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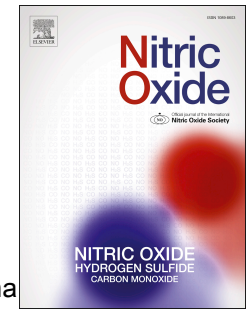

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Reactions of superoxide dismutases with HS^-/H_2S and superoxide radical anion: an *in vitro* EPR study

Bojana Bolić [a], Ana Mijušković [b], Ana Popović-Bijelić [c], Aleksandra Nikolić-Kokić [b], Snežana Spasić [d], Duško Blagojević [b], Mihajlo B. Spasić [b], Ivan Spasojević [e,*]

[a] *Abbott Laboratories S.A., Belgrade, Serbia*

[b] *Department of Physiology, Institute for Biological Research "Siniša Stanković", University of Belgrade, Belgrade, Serbia*

[c] *Faculty of Physical Chemistry, University of Belgrade, Belgrade, Serbia*

[d] *Department of Chemistry, Institute of Chemistry, Technology and Metallurgy, University of Belgrade, Belgrade, Serbia*

[e] *Department of Life Sciences, Institute for Multidisciplinary Research, University of Belgrade, Belgrade, Serbia*

***Corresponding author at:** Institute for Multidisciplinary Research, University of Belgrade, Kneza Višeslava 1, 11030 Belgrade, Serbia

Tel: +381 11 2078459; Fax: +381 11 3055289; E-mail: ivan@imsi.rs; redoxsci@gmail.com

Abstract

Interactions of hydrogen sulfide (HS^-/H_2S), a reducing signaling species, with superoxide dimutases (SOD) are poorly understood. We applied low-T EPR spectroscopy to examine the effects of HS^-/H_2S and superoxide radical anion ($O_2^{\bullet-}$) on metallocenters of FeSOD, MnSOD, and CuZnSOD. HS^-/H_2S did not affect FeSOD, whereas active centers of MnSOD and CuZnSOD were open to this agent. Cu^{2+} was reduced to Cu^{1+}, while manganese appears to be released from MnSOD active center. Untreated and $O_2^{\bullet-}$-treated FeSOD and MnSOD predominantly show 5 d-electron systems, *i.e.* Fe^{3+} and Mn^{2+}. Our study provides new details on the mechanisms of (patho)physiological effects of HS^-/H_2S.

Keywords: Superoxide dismutase; EPR; H_2S; Iron; Manganese, Copper

Abbreviations: CuZnSOD, copper-zinc superoxide dismutase; EPR, electron paramagnetic resonance; FeSOD, iron superoxide dismutase; HS^-/H_2S, hydrogen sulfide; MnSOD, manganese superoxide dismutase, $O_2^{\bullet-}$, superoxide radical anion.

Highlights

- FeSOD from *E. coli* and *P. leiognathi* are resistant to HS^-/H_2S-induced reduction
- HS^-/H_2S appears to provoke a release of manganese from MnSOD
- HS^-/H_2S reduced Cu^{2+} to Cu^{1+} in CuZnSOD
- 5 d-electron systems predominate in MnSOD and FeSOD

1. Introduction

Hydrogen sulfide (HS^-/H_2S) represents the third gaseous signaling molecule, in addition to nitric oxide and carbon monoxide [1]. H_2S is a reducing agent and a weak acid with approximately 4:1 HS^-/H_2S ratio at physiological pH [2]. Recent studies have underscored the fact that HS^-/H_2S and reactive oxygen species signaling systems are intertwined [3,4]. For example, superoxide radical anion ($O_2^{\bullet-}$) reacts very rapidly with H_2S, whereas HS^- can reduce metal centers which in some cases (such as cytochrome c) might lead to production of $O_2^{\bullet-}$ from molecular oxygen [4]. Importantly, they share common targets, including superoxide dismutases (SOD). It has been shown that HS^- enhances $O_2^{\bullet-}$ scavenging activity of the bovine erythrocyte copper-zinc SOD (CuZnSOD) by about twofold [5]. Binding of HS^- to the enzyme is rapid, with $k > 10^7\ M^{-1}\ s^{-1}$. These observations suggest that HS^- binds to SOD at the catalytic Cu center and that it might represent a genuine substrate of the enzyme. It has been shown that NaHS increases the activity of CuZnSOD and manganese SOD (MnSOD) *in vivo* [6]. Further examination indicated that HS^-/H_2S up-regulates the expression of MnSOD but not of CuZnSOD. Finally, using a cell-free system, it has been documented that HS^-/H_2S causes increased CuZnSOD activity. Other than this, the interactions between SODs and HS^-/H_2S are poorly understood. For example, H_2S is converted in mitochondria to thiosulfate, followed by further conversion to sulfite, and finally to sulfate, the major end product of H_2S metabolism [7], but a potential role of mitochondrial MnSOD in this process is still unknown. Gut bacteria release large amounts of hydrogen sulfide [8]. It is clearly of interest to elucidate the effects of such settings on MnSOD and CuZnSOD in colonic epithelium and on primitive iron SOD (FeSOD) that is present in bacteria and some parasites [9]. Finally, although SOD research begun almost a half century ago [10], not all the pieces of the puzzle of SODs' interactions with $O_2^{\bullet-}$, have been gathered. Pertinent to this, we

examined and compared the reactions of metallocenters of FeSOD (from *E. coli* and *P. leiognathi*), MnSOD (from *E. coli*), and CuZnSOD (from rat) with HS^-/H_2S (donor: Na_2S) and $O_2^{\bullet-}$ (donor: KO_2), using low-T electron paramagnetic resonance (EPR) spectroscopy. The majority of studies on hydrogen sulfide utilize Na_2S (and NaHS) as exogenous donors. HS^-/H_2S release is rapid upon reaction of Na_2S with water, due to its high solubility.

2. Materials and methods

SODs were isolated and purified using previously established techniques [11]. The isolates were confirmed by gel electrophoresis. Specific activities were: 1500–1600 units/mg for *E. coli* and *P. leiognathi* FeSODs and *E.Coli* MnSOD, and 3000 NBT/riboflavin units/mg for rat CuZnSOD. SODs were dissolved in HEPES buffer (50 mM, pH = 7.4) to a final concentration of 100 μM. Enzymes were either untreated or exposed to Na_2S (Merck, Darmstadt, Germany) or KO_2 (Sigma–Aldrich, St. Louis, MO, USA) at final concentrations of 2 mM and 1 mM, respectively. Of note, Na_2S and KO_2 release HS^-/H_2S and $O_2^{\bullet-}$ in 1:1 ratio. KO_2 is rapidly decomposed in water to give $O_2^{\bullet-}$. Pertinent to this, KO_2 has to be prepared in an organic water-free solvent. Chlorinated/halogenated organic solvents should be avoided because they create settings for production of singlet oxygen [12]. The best choice was ultrapure water-dried DMSO (Sigma–Aldrich, Product No. 34943), although KO_2 shows a limited solubility in DMSO (< 2mM) [13]. In order to achieve the final concentration of 1 mM and to minimize the amount of DMSO in samples (5%), we prepared an oversaturated solution of KO_2 (equivalent of 20 mM). The solution was freshly prepared before each set of experiments, and vortexed immediately before each pipetting (*i.e.* addition of aliquots to samples). It is important to note that the enzymatic $O_2^{\bullet-}$-generating system (xanthine oxidase + (hypo)xanthine) could not be applied here, because xanthine oxidase contains EPR-active metals – Fe and Mo. Na_2S stock was prepared in water and

used immediately. In all experiments bidistilled deionised ultrapure (18 MΩ) water was used. Samples were incubated for 30 s at room temperature, placed in quartz EPR tubes, and quickly frozen in cold isopentane.

EPR spectra were recorded at 20K on a Bruker Elexsys-II EPR spectrometer with an Oxford Instruments ESR900 helium cryostat, operating at X-band (9.4 GHz) under the following conditions: modulation amplitude, 5 G; modulation frequency, 100 kHz; microwave power, 3.2 mW; scan time, 2 min; number of accumulations, 4 (*E. coli* FeSOD, MnSOD, and CuZnSOD) or 8 (*P. leiognathi* FeSOD). All spectra were baseline corrected. All experiments were performed in triplicate. Characteristic spectra are presented.

3. Results and discussion

Fig. 1 shows characteristic spectra of high-spin Fe^{3+} with a distorted trigonal bipyramidal electronic structure in the active center of prokaryotic FeSOD [14,15], combined with the signal of non-specifically bound Fe^{3+} ('dirty iron'; g = 4.25). g-Values for *E. coli* FeSOD match perfectly with those previously reported [14]. Fe^{3+} in the active centers of both FeSOD enzymes showed to be resistant to HS^-/H_2S-provoked reduction. This is in line with the available data on redox potentials. Namely, redox midpoint potential of FeSOD (~100 mV) is lower compared to redox potential of HS^- (920 mV at pH 7.4; reaction: $HS^- \rightarrow HS^{\bullet} + e^-$; the same potential applies to $H_2S \rightarrow HS^{\bullet} + H^+ + e^-$) [9,16,17]. The resistance of FeSOD to reduction appears to be in line with its role in the early evolution of life that took place under the reducing conditions [9]. In a nutshell, the metallocenter of FeSOD had to be protected from reducing agents in order to maintain the function. On the other hand, there is no doubt that $O_2^{\bullet-}$ can react with FeSOD active center. A modest decrease of the level of Fe^{3+}SOD following exposure to $O_2^{\bullet-}$ donor might be explained by the fact that the first half-reaction (Fe^{3+}SOD → Fe^{2+}SOD) in $O_2^{\bullet-}$ dismutation is

faster compared to the second half-reaction (Fe^{2+}SOD → Fe^{3+}SOD) [18]. In this way, some quantity of the enzyme remains in the Fe^{2+}SOD form. It is also important to address the effects of the examined agents on non-specifically bound Fe^{3+}. Fe^{3+}/Fe^{2+} redox pair has a redox potential of ~110 mV at pH 7. Hence it could not be reduced by hydrogen sulfide, but it can be reduced by $O_2^{\bullet-}$, because $O_2/O_2^{\bullet-}$ pair shows a lower redox potential (-330 mV) [19]. The fact that the exposure to $O_2^{\bullet-}$ did not result in reduction of non-specifically bound Fe^{3+} might be explained by the binding of iron to highly negative domains on the surface of the protein, which could repulse agents of the same charge. It worth mentioning that some amount of H_2O_2 might have been present in the system either because of SOD activity or due to non-enzymatic dismutation of $O_2^{\bullet-}$. Fe^{2+} is oxidized by H_2O_2 (Fenton reaction) to produce Fe^{3+}, so this might be an alternative explanation for the absence of changes in the level of non-specifically bound Fe^{3+} following the exposure to KO_2.

In contrast to FeSOD, MnSOD active center underwent significant changes following the exposure to HS^-/H_2S and $O_2^{\bullet-}$. Fig. 2A shows characteristic six-line EPR spectra of high-spin Mn^{2+} in MnSOD active center [20,21]. In addition, there are five pairs of weak lines from forbidden $\Delta m_I = \pm 1$ transitions. The hyperfine splitting of the half-field and low-field transitions is also observed (data not shown). It appears that isolated MnSOD contains manganese in both Mn^{2+} and Mn^{3+} forms, and that $O_2^{\bullet-}$ reduced (some of) Mn^{3+} to Mn^{2+} resulting in the increased intensity of MnSOD signal. In addition, it has been shown that $O_2^{\bullet-}$ at high concentrations might cause inhibition of MnSOD via formation of a so called "dead-end" complex between Mn^{2+}SOD and $O_2^{\bullet-}$ [18,22]. Although FeSOD and MnSOD belong to the same SOD family, electronic configurations of their 3d orbitals undergo substantially different changes following the reaction with $O_2^{\bullet-}$. In FeSOD, 5 d-electron system (Fe^{3+}) is converted to 6 d-electron system (Fe^{2+}),

whereas Mn^{3+} reduction to Mn^{2+} corresponds to conversion of 4 to 5 d-electron system [9]. Our results imply that a 5 d-electron system, which has one electron in each d orbital, represents a more stable redox state of both of these enzymes. Taking into account that MnSOD redox midpoint potential is approximately 300 mV [9], it could be expected that HS^-/H_2S would not induce Mn^{3+}SOD reduction and an increase of Mn^{2+}SOD signal. However, a decrease was not expected as well. A plausible explanation for decreased Mn^{2+}SOD signal might be that HS^-/H_2S caused a release of Mn^{2+} from the enzyme. Mn^{2+} in buffer solutions shows a very weak six-line signal centered at $g \approx 2$ [23], which overlaps with the strong signal of MnSOD. This finding is in agreement with previously reported *in vivo* effects of NaHS. It upregulated the expression of MnSOD, which might be a consequence of HS^-/H_2S-provoked irreversible inhibition of MnSOD. The inhibition of MnSOD might at least partially account for previous 'paradoxical' findings that this reducing agent might induce oxidative stress in mammalian cells [24].

Fig. 2B shows EPR spectra of Cu^{2+} in rat CuZnSOD [25,26]. Superoxide radical anion did not cause any EPR changes. According to Perry *et al*., the first half-reaction of superoxide dismutation begins with the $O_2^{\bullet-}$ binding to Cu^{2+}. Cu^{2+} is then reduced to Cu^{1+}, while $O_2^{\bullet-}$ is oxidized to O_2 [27]. The Cu ion-bridging histidine (HsCu,ZnSODHis63) bond is broken, leaving His63Nε1 protonated. In the second half-reaction, a proton from His63Nε1 and an electron from Cu^{1+} are donated to $O_2^{\bullet-}$. Cu^{1+} is oxidized to Cu^{2+}, and $O_2^{\bullet-}$ is reduced to H_2O_2 or HO^{2-}. The rates of two half-reactions at physiological pH are similar [18]. This balance can explain the same amount of Cu^{2+}ZnSOD that was found here before and after the exposure to $O_2^{\bullet-}$-generating system. It is important to point out that CuZnSOD and FeSOD are susceptible whereas MnSOD is resistant to H_2O_2-induced inhibition [28]. Apparently, small amounts of H_2O_2 that most likely emerged in our system did not affect CuZnSOD and FeSOD. Effects of H_2O_2 on CuZnSOD

encompass the oxidation of His residues involved in the coordination of Cu [29]. Pertinent to this, it has been shown using low-T EPR that H_2O_2 modifies the structure of catalytic center of CuZnSOD [30]. This was not the case here, since no changes in the shape of the low-T EPR spectrum of CuZnSOD (and FeSOD) were observed following the exposure to KO_2.

HS^-/H_2S induced the reduction of Cu^{2+} to EPR 'silent' Cu^{1+}, which is observed as the loss of the CuZnSOD signal. This clearly implies that the active center of CuZnSOD is open to the effects of HS^-/H_2S. It has been proposed previously that CuZnSOD catalyzes HS^- oxidation via reaction: $HS^- + H^+ + O_2 \rightarrow S^0 + H_2O_2$, with concomitant reduction of Cu^{2+} to Cu^{1+} [31]. Altogether this two electron transfer is a very slow process, but the first half-reaction in which HS^- is bound to Cu^{2+} and Cu^{2+} is reduced to Cu^{1+} is fast with $t_{1/2}$ = 6 s [5]. Our results speak in favor of this mechanism which includes two electron oxidation of HS^-. A wide range of redox midpoint potentials (120–525 mV) has been reported for bovine CuZnSOD [32]. Although we could not find a reliable information on redox potential for rat CuZnSOD, it is unlikely to be higher than 920 mV, which is the redox potential for one electron oxidation of HS^- [16]. On the other hand, two-electron redox potential of HS^- (reaction: $HS^- \rightarrow S^0 + H^+ + 2e^-$) at pH 7 is 170 mV [33], which makes HS^--induced reduction of Cu^{2+}ZnSOD feasible. In addition, one-electron oxidation of HS^- would result in the production of $HS^\bullet$ radical, which we did not detect using EPR spin-trapping and DEPMPO spin-trap. Still, we cannot be absolutely sure that $HS^\bullet$ radical is not produced in the system, because spin adduct (DEPMPO/SH) might show a very brief life-time due to strongly reducing conditions. Such problem has been observed previously for cysteine thiyl radical [34]. Finally, a possibility that HS^-/H_2S could provoke a release of copper from CuZnSOD and to maintain it in 'free' Cu^{1+} form (Cu^{2+} in buffer solution shows a strong EPR signal [35]) can be excluded in accordance with findings that hydrogen sulfide promotes the

activity of this enzyme [5,6]. All relevant redox potentials and proposed mechanisms of reactions of HS^-/H_2S with SODs are sublimed in Fig. 3. It is worth mentioning that hydrogen sulfide might interfere with specific SOD activity assays (*e.g.* cytochrome c reduction) [4]. However, Searcy and colleagues applied a diverse selection of SOD activity assays with consistent outcome [5], whereas Sun and co-workers tested the effects of HS^-/H_2S on their assay (reduction of a tetrazolium salt) in the absence of SOD [6].

The concentration of free HS^-/H_2S in cells under physiological conditions is in the lower micromolar or higher nanomolar range [37]. However, there are two large pools of stored hydrogen sulfide: (i) acid-labile sulfur (40–160 μM) that is mainly localized in mitochondria; and (ii) bound sulfane sulfur (up to 450 μM) which is localized in the cytosol [38]. It has been proposed that HS^-/H_2S might be released from the stores in response to specific (patho)physiological stimuli, such as a decrease of pH in mitochondria, increase of cytosolic pH, changes in the redox conditions, and others [37]. For example, it has been shown that HS^-/H_2S is released from bound sulfane sulfur in astrocytes in response to the excitation of co-cultured neurons [38]. The concentration of CuZnSOD ranges between 1 and 20 μM in different human tissues [39], whereas the activity of MnSOD is generally 10–15% of total SOD activity in mammalian cells [40]. It can be observed that SOD/hydrogen sulfide concentration ratio applied here (1:20) reflects *in vivo* settings in which HS^-/H_2S is released from intracellular stores. Finally, our results might be important for understanding: (i) The interplay between reducing and oxidizing agents. *E.g.* MnSOD, which is affected by HS^-/H_2S, represents the main intracellular site of H_2O_2 generation [41]. (ii) Events in gut microflora that affect the population profile and dynamics. Gut bacteria produce large amounts of H_2S [42]. Many bacterial species contain either FeSOD or MnSOD (although some, like *E. coli*, employ both) [40]. The former is not affected

whereas the latter is affected by HS^-/H_2S. (iii) Pathological processes in colon cancer. Human colon cancer specimens show 10-fold higher production of hydrogen sulfide and two-fold higher MnSOD expression compared to normal mucosa [43,44].

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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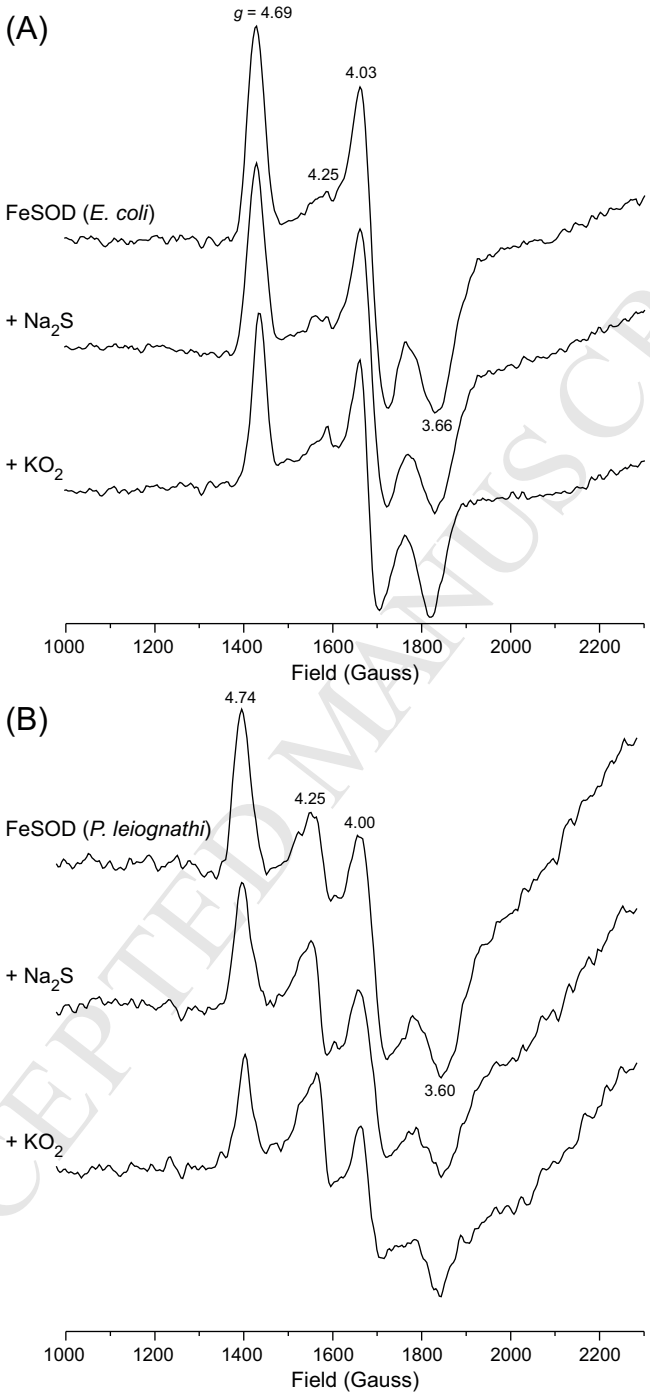
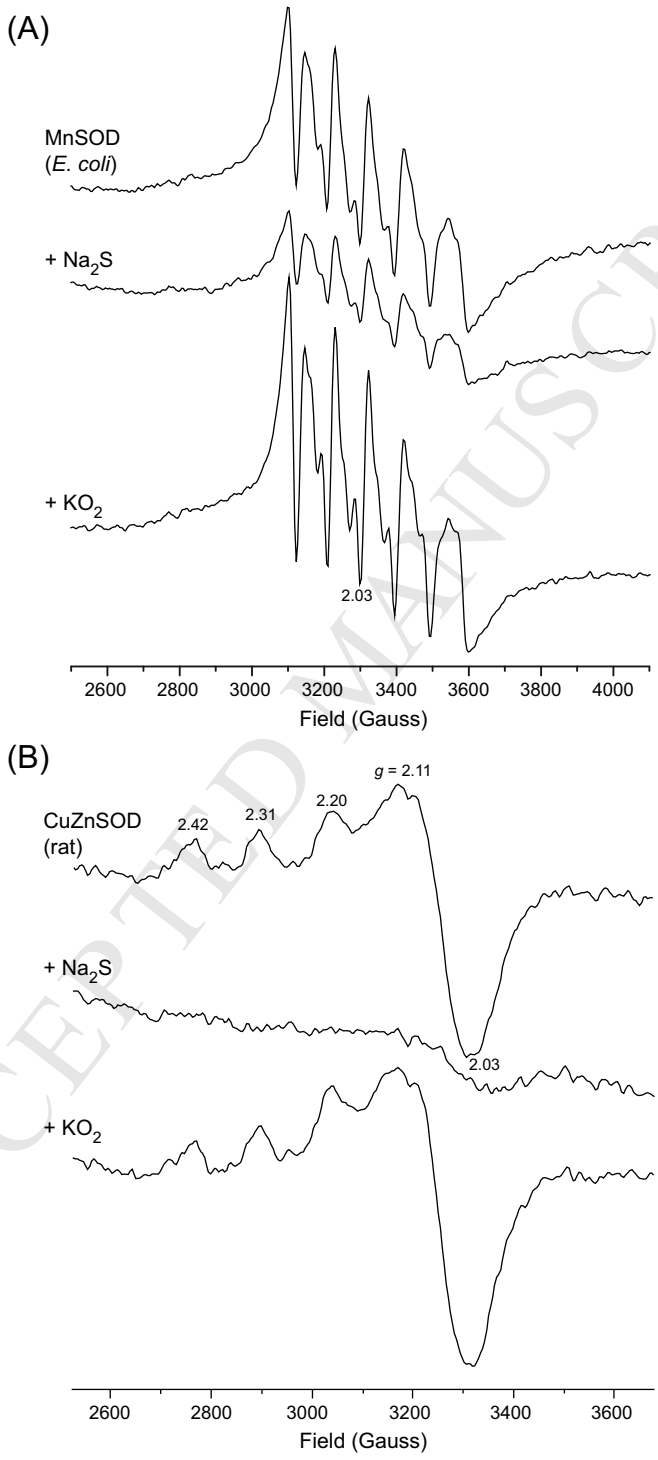
Figure legends

Fig. 1. 20K EPR spectra of FeSOD. (A) FeSOD from *E. coli*; (B) FeSOD from *P. leiognathi*. The concentration of enzyme was 100 μM in HEPES buffer (50 mM, pH = 7.4). The concentrations of Na_2S and KO_2 were 2 mM and 1 mM. Signal of non-specifically bound Fe^{3+} is at g = 4.25.

Fig. 2. 20K EPR spectra of MnSOD and CuZnSOD. (A) MnSOD from *E. coli*. Signal of non-specifically bound Fe^{3+} is at g = 4.25. (B) CuZnSOD from rat. The concentration of enzyme was 100 μM in HEPES buffer (50 mM, pH = 7.4). The concentrations of Na_2S and KO_2 were 2 mM and 1 mM, respectively.

Fig. 3. Proposed mechanisms of reactions of HS^-/H_2S with SODs. (A) Redox potentials of interest. (B) The reaction of HS^-/H_2S with MnSOD. Mn^{2+} is released from the active center. Details, such as the products of HS^-/H_2S are not known. (C) The proposed reactions of HS^- with CuZnSOD. HS^- binds to Cu^{2+} in the active center and causes reduction. $HS^{\bullet}$ is released and probably undergoes rapid deprotonation and reaction with another HS^- [36].

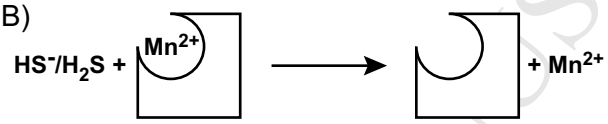




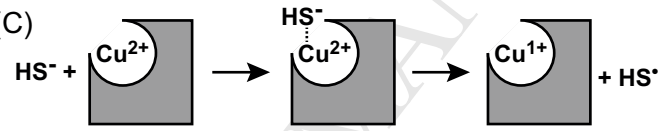
(A)

$HS^- \rightarrow HS^\bullet + e^-$	$E^o = 920$ mV
$H_2S \rightarrow HS^\bullet + H^+ + e^-$	$E^o = 920$ mV
$HS^- \rightarrow S^0 + H^+ + 2e^-$	$E^o = 170$ mV
$Fe^{3+}SOD + e^- \rightarrow Fe^{2+}SOD$	$E^o = 110$ mV
$Mn^{3+}SOD + e^- \rightarrow Mn^{2+}SOD$	$E^o \approx 300$ mV
$Cu^{2+}ZnSOD + e^- \rightarrow Cu^{1+}ZnSOD$	$E^o \sim 120–525$ mV

(B)



(C)



Highlights

- FeSOD from *E. coli* and *P. leiognathi* are resistant to HS^-/H_2S-induced reduction
- HS^-/H_2S appears to provoke a release of manganese from MnSOD
- HS^-/H_2S reduced Cu^{2+} to Cu^{1+} in CuZnSOD
- 5 d-electron systems predominate in MnSOD and FeSOD