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Is nitrogen liganded to manganese in the photosynthetic oxygen-evolving system? EPR studies after isotopic replacement with ^{15}N

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The nature of the ligands to manganese in the photosynthetic oxygen-evolving system was investigated by the effect of isotopic substitution with ^{15}N on the spectral properties of the multiline EPR signal of the S_2 state. No changes were observed in the general shape of the signal or in the linewidth. The results show that the resolved fine structure in the multiline signal cannot be explained as nitrogen superhyperfine structure but most likely arises from manganese hyperfine interaction. The large difference between the highest value for the nitrogen coupling constant consistent with the results and that earlier observed in a binuclear, antiferromagnetically coupled manganese model complex strongly favors non-nitrogen ligands such as oxygen in carboxylic amino acid side chains.

In oxygenic photosynthesis, water is oxidized in Photosystem II in a process which requires the participation of four manganese ions. The successive removal of electrons from the oxygen-evolving system leads to a cyclic interconversion of different states of the system, called S-states, which differ with the respect to the oxidation state of manganese. One of the states, S_2, is associated with two EPR signals (electron paramagnetic resonance), a derivative-type signal at $g = 4.1$ and a complex multiline signal at $g = 2$, originating from manganese (for recent accounts of the role of manganese in photosynthetic oxygen evolution, for reviews, see Refs. 1–3). The interpretation of the signal properties has prompted formulations of structural models of the water-splitting complex either consisting of a dimer of strongly interacting manganese ions with associated accessory manganese ions [4] or a cluster of four interacting ions [5].

Very little is known about the binding of the manganese to protein and the nature of the ligands. However, EXAFS (extended X-ray absorption fine structure) studies indicate the presence of several nitrogen or oxygen nuclei at distances typical of ligands [6].

So far, oxygen, through the broadening effect of ^{17}O on the multiline spectrum, is the only nucleus which has been positively identified as a ligand to manganese in the S_2 state [7], such as liganded water or as a bridging ligand.

The resolved narrow lines, seen in addition to the main 16–19 manganese hyperfine lines of the multiline signal, have been proposed to be due to superhyperfine structure from coordinating ligands such as nitrogen [8,9]. A coordinated ligand with a nuclear spin of $I = 3/2$ has also been implicated [10]. Comparisons with the line widths in the EPR spectra of model compounds have also suggested the presence of nitrogen ligands [8].

In addition, chloride has been proposed as a ligand, but both EXAFS [6] and high-resolution EPR [9] seem to argue against this possibility.

In an attempt to resolve some of the questions concerning the nature of the ligands to manganese the effect of isotopic substitution with ^{15}N on the shape of the multiline signal was studied. With nitrogen as a ligand to manganese, changes in the structural details the signal and/or improved resolution in the spectrum are expected, due to the differences in nuclear spin of ^{14}N and ^{15}N.

Spinach was grown hydroponically with $K^{15}NO_3$ (Amersham, 99% abundance) as the sole nitrogen source. Broken chloroplasts were isolated as in Ref. 7 and washed once in 20 mM Mes (4-morpholineethanesulfonic acid), 15 mM NaCl (pH 6.3), before resuspension in 20 mM Mes (pH 6.3), with 15 mM NaCl, 5 mM $MgCl_2$ and 400 mM sucrose.

Abbreviations: PPBQ, phenyl-*p*-benzoquinone; Mes, 4-morpholineethanesulfonic acid.

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For EPR, broken chloroplasts in EPR tubes were dark-adapted on ice for 1 h. Prior to freezing of the sample tubes 2 mM phenyl-*p*-benzoquinone (PPBQ) was added to fully oxidize the photosystem II acceptor side. PPBQ was added dissolved in ethanol which increases the resolution of the multiline signal [4,11]. The S_2 state of the oxygen-evolving system was generated by illumination at 200 K for 4 min. Low-temperature EPR spectra were obtained as in [7].

Broken chloroplasts illuminated at 200 K showed the normal multiline EPR spectrum of the S_2 state (Fig. 1). A comparison between spectra obtained from control (^{14}N) and ^{15}N-substituted chloroplasts did not reveal any significant differences in the appearance of the multiline signal in highly resolved spectra (Fig. 2), i.e., all features present in the control were reproduced in the spectra from the ^{15}N-substituted chloroplasts. In addition, no significant consistent differences in linewidth were found between signals from material with different isotopic composition. Lowering the modulation amplitude of the spectrometer below 0.63 mT did not improve the resolution in either spectrum.

One conclusion which may be drawn immediately from these results is that the resolved structural details in the EPR spectrum of the S_2 state cannot originate from resolved nitrogen-ligand superhyperfine structure as has sometimes been proposed [8,9]. Because of differences in nuclear spin, the two nitrogen isotopes should give rise to different splitting patterns which should have changed the positions of the fine-structure lines in a recognizable manner. Thus, the resolved narrow lines in the multiline signal most likely arise from manganese

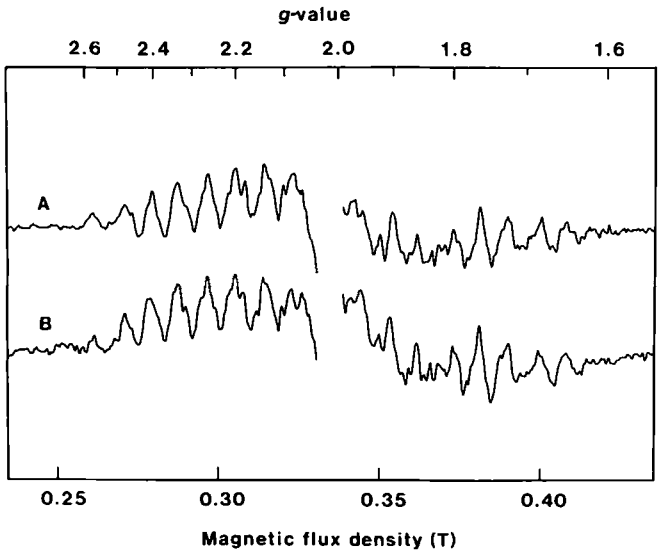

Fig. 1. The effect of isotopic N-substitution on the multiline EPR signal from the S_2 state of the oxygen-evolving system. (A) Sample containing spinach thylakoids grown on ^{14}N. (B) As (A), but grown on ^{15}N. For details, see text. Conditions for EPR: microwave frequency, 9.46 GHz; power, 20 mW; modulation amplitude, 0.63 mT; temperature, 11 K.

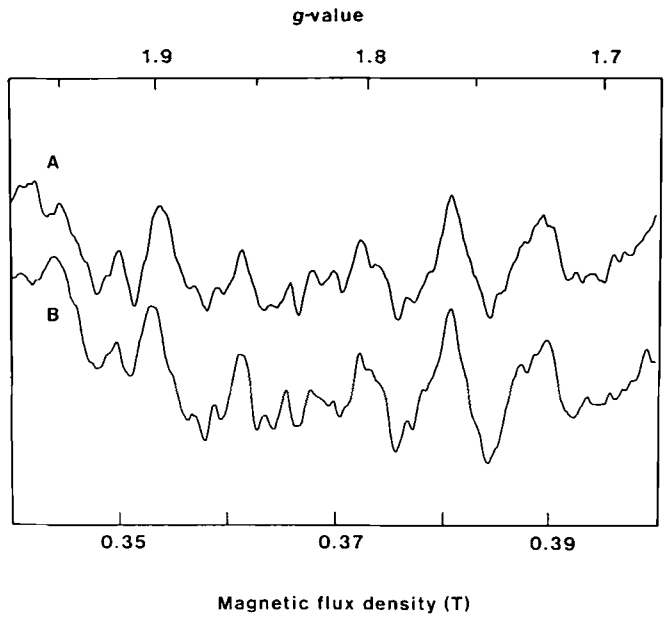

Fig. 2. Detail of the spectrum in Fig. 1.

hyperfine interaction. This has earlier been discussed as an alternative explanation for the fine structure [9,12].

The lack of effects on the linewidth of the multiline signal after isotopic substitution with ^{15}N also offers arguments against nitrogen as a ligand to photosynthetic manganese. Due to its somewhat larger magnetic moment the ^{15}N nucleus is expected to be associated with a hyperfine-splitting constant about 40% higher than that of the ^{14}N nucleus. However, the nuclear-spin change from $I = 1$ (^{14}N) to $I = 1/2$ (^{15}N) more than sufficiently compensates for the effect of the magnetic moment so a substitution of ^{15}N for ^{14}N should result in a net narrowing and a concomitant increase in the intensity of EPR lines broadened by unresolved nitrogen superhyperfine structure.

To estimate the absolute effect of isotopic replacement of nitrogen ligands on the linewidth one must know the magnitude of the hyperfine-splitting constant associated with nitrogen. Dismukes et al. [8], when replacing oxygen ligands (2,2′-bipyridine-*N*,*N*′-dioxide) with nitrogen (2,2′-bipyridine) in a binuclear, antiferromagnetically coupled, mixed-valence manganese (III/IV) complex of a type which has been discussed as a possible model for the water-splitting site, found a 2–3-fold broadening (from about 1.7 to about 4.0 mT) of the hyperfine lines of the multiline-type EPR signal compared to the original (oxygen-liganded) complex. The broadening, which was ascribed to unresolved nitrogen superhyperfine structure, corresponds to an average hyperfine-splitting constant of about 1 mT for the eight nitrogens in the model compound.

The most narrow lines in the multiline spectrum have widths of 1 mT or less. Such narrow lines are incompatible with the presence of unresolved nitrogen superhy-

perfine structure with hyperfine-coupling constants of the order of 1 mT as in the model compound. Therefore, if nitrogen ligands are present they must be associated with significantly smaller coupling constants. Simulations of the effect of isotopic substitution on the width of gaussian lines suggest that, even with isotropic coupling constants as small as a few tenths of a mT, lines, with a final width of 1 mT due to unresolved superhyperfine structure from one or two ^{14}N ligands, will split after replacement with ^{15}N. As an example, two ^{14}N nuclei with a hyperfine coupling of 0.32 mT will broaden an EPR line with an intrinsic width of 0.45 mT to an unresolved line with a final width of 1 mT. Replacement with ^{15}N would result in the appearance of resolved superhyperfine structure in this line in addition to an increase in amplitude by about 20%. Increasing the number of nitrogen nuclei would require even smaller values for the coupling constant to avoid an observable splitting with ^{15}N. Such simulations suggest maximum values for the hyperfine-coupling constant from below 0.2 to about 0.3 mT, depending on the number of nuclei involved. These values are much smaller than that found for the nitrogen ligands in the model compound referred to above and seriously question the presence of nitrogen ligands in the manganese complex responsible for the multiline EPR signal.

These isotope substitution experiments and comparisons with the nitrogen-hyperfine interactions in one relevant type of model compound, therefore, favor non-nitrogen ligands to manganese, which would seem to exclude histidine which has often been suggested in this role [13–15]. On the other hand, the results are in line with recent electron spin-echo studies of oxygen-evolving Photosystem II membranes which were unable to reveal the presence of nitrogen ligands [16]. The experimental arguments in favor of non-nitrogen ligands limit the number of choices among possible involved amino acids and lend some support to proposals based on sequence analysis that carboxylic side chains on the D1 and D2 polypeptides play a major role as ligands to manganese (Ref. 17; but see also Ref. 15).

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