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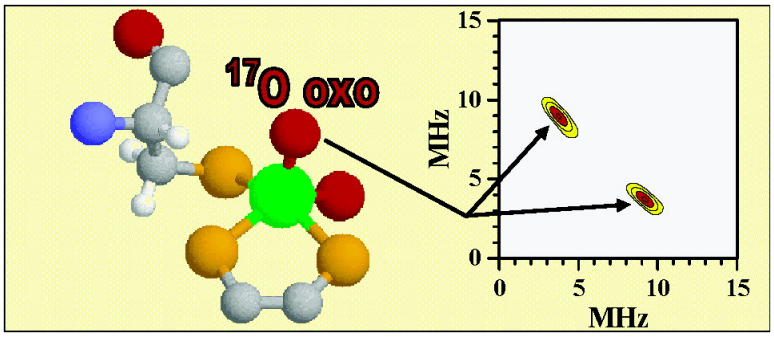
J. Am. Chem. Soc., **2005**, 127 (2), 502-503• DOI: 10.1021/ja0461466 • Publication Date (Web): 21 December 2004



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Published on Web 12/21/2004

^{17}O ESEEM Evidence for Exchange of the Axial Oxo Ligand in the Molybdenum Center of the High pH Form of Sulfite Oxidase

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Sulfite oxidase (SO) is a vital molybdenum enzyme that is essential for normal neonatal neurological development,[1] and several point defects in human SO have been shown to result in SO deficiency.[2] Sulfite oxidation occurs at the dioxomolybdenum center; the equatorial oxo ligand is exposed to solvent and positioned to be transferred to the substrate, sulfite.[3,4] The axial oxo ligand is less accessible to solvent and is proposed to act as a "spectator" group[5] to effectively labilize the equatorial oxo ligand that reacts with substrate.[6,7] Our recent high-resolution variable frequency pulsed electron paramagnetic resonance (EPR) studies show that the transient Mo(V) species produced upon reduction by sulfite[8,9] contain a coordinated equatorial OH or OH_2 group (Figure 1).[10] The low pH/high chloride (*lpH*) form has a single exchangeable proton with a large isotropic hyperfine interaction (*hfi*) constant of ~26 MHz,[11] whereas the high pH/low chloride (*hpH*) form has two exchangeable protons with the anisotropic *hfi* constant being distributed from ~−4 to ~−7 MHz and no detectable isotropic *hfi*.[10] The additional proton in *hpH* SO is assigned to an OH (or H_2O) that is hydrogen bonded to the coordinated equatorial OH group.[10] For samples of SO prepared in $H_2{}^{17}O$, the *hfi* of ^{17}O of the equatorial ligand results in EPR splittings of ~20 MHz in the *lpH* form and ~30 MHz in the *hpH* form.[12] Similar Mo(V) ^{17}O *hfi* constants were observed in various types of xanthine oxidase[13−16] and in model systems with equatorial oxygen ligands.[15] Despite numerous attempts, however, exchangeable oxygen ligands in molybdenum enzymes, *other* than the strongly coupled equatorial OH or OH_2 group, have thus far escaped definitive detection by CW EPR or high-resolution EPR techniques of electron−nuclear double resonance (ENDOR)[17] and electron spin−echo envelope modulation (ESEEM) spectroscopy. In this work, as part of a systematic study of oxygen trafficking in the catalytic cycles of molybdenum enzymes, we have performed a ^{17}O ESEEM investigation of *hpH* SO using hyperfine sublevel correlation (HYSCORE) spectroscopy at 29.252 GHz. The HYSCORE spectra have revealed a new type of exchangeable oxygen ligand that is assigned to the axial "spectator" oxo group.

The ^{17}O-enriched *hpH* buffer (100 mM Bis-Tris propane) was prepared by mixing 7 mg of Bis-Tris propane and 0.7 mg of Trizma-HCl with 250 μL of $H_2{}^{17}O$ (70 at. % ^{17}O, Isotec), pH = 9.7. An aliquot (~15 μL) of chicken SO (3 mg, A_{414}/A_{280} = 0.95) in ^{16}O *hpH* buffer was mixed with 110 μL of ^{17}O-enriched *hpH* buffer and then incubated at 4 °C for about 20 h. The solution was concentrated to ~10 μL and then incubated with 60 μL of $H_2{}^{17}O$ buffer at 4 °C for another 2 h. An aliquot (2.5 μL) of 0.6 M Na_2SO_3 in ^{16}O *hpH* buffer was then added to the chicken SO in the ^{17}O-enriched *hpH* buffer (~70 μL). The solution was transferred to an EPR tube and frozen in liquid nitrogen within 60 s. The final EPR sample had a total [SO] of ~1 mM with a 20-fold excess of sulfite and pH 9.7. The final concentration of the ^{17}O label was about 62%. The X-band CW EPR spectrum of this ^{17}O-enriched sample of *hpH* SO was similar to that described elsewhere.[12]

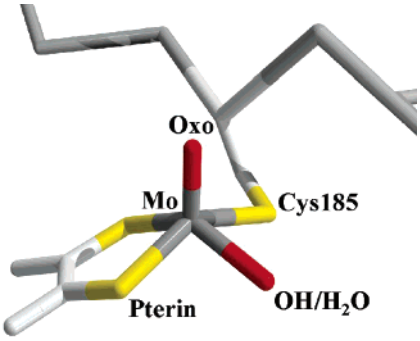

Figure 1. Stick diagram of the Mo(V) coordination in the active site of chicken SO derived from X-ray crystallography[3] and pulsed EPR spectroscopy.[10] Only part of the pterin ligand and protein α-C chain are shown for clarity; the color scheme is red = O, yellow = S, white = C.

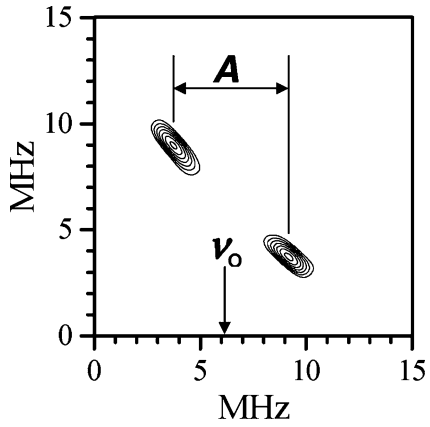

Figure 2. (++) Quadrant of the ^{17}O HYSCORE spectrum of *hpH* SO in $H_2{}^{17}O$ obtained at the g_Y turning point of the EPR spectrum. Experimental conditions: mw frequency, 29.252 GHz; B_o = 1063.2 mT; mw pulses, 4 × 15 ns; time interval τ between the first and second mw pulses, 200 ns; measurement temperature, 20 K; accumulation time, ~1 h. The value of the ^{17}O Zeeman frequency, ν_O, is indicated by an arrow. The measurement of the $A(^{17}O)$ *hfi* constant is also shown. HYSCORE spectra at the g_Z and g_X turning points are given in the Supporting Information.

The HYSCORE experiments were performed at a microwave (mw) frequency of 29.252 GHz using a home-built K_a-band pulsed EPR spectrometer operating in the mw frequency range from 26.5 to 40 GHz. The measurement temperature was about 20 K.

The HYSCORE spectra obtained at several EPR positions (see a typical spectrum in Figure 2) show ^{17}O correlation lines in the (++) quadrants that are absent in the sample with $H_2{}^{16}O$. The transition frequencies of ^{17}O between the states with the nuclear spin projections $m_I = {}^1/_2$ and $m_I = -{}^1/_2$ are those least affected by the nuclear quadrupole interaction (*nqi*). Therefore, these transitions are the most intense ones, and their frequencies are approximately (to first order in *hfi* and *nqi*) given by $\nu = \nu_O \pm A/2$, where ν_O is the ^{17}O Zeeman frequency and A is the secular component of the

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^{17}O *hfi*. The values of *A* estimated from the HYSCORE spectra obtained at the g_Z, g_Y, and g_X EPR turning points are 4.3, 5.3, and 4.8 MHz, respectively (cf. Figure 2 and Supporting Information). These values indicate that the isotropic *hfi* constant is on the order of 5 MHz, while the anisotropic *hfi* constant is on the order of 1 MHz. The latter value may be affected to some degree by the second-order effects of the *nqi*. However, these effects should not be significant because in all of the HYSCORE spectra the half-sum of the frequencies of the correlation lines nearly coincides with ν_O.

The ^{17}O *hfi* of ~5 MHz observed in HYSCORE spectra is much weaker than that of the equatorial $OH_{(2)}$ ligand that gives ~30 MHz splittings in the CW EPR spectrum.[12] One possibility is that the 5 MHz *hfi* is due to a nearby $^{17}OH_{(2)}$ species that is hydrogen-bonded to the equatorial $OH_{(2)}$ ligand in *hpH* SO.[10] However, 5 MHz is about 1 order of magnitude greater than the *hfi* expected for such a hydrogen-bonded $^{17}OH_{(2)}$ species.[18] A similar estimate can be obtained from analysis of the nitrogen *hfi* in a NH group that is hydrogen-bonded to a quinone radical.[19,20] We conclude, therefore, that the HYSCORE spectra of *hpH* SO reveal the presence of an additional exchangeable ^{17}O ligand, distinct from that detected by CW EPR.

Previous studies of ^{17}O *hfi* in model Mo(V) compounds[15] showed that the ^{17}O *hfi* parameters are highly dependent on the coordination position of the oxygen ligand. For an axial oxo group the ^{17}O *hfi* constant is ~4−7 MHz, whereas an equatorial ^{17}OR ligand shows a much stronger *hfi*, with the isotropic part being up to 35 MHz and the anisotropic tensor components being as large as ~10 MHz.[15] Accordingly, for *hpH* SO, the strongly (in the *hfi* sense) coupled ^{17}O detected by CW EPR[12] was assigned to an exchangeable Mo−OH group. The more weakly magnetically coupled ^{17}O observed here is then assigned to the axial oxo ligand (Figure 1). The ^{17}O HYSCORE spectra of the *hpH* form of SO thus provide the first direct evidence of an exchangeable oxo group in *any* molybdenum enzyme.

One possible pathway for introduction of the axial ^{17}O ligand in SO is via the *oxidized* Mo(VI) form during the ~22 h incubation with $H_2{}^{17}O$ (to replace normal $H_2{}^{16}O$ water from the buffered solution) prior to reduction to Mo(V). Precedence for such exchange comes from a ^{17}O NMR study of asymmetric six-coordinate dioxo-Mo(VI) compounds, which showed that *both* of the oxo groups exchanged rapidly with ^{17}O (within about an hour) when only a few drops of $H_2{}^{17}O$ were added to an acetonitrile solution of the compound.[21]

Another possible pathway for introducing the axial ^{17}O ligand is via the *reduced* Mo(V) form (Figure 1). Mo−OH and $Mo-OH_2$ species exchange rapidly with water, and proton transfer between these groups and an adjacent Mo═O group has been shown to facilitate exchange of oxo ligands in coordination compounds.[22−24] However, a resonance Raman study of the molybdenum domain of recombinant human SO that had been redox cycled in $H_2{}^{18}O$ indicated that only one oxo group exchanged with ^{18}O at pH 8.[25] On the other hand, the higher pH (9.7) and different reduction process in our study might facilitate exchange of the axial oxygen of *hpH* SO during the ~1 min reaction with sulfite.

The possibility that the weakly magnetically coupled ^{17}O is due to an OH (or OH_2) ligand in the vacant sixth coordination position (Figure 1) was also considered. However, analysis of the crystal structure of chicken SO[3] indicates that addition of a sixth ligand would require extensive structural change of the protein around the active site, and thus this possibility seems unlikely.

In summary, this initial study of oxygen trafficking in molybdenum enzymes by K_a band HYSCORE spectroscopy has provided the first evidence that the axial "spectator" oxo ligand of SO exchanges in $H_2{}^{17}O$. This result raises the intriguing possibility that the axial oxo group plays a more active role in the catalytic cycle of these enzymes than previously thought. Ongoing studies of ^{17}O exchange in other molybdenum enzymes will be the subject of future reports.

Acknowledgment. We gratefully acknowledge support by the National Institutes of Health (GM-37773 to J.H.E.) and grants from the National Science Foundation (DBI-0139459, DBI-9604939 and BIR-9224431) for construction of the pulsed EPR spectrometers. We thank Professors Michael K. Johnson and Caroline Kisker for helpful discussions.

Supporting Information Available: The ^{17}O HYSCORE spectra of *hpH* SO at the g_Z and g_X turning points of the EPR spectrum; scheme for axial oxo exhange in Mo(V). This material is available free of charge via the Internet at http://pubs.acs.org.

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JA0461466