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Implications for the mechanism of sulfite oxidizing enzymes from pulsed EPR spectroscopy and DFT calculations for “difficult” nuclei†

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Abstract

The catalytic mechanisms of sulfite oxidizing enzymes (SOEs) have been investigated by multi-frequency pulsed EPR measurements of “difficult” magnetic nuclei ($^{35,37}\text{Cl}$, ^{33}S , ^{17}O) associated with the Mo(V) center. Extensive DFT calculations have been used to relate the experimental magnetic resonance parameters of these nuclei to specific active site structures. This combined spectroscopic and computational approach has provided new insights concerning the structure/function relationships of the active sites of SOEs, including: (i) the exchange of oxo ligands; (ii) the nature of the *blocked* forms; and (iii) the role of Cl^- in low pH forms.

1. Introduction

Sulfite-oxidizing enzymes (SOEs) are ubiquitous and can be found in vertebrates, plants and bacteria. The general function of SOEs is to catalyze the oxidation of sulfite to sulfate as represented by eqn (1). A unique feature of molybdenum-containing oxidase enzymes is that the oxygen atom that is incorporated into substrate comes from water rather than dioxygen.

In the fully oxidized resting state of the enzymes, the catalytic site is a nearly square-pyramidal dioxo-molybdenum center that has three equatorial sulfur ligands (one from the conserved cysteinyl side chain and two from the molybdopterin (MPT) cofactor) and two oxo ligands—one equatorial and one axial (Fig. 1).^{1–4}

A simplified version of the proposed catalytic cycle for SOEs is shown in Scheme 1. The first step involves nucleophilic attack of sulfite on the solvent-exposed equatorial Mo=O group of **1**. This leads to **2**, the enzyme product complex in which sulfate is bound to Mo(IV). Hydrolysis of **2** and oxidation by one electron gives **3**, a Mo(V) center that can be studied by electron paramagnetic resonance (EPR) spectroscopy. Oxidation of **3** by one electron regenerates **1** and completes the catalytic cycle. At high pH (pH ≥ 9), continuous wave (CW) EPR shows that species **3** is formed for all wild type (*wt*) SOEs. At lower pH

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values and for various mutant forms of SOEs, however, other Mo(V) species such as **3A** and **3B** can form, which are discussed in Section 2.

The actual stepwise process whereby the Mo center catalyzes the transformation of eqn (1) continues to be an area of intense study for both fundamental mechanistic insight and for practical understanding of why certain point mutations of human sulfite oxidase (hSO) are fatal to neonatal children.^{3,4} Fortunately, the catalytic cycle of SOEs can easily be interrupted at the Mo(V) state for detailed interrogation by EPR methods. For vertebrate SOs, two-electron reduction by the substrate (sulfite) is followed by intramolecular electron transfer which redistributes the electrons between the integral *b*-type heme of the enzyme and the Mo center so that about 40% of the active sites are in the intermediate paramagnetic Mo(V) state, which is stable indefinitely in frozen solution. This d^1 system is ideally suited for investigation by all types of EPR methods, and the results can be used to elucidate the structure of the active site and its reaction mechanism through interactions between the unpaired electron of the Mo(V) center and nearby magnetic nuclei. Pulsed EPR studies, in particular, have shown that the structure of the Mo(V) center and the catalytic cycle depend upon the pH of the solution, the presence of anions (type and concentration), mutation of active site amino acid residues and the type of reductant used to generate Mo(V).

The virtues of EPR for investigating the Mo(V) sites of enzymes have been recognized since the early 1980s, with the identification of three distinct CW EPR forms of vertebrate SO: low pH (*lpH*); high pH (*hpH*); and phosphate inhibited (P_i).^{5–7} Although chloride and phosphate were proposed to be directly coordinated to Mo(V) for the *lpH* and P_i forms, respectively, no $^{35,37}\text{Cl}$ ($I = 3/2$) or ^{31}P ($I = 1/2$) hyperfine interactions (*hfi*) were directly detected for these nuclei by CW EPR.^{5–9} In 1996, electron spin echo envelope modulation (ESEEM) studies between 8–18 GHz detected ^{31}P *hfi* for the P_i form of vertebrate SO and demonstrated that it has an equatorial monodenate phosphate group.⁹ However, until recently, the role of Cl^- in *lpH* SO remained elusive. A multi-frequency CW EPR experiment using hSO and $^{35}\text{Cl}^-$ and $^{37}\text{Cl}^-$ -enriched solutions showed very small spectral differences that were interpreted as indicating the presence of an axial Cl^- ligand *trans* to the terminal oxo group. However, the limited resolution of the CW EPR spectra precluded reliable determination of the *hfi* and nuclear quadrupole interaction (*nqi*) parameters required to define the structure of *lpH* SO.¹⁰ Very recently, the magnetic resonance parameters (MRP) of $^{35,37}\text{Cl}$ ($I = 3/2$) in *lpH* SO were finally detected by ESEEM methods, and density functional theory (DFT) calculations favored a structure in which the Cl^- anion is hydrogen bonded to the equatorial OH ligand and surrounded by several proton donors from nearby water molecules and charged amino acid side chains.¹¹

At some stage of the catalytic cycle of Scheme 1, the product, SO_4^{2-} , has to be released from the active site. This release might occur from either the Mo(IV) (**2**) or Mo(V) (**3A**) states. In 1982 Bray *et al.* reported that the presence of excess sulfite leads to another EPR-active Mo(V) species at low pH, which they attributed to a sulfite complex of the Mo center (**3B** in Scheme 1).¹² However, evidence for a Mo(V) species with a sulfur-containing ligand was obtained only recently from our pulsed EPR investigations of samples reduced with sulfite labeled with ^{33}S ($I = 3/2$) at low pH, which allowed, for the first time, direct detection of coupling to ^{33}S . This coupling was initially observed in plant SO from *Arabidopsis thaliana* (At-SO) and was assigned to a Mo(V)– SO_4 complex.¹³

Based on the postulated catalytic cycle for SO,^{1,14} it seemed reasonable to assign the observed ^{33}S couplings to a sulfate (rather than sulfite) complex, and we suggested that a conformational change of some of the nearby amino acid residues created a steric *block* that prevented hydrolysis of the sulfate ligand.¹³ Subsequently, numerous examples of this sulfate *blocked* form have been observed for mutants of hSO and of SDH.^{15,16} Similar to the

P_i form,⁹ the sulfate *blocked* form has been proposed to have a monodentate coordinated oxyanion. Very recently, we have shown that the *blocked* form can also be detected by ESEEM methods in chloride-depleted samples of *wt* hSO that have been reduced with ^{33}S -labeled sulfite.¹⁷

This work includes a description of the ESEEM experiments for detecting the *blocked* form of various SO enzymes using ^{33}S -labeled sulfite, a re-evaluation of the nature of the sulfur-containing ligand (sulfate *vs.* sulfite) bound to Mo(V) in the *blocked* form, based upon DFT calculations, and an investigation of the conditions for conversion of the *blocked* form into the *lpH* form upon Cl^- addition.

Another subject of this paper is “oxygen trafficking” at the Mo center during the proposed catalytic cycle of Scheme 1. Early CW EPR studies of vertebrate SO in H_2^{17}O showed ^{17}O *hfi* that were sufficiently large to be detected by CW EPR at X-band. Moreover, the magnitude of the ^{17}O *hfi* was greater for the *hpH* form of SO compared to the *lpH* form.¹⁸

Once the crystal structure of chicken SO (cSO) became available,³ the equatorial oxygen, having greater exposure to solvent, was assigned to be the one that becomes reduced to Mo-OH and readily exchanged with H_2^{17}O water. The axial oxo ligand, which is less exposed to solvent in the crystal structure, was presumed to be inert to exchange with bulk water. However, subsequent pulsed EPR studies on several SOEs at high pH revealed a second, weakly coupled, ^{17}O ligand ($a_{\text{iso}} \approx 5\text{--}6$ MHz) that was assigned to the axial oxo ligand.²² These results implied that the more buried axial oxo ligand also exchanged with H_2^{17}O . Here we investigate possible alternative explanations for the weakly coupled ^{17}O atoms in samples prepared under a variety of conditions.

The *nqi* of ligand nuclei is an important spectroscopic parameter that can give more definitive information about the type of ligand and, ultimately, about the catalytic mechanism itself. This interaction, however, is not directly detectable by CW EPR. We report here our findings on the MRP for different types of ^{17}O ligands, which allow us to hypothesize about the intimate details of oxygen exchange, including indications of a “dead end” in the catalytic cycle. All of the studies of SOEs outlined above involve “difficult” high spin nuclei that present challenges for detection by ESEEM and for extraction of their MRP from the spectra. Background material on the basics of pulsed EPR theory and the experimental methods used in variable frequency multi-pulse EPR studies have been described previously.^{19,20}

2. Materials and methods

The preparation of EPR samples of different forms of SOEs is described elsewhere, including methods for samples in ^{17}O -enriched water.^{15,16,21,22}

X-band CW EPR experiments were performed on a Bruker ESP-300 EPR spectrometer at a temperature of 77 K. The resonator used in these experiments was a standard rectangular cavity operated in TE_{102} mode. The principal *g*-values of the Mo(V) center were determined using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical standard ($g = 2.0036$).

Pulsed EPR experiments were performed on home-built pulsed EPR spectrometers operating at the microwave (mw) frequencies from 8 to 18 GHz (X- and K_u mw bands),²³ from 26 to 40 GHz (K_a band),²⁴ and 94.897 GHz (W-band).²⁵ The measurement temperature was about 20 K. The pulse sequences used for various ESEEM experiments have been extensively described previously.²⁰

DFT calculations were performed with the ORCA computational package²⁶ (version 2.7.0), following the methods described previously, using the BP86²⁷ and B3LYP²⁸ functionals in conjunction with the all-electron TZVP basis,²⁹ for geometry optimization and property calculations, respectively.¹¹ Starting coordinates for the sulfate- and sulfite-bound Mo(V) models were prepared from the cSO crystal structure coordinates (pbd 1SOX),³ with the solvent-exposed oxo ligand of the Mo center modified to either a sulfato or sulfito group. The optimized atom coordinates for each model, from which all properties were calculated, are provided in the ESI.[†]

3. Results

3a. Equatorial ¹⁷O ligands

For Species **3** in Scheme 1, the equatorial oxygen atom of the Mo(V)-OH unit is derived from bulk water. The main isotope of oxygen (¹⁶O, 99.963% natural abundance) is not magnetic; however, addition of water enriched in ¹⁷O (*I* = 5/2) results in large ¹⁷O *hfi* (20–40 MHz) that can be observed and approximately evaluated, even from CW EPR spectra.^{5,6,18}

This ¹⁷O *hfi* is pH dependent and can be assigned to an equatorial Mo(V)-¹⁷OH unit. Naturally, the relatively strong *hfi* of the equatorial ¹⁷O of the Mo(V)-OH units makes their detection by high resolution pulsed EPR very challenging instrumentally due to the limited amplitude of the mw pulses.^{24,25} For example, analysis of the CW EPR spectra for the *hpH* forms of SO and SDH indicates that the expected ¹⁷O *hfi* constants are too large to allow the HYSCORE correlation peaks to be generated with current instrumentation because incomplete excitation results in the amplitude of the high-frequency lines being too small to be detectable. We have found, however, that in 1D ESEEM spectra, the low-frequency line is observable, and from the position of this line, the *hfi* constants at different EPR positions can be determined. In order to simplify the observation of this line, we have obtained in our study the three-pulse (stimulated) ESEEM spectra, which only contain the fundamental lines of nuclear transitions. To minimize possible contributions from axial ¹⁷O-oxo ligand exchange (see below), samples prepared with short incubation time were used. To eliminate the blind spots that distort the spectra, the τ -integrated stimulated ESEEM was used in combination with the pulse-swapping technique suggested elsewhere.³⁰

The τ -integrated stimulated ESEEM spectra of *hpH wt* cSO are shown in Fig. 2. The low-frequency fundamental line of the equatorial ¹⁷O in each spectrum of Fig. 2 is marked by a diamond. This line belongs to the $\Delta m_I = 1$ transitions of ¹⁷O within one of the electron spin manifolds. While the frequency of the $-\frac{1}{2} \leftrightarrow \frac{1}{2}$ nuclear transition does not depend on the *nqi* to first order, the frequencies of all other transitions do. However, the largest splitting between the separate lines is only about $\Delta\nu_{\max} \sim 0.15 e^2 Qq/h$,³² where $e^2 Qq/h$ is the nuclear quadrupole coupling constant, *nqcc*. For an *nqcc* of ~ 7 MHz,³³ $\Delta\nu_{\max}$ would be ~ 1 MHz. This splitting is smaller than the experimental linewidth (1.5 MHz), and is likely to be masked by even a slight statistical distribution of the *hfi* constant about the mean value of ~ 30 MHz (see below). As a result, all of the $\Delta m_I = 1$ fundamental lines within the same electron spin manifold merge into a single feature observed in the spectra of Fig. 2. The *hfi* constant at each EPR position can be found from the frequency of this feature as $|A| = 2(|\nu_a| + |\nu_O|)$, where ν_a is the assumed assignment of the experimental ESEEM frequency and ν_O is the Zeeman frequency of ¹⁷O.

[†] Electronic supplementary information (ESI) available: Optimized XYZ atom coordinates of the sulfate- and sulfite-bound Mo(V) computational models. See DOI: 10.1039/c004404k

Fig. 3 shows the dependence of the hfi constant (A) on the EPR position obtained for hpH *wt* cSO (black squares). From the full range of the variation of A , one can estimate the anisotropic hfi constant as $T_{\perp} \cong 2.5$ MHz (assuming the interaction to be axial). The fact that the hfi constant reaches its minimum at g_Z shows that g_Z approximately corresponds to the canonical orientation of the anisotropic hfi . The isotropic constant is estimated as $a_{iso} = 37$ MHz. Similar measurements were performed for the samples of hpH *wt* and Y236F SDH (empty and filled circles in Fig. 3). For *wt* SDH, the ^{17}O hfi parameters of the equatorial OH ligand are similar to those obtained for hpH cSO, while for hpH Y236F SDH the isotropic hfi constant is slightly larger (39 MHz). The ^{17}O hfi results for SOEs at high pH are consistent with previous investigations, which showed that the hfi parameters for the nearby proton of the hpH forms of SOEs are very similar for all organisms.^{14,34} Thus, we conclude that the hpH forms of SOEs from all organisms have similar Mo(V) structures. The variation of the MRP with the orientation of the OH group has been analyzed by DFT calculations.²¹

In contrast to the common hpH form of SOEs, a diverse range of species can be observed at low pH, depending upon the organism, mutations near the active site, and anions in the medium. For lpH cSO (at least in the presence of Cl^- , see Section 3), the relatively large ^{17}O hfi constant is always accompanied by a characteristic large isotropic hfi constant of the equatorial OH proton ($a_{iso} \sim 30$ MHz).⁵ In most cases, the ^{17}O hfi constant of low pH forms is substantially smaller than that of the hpH form. Consequently, the incomplete excitation problems caused by the limited mw power of the available instrumentation become less restrictive, and it is possible to generate HYSCORE correlation peaks from the equatorial ^{17}O . Because of the strong hfi ($A > 2\nu_O$), these peaks appear in the $(-+)$ quadrant of the HYSCORE spectrum and are well separated from the peaks caused by the weakly-coupled ^{17}O ligand and/or second-sphere oxygens that appear in the $(++)$ quadrant. As an example, Fig. 4 presents the HYSCORE spectrum of mutant R160Q hSO at K_a band. The hfi constant estimated for the equatorial ^{17}O of R160Q (~ 18 – 19 MHz) is only about half of that observed for equatorial ^{17}O in hpH forms, and this oxygen is assigned to the S-containing ligand of the *blocked* form (Section 2).

In the above discussion of ^{17}O equatorial ligands, we did not mention the nqi parameters, which are important for understanding the type of ^{17}O ligand and its orientation in the molecular frame. In principle, one can estimate the nqi by measuring the splittings of the sum combination line in 1D ESEEM spectra or the splittings of the correlation peaks in a HYSCORE spectrum. In practice, however, detecting the nqi for ^{17}O ligands turns out to be substantially more complicated than detecting the hfi for two reasons.²⁰ First, as mentioned earlier, the ^{17}O hfi constants for hpH SOEs are too large to allow the HYSCORE correlation peaks to be generated with current instrumentation because incomplete excitation results in the amplitude of the high-frequency lines being too small to be detectable. Second, even when sufficient excitation was possible (Fig. 4), the quadrupole splittings were not resolved because of the unfavorable combination of the low fundamental frequency ν_a (comparable with strong anisotropic hfi) and a possible static distribution of equatorial ligand orientations.

This problem can be overcome by performing the experiments at W-band (95 GHz), where the Zeeman frequency of ^{17}O is ~ 20 MHz and the weak hyperfine interaction condition ($\nu_O \geq A/2$) is achieved for all known SOE equatorial ^{17}O ligands. However, due to the limited mw power of most W-band spectrometers, only the ^{17}O -ligand in R160Q hSO was a feasible target for this method. The ^{17}O hfi of this ligand ($A \sim 18$ MHz) is smaller than that of any of other SOEs, and its highest fundamental frequency is only ~ 30 MHz, within the range of instrument capabilities.²⁵ The W-band two-pulse ESEEM spectrum of ^{17}O in R160Q hSO, collected at g_X , is shown by the solid trace in Fig. 5.

The key feature observable in this spectrum is the quintet of lines with negative amplitude, located at the double Zeeman frequency of ^{17}O , $2\nu_1 \approx 40$ MHz. These lines are naturally assigned to sum combinations of the fundamental lines, ν_0 . The $nqcc$, determined from analyzing the two-pulse ESEEM and additional HYSCORE experiments, was about 6.8 MHz. To our knowledge, this is the first direct observation of an nqi of an equatorial ^{17}O in a metalloenzyme. There are few experimental nqi measurements for ^{17}O coordinated to a metal ion. The only previous direct measurements of this parameter are for the ^{17}O aqua ligands of aqueous Gd(III) ,³³ where the $nqcc$ was found to be 6.5 MHz, close to that found here for R160Q hSO.

In principle, the $nqcc$ can be used to choose between possible structures for the *blocked* form of R160Q hSO (Section 2) and shed light on intimate details of the structure and function of SOEs at low pH. In practice, however, the lack of ^{17}O nqi reference data from well-characterized model compounds and the still insufficient (for this purpose) accuracy of DFT calculations (see Section 1) make a conclusive choice for the structure of R160Q hSO problematic (Sections 1 and 2). Nonetheless, the W-band ^{17}O nqi measurement is an encouraging first step in implementing additional MRP for understanding the mechanisms of SOEs.

3b. The axial ^{17}O ligand

In the above discussion, the properties of the axial oxo ligand, which is not exposed to solvent, were not explicitly considered. Our initial experimental protocol for *hpH* SO involved incubating the oxidized enzyme (dioxo-Mo(VI) state) with buffered H_2^{17}O solution, reduction by excess sulfite, then rapid freezing of the solution (within one minute).²² The experiments performed for samples incubated for a short time (2 min) and long time (3 h) at high pH demonstrated substantial differences in their ^{17}O ESEEM spectra. This observation indicates that other nearby oxygen atoms, in addition to the equatorial OH ligand, can also exchange with H_2^{17}O . Numerous K_a -band pulsed EPR studies of several *hpH* SOEs in buffered H_2^{17}O solutions, using various exchange times and difference spectroscopy, revealed a weakly coupled ^{17}O ($a_{\text{iso}} \approx 6$ MHz) in addition to the strongly coupled ^{17}O discussed above for the equatorial OH ligand. As an example, the ESEEM spectra of this weakly coupled ^{17}O obtained for *hpH* cSO are shown in Fig. 6. Similar experiments were performed for the *hpH* forms of other SOEs (*wt* SDH, R55M SDH, Y236F SDH).^{15,21}

The MRP obtained from analysis of these spectra, presented in Table 1, show that all of these *hpH* SOEs have a weakly coupled ^{17}O with similar hfi ($a_{\text{iso}} \sim 6$ MHz and anisotropic $hfi \sim 1.5$ MHz). The $nqcc$ values were found to be small (≤ 1.5 MHz). This weakly coupled ^{17}O was assigned to the axial oxo ligand. Support for this assignment was provided by detailed experimental and theoretical studies of $[\text{Mo(V)}^{17}\text{O}(\text{SPh})_4]^-$, whose ^{17}O MRP (Table 1) are in close agreement with those found for the weakly coupled ^{17}O in all *hpH* SOEs.

This assignment suggested that exchange of the axial oxo group occurs in all *hpH* SOEs. However, comparison of the simulated and experimental ^{17}O ESEEM amplitudes of the weakly coupled ^{17}O indicates that this exchange is quite inefficient. In buffer solutions, which contain $\sim 60\%$ H_2^{17}O , only $\sim 10\%$ of the oxo ligands are exchanged. The much slower rate of exchange of the axial oxo ligand relative to the equatorial OH group probably reflects the diminished access of water to the axial oxo-group, as is indicated by the X-ray structures of SOEs.

The apparent exchange of the Mo axial and equatorial oxygen atoms with H_2^{17}O raises questions about its mechanism. The dependence of axial ^{17}O exchange on the incubation

time suggests that it occurs in the resting state of the enzyme. This conclusion is supported by an early ^{17}O NMR experiment which indicates that the oxygen atoms of a six-coordinate model dioxo-Mo(VI) compound (in acetonitrile solution) exchange with H_2^{17}O within hours.³⁵ However, recent high-resolution mass spectrometry experiments in our laboratory for the several six-coordinate model dioxo-Mo(VI) complexes show no oxygen exchange with H_2^{18}O water (at least at pH = 7 in pure water) even after more than 24 h.³⁶ On the other hand, the five-coordinate model compound $[\text{Mo}(\text{V})^{17}\text{O}(\text{SPh})_4]^-$ exchanges its oxo-group with H_2^{17}O within minutes.³⁷ Earlier studies of simple inorganic complexes showed that facile exchange of oxo ligands can occur when there are *cis* OH ligands.³⁸ Although this process can be envisaged for the *hpH* form of SOEs, the shielding of the axial oxo ligand by the protein matrix would be expected to result in much slower exchange of this oxygen atom.

Two resonance Raman studies of SOEs have investigated the effect of turning over the enzyme in H_2^{18}O water on the $\text{Mo}=\text{O}$ vibrational frequencies.³⁹ These enzyme turnover studies in H_2^{18}O are inconclusive as to whether one, or both, terminal oxo ligands incorporate label, but the resonance Raman results are consistent with the proposed mechanism of Scheme 1, which involves oxygen from water being incorporated into the active site during turnover for incorporation into substrate on a subsequent cycle. The resonance Raman results do not address whether exchange of oxo ligands occurs in the absence of catalysis.

Our attempts to directly generate and/or observe weakly coupled ^{17}O in *lpH* forms of SO of vertebrates and the *lpH* form of plant SO that have the same parameters as the “axial” oxo group have not been successful. For instance, the only way to generate an “axial” ^{17}O signal in *lpH* cSO was to prepare it at high pH and then decrease the pH to 6–7.¹¹ This preparation procedure transforms the EPR spectrum to the typical *lpH* form, which suggests that the geometry of active site also transforms accordingly. The MRP of the oxo ligand remained, however, nearly identical to those found for *hpH* cSO (see Table 1), indicating that for this particular case the variation of the equatorial ligand orientation has no visible effect on the MRP of the axial ligand.

A different situation was observed for *blocked* forms. As was already shown in Fig. 4, the *blocked* form of R160Q hSO has two types of coordinated oxygen atoms. One of them was already assigned as the equatorial ligand. In addition, the (++) quadrant of the HYSCORE spectrum shows two off-diagonal lines centered at the Zeeman frequency of ^{17}O , $\nu_{\text{O}} \approx 6$ MHz. The splitting between these lines is about 5.5 MHz, which gives an estimate of the *hfi* constant similar to that observed earlier for the oxo- ^{17}O in *hpH* chicken SO and the model oxomolybdenum complex $[\text{Mo}(\text{V})^{17}\text{O}(\text{SPh})_4]^-$. To determine the *nqcc* of the oxo- ^{17}O , τ -integrated four-pulse ESEEM has been used.

Fig. 7 shows the sum combination line region of the τ -integrated four-pulse ESEEM spectrum of R160Q hSO obtained at g_z , which clearly shows quadrupole splitting of the sum combination line, $\Delta\nu_{\text{O}} \approx 1.5$ MHz. From this splitting, the *nqcc* of the oxo- ^{17}O can be estimated as $e^2Qq/h \sim (40/12) \times \Delta\nu_{\text{O}} \sim 5$ MHz. This value is about 3 times larger than the *nqcc* of an axial oxo group, which is ~ 1.5 MHz, as described above. Therefore, in the case of the *blocked* form of R160Q hSO we encounter a puzzling situation: the *hfi* parameters of the weakly coupled ^{17}O are similar to those observed for an axial oxo group, but the *nqcc* more closely resembles that of an equatorial ligand rather than an axial one. This observation raises concerns as to whether this weakly coupled ^{17}O is actually an axial oxo group.

To further address this question, we investigated the weakly coupled ^{17}O in two other known *blocked* forms: R55Q SDH (the bacterial analog of R160Q hSO)¹⁵ and At-SO. In the

former, the $nqcc$ was found to be ~ 4 MHz and the hfi constants were similar to those found for R160Q hSO. For the *blocked* form of At-SO, the $nqcc$ of this weakly coupled ^{17}O from HYSCORE and four-pulse ESEEM spectra collected in K_a and W bands resulted in a similar value, ~ 4 MHz. The hfi parameters, however, as evaluated from HYSCORE spectra, are found to be rather small and do not exceed ~ 3 MHz, about half of those observed for R160Q hSO and R55Q SDH. Therefore, the common feature found for all of the *blocked* forms investigated to date is their $nqcc$, which is much larger than that of the established axial oxo ligand in $[\text{Mo}(\text{V})^{17}\text{O}(\text{SPh})_4]^-$. This difference in $nqcc$ prompts us to consider alternative explanations for the weakly coupled ^{17}O observed for *blocked* forms.

First, we consider distortions in molecular geometry that can lead to a substantial variation of the $nqcc$ of an axial oxygen. One possible distortion, suggested by Doonan *et al.*,⁴⁰ is that the Mo-center of R160Q hSO has a sixth ligand, *trans* to the axial oxo ligand. To check whether such a distortion can produce an observable increase in the $nqcc$ of the axial oxo ligand, the model compound $(\text{dtd})\text{Mo}^{17}\text{O}^{17}\text{Otms}$ was synthesized (H_2dtd is 2,3,8,9-dibenzo-1,4,7,10-tetrathia-decane).¹⁶ This compound has a sulfur atom coordinated at the axial position *trans* to the oxo ligand. The HYSCORE and refocused primary ESEEM experiments on $(\text{dtd})\text{Mo}^{17}\text{O}^{17}\text{Otms}$ gave a hfi constant for the oxo ligand (~ 5 MHz) that is similar to that in *hpH* SO and $[\text{Mo}(\text{V})^{17}\text{O}(\text{SPh})_4]^-$. The integrated four-pulse ESEEM experiments reveal that the $nqcc$ for $(\text{dtd})\text{Mo}^{17}\text{O}^{17}\text{Otms}$, ~ 3 MHz, is less than that found in all *blocked* forms, 4–5 MHz, but still about twice as large as that in *hpH* SO and in $[\text{Mo}^{17}\text{O}(\text{SPh})_4]^-$. Therefore, these experiments provide strong, if indirect, support for the hypothesis that the increase of the $nqcc$ of the oxo- ^{17}O in the *blocked* forms can be caused by axial coordination *trans* to the oxo ligand. However, this explanation for the weakly coupled ^{17}O does not provide insight as to a mechanism for exchange of the axial oxo ligand in the *blocked* forms.

Our initial analysis of the data for the *blocked* form in ^{17}O -enriched water¹⁶ did not explicitly consider the rapid exchange of the oxygen atoms of sulfite with ^{17}O .⁴⁷ This known process means that the *remote* oxygen atoms of both **3A** and **3B** of Scheme 1 will also be labeled with ^{17}O (sulfate is produced from sulfite in the generation of the $\text{Mo}(\text{V})$ state). Thus, an alternative assignment of the observed weakly coupled oxygen of the *blocked* form is to one of the remote oxygen atoms of a $^{17}\text{O}-\text{S}^{17}\text{O}_3^{2-}$ or $^{17}\text{O}-\text{S}^{17}\text{O}_2^{2-}$ group, (O-bound sulfate or O-bound sulfite, respectively). In this interpretation, the axial oxygen of the *blocked* form is not exchangeable. Assignment of the weakly coupled ^{17}O of the *blocked* form to remote oxygen atoms of sulfite (or sulfate) is supported by the DFT calculations summarized in Fig. 8 and discussed below.

The calculated nqi and hfi parameters of the remote oxygens of sulfate and sulfite, as shown in Fig. 8a and 8b, are in reasonable agreement with those estimated from the experimental ESEEM spectra. For instance, the remote oxygens in the $^{17}\text{O}-\text{S}^{17}\text{O}_2^{2-}$ group in Fig. 8b have a calculated $nqcc$ that is three times larger than that of an axial oxygen, very similar a_{iso} to an axial oxygen, and anisotropic hfi constants that are only about two times larger than would be expected for a typical axial oxo ligand.

It is also instructive to consider the ratios between the calculated $nqcc$ for an axial oxo group and those for the remote ^{17}O nuclei of the O-bound sulfite and sulfate ligands of Fig. 8a and 8b. For the $^{17}\text{O}-\text{S}^{17}\text{O}_3^{2-}$ group (O-bound sulfate), the ratio of the calculated $nqcc$ of the remote oxygens to that of the axial oxo group is 10–12; the largest calculated a_{iso} for a remote ^{17}O is about half of that observed for an axial oxo (Table 1), while the largest calculated anisotropic hfi constant is about two times larger. For the $^{17}\text{O}-\text{S}^{17}\text{O}_2^{2-}$ group (O-bound sulfite) the ratio of the calculated $nqcc$ of the remote oxygens to that of the axial oxo group is 3–4; the calculated a_{iso} for the remote ^{17}O atoms are again about half of that

observed for an axial oxo ligand (Table 1), while the largest calculated anisotropic hfi constant is 3–4 times larger.

These DFT calculations show that the large $nqcc$ values that are observed experimentally for the oxygens of *blocked* forms (three to four times greater than those for an axial oxo ligand) and the simultaneously large (R160Q hSO; R55Q SDH) or small (At-SO) a_{iso} values can be explained without invoking a six-coordinate active site.^{16,40} Additionally, there is no need to assume a labile axial oxo ligand at low pH. Therefore, on the basis of these new DFT calculations, assignment of the weakly coupled ^{17}O to remote O atoms of the coordinated sulfate (or sulfite) group is a plausible and attractive alternative to the earlier hypothesis that the axial oxo ligands exchange with solvent at low pH.¹⁶

Thus, if the above proposal is correct, then the combined experimental and DFT data on $nqcc$ can be used to choose between sulfite and sulfate ligands in the *blocked* forms. Indeed, the ratio of the experimental nqc constants of weakly coupled ^{17}O in the *blocked* forms and hpH forms or five-coordinate model compounds is about 2–4. This value is significantly closer to that found for sulfite than for the sulfate ligand, suggesting that the equatorial ligand in the *blocked* form is sulfite. This hypothesis is further supported by the experimental and DFT data on ^{33}S nqi discussed below.

4. ^{33}S ESEEM for the Blocked Form of SOEs

The CW EPR spectra of SO under low pH, high chloride conditions show the well-known lpH signal with strong coupling from a single exchangeable proton. However, Bray showed that this characteristic proton splitting was not observed in samples prepared at low pH in MES buffer with excess sulfite. He ascribed this atypical behavior to the formation of a sulfite-bound species, analogous to the P_i species.¹² More recently, it was shown that At-SO gives a similar EPR signal at low pH. It was proposed that the signal was due to a Mo(V)-OSO₃ species, in which the product of the catalytic reaction, sulfate, was sterically *blocked* from leaving the active site.⁴¹ The proposed Mo(V)-OSO₃ unit has no exchangeable protons to produce the strong 1H coupling seen in the typical lpH spectra. However, because the major naturally occurring sulfur isotope (^{32}S , 95% abundant) has no nuclear spin ($I = 0$), direct verification of the presence of a sulfur-containing ligand required enrichment with ^{33}S ($I = 3/2$). This was conveniently achieved by using ^{33}S -labeled sulfite as the reductant in Scheme 1, and K_a-band ESEEM experiments on At-SO unambiguously showed magnetic couplings to ^{33}S , providing direct evidence for a sulfur-containing ligand.¹³ Subsequent ^{33}S ESEEM studies of R160Q hSO¹⁶ and R55Q SDH¹⁵ showed that mutation of this conserved active site arginine also generates the *blocked* form at low pH. More recently, we have carried out a ^{33}S ESEEM investigation of the Y343F mutant of hSO. On the basis of previous 1H ESEEM and pulsed ENDOR investigations of this mutant, which showed no proton splittings at low pH, we hypothesized that at low-pH Y343F hSO might also be in a *blocked* form,⁴² similar to that found for At-SO.¹³

Fig. 9 shows a HYSCORE spectrum of Y343F hSO reduced with ^{33}S -enriched sulfite at pH 5.8. The prominent lines in the (++) quadrant of this spectrum (the doublet at the frequencies (8.8, 3.5) and (3.5, 8.8) MHz and a single peak on the main diagonal at the frequency of 17.8 MHz are only observed for the sample containing ^{33}S , and therefore these lines are caused by the interaction of the unpaired electron of Mo(V) with ^{33}S . This ^{33}S HYSCORE spectrum is similar to the spectra obtained previously for At-SO,¹³ which was explained by the combination of weak hfi and strong nqi . In such a situation, the strong nqi splits the four spin projection states of $I = 3/2$ into two doublets with $m_I = \pm 1/2$ and $\pm 3/2$ (see Fig. 10 for the energy level diagram).

The energy separation between the two doublets, the so-called interdoublet transition frequency, ν_{id} , is approximately equal to $e^2Qq/2h$. The Zeeman and hyperfine interactions lift the degeneracy within the doublets. The frequencies of $|1/2\rangle \leftrightarrow |-1/2\rangle$ transitions within the electron spin manifolds with $m_S = \pm 1/2$ are equal to $c_{hq}(\nu_I \pm A/2)$, where the factor $c_{hq} \in [1, 2 + \eta]$ depends on the orientation of magnetic field with respect to principal axes of the nqi tensor (η is the asymmetry parameter of the nqi tensor).¹⁹ The frequencies of $|3/2\rangle \leftrightarrow |-3/2\rangle$ transitions are described by a similar expression, with the proportionality factor $k_{hq} \in [\eta, 3]$. The lines of these transitions in ESEEM spectra usually have low amplitude and are not readily observable.

In the experimental spectrum of Fig. 9, the line on the main diagonal corresponds to the interdoublet transition, and from its frequency one can estimate $e^2Qq/h = 35.6$ MHz. The doublet of lines at (8.8, 3.5) and (3.5, 8.8) MHz, located symmetrically with respect to the main diagonal, corresponds to the $|1/2\rangle \leftrightarrow |-1/2\rangle$ transitions and allows one to estimate the hfi constant: $A = 2\nu_I|\nu_{+1/2} - \nu_{-1/2}|/(\nu_{-1/2} + \nu_{+1/2}) \approx 3$ MHz (for $\nu_I \approx 3.5$ MHz).

The ^{33}S ESEEM experiments described above prove that the Mo(V) EPR signal obtained for Y343F hSO at low pH indeed belongs to the *blocked* species, as we hypothesized earlier based on less direct evidence.⁴² Characteristic of all of the *blocked* species investigated to date is a large $nqcc$ (27–40 MHz) and a small hfi constant of ~ 3 MHz. However, details of the ^{33}S MRP among the various *blocked* species show substantial variation (Table 2).

The assignment of the equatorial ligand of the *blocked* form as sulfate or sulfite is aided by the DFT calculations shown in Fig. 8. The calculated $nqcc$ for O-bound sulfite Fig. 8a is 24 MHz compared to 11 MHz for bound sulfate. Both values are substantially smaller than the experimental values for ^{33}S $nqcc$ of the *blocked* SOEs (27–40 MHz), but the calculated $nqcc$ for O-bound sulfite is in better agreement with experiment. Thus, we now favor the original proposal of Bray that this species contains bound sulfite,¹² rather than our earlier suggestions that the *blocked* form retains bound sulfate.^{13,16,42} However, this selection cannot be definitive since no well-defined model compounds with ^{33}S -labeled sulfite and sulfate ligands have been reported to date.

5. Recent studies of $^{35,37}\text{Cl}^-$ in SOEs

The Introduction summarizes the pH-dependent interconversions of the *hpH* and *lpH* forms of SO as a function of Cl^- concentration and the use of CW^{8,10} and pulsed EPR¹¹ to study samples prepared with Na^{35}Cl and Na^{37}Cl to obtain experimental evidence regarding the possible coordination of Cl^- to *lpH* SO. The first successful direct observation of Cl^- in the vicinity of the *lpH* species of SO was done using ESEEM at K_a band and samples enriched with ^{35}Cl and ^{37}Cl .¹¹ The ^{35}Cl HYSCORE spectra enabled the hfi to be estimated and showed that the nqi is weak. From the integrated 4-pulse ESEEM spectrum, the ^{35}Cl nqi could be estimated as $e^2Qq/h \sim 3$ MHz. This is a very weak nqi for ^{35}Cl and is indicative of a very symmetric environment with multiple H-bonds. Typical values of e^2Qq/h for ^{35}Cl with a single covalent bond are in the tens of MHz.⁴³

Two structural models of the *lpH* form were considered: (1) first-sphere coordination of Cl^- in the axial position of the Mo center, *trans* to the oxo ligand, as proposed previously,¹⁰ and (2) second-sphere coordination, where Cl^- is H-bonded to the equatorial OH ligand, surrounding water molecules, and charged protein side chains. Using DFT calculations, we established that the axial coordination of Cl^- is not possible, and that the second-sphere coordination model with multiple H-bonds to Cl^- gives reasonable agreement with the experiment while also being structurally possible within the protein.¹¹

In the absence of inhibiting anions (PO_4^{3-} , AsO_4^{3-}), *wt* vertebrate SOs typically show the formation of *lpH* and *hpH* species only. Recently, however, we started purifying the *wt* hSO using a large desalting column (about two times larger than those used elsewhere). At low pH in the absence of added chloride the CW EPR spectra of these preparations indicate the formation of the *blocked* form instead of the usual *lpH* species; this was proved by ^{33}S ESEEM experiments similar to those described above. The formation of the *blocked* form in the recent preparations of *wt* hSO was ascribed to more efficient removal of Cl^- from the enzyme during the purification process. Indeed, the concentration of Cl^- in hSO prepared by the new procedure was ~ 5 mM compared to 10–20 mM for the previous purification protocol.¹⁷

In order to test this hypothesis, we added 100 mM NaCl to the hSO solution at pH 5.8 before adding sulfite; the usual *lpH* form was generated by this procedure. Furthermore, addition of 100 mM NaCl to samples in which the *blocked* species was already present resulted in the disappearance of the *blocked* species and formation of the usual *lpH* species (Fig. 11).

Similar chloride addition experiments were performed with samples of other SOEs where the *blocked* species was formed. In most of these samples, the *blocked* form was at least partially converted to the *lpH* form. The only exception was the *blocked* form of R160Q hSO; addition of up to 300 mM NaCl did not result in any changes of its EPR spectrum.

Although $^{35},^{37}\text{Cl}$ ESEEM is detected for the *lpH* form of SOEs, it is not observed for other forms. This does not necessarily mean that there is no Cl^- near the active center since it is also possible that there is simply no spin density propagation pathway between Mo(V) and Cl^- ions in the other forms.

6. Discussion

The generally accepted mechanism for SOEs involves attack of sulfite on the electrophilic equatorial oxo ligand that is exposed to solvent, followed by hydrolysis of sulfate and two sequential one-electron oxidations to return the enzyme to the fully oxidized resting state.^{1,14} This is summarized in abbreviated fashion in Scheme 1 with the steps $1 \rightarrow 2 \rightarrow 3 \rightarrow 1$. However, the direct detection of a coordinated sulfur-containing ligand by ^{33}S ESEEM spectroscopy¹³ shows that one-electron oxidation of **2**, the Mo(IV)-product complex, to give **3A** or **3B** may precede hydrolysis of **2** to release the product (sulfate). This observation is consistent with an earlier hypothesis based upon the fact that the intramolecular electron transfer rates in vertebrate SO and bacterial SDH are much faster than enzyme turnover.⁴⁴

Previously we proposed that the sulfur-containing species detected by ^{33}S ESEEM was **3A** in Scheme 1, with bound sulfate (product) that was sterically *blocked* from leaving the active site.^{13,16,42} Coordinated sulfate should have a nearly tetrahedral environment with respect to the sulfur atom, and therefore the ^{33}S *nqi* is expected to be weak. The typical *nqcc* values of 27–40 MHz determined for the *blocked* species seem rather large, but because of the high cost of ^{33}S , few data on ^{33}S *nqi* are available for comparison. The largest values in the literature are ~ 50 MHz,⁴⁵ no more than two times greater than those determined for the *blocked* form of SOEs. Sulfite is less symmetric than sulfate, and the ^{33}S *nqcc* for sulfite is expected to be larger, but again, it is difficult to differentiate between these based on qualitative considerations only. However, the DFT calculations presented here for an SOE active site suggest that the ^{33}S parameters most closely fit an O-bound sulfite (Fig. 8a).

The results from ^{33}S ESEEM and the DFT calculations can also be interpreted within the mechanism of Scheme 1. Sulfate is a very weak ligand, and the Mo(IV)-sulfate species (**2**) is readily hydrolyzed at high pH to give **3**. At low pH, **2** is oxidized by one electron and sulfate is replaced by sulfite, which is present in excess, to give **3b**, which is seen as the *blocked*

form by ^{33}S ESEEM. In the presence of excess chloride; however, the *blocked* form is not observed in *wt* vertebrate SO, but rather the usual *lpH* form with a second sphere Cl^- that is hydrogen bonded to the active site is seen by $^{35,37}\text{Cl}$ ESEEM.¹¹ Thus, on the basis of ^{33}S and $^{35,37}\text{Cl}$ ESEEM and DFT calculations, we now favor describing the *blocked* form as a Mo(V) species with bound sulfite, rather than bound sulfate, as in our previous publications.^{13,16,42} This revised formulation follows the original suggestion of Bray, from CW EPR studies of SO, that a Mo(V)-sulfite species can be formed at low pH.¹²

The *blocked* forms of other SOEs show similar behavior to that of *wt* hSO, and they are at least partially converted to the *lpH* form upon addition of chloride. The exception is R160Q hSO, which remains in the *blocked* form, even in 300 mM chloride.¹⁷ This stability of the *blocked* form of R160Q hSO may be an important factor in the lethality of this mutation.¹⁶

Molybdenum enzymes such as SOEs are chemically unique in that the oxygen atom that is incorporated into substrate comes from water (Scheme 1). Thus, reducing SOEs in water enriched in H_2^{17}O provides an easy route for incorporating ^{17}O ($I = 5/2$) into their Mo(V) forms. Although ^{17}O *hfi* were detected for SO over 30 years ago, elucidation of the active site structure from ^{17}O MRPs remains challenging. Weakly coupled ^{17}O atoms were discovered by ESEEM studies at K_a -band for several types of SOE samples.^{13,15,16} Originally these spectral features were assigned to axial oxo groups, but the wide range of *nqcc* for these weakly coupled ^{17}O atoms now suggests that they have more than one source. The very rapid exchange of the oxygen atoms of sulfite with H_2^{17}O complicates the interpretation of the ^{17}O ESEEM results. For the *blocked* forms of SOEs, this work has used DFT calculations to assign the weakly coupled ^{17}O signals to the remote oxygen atoms of an O-bound equatorial sulfite (or less likely, sulfate) ligand (Fig. 8). For the *hpH* SOEs, the original assignment of the weakly coupled oxygen to an axial oxo group²² remains consistent with the data from model compounds and DFT calculations.^{21,32} However, a possible mechanism whereby the axial oxo ligand of SOEs, which is shielded by the protein matrix, appears to slowly exchange with H_2^{17}O is not yet understood.

Throughout this work, DFT calculations using the ORCA computational package²⁶ have provided valuable insight into the interpretation of the ^1H , ^{33}S , ^{35}Cl and ^{17}O spectroscopic data and their relation to electronic and molecular structure. The *hfi* parameters for ^1H of the OH ligand were reliably calculated for rotation of the Mo–OH group about the Mo–O bond for both the *lpH* and *hpH* forms.²¹ For ^{17}O , the accuracy of the calculated MRPs is lower; for oxo ligands the calculated *A* and e^2Qq/h are 60–70% of the experimental values. For equatorial OH ligands the calculated ^{17}O MRP as a function of the rotational angle about the Mo–O bond predicted a small $A(^{17}\text{O})$ for the *hpH* form, contrary to experiment.²¹ However, the structural model in this case is very truncated, and outer sphere interactions may also have a strong effect on $A(^{17}\text{O})$.

For ^{33}S , the calculated *nqi* are much smaller than the experimental values, the case of a monodentate O-bound SO_3^{2-} ligand was comparatively better ($e^2Qq/h \sim 20$ MHz) than SO_4^{2-} ($e^2Qq/h \sim 10$ MHz). At this point, however, the lack of experimental ^{33}S *nqi* data for well-characterized model systems precludes directly correlating the observed MRP of SOEs with known structures. Model compounds labeled with ^{33}S are also needed for validating the DFT predictions of ^{33}S MRP.

DFT calculations for Cl clearly demonstrated the unfeasibility of Cl^- coordination in the otherwise vacant axial position, contrary to the assumptions made by George and coworkers¹⁰ and also by us initially.⁴⁶ The calculations showed that Cl^- is most likely located in the second coordination sphere (as in PDB 2A99)⁴ and that this structure gives the best agreement of the derived and experimental spectroscopic parameters.¹¹ In summary,

this work has shown that insight into the detailed chemical transformations that can occur at the active sites of SOEs can be obtained from ESEEM spectra of “difficult” nuclei, ^{17}O , $^{35,37}\text{Cl}$ and ^{33}S , in conjunction with high level DFT calculations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. X-ray crystallographic structural representation of the chicken sulfite oxidase (cSO) active site with Cl^- occupying the positively charged substrate binding pocket formed by R138, W204 and R190 (PDB 2A99).⁴

Fig. 2.

Traces 1 through 5, τ -integrated three-pulse ESEEM spectra (cosine FT) of *wt hpH* cSO for the samples incubated for 2 min in H_2^{17}O buffer, obtained at $B_0 = 1071.8$ mT (g_Z), 1079.5 mT, 1083.4 mT (g_Y), 1086 mT and 1088.6 mT (g_X), respectively. Before the FT, the experimental ESEEM traces were extrapolated into the dead time using the FTS algorithm.
³¹ Experimental conditions: $\nu_{\text{mw}} = 29.810$ GHz; mw pulses, 3×13 ns; $T = 21$ K. Diamonds and triangles indicate the $\Delta m_I = 1$ fundamental lines of the equatorial ^{17}O (from the OH ligand) and weakly coupled 2nd sphere ^{17}O , respectively. The higher-frequency lines belong to $\Delta m_I = 2$ and $\Delta m_I = 3$ fundamental lines of the equatorial ^{17}O .

Fig. 3. Hyperfine constants of the equatorial ^{17}O ligand of several different *hpH* SOEs as a function of the EPR position, calculated from the position of the experimental fundamental line ν_a as $A = 2(\nu_a + \nu_O)$.

Fig. 4. HYSCORE spectrum of the *blocked* form of ^{17}O -R160Q SO showing the lines of weakly coupled ^{17}O in the $(++)$ quadrant (top) and strongly coupled equatorial ^{17}O in the $(+-)$ quadrant (bottom). Experimental conditions: $\nu_{\text{mw}} = 29.530$ GHz; $B_0 = 1070$ mT (g_Y); mw pulses, 10, 10, 20 and 10 ns; time interval between the first and second mw pulses, $\tau = 180$ ns; $T = 20$ K.

Fig. 5.

Cosine FT of the two-pulse ESEEM collected in (a) W-band and (b) K_a -band at the g_X field position for the low pH, *blocked* form of R160Q hSO (pH = 5.8). The amplitude of (b) is recalculated for the W-band. Experimental conditions: $T = 20$ K; pulse durations: 2×25 ns and 2×15 ns; magnetic field = 3.4747 T and 1.081 T; $\nu_{mw} = 94.897$ GHz and 29.523 GHz for the W-band and K_a -band, respectively.

Fig. 6. ^{17}O sum combination line regions of τ -integrated four-pulse ESEEM spectra (cosine FT). Solid traces, experimental for *hpH wt cSO*, obtained as a difference between the spectra of samples incubated for 3 h and 2 min in H_2^{17}O buffer. Magnetic fields for traces 1 through 3, $B_0 = 1056.5$ mT (g_Z), 1067.7 mT (g_Y) and 1073 mT (g_X), respectively. Experimental conditions: mw frequency = 29.38 GHz; mw pulses = 14, 14, 22 and 14 ns; $T = 21$ K. Dashed traces, simulations.

Fig. 7. ^{17}O sum combination line region of the τ -integrated four-pulse ESEEM spectrum (cosine FT) of *blocked* form of ^{17}O -R160Q SO obtained at g_z . Experimental conditions: $\nu_{\text{mw}} = 29.530$ GHz; $B_0 = 1052$ mT; mw pulses, 10, 10, 20 and 10 ns; starting time interval between the first and second mw pulses, $\tau = 180$ ns; $T = 20$ K.

Fig. 8. DFT-calculated optimized structures and *hfi* and *nqi* parameters for ^{17}O and ^{33}S of sulfate- and sulfite-bound Mo(V) of the SO active site (a and b, respectively). All values are in MHz.

Fig. 9. HYSCORE spectrum of Y343F hSO reduced with ^{33}S -enriched sulfite at pH 5.8 (*blocked* form). Experimental conditions: $\nu_{\text{mw}} = 29.592$ GHz; $B_0 = 1077.1$ mT (g_Y); mw pulses, 15, 15, 27 and 15 ns, $\tau = 170$ ns; $T = 21$ K.

Fig. 10.

Energy level diagram for $I = 3/2$ in the case of strong nqi . The factors $c_{\text{hq}} \in [1, 2 + \eta]$ and $k_{\text{hq}} \in [\eta, 3]$ depend on the orientation of magnetic field with respect to principal axes of the nqi tensor (η is the asymmetry parameter of the nqi tensor).

Fig. 11. X-band CW EPR spectra of *wt* hSO at pH 5.8 without adding NaCl (trace 1), with 10 mM NaCl (trace 2) and with 100 mM NaCl (trace 3). Experimental conditions: mw frequency = 9.455 GHz; mw power = 2 mW; magnetic field modulation amplitude = 0.1 mT; $T = 77$ K.

Scheme 1.

Simplified catalytic cycle of SO and SDH. Stage **1** corresponds to the resting state of the enzyme. The pathway **1** $\rightarrow$ **2** $\rightarrow$ **3** $\rightarrow$ **1** corresponds to the normal catalytic turnover. Depending on buffer pH, stage **3** corresponds to the *hpH* or *lpH* form of the active center. Stages **3A** and **3B** (typically observed at pH < 7) are the two debated alternatives which correspond to the *blocked* form of the active center. In *wt* vertebrate SO, the *blocked* form is generated in chloride-depleted samples at low pH. Addition of chloride results in hydrolysis of the sulfur-containing ligand (transition **3A** $\rightarrow$ **3** or **3B** $\rightarrow$ **3**).

Table 1

The hfi and nqi parameters of weakly coupled ^{17}O nuclei in the vicinity of the Mo(V) center of *hpH* SOEs and related model compounds, as determined by ESEEM

Species	$a_{\text{iso}}/\text{MHz}$	$(T_{11}, T_{22}, T_{33})/\text{MHz}$	$e^2Qq/h/\text{MHz}$	Ref.
<i>hpH</i> cSO	6.3	2.4, 1, -3.4	1.6	21
$[\text{Mo(V)}^{17}\text{O}(\text{SPh})_4]^-$	6.44	1.6, 1.6, -3.2	1.45	32
SDH R55M ^a	5.7	1.2, 0.6, -1.8	~0.7	15
<i>lpH</i> cSO ^b	6.4	1.2, 1.6, -2.8	~1.6	11
<i>hpH</i> SDH Y236	5.6	1.0, 0.3, -1.3	~1.1	21
low-pH (<i>blocked</i>)				
R160Q hSO	5.5	1.4, -0.3, -1.0	~5	16
low-pH (<i>blocked</i>)				
R55Q SDH	5.2	1.3, 0.6, -1.9	~4	15
low-pH (<i>blocked</i>)				
At-SO	~2	1, 1, -2	~4	16
(dttd)Mo ¹⁷ O(¹⁷ Otms)	6.7	1.1, 1.1, -2.2	~3	16

^aThis enzyme exists only in *hpH* form.

^bThe ^{17}O exchange was performed at pH 9.0 with subsequent pH decrease to 6.0.

Table 2

Enzyme	$a_{\text{iso}}/\text{MHz}$	$(T_{11}, T_{22}, T_{33})/\text{MHz}$	$e^2Qq/h/\text{MHz}$	η	Ref.
low-pH (<i>blocked</i>) At-SO	3.3	(1.3, 1.5, -2.8)	40	0	13
low-pH (<i>blocked</i>) R160Q hSO	2.1	(1.6, 2.5, -4.1)	36	0.2	16
low-pH (<i>blocked</i>) R55Q SDH	$A_X = 0.6 \text{ MHz}; A_Y = iA_Z; A_Z = 1.7 \text{ MHz}$		27	~ 0	15
low-pH (<i>blocked</i>) Y343F hSO	2.2	(0.8, 1.4, -2.2)	36	0	This work

^dThe HYSCORE correlation peaks at gY were not observed. Therefore, the complete A tensor could not be determined, and the separation into the isotropic and anisotropic parts was impossible.