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Review

# Applications of pulsed EPR spectroscopy to structural studies of sulfite oxidizing enzymes☆

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ABSTRACT

Sulfite oxidizing enzymes (SOEs), including sulfite oxidase (SO) and bacterial sulfite dehydrogenase (SDH), catalyze the oxidation of sulfite ($SO_3^{2-}$) to sulfate ($SO_4^{2-}$). The active sites of SO and SDH are nearly identical, each having a 5-coordinate, pseudo-square-pyramidal Mo with an axial oxo ligand and three equatorial sulfur donor atoms. One sulfur is from a conserved Cys residue and two are from a pyranopterindithiolene (molybdopterin, MPT) cofactor. The identity of the remaining equatorial ligand, which is solvent-exposed, varies during the catalytic cycle. Numerous *in vitro* studies, particularly those involving electron paramagnetic resonance (EPR) spectroscopy of the Mo(V) states of SOEs, have shown that the identity and orientation of this exchangeable equatorial ligand depends on the buffer pH, the presence and concentration of certain anions in the buffer, as well as specific point mutations in the protein. Until very recently, however, EPR has not been a practical technique for directly probing specific structures in which the solvent-exposed, exchangeable ligand is an O, $OH^-$, $H_2O$, $SO_3^{2-}$, or $SO_4^{2-}$ group, because the primary O and S isotopes ($^{16}O$ and $^{32}S$) are magnetically silent ($I=0$). This review focuses on the recent advances in the use of isotopic labeling, variable-frequency high resolution pulsed EPR spectroscopy, synthetic model compounds, and DFT calculations to elucidate the roles of various anions, point mutations, and steric factors in the formation, stabilization, and transformation of SOE active site structures.



## 1. Introduction

Sulfite oxidizing enzymes, including sulfite oxidase (SO) and sulfite dehydrogenase (SDH), catalyze the physiologically essential two-electron oxidation of sulfite to sulfate (Eq. (1)) [1,2]. In plants and vertebrates, SO functions in the final metabolic degradation step of sulfur-containing compounds and serves to eliminate toxic sulfite from the organism [3]. The electrons from this process are ultimately passed on to either ferricytochrome *c* (cyt *c*) in vertebrates or to molecular oxygen in plants [4]. In contrast, certain bacteria use SDH to catalyze the oxidation

*Abbreviations:* SO, sulfite oxidase; SDH, sulfite dehydrogenase; SOE, sulfite oxidizing enzyme; EPR, electron paramagnetic resonance; ESEEM, electron spin echo envelope modulation; IET, intramolecular electron transfer; DFT, density functional theory; *hpH*, high pH; *lpH*, low pH; $P_i$, phosphate inhibited; *hfi*, hyperfine interaction; *nqi*, nuclear quadrupole interaction; HYSCORE, hyperfine sublevel correlation; Moco, molybdenum cofactor.

☆ In celebration of the 65th birthday of Prof. Edward I. Solomon.

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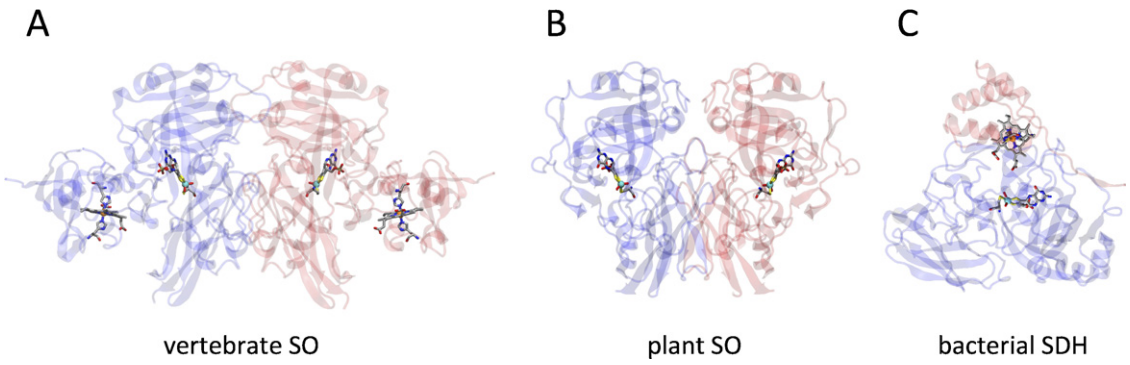


**Fig. 1.** Representative wild-type protein structures of vertebrate SO, plant SO, and bacterial SDH. (A) Vertebrate SO (1.7 Å chicken liver SO; pdb ID = 1SOX) is a 110 kDa $\alpha_2$-dimeric protein located in the mitochondrial intermembrane space. Each subunit consists of a *b*-type-heme domain and a Moco domain, which are connected to each other by a flexible tether that is disordered in the crystal structure. (B) Plant SO (2.6 Å *A. thaliana* SO; pdb ID = 1OGP) is a 90 kDa $\alpha_2$-dimeric protein located in the peroxisome. In contrast to all other SOEs, plant SO contains no heme centers since molecular oxygen serves directly as the terminal electron acceptor in plants. (C) Bacterial SDH (1.8 Å *S. novella* SDH; pdb ID = 2BLF) is a 50 kDa αβ-dimeric protein located in the periplasm that consists of a Moco subunit and *c*-type-heme subunit. The metal cofactors and metal-coordinated amino acids in each structure are displayed as ball-and-stick figures for clarity.

of sulfite from thiosulfate, which they use as an energy source [5].

$$SO_3^{2-} + H_2O \rightarrow SO_4^{2-} + 2H^+ + 2e^- \quad (1)$$

Although the tertiary structures of the SO [6,7] and SDH [8] proteins differ considerably from each other (Fig. 1), the active site structures of these enzymes, at least with respect to their catalytic Mo centers, are practically identical (Fig. 2a). In each case, Mo is coordinated by a total of five ligand donor atoms

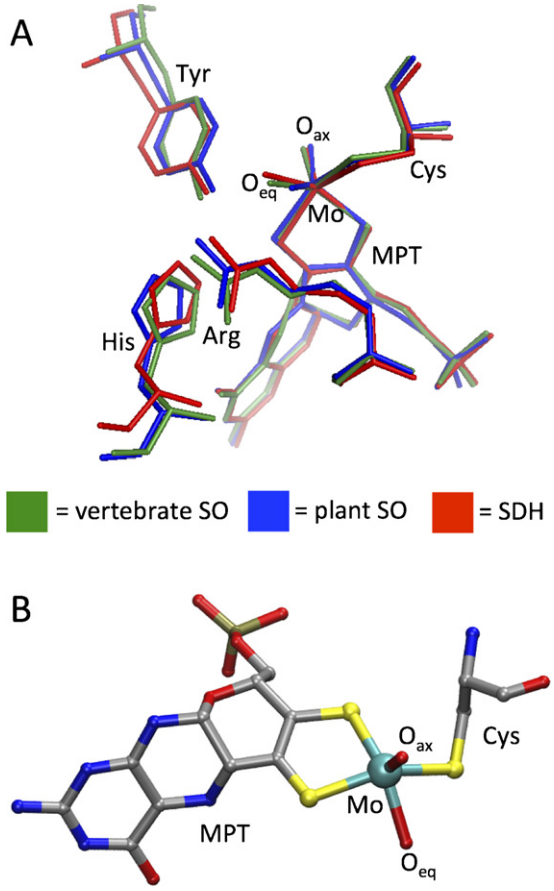


**Fig. 2.** The active site structure of SOEs. (A) Overlay view of the SO and SDH active sites, including selected nearby conserved residues. (B) Ball-and-stick representation of the fully oxidized Moco center, shown with the conserved Cys residue coordinated to Mo.

in the same pseudo-square pyramidal geometric arrangement: an axial oxo group ($O_{ax}$), an equatorial sulfur from a conserved Cys residue, two sulfurs from a pyranopterindithiolene cofactor (molybdopterin, MPT), and an exchangeable equatorial ligand whose identity depends on the stage of the catalytic cycle, specific protein point mutations, and the buffer conditions in which the enzyme is prepared (Fig. 2b). In the fully oxidized Mo(VI) resting state of SOEs, this equatorial ligand is also an oxo group ($O_{eq}$) [9]. For the pathological R160Q mutant of human SO (hSO), it has been suggested that a nearby Gln residue (substituting the conserved non-coordinating Arg residue that is located immediately *trans* to $O_{ax}$ in the *wt* enzyme) also coordinates to form a 6-coordinate Mo center [10]. However, recent results obtained using pulsed electron paramagnetic resonance (EPR) techniques in conjunction with isotopic labeling and density functional theory (DFT) argue that the Mo center of this mutant remains five-coordinate in a pseudo-square-pyramidal geometry (*vide infra*) [11].

The boxes of Scheme 1 show a simplified catalytic cycle for the oxidation of sulfite by SOEs [1]. The initial reaction of sulfite with the dioxo-Mo center (**1**) reduces the fully oxidized Mo(VI) ($d^0$) state to Mo(IV) ($d^2$), forming the enzyme-product (EP) complex (**2**). Mo(IV) is reoxidized to the Mo(VI) resting state by two sequential one-electron oxidations, passing through the paramagnetic Mo(V) ($d^1$) intermediate (**3**). In the vertebrate and bacterial enzymes these sequential one-electron oxidations involve intramolecular electron transfer (IET) to their integral heme centers [3].

The exact order of IET and product release in going from **2** → **3** in Scheme 1 is still a matter of debate. A mechanism in which hydrolysis of the product, sulfate, occurs prior to any Mo(IV) oxidation step has been most frequently invoked [1]. However, pulsed EPR studies of the paramagnetic Mo(V) state of plant SO at low pH suggested that electron transfer from Mo(IV) could precede hydrolysis [12,13], as indicated by **2** → **4** in Scheme 1. The formation of this so-called "blocked" Mo(V) form (**4**) is also consistent with the fact that the enzyme turnover rates of SOEs are known to be much slower than their IET rates [3,14,15]. Because of the large excess of substrate (sulfite) present in the EPR studies, structure **5** with bound sulfite is another possible species that may be formed. Indeed, a sulfite-containing Mo(V) species was originally proposed in the early 1980s by Bray et al. [16]. Evidence for **4** and **5** is discussed in Section 3.

Historically, EPR has been an extremely important technique for probing the Mo(V) states of SOEs and for obtaining information about specific Mo(V) structures [16–19]. However, it can be difficult to distinguish among structures in which the solvent-exposed, exchangeable ligand is a $O^{2-}$, $OH^-$, $H_2O$, $SO_3^{2-}$, $SO_4^{2-}$, or $PO_4^{3-}$