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OBSERVATION OF ^{17}O EFFECTS ON Mo^{V} EPR SPECTRA IN SULFITE OXIDASE, XANTHINE DEHYDROGENASE, AND $MoO(SC_6H_5)_4^-$

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Summary: ^{17}O effects have been observed on the Mo^{V} EPR signals from sulfite oxidase, xanthine dehydrogenase, and $MoO(SC_6H_5)_4^-$. The results point to the presence of a rapidly exchangeable oxygen ligand in the molybdenum coordination sphere of the enzymes. Average splittings were on the order of 10 gauss for the enzymes, but only about 2 gauss for $MoO(SC_6H_5)_4^-$.

INTRODUCTION. With the significant exception of nitrogenase, all of the known molybdenum enzymes exhibit EPR spectra assignable to Mo^{V}. (1,2) These signals are characterized by high g-values, significant anisotropy, and hyperfine splitting by an exchangeable proton. As early as 1966, Meriwether suggested that the high g-values were the result of sulfur ligation. (3) Recent EXAFS studies of sulfite oxidase(4) and xanthine oxidase(5) have confirmed the presence of sulfur ligands to molybdenum and have suggested the presence of terminal oxo groups as well. In order to further elucidate the nature of the molybdenum site present in these enzymes, the effects of $H_2^{17}O$ on the EPR spectra of sulfite oxidase, xanthine dehydrogenase, and $MoO(SC_6H_5)_4^-$ have been examined.

MATERIALS AND METHODS. Sulfite oxidase was purified from chicken liver as previously described (6). Low pH samples were prepared by concentrating enzyme in 1 M Tris HCl buffer, pH 6.5, to approximately 100 mg/ml in an Amicon stirred cell using a PM-10 ultrafiltration membrane. Aliquots of 0.1 ml were diluted with 1 ml of 54.7% enriched $H_2^{17}O$ (Mound Research Labs) or

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with 1 ml of unlabelled H_2O, and 0.2 ml aliquots of diluted enzyme were transferred to quartz EPR tubes (4 mm i.d.). The samples were reduced with 4µl of 0.1 M Na_2SO_3 for 1 min and frozen in liquid nitrogen. Preparation of high pH samples was identical except that 1 M Tris HCl, pH 9.2, was used.

Xanthine dehydrogenase was purified from chicken liver by a combination of previously published procedures (7,8). Partial deflavination was achieved using the method of Kanda et al.(9) The enzyme was concentrated to 54 mg/ml in 0.2 M potassium phosphate buffer, pH 7.8, with 0.1 mM EDTA, and 0.1 ml aliquots were diluted with 0.3 ml $H_2^{17}O$ (54.7% enriched) or 0.3 ml unlabelled H_2O. Samples of 0.2 ml were transferred to EPR tubes, reduced with 0.25 mM NADH for 1 min and frozen in liquid nitrogen.

$[(C_2H_5)_4N]\,[MoO(SC_6H_5)_4]$ was synthesized by the literature method.(10) An ^{17}O-enriched sample was prepared by treating $MoCl_5$ with $H_2^{17}O$ in CH_3CN before adding benzenethiol according to the published procedure. The infrared spectrum (KBr) showed ν $Mo-^{17}O$ at 911 cm^{-1} (calc 909 cm^{-1}).

The EPR spectra were recorded on a Varian E-12 with a Nicolet computer for digitizing, averaging, and storing spectra.

RESULTS. The most dramatic ^{17}O effects are observed on the g_1 = 2.004 feature of the high pH sulfite oxidase spectrum (Figure 1a,b). Three of the six expected hyperfine lines appear as distinct new peaks in the spectrum, while the two innermost lines broaden the central peak, and the high field hyperfine feature overlaps with the g_2 and g_3 features. The average splitting of these hyperfine lines yields a value of 11 gauss for A_1. The g_2 and g_3 features of this spectrum are broadened by ^{17}O, but no distinct new lines are observed.

In the low pH spectrum (Figure 1c,d), the low field doublet is merely broadened upon ^{17}O incorporation, and it is the high field region where distinct new features occur. Analysis of these features gives an average value for A_2 and A_3 of 8 gauss.

The emergence of the ^{17}O effects was rapid and independent of the path of reduction. Samples reduced after 5 minutes, 20 minutes, or 1 day of mixing with $H_2^{17}O$ all gave the same spectrum. Reduction with methyl viologen gave the same pattern as reduction with sulfite. Equilibration at pH 7, followed by raising the pH to 9, gave the typical high pH ^{17}O spectrum.

Turning to the xanthine dehydrogenase spectra (Figure 2a,b), the major effect of ^{17}O is a broadening of the features of the Mo(V) signal. The lack of resolved hyperfine structure is due to the lower ^{17}O enrichment

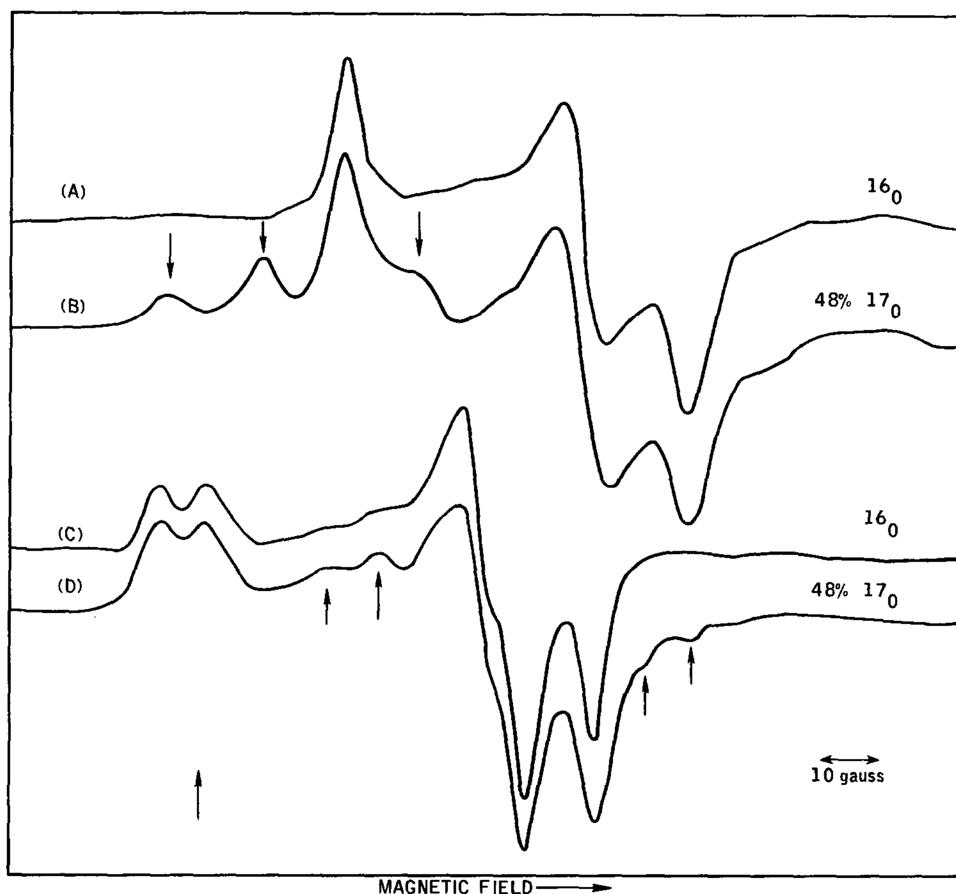

Figure 1a) Sulfite oxidase, high pH form. EPR parameters: Gain-2.5 x 10^3, Scan Rate-50 gauss/min, Time Constant - 0.25 s, Modulation 1 gauss, Power - 10 mW, Temperature - 173°K

b) Same as a), but 50% ^{17}O in solvent

c) Sulfite oxidase, low pH form. Same EPR parameters as a)

d) Same as c), but 50% ^{17}O solvent

(The arrow at lower left marks g = 2.0030.)

attained with this sample (40%) and interference by the flavin radical signal. Detailed analysis of ^{17}O splittings in this enzyme must await computer simulation of a difference spectrum.

Finally, in an attempt to reproduce these observed ^{17}O effects in a well-defined oxo-molybdenum compound, ^{17}O-labelled $[N(C_2H_5)_4]$ $[MoO(SC_6H_5)_4]$

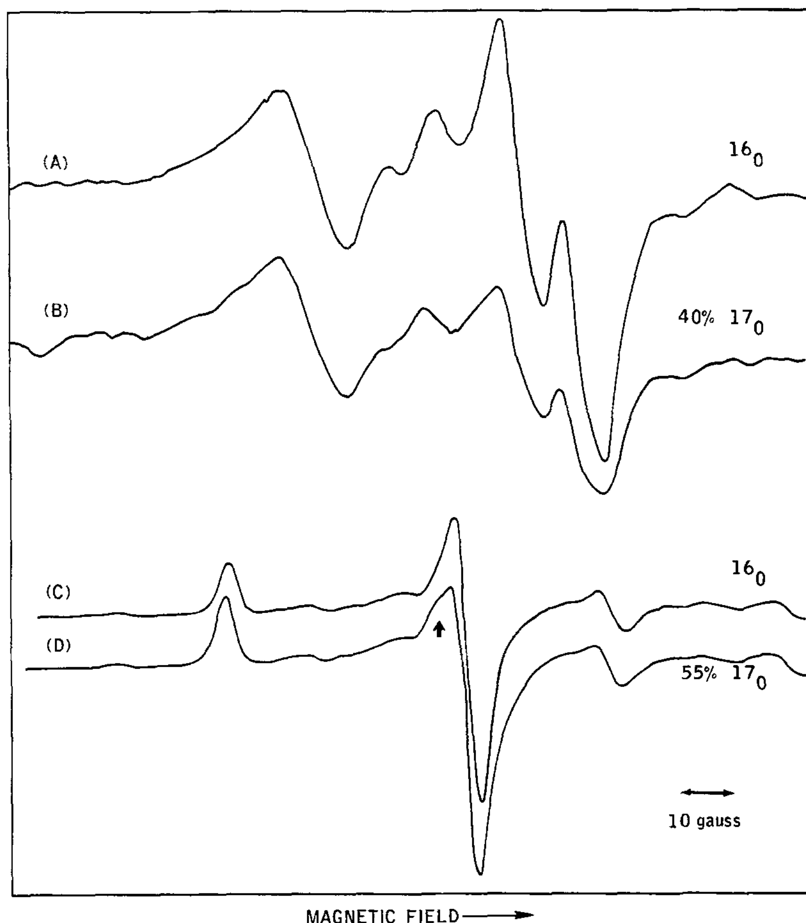

Figure 2a) Xanthine dehydrogenase control, pH 7.8

b) Same as a), but 40% ^{17}O in solvent

c) $[N(C_2H_5)_4]$ $[MoO(SC_6H_5)_4]$ in 4:1 v/v CH_3CN/DMF

d) Same, but 55% ^{17}O-enriched

was prepared, and its EPR compared with unlabelled material. The observed effects (Figure 2c,d) were surprisingly small in comparison with the enzyme results. The $g_{\parallel}$ peak was broadened slightly (about 1 gauss after subtracting out contributions from ^{16}O), and a shoulder appeared on the low field side of the $g_{\perp}$ peak. Inspection of this shoulder shows $A_{\perp}$ to be roughly 2 gauss. This is in agreement with other solution studies of this compound.(11)

DISCUSSION. The observation of ^{17}O hyperfine splittings upon diluting sulfite oxidase or xanthine dehydrogenase in $H_2^{17}O$ demonstrates

the presence of an exchangeable oxygen ligand in the coordination sphere of molybdenum in these enzymes. The distinct 6-line pattern for high-pH sulfite oxidase indicates substantial splitting by only a single exchangeable oxygen. An obvious candidate for this oxygen ligand is a terminal oxo group, evidence for which was obtained from EXAFS.(12) However, the effects might just as well be due to a bound water molecule. A larger body of data on ^{17}O splittings in molybdenum compounds is needed before any detailed structural conclusions can be made from these data.

The difference in coupling constants between the enzymes and $[MoO(SC_6H_5)_4]^-$ is substantial, and it illustrates the sensitivity of ^{17}O effects to molecular structure. Variability in ^{17}O effects has also been observed in copper EPR studies. $H_2^{17}O$ as an axial ligand in aqueous Cu(II) $(acetylacetonate)_2$ introduced negligible splitting, but after chemical exchange into an equatorial position, a 12 gauss splitting was observed.(13) A similar splitting of 12 gauss was observed on the low field line of fungal laccase.(13) Even larger splittings have been observed on ^{17}O-enriched samples of $[Cu(II)(H_2O)_6]^{2+}$ in doped single crystal studies.(14)

The current results have revealed a new physical property of enzymatic molybdenum, which may be of use in structural kinetic and mechanistic studies. One test of appropriate model compounds for the non-nitrogenase molybdenum enzyme should be comparable ^{17}O hyperfine effects.

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12. Preliminary EXAFS results on sulfite oxidase have already indicated the presence of oxo groups in this enzyme. However, the original spectra on sulfite-reduced sulfite oxidase were interpreted as indicating two oxo groups on the Mo^{V} species. Subsequent quantitative EPR titrations have shown that only about 50% of the enzyme is converted to Mo^{V} under these conditions. Thus, further EXAFS analysis of the Mo^{V} state is required, possibly by difference spectrum analysis of samples with known Mo^{IV},Mo^{V}, and Mo^{VI} concentrations.

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