, 10 mM $CaCl_2$, 8 mM $MgCl_2$ and 1.6 mM K_2HPO_4, and were stored at 77 K until use. The activity, measured as in [5] with 1 mM phenyl-*p*-benzoquinone and 1 mM $K_3Fe(CN)_6$ as acceptors, was typically more than 3000 μmol O_2/mg Chl per h at 26°C.

O_2-evolving PS II membranes from spinach were prepared in darkness as described by Beck et al. [6], except that the grinding medium used for isolating the chloroplasts contained 20 mM Mes/NaOH, pH 6.3, 350 mM sorbitol, 10 mM

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00145793/87/$3.50

NaCl, 5 mM EDTA and 1 g/l bovine serum albumin. The PS II membranes were suspended at 10 mg Chl/ml in 20 mM Mes/NaOH, pH 6.0, 15 mM NaCl and 30% ethylene glycol. The activity measured as in [7] was 600 μmol O_2/mg Chl per h.

Ammonia modification of the S_2 state was accomplished as in [8] in 20 mM Hepes/NaOH, pH 7.5, with 80 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and 50 mM $(NH_4)_2SO_4$ present. Otherwise the composition of the media was the same as that of the suspension media described above.

2.2. *EPR samples*

Metmyoglobin (150 μM) was added to the PS II material in EPR tubes, and the samples were illuminated at about 100 W/m^2 and 0°C for 2 min [6] with stirring to expose the whole sample to light. After dark adaptation at 0°C (6 min for the 'active' state and 4 or 2 h for the 'resting' state [6] for spinach and *A. nidulans*, respectively) the samples were frozen in ethanol-dry ice, illuminated for 5 min at 200 K and 1 kW/m^2 to generate the S_2 state and transferred to liquid nitrogen.

2.3. *EPR spectroscopy*

EPR spectra were recorded with a Bruker ER 200 D-SRC X-band spectrometer equipped with a standard TE_{102} rectangular cavity and an Oxford Instruments ESR-10 helium flow cryostat.

As the temperature-measuring system in the cryostat is not reliable at very low temperatures, we measured the sample temperature by an alternative method. The high-spin Fe^{3+} EPR signal from metmyoglobin, directly dissolved in the EPR tube, was used as an internal temperature indicator. Metmyoglobin was chosen because it has a strong line at $g = 6$ which is not easily power-saturated and does not disturb the multiline Mn signal. Furthermore, under our conditions myoglobin does not interfere or react with the photosynthetic system. The amplitude of the $g = 6$ signal, recorded under non-saturating conditions and corrected for the Boltzmann distribution among the three zero-field splitting levels (D = 9.14 cm^{-1} [9]), gave a direct measure of the relative sample temperature. In order to obtain an absolute value this internal thermometer was calibrated at about 8 K, using two carbon resistors, placed at the top and bottom of the cavity in a separate tube. The temperature gradient over that region was found to be about 0.3 K at 8 K. We estimate that the overall accuracy of the temperature measurements is ± 0.2 K.

3. RESULTS AND DISCUSSION

3.1. *EPR spectra*

The spectra of the resting S_2 multiline signal from the *A. nidulans* and spinach PS II preparations (fig.1) are remarkably similar in about every detail, although clearly better resolved in the *A. nidulans* spectrum. The relaxation behavior, as judged from the response to microwave power, was also similar for both spectra. These consist of about 18 lines if only the main structures are counted and two lines are assumed hidden under the strong radical signal (signal II). Spectra identical to those in fig.1B were also obtained from a spinach preparation from which the 16 and 24 kDa polypeptides had been removed by a salt wash (2 M NaCl [10]). Thus, in contrast to what has been reported [3], removal of these polypeptides from the spinach preparation or their absence in the cyanobacterial sample [11] does not affect the multiline signal.

Ammonia, which binds to the oxygen-evolving complex and modifies the multiline signal [8], induces similar effects in spinach and *A. nidulans*, i.e. a decrease in mean separation between the lines of the multiline signal from about 9 to approx. 7 mT (fig.2).

3.2. *Relaxation and temperature behavior*

EPR spectra were recorded for a number of temperatures between 2.2 and 15 K. At each temperature the microwave power was lowered until a nonsaturated spectrum was obtained. This power was found to vary from 2 mW to about 1 μW in the temperature interval used. As a measure of the signal intensity we used the average of the amplitudes of the three strongest lines between 0.385 and 0.405 T at the high-field side of the spectrum (fig.1). The low-field lines were not used because of the possible interference with the underlying g_y-line from cytochrome *b*-559.

Figs 3 and 4 show the temperature dependence of the multiline signal from spinach and *A. nidulans*, respectively. It is clear that no significant

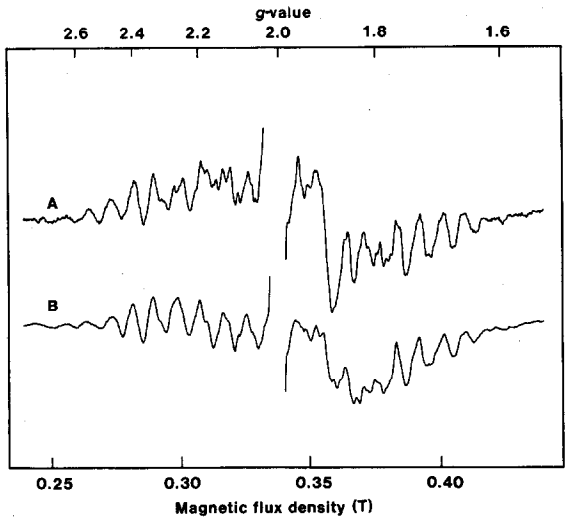

Fig.1. The light-minus-dark multiline EPR signal from the resting S_2 state in (A) *A. nidulans* and (B) spinach. The samples were prepared as described in section 2. EPR conditions: temperature, 11 K; microwave power, 20 mW; modulation amplitude, 1.25 mT; microwave frequency, 9460 MHz.

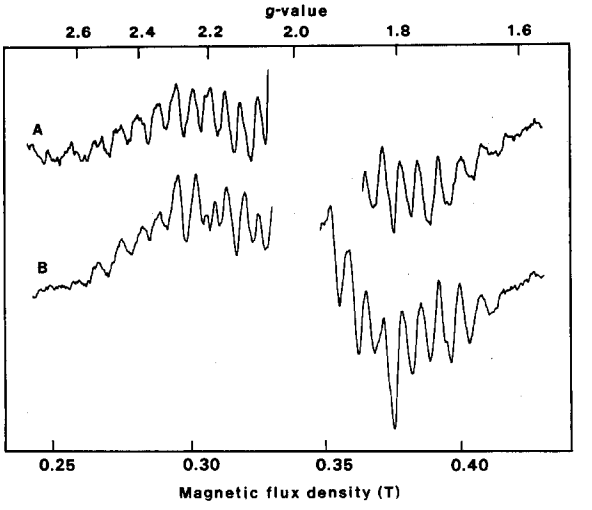

Fig.2. Comparison between the multiline EPR signals from (A) *A. nidulans* and (B) spinach after reaction with ammonia. Modulation amplitude, 2.5 mT. Other conditions for EPR as in fig.1.

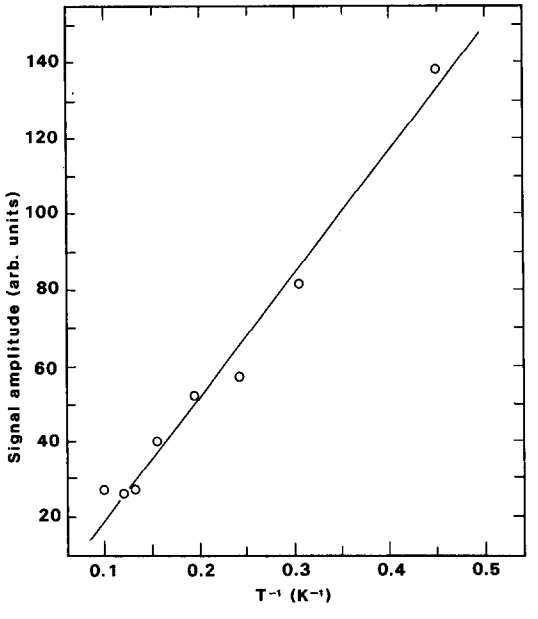

Fig.3. Temperature dependence of the unsaturated multiline EPR signal from the resting S_2 state in spinach. The sample is the same as that in fig.1B.

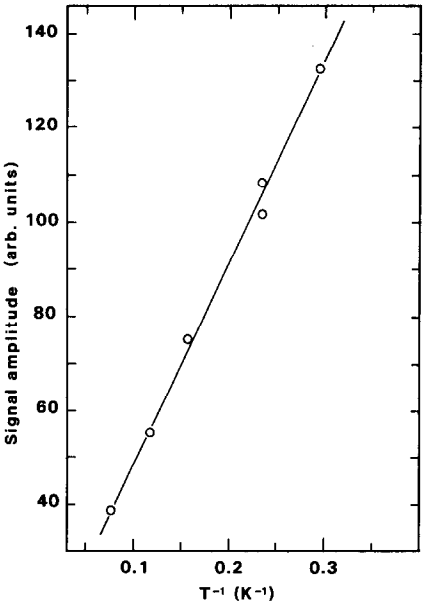

Fig.4. Temperature dependence of the unsaturated multiline EPR signal from the resting S_2 state in *A. nidulans*. The sample is the same as that in fig.1A.

deviation from Curie behavior can be observed. Also, a spinach sample prepared to be in an active S_2 state gave the same type of temperature dependence. Thus, our results are strikingly in conflict with the data presented by other laboratories [2,12], who report non-Curie behavior for samples prepared in the same way as ours (see, for example, fig.3a in [2]). We cannot offer any obvious explanation for this discrepancy, although the stated non-saturating conditions [2] seem somewhat questionable for the lowest temperatures.

The Curie behavior of the multiline signal intensity means that it may arise from a ground spin multiplet of a manganese dimer as suggested earlier [13]. Thus, in contrast to De Paula et al. [2] we need not explain our results in terms of a tetrameric manganese cluster.

3.3. *Comparison between the signals from spinach and* A. nidulans

There is a striking similarity between the multiline signal from spinach and *A. nidulans* in both spectral shape (fig.1), its response to ammonia (fig.2) and its temperature dependence (figs 3,4). This must mean that the molecular structure of the manganese cluster involved in oxygen evolution is very similar with respect to ligands and distances in both organisms. Such a highly conserved structure, despite many hundred million years of possible evolution, shows that nature here has devised a very efficient solution to a difficult chemical problem, the splitting of water.

ACKNOWLEDGEMENTS

This work was supported by grants from the Swedish Natural Science Research Council.

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