Consensus structures of the Mo(V) sites of sulfite-oxidizing enzymes derived from variable frequency pulsed EPR spectroscopy, isotopic labelling and DFT calculations

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Abstract Sulfite-oxidizing enzymes from eukaryotes and prokaryotes have five-coordinate distorted square-pyramidal coordination about the molybdenum atom. The paramagnetic Mo(V) state is easily generated, and over the years four distinct CW EPR spectra have been identified, depending upon enzyme source and the reaction conditions, namely high and low pH (*hpH* and *lpH*), phosphate inhibited (*P_i*) and sulfite (or blocked). Extensive studies of these paramagnetic forms of sulfite-oxidizing enzymes using variable frequency pulsed electron spin echo (ESE) spectroscopy, isotopic labeling and density functional theory (DFT) calculations have led to the consensus structures that are described here. Errors in some of the previously proposed structures are corrected.

Introduction

Recent comprehensive reviews of molybdenum-containing enzymes include information about the sulfite oxidase family, which contains sulfite-oxidizing enzymes from both eukaryotes (sulfite oxidases, SOs) and prokaryotes (sulfite dehydrogenases, SDHs), and the assimilatory nitrate reductases from eukaryotes.^{2,3} The active site structures of members of this family are nearly identical, each having a five-coordinate, pseudo square-pyramidal Mo center with

of the remaining equatorial ligand, which is solvent-exposed, varies during the catalytic cycle.

In the generally accepted catalytic cycle for SOs (Figure 2), the Mo center passes through the paramagnetic Mo(V) state with a 4d¹ electronic configuration that can be conveniently studied by electron paramagnetic resonance (EPR) spectroscopy.^{1,4-8}

Figure 1 Ball-and-stick representation of the fully oxidized molybdenum center of sulfite-oxidizing enzymes, showing the dithiolene coordination of the MPT unit and the conserved equatorial Cys residue. The identity of O_{eq}, which is solvent-exposed, varies during the catalytic cycle.

an axial oxo ligand and three equatorial sulfur donor atoms (Figure 1). One sulfur is from a conserved Cys residue and two are from a pyranopterin dithiolene (molybdopterin, MPT) cofactor. The identity

Figure 2 General view of Mo center oxidation state changes during turnover. Reproduced from reference 4 by permission of the Royal Society of Chemistry.

Several types of continuous wave (CW) EPR spectra have been observed for vertebrate SOs, depending upon buffer pH, the presence and concentration of certain anions in the buffer, as well as specific point mutations in the protein (Figure 3, left hand side). The corresponding structures deduced from electron spin echo (ESE) experiments on these forms, in conjunction with isotopic labeling, density functional theory (DFT) calculations,^{9,10} and

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studies of model compounds, are shown on the right hand side of Figure 3.¹¹ Elucidation of the specific structures associated with the various CW EPR spectra has been especially challenging because the Mo(V) coordination sphere involves only O and S atoms, whose primary isotopes (^{16}O and ^{32}S) are magnetically silent ($I = 0$). Determining whether the solvent-exposed, exchangeable equatorial ligand is an O, OH^- , H_2O , SO_3^{2-} , or SO_4^{2-} group, has required isotopic labeling with ^{17}O ($I = 5/2$) or ^{33}S ($I = 3/2$) in order to obtain experimental hyperfine interaction (*hfi*) and nuclear quadrupole interaction (*nqi*) constants for these coordinated nuclei using variable-frequency high resolution pulsed EPR spectroscopy.

Preparing appropriately isotopically labeled materials can be difficult due to the expense of the isotopes and the chemical form in which they are available. A useful source of ^{17}O (0.037% natural abundance) for studies of SOs is highly enriched H_2^{17}O , which is commercially available from several companies as a byproduct of the larger scale production of H_2^{18}O for use in mass spectrometry of biomolecules. However, ^{33}S (0.76% natural abundance) has limited availability as elemental sulfur; thus, small amounts of this costly material must be efficiently converted into useful ^{33}S -labeled reagents for subsequent structural studies of SOs using pulsed EPR spectroscopy.

The *hfi* and *nqi* constants of a magnetic nucleus can be used to help assign the corresponding atom to a specific fragment of the molecular structure of the Mo(V) active site of an SO. An experimental approach to such an assignment is based on a similarity of the obtained *hfi* and *nqi* constants with those determined for the same atom in a structurally defined Mo(V) model compound. An excellent example of this is the close similarity of the *hfi* and *nqi* constants for the axial ^{17}O of the $[\text{Mo}^{17}\text{O}(\text{SPh})_4]^-$ anion and the weakly coupled ^{17}O of the high pH form of chicken SO (cSO), which can then be confidently assigned to

the axial O atom of the SO active site (Figure 1).^{12, 13}

However, the relative paucity of experimental *hfi* and *nqi* constants for ^{17}O and ^{33}S ligands in paramagnetic metal systems can result in ambiguities for selecting the preferred structure for a Mo(V) active site. In the absence of closely related Mo(V) model compounds, the next best way to relate the *hfi* and *nqi* constants of the Mo(V) active site to structure is through high level density functional theory (DFT) calculations using the ORCA suite of programs,¹⁴ which have been previously benchmarked with known model systems, such as the $[\text{Mo}^{17}\text{O}(\text{SPh})_4]^-$ anion.¹³ Nevertheless, preconceived ideas about the mechanism of SOs (Figure 2), the steric constraints of the coordination of the Mo(V) center, and the properties of the exchangeable ligands themselves, have resulted in some structural and reactivity proposals which have been subsequently been shown to be incorrect. This manuscript addresses the various inconsistencies in the reported structures for the Mo(V) centers of SOs, especially vertebrate SOs, and attempts to provide a self-consistent description of all of the structures in Figure 3 that agrees with the electron spin echo envelope modulation (ESEEM) spectra, model compound results, DFT calculations, and properties of the exchangeable ligands themselves.

A unique feature of all Mo-containing enzymes (except nitrogenase) is coordination by the S atoms of a pyranopterindithiolate (also called molybdopterin, MPT, Figure 1). Dithiolene ligands are known to stabilize multiple oxidation states of transition metals,^{15, 16} and it is proposed that the dithiolene moiety of the MPT unit plays an important role in redox catalysis by Mo enzymes. Investigation of the spin density on the sulfur atoms of a paramagnetic metal-dithiolene center could provide significant insight about the possible role of dithiolenes in redox catalysis. However, as noted earlier, naturally abundant ^{32}S is magnetically silent ($I = 0$), and provides no information about delocalization of spin density onto the S atoms for either model compounds or for Mo enzymes. Recently, preparation of catalytically active bioengineered sulfite oxidase with a ^{33}S -labeled molybdenum cofactor has enabled pulsed EPR spectroscopy to be used for the first direct measurement of the spin density on the S atoms of a coordinated dithiolene.¹⁷

Results and discussion

High pH (*hpH*) and low pH (*lpH*) forms

CW EPR experiments on vertebrate SOs in the 1980s showed that the spectra of the enzymes prepared in high pH buffers ($\text{pH} \geq 9$) differ considerably from those obtained at neutral or slightly acidic pH values (Figure 3).¹ The *lpH* spectrum shows a distinct doublet at low field, which collapses to a singlet in D_2O , indicative of an exchangeable Mo-OH proton with an isotropic hyperfine interaction constant of $a_{\text{H}} \sim 25$ MHz. No isotropic proton *hfi* is observed in the *hpH* CW EPR spectrum, but George proposed that a Mo-OH group could also be present at high pH.¹⁸ The detection of this proton was eventually accomplished through a series of pulsed EPR

Figure 3 Left hand side; different types of CW EPR spectra observed for vertebrate SOs, originally investigated by Bray and coworkers in the 1980s.¹ Right hand side; structures elucidated for the different EPR species from ESE spectroscopies, isotopic labeling and DFT calculations. Adapted from reference 11 with permission of Springer International Publishing AG.

experiments.^{19, 20} Early CW EPR spectra of chicken liver SO in ¹⁷O-enriched water showed that both the *lpH* and *hpH* forms exhibited distinct, but different, ¹⁷O *hfi* splittings.²¹

The relationship between the *hpH* and *lpH* forms was recently elucidated by combining their observed isotropic ¹⁷O and ¹H *hfi* with a series of DFT calculations (Figure 4). The right hand side of Figure 4 shows the model for the DFT calculations. The view is along the O-Mo bond of the exchangeable Mo-OH group of the active site (Figure 1). The axial oxo ligand is approximately vertical; the three S atoms complete the coordination in the equatorial plane of the Mo(V) active site (Figure 1). The S_{Cys} is to the right; one dithiolene S is to the left and the other towards the back. The DFT calculations involve counter clockwise rotation of the exchangeable equatorial Mo-OH group about its Mo-O bond. Having the projection of the OH bond parallel to the axial oxo group defines the torsion angle $\vartheta_{OH} = 0$. As the Mo-OH group is rotated counter clockwise both a_O and a_H are calculated. The left side of Figure 4 combines the experimental values of a_O and a_H for the *lpH* and *hpH* forms with the values calculated by DFT for the variation in Mo-OH torsion angle. The horizontal bars at the top half of the left hand diagram show the experimental a_H values (*lpH* form, light grey horizontal bar, ~25 MHz; *hpH* form, dark grey horizontal bar, ~0 MHz). The bottom half of the left hand side shows the experimental a_O values (*lpH* form, light grey horizontal bar, ~-26 MHz; *hpH* form dark grey horizontal bar, ~-12 MHz). The oscillating curves with open circles are the values calculated for a_H (top) and a_O (bottom) by DFT as a function of the torsion angle, ϑ_{OH} . Blue circles show where calculated and experimental *hfi* agree for **both** H and O for the *hpH* form. The dark grey vertical bars show that these intersections are at torsion angles of ~25 and 150 deg, and these orientations of the H atom of the Mo-OH group are indicated by blue arrows on the right hand side of Figure 4. The orange circles show where calculated and experimental *hfi* agree for **both** H and O for the *lpH* form. The light grey vertical bars show that these intersections are at ~230 and 300 deg, and these orientations of the equatorial Mo-OH group are shown with orange arrows on the right hand side of Figure 4. The combination of pulsed EPR results and DFT calculations shown in Figure 4 enables the dramatic differences between the CW EPR spectra of the *lpH* and *hpH* forms (Figure 3) to be readily understood. In a tetragonal oxo-Mo(V) center the unpaired electron is primarily in the equatorial plane and localized between the ligand atoms.²² The favored orientations for the *lpH* form (orange circles and arrows) place the H atom of the Mo-OH unit close to the equatorial plane which allows a strong isotropic interaction with the unpaired electron, and the proton *hfi* is easily observed in the CW EPR spectrum. This orientation is also consistent with the previously proposed hydrogen bonding interaction between the Mo-OH group and the S_{Cys} atom.¹⁹

For the *hpH* form, on the other hand, the favored orientations (blue circles and arrows) place the H atom approximately perpendicular to the equatorial plane, which contains the unpaired electron. The isotropic *hfi* is then ~0 MHz, and no proton hyperfine splitting is

Figure 4 *Right hand side*: coordination about the Mo center of SO (Figure 1) viewed along the O_{eq}-Mo bond of the exchangeable Mo-O_{eq}H group; O_{ax} is vertical; S_{Cys} is to the right; one dithiolene S is to the left and the other towards the back. White spheres are the H-atom positions for the *lpH* (orange arrows) and *hpH* (blue arrows). *Left hand side*: horizontal bars at the top half show the experimental a_H values (*lpH* form, light grey horizontal bar, ~25 MHz; *hpH* form, dark grey horizontal bar, ~0 MHz); horizontal bars at the bottom half show the experimental a_O values (*lpH* form, light grey horizontal bar, ~-26 MHz; *hpH* form dark grey horizontal bar, ~-12 MHz). The oscillating curves with open circles are the values calculated for a_H (top) and a_O (bottom) by DFT as a function of the torsion angle, ϑ_{OH} , upon counter clockwise rotation of the Mo-OH group about the Mo-O_{eq} bond. Blue circles show where calculated and experimental *hfi* agree for **both** H and O for the *hpH* form. The dark grey vertical bars show that these intersections are at torsion angles of ~25 and 150 deg; on the right hand side, these orientations of the Mo-OH group are indicated by blue arrows. The orange circles on the left hand side show where calculated and experimental *hfi* agree for **both** H and O for the *lpH* form. The light grey vertical bars show that these intersections are at ~230 and 300 deg, and these orientations of the equatorial Mo-OH group are shown with orange arrows on the right hand side.

seen in the *hpH* CW EPR spectrum. However, there is a substantial dipolar interaction between the proton and the unpaired electron, which can be detected by pulsed EPR experiments.^{19, 20} The different orientations depicted for the Mo-OH group in the *lpH* and *hpH* forms also affect the interactions of the oxygen orbitals with the unpaired electron in the Mo d_{xy} orbital in the equatorial plane, and hence differing values of a_O are observed for the two forms.^{9, 21}

Effects of surroundings on the *hpH* and *lpH* forms

Figure 4 concisely and logically explains the structural and electronic basis for the differences observed in the CW EPR spectra of the *hpH* and *lpH* forms of vertebrate SOs. However, Figure 4 provides no insight as to why two different forms, with the same composition, are observed spectroscopically. The first clues came from studies of Bray and coworkers in the 1980s, who found that the *lpH* form was preferred in the presence of chloride (~100 mM).¹ Much later ³⁵Cl (*I* = 3/2) *hfi* and *nqi* were detected for the *lpH* form by pulsed EPR,²³ and unambiguous couplings to Br and I were observed in CW EPR spectra.²⁴ These results led to the initial suggestions that the halides were directly coordinated to the Mo(V) center, trans to the axial oxo ligand.^{23, 24} However, DFT calculations showed that neither the *g*-values nor the ³⁵Cl *hfi* and *nqi* values were consistent with such a model.²⁵ In particular, the small *nqi* value (3 MHz) did not support axial coordination by Cl⁻. Removing the axial constraint on the position of the Cl⁻ led to a structure in

which the Cl^- was in the equatorial plane and surrounded by a nearly spherical array of protons from nearby water molecules and side chains of the protein. Sufficient spin density was transferred through the $\text{Mo}-\text{OH}\cdots\text{Cl}$ interaction to account for the observed ^{35}Cl *hfi* (4.5 MHz), and the nearly spherical electrostatic environment of the Cl^- was consistent with the small *nqi*.²⁵ In retrospect, trans axial coordination of Cl^- to tetragonal oxo-Mo(V) is strongly disfavored because the short $\text{Mo}\equiv\text{O}$ bond results in severe non-bonded contacts between the equatorial ligand atoms and any trans axial ligand. This effect will be especially problematic for relatively large halide ligands binding to the active site of SO, which includes three bulky S atoms in the equatorial plane.

Not all sulfite-oxidizing enzymes exhibit the *lpH* CW EPR spectrum observed for vertebrate SOs in the presence of Cl^- . Bacterial SorAB (sulfite dehydrogenase from *Starkeya novella*) shows only the *hpH*-type spectrum, even in 200 mM Cl^- .²⁶ However, pulsed EPR experiments clearly showed anisotropic coupling to an exchangeable proton of an $-\text{OH}$ group, as occurs in the *hpH* form of vertebrate SOs.²⁷ Thus, even though all sulfite-oxidizing enzymes have the same basic coordination structure (Figure 1), the interaction of the entire Mo(V) active site assembly with anions in the media and the nature of the surrounding protein modulate the observed EPR spectra.

Oxanyion forms

Phosphate inhibited (P_i) form

Phosphate and other anions are inhibitors of cSO.²⁸ The CW EPR spectrum for the phosphate inhibited (P_i) form at low pH (Figure 3) shows no observable hyperfine interaction with ^{31}P ($I = 1/2$, 100 % abundant)⁸, but 1D²⁰ and 2D⁹ pulsed EPR experiments clearly exhibit ^{31}P hyperfine coupling, consistent with the coordination of phosphate via an oxygen atom, as shown in Figure 3. It is interesting that no P_i form is observed for SorAB SDH or for plant sulfite oxidase from *Arabidopsis thaliana* (At-SO).^{26, 29}

Sulfite (“blocked”) forms

Early CW EPR studies of At-SO showed that the observed spectrum depended upon the method of generation of the Mo(V) state.³⁰ Reduction of At-SO with Ti(III) citrate at pH 6 generated a Mo(V) signal with large hyperfine splittings from a single exchangeable proton, as is typically observed for *lpH* SO from vertebrates. However, reduction by sulfite followed by partial reoxidation with ferricyanide generated an EPR spectrum with *g*-values similar to the low-pH (*lpH*) form of vertebrate SOs, but with *no* detectable exchangeable protons. From the proposed catalytic cycle for SOs (Figure 2) it was suggested that the latter result was due to the sulfate product being trapped in the active site of the Mo(V) state.³⁰ Naturally abundant ^{32}S has $I = 0$ and is magnetically silent. To test this hypothesis experimentally, sulfite labeled with ^{33}S ($I = 3/2$) was prepared and reacted with At-SO. ESEEM spectroscopy showed well-defined modulations for the ^{33}S -labeled system that were not

present for the naturally abundant ^{32}S -containing sample,³¹ consistent with the proposed coordination of sulfate to Mo(V).³⁰ The interpretation of the CW EPR and ESEEM spectra was facilitated by a theoretical analysis of the nuclear transition frequencies expected for the situation of the nuclear quadrupole interaction being much stronger than the Zeeman and hyperfine interactions, so that the spectra are dominated by the “interdoublet” transition arising from the strong nuclear quadrupole interaction.³¹ Analysis and simulation of the spectroscopic parameters for a ^{33}S -labeled sulfate ligand led to a small ^{33}S isotropic *hfi* constant ($a_s \sim 3$ MHz). The observed ^{33}S quadrupole coupling constant ($e^2 Qq/h \sim 40$ MHz) seemed quite large for a tetrahedral environment, but there were no other experimental or theoretical results then available for direct comparison. This sulfite-reduced form of At-SO was called the “blocked” form because the postulated bound sulfate product presumably prevented access of water to the Mo(V) active site to produce the *lpH* form.³¹

Subsequently it was shown that reduction of human sulfite oxidase (hSO) with ^{33}S -labeled sulfite at low pH in the *absence* of chloride also produces the “blocked” EPR spectra for wild type hSO, as well as for all known variants,³² including the fatal R160Q mutation.³³ Thus, all of these species were initially described as having coordinated sulfate as the equatorial oxanyion. A more detailed analysis of the structure of the “blocked” form appears in a later section, but it should be noted here that the CW EPR spectrum of the “blocked” form is essentially the same as that shown for the “sulfite” form of vertebrate SO in Figure 3, which was originally described by Bray and co-workers.⁷ The “blocked” forms are converted to the *lpH* form upon addition of excess chloride to wild type hSO and several known variants,³² or by addition of CaCl_2 solution to R160Q.³⁴

Axial oxo ligand

As noted above, facile exchange of the equatorial $-\text{OH}_n$ ligand of SOs for both the *lpH* and *hpH* forms has been known since 1979 from CW EPR studies in ^{17}O -enriched water.²¹ In 2005 a new type of weakly coupled exchangeable oxygen ligand with an isotropic *hfi* constant of $a_o \sim 5$ MHz was revealed in a ^{17}O ESEEM investigation of *hpH* cSO using hyperfine sublevel correlation (HYSCORE) spectroscopy at 29 GHz.¹² This new feature was assigned to the axial oxo ligand, and this assignment was supported by preparing the known model compound $[\text{Mo}^{17}\text{O}(\text{SPh})_4]^-$ with an axial oxo ligand and showing that its ^{17}O *hfi* and *nqi* constants were nearly identical to those found for *hpH* cSO.^{12, 13} DFT calculations on $[\text{Mo}^{17}\text{O}(\text{SPh})_4]^-$ were also consistent with the assignment of the weakly coupled ^{17}O to the axial oxo group.¹³

The observation of exchange of the axial oxo ligand in *hpH* cSO stimulated a search for axial oxo ligand exchange in *lpH* forms of vertebrate SOs, particularly the fatal R160Q mutant of hSO. The enzyme was dissolved in H_2^{17}O and reacted with a 20-fold excess of ^{33}S -labeled sulfite. Pulsed EPR spectra showed ^{33}S *hfi* and *nqi* constants characteristic of the “blocked” form described above, and

several ^{17}O features were also observed.³³ Unfortunately, however, when this work was initiated the authors were not aware that *the O atoms of sulfite exchange very rapidly in water*.³⁵ Thus, all ^{33}S -labeled sulfite would have immediately become ^{17}O -labeled, $[\text{S}^{33}\text{S}^{17}\text{O}_3]^{2-}$, by the large excess of H_2^{17}O in the solvent. In addition, the reaction would have resulted in the sulfate (product) having all of its O atoms present as ^{17}O . Finally, the 20-fold excess of sulfite relative to protein would favor replacement of any coordinated sulfate product by excess sulfite reagent in the “blocked” form of the enzyme. This leaves two questions. 1) Does the “blocked” form contain coordinated sulfate or coordinated sulfite? 2) Does the axial oxo group exchange at low pH?

These questions have been answered by a combination of DFT calculations and additional pulsed EPR experiments. DFT calculations for coordinated ^{33}S -labeled sulfate gave a quadrupole coupling constant of 4.4 MHz; whereas the calculated value for coordinated sulfite was 27.9 MHz. The experimental ^{33}S quadrupole coupling constant for R160Q hSO is 36 MHz, which clearly indicates coordinated sulfite for the “blocked” form.³⁴

The right hand side of Figure 5 shows that the Mo(V) center with bound sulfite can have three different types of oxygen atoms: 1) a coordinated O atom from the sulfite ligand; 2) two remote O atoms from the sulfite; 3) an axial oxo ligand. The left hand side shows the W-band (95 GHz) ^{17}O HYSCORE spectra at the g_y (intermediate-field) and g_x (high-field) positions and the assignments of the features to the coordinated and remote ^{17}O atoms of the sulfite ligand. The nq_i constants for the coordinated and remote ^{17}O atoms are 7 and 5 MHz, respectively, considerably larger than the ^{17}O nq_i constant for the axial oxo group in the model compound, $[\text{Mo}^{17}\text{O}(\text{SPh})_4]^-$ (1.45 MHz)¹³ and that for the *hpH* form of cSO (1.5 MHz).¹² In addition, DFT calculations predict a nq_i constant of 1.1 MHz for an axial oxo ligand in R160Q hSO at low pH.³⁴ Thus, it is clear that the axial oxo

Figure 5 W-band (95 GHz) HYSCORE of sulfite-reduced R160Q hSO in ^{17}O -enriched water at pH 6. The ball-and-stick structure shows the three types of ^{17}O oxygen atoms that are possible if the “blocked” form has O-bound sulfite: 1) coordinated O atom of sulfite; 2) two remote O atoms from the sulfite; 3) an axial oxo ligand. The assignments of the spectral features to the coordinated and remote ^{17}O atoms of the sulfite ligand are discussed in the text.

ligand of R160Q hSO does *not* undergo facile exchange in H_2^{17}O at low pH.

Further evidence that the ^{17}O features in the HYSCORE spectra of Figure 5 arise from coordinated sulfite is provided by Figure 6, Ka-band two-pulse ESEEM spectra of R160Q hSO at low pH (recorded at the low field edge of the spectrum).³⁴ The top trace shows strong modulations from ^{17}O below 20 MHz. Addition of 200 mM phosphate, $[\text{PO}_4]^{3-}$, a strong inhibitor of SO whose O atoms do not readily exchange in H_2^{17}O , gives rise to the bottom trace of Figure 6. The strong ^{17}O modulations have disappeared, and the new sum combination line at ~ 37.5 MHz is indicative of coordinated phosphate and replacement of the ^{17}O -labeled sulfite ligand.

Dithiolene group of the pyranopterin

The pyranopterindithiolate group (molybdopterin, MPT) is one of the oldest known organic cofactors in biology.³⁶ This unit coordinates to the Mo atom of SOs through the sulfur atoms of its dithiolene moiety (Figure 1). Dithiolene ligands are known to stabilize multiple oxidation states of transition metals,^{15,16} and it has been proposed that the conformation of MPT and delocalization of electron density between the dithiolene moiety of the MPT unit and the Mo atom play important roles in redox catalysis by SOs.³⁷ However, direct experimental evidence about the spin density on the S atom of a dithiolene coordinated to a paramagnetic Mo(V) center has been lacking because naturally abundant ^{32}S is magnetically silent ($I = 0$). Recently, direct incorporation of ^{33}S ($I = 3/2$) into the MPT moiety of a catalytically active bioengineered sulfite oxidase has enabled pulsed EPR spectroscopy to be used to make the *first* direct measurement of the spin density on the S atoms of a coordinated dithiolene.¹⁷ The ESEEM spectra of ^{33}S -labeled MPT in this catalytically active SO

Figure 6 Ka-band (29 GHz) two-pulse ESEEM spectra of R160Q hSO at low pH (recorded at the low field edge of the spectrum). The top trace shows strong modulations from ^{17}O below 20 MHz. Addition of 200 mM phosphate, $[\text{PO}_4]^{3-}$, a strong inhibitor of SO whose O atoms do not readily exchange in H_2^{17}O , gives rise to the bottom trace in which the strong ^{17}O modulations have disappeared; the new sum combination line at ~ 37.5 MHz is indicative of coordinated phosphate and replacement of the ^{17}O -labeled sulfite ligand. Reproduced from reference 34 with permission. Copyright 2012 American Chemical Society.

variant (Figure 7) are dominated by the “interdoublet” transition

arising from the strong nuclear quadrupole interaction, as also

Figure 9 Canonical Kohn-Sham molecular orbital diagram of the frontier orbital region for the computational SO active site model. HOMO = Highest Molecular Orbital; LUMO = Lowest Unoccupied Molecular Orbital. For the SOMO (Singly Occupied Molecular Orbital), which is highlighted, spin-polarization mixes the in-plane Mo d_{xy} orbital and out-of-plane S_{MPT} p orbitals to give rise to the experimentally observed ^{33}S hfi and nqi data. Reproduced from reference 17 with permission. Copyright 2014 American Chemical Society.

occurs for the ^{33}S -labeled exchangeable equatorial sulfite ligand in R160Q HSO.³⁴ The estimated experimental hfi and nqi parameters for ^{33}S ($a_{iso} = 3$ MHz and $e^2qQ/h = 25$ MHz) are in good agreement with those predicted by DFT (Figure 8). In addition, the DFT calculations show that the two ^{33}S atoms are indistinguishable by EPR and reveal a strong intermixing between their out-of-plane p_z orbitals and the singly occupied in-plane d_{xy} orbital of Mo(V) (Figure 9).

Figure 10 shows the active site SO model that was used for a series of geometry-optimized DFT calculations that gave the ^{33}S hfi and nqi parameters plotted in Figure 8. The dihedral angle (ϑ) is defined by two vectors involving the coordinated dithiolene unit. One vector is from the Mo atom to the centroid between the dithiolene S atoms; the second vector is from the centroid between the dithiolene S atoms to the centroid between the dithiolene C atoms. The calculated energy minimum has $\vartheta \sim 152^\circ$ and is shown in the ball-and-stick representation. This structure also most closely corresponds that of the active site of native enzymes (Figure 1).

Figure 7 Primary ESEEM spectra at the g_z (low-field) and g_x (high-field) positions of the MPT moiety of a catalytically active bioengineered SO containing ^{32}S ($l = 0$) and ^{33}S ($l = 3/2$). The prominent line at ~ 9.5 MHz in the ^{33}S spectra is due to the interdoublet quadrupole transition. Experimental conditions: K_α -band (35.1 GHz); temperature 21 K; mw pulse durations, 12 ns.

Figure 8 Calculated values of the ^{33}S isotropic hfi (a_{iso}), the anisotropic or spin-dipole (SD) hyperfine components, and the nqi (e^2qQ/h) as a function of the dihedral angle defined in Figure 10 for the SO active site model. The vertical dashed line indicates the dihedral angle corresponding to the minimum-energy structure ($\vartheta = 152^\circ$), which also most closely represents the geometry of the native enzyme. Reproduced from reference 17 with permission. Copyright 2014 American Chemical Society.

Figure 10 Overlay of a series of geometry-optimized active site models used to explore the relationship between the MPT dihedral angle (θ), defined as the angle between Mo (cyan), a centroid between the dithiolene ^{33}S atoms, and a centroid between the dithiolene C atoms. The ball-and-stick representation is the minimum-energy calculated by DFT for the active site structure ($\theta \sim 152^\circ$), and leads to the calculated *hfi* and *nqi* results of Figure 8. Reproduced from reference 17 with permission. Copyright 2014 American Chemical Society.

The paramagnetic ^{33}S -labelled catalytically active bioengineered sulfite oxidase formally contains a Mo(V) $4d^1$ center. It has long been known that the dihedral angle of the coordinated dithiolene ligand of organometallic compounds of the type $\text{Cp}_2\text{M}(\text{bdt})$ (Cp = cyclopentadienyl; M = Mo, V, Ti; bdt = benzenedithiolate), depends on the electronic configuration of the formally 4+ metal center.^{38, 39} $\text{Cp}_2\text{V}(\text{bdt})$ contains V(IV) and a d^1 metal center; its dihedral angle, $\theta \sim 142^\circ$ (see Figure 11),⁴⁰ is similar to that of the minimum energy structure for the SO model shown in Figure 10. It has been proposed that the d^1 electronic configuration of the Mo(V) state of SOs plays a key role the overall catalytic cycle (Figure 2) by providing a low-energy pathway for reoxidation and geometric reorganization.³⁹

Coordinated cysteine (S_{cys}) Figure 11 Structure of $\text{Cp}_2\text{V}(\text{bdt})$ showing the dihedral angle of $\sim 142^\circ$ between the plane containing the V and S atoms and the plane of the dithiolene ligand. Alternatively described as a "fold angle" of 38° .[§] Reproduced

Figure 11 Structure of $\text{Cp}_2\text{V}(\text{bdt})$ showing the dihedral angle of $\sim 142^\circ$ between the plane containing the V and S atoms and the plane of the dithiolene ligand. Alternatively described as a "fold angle" of 38° .[§] Reproduced from reference 40 with permission. Copyright 1992 American Chemical Society.

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It is not feasible to carry out ^{33}S -labeling of the coordinated cysteine of SOs. However, pulsed electron nuclear double resonance (ENDOR) spectra of nonexchangeable protons in the vicinity of the Mo(V) center have provided considerable insight about the dependence of the geometry of the S_{cys} ligand upon enzyme source and pH of the medium.⁴¹ The close similarity of these spectra for cSO and hSO indicates that the structures of their molybdenum centers are essentially identical. From the *lpH* SO spectra, the closest nonexchangeable proton is $\sim 2.8 \text{ \AA}$ from the Mo atom. From comparison with the X-ray structure of cSO,⁴² this closest nonexchangeable proton is assigned to the α -proton of the coordinated conserved cysteinyl residue (Cys185 in cSO, Cys207 in hSO). The closest Mo...H distance for the nonexchangeable protons of *hpH* SO is $\sim 3.3 \text{ \AA}$. This increase in the Mo...H distance for the cysteinyl α -proton is meaningfully different from the X-ray and *lpH* SO distances, but requires only small changes in torsional angles. The minimum structural modifications that account for the Mo...H distance change between *lpH* and *hpH* SO can be described by two $\sim 15^\circ$ rotations around $\text{C}_\alpha\text{-C}_\beta$ and $\text{C}_\beta\text{-S}_\gamma$ bonds of the coordinated cysteinyl residue, which may reflect small pH dependent changes in the folding of the SO protein near the active site.

Conclusions

1. All members of the sulfite oxidase family have a nearly identical, five-coordinate, pseudo square-pyramidal Mo center (Figure 1). Coordination of a sixth ligand trans to the short $\text{Mo}=\text{O}$ bond is extremely unlikely because severe non-bonded contacts with the equatorial ligand atoms will result.
2. The paramagnetic Mo(V) state can be easily generated and investigated by CW and pulsed EPR spectroscopies. The high pH (*hpH*) signal is seen for all known sulfite-oxidizing enzymes. Vertebrate SOs also exhibit the low pH (*lpH*), phosphate inhibited (*P_i*) and sulfite (or blocked) signals. The structures of all of these forms have been determined by a combination of isotopic labelling, variable frequency electron spin echo (ESE) spectroscopy, and density function theory (DFT) calculations.
3. The so-called "blocked" form of SOs does not contain coordinated sulfate as initially proposed,^{30, 31} but rather contains coordinated sulfite, as shown in Figure 5.
4. The axial oxo ligand of *hpH* SO exchanges slowly in H_2^{17}O ; however, there is no evidence that the axial oxo ligand of *lpH* SO undergoes facile exchange in H_2^{17}O .
5. Application of variable frequency ESE spectroscopy to quadrupolar nuclei with large nuclear spin (^{17}O , $I = 5/2$; $^{35,37}\text{Cl}$, $I = 3/2$; ^{33}S , $I = 3/2$) is now routine.

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Notes and references

§ The organometallic literature uses “fold angle” to describe the geometry of the dithiolene ligand. The “fold angle” is the angle between the MS₂ plane and the S₂C₂ plane of the dithiolene. The dihedral angle of Figure 10 is the complement of the “fold angle”. In the text, the “fold angle” for Cp₂V(bdt) has been converted to the dihedral angle to be consistent with the nomenclature of Figure 10.

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1