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

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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 ($O_2^{\cdot -}$) 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 $O_2^{\cdot -}$ 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.

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 ($O_2^{\cdot -}$) 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 $O_2^{\cdot -}$ from molecular oxygen [4]. Importantly, they share common targets, including superoxide dismutases (SOD). It has been shown that HS^- enhances $O_2^{\cdot -}$ 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 $O_2^{\cdot -}$, have been gathered. Pertinent to this, we examined and compared the reactions of metallocenters of FeSOD

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.

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(from *Escherichia coli* and *P. leiognathi*), MnSOD (from *E. coli*), and CuZnSOD (from rat) with HS^-/H_2S (donor: Na_2S) and $O_2^{\cdot -}$ (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 $O_2^{\cdot -}$ in 1:1 ratio. KO_2 is rapidly decomposed in water to give $O_2^{\cdot -}$ 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 (<2 mM) [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 $O_2^{\cdot -}$ 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 20 K 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 $O_2^{\cdot -}$ can react with FeSOD active center. A modest decrease of the level of Fe^{3+}SOD following exposure to $O_2^{\cdot -}$ donor might be explained by the fact that the first half-reaction (Fe^{3+}SOD → Fe^{2+}SOD) in $O_2^{\cdot -}$ 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

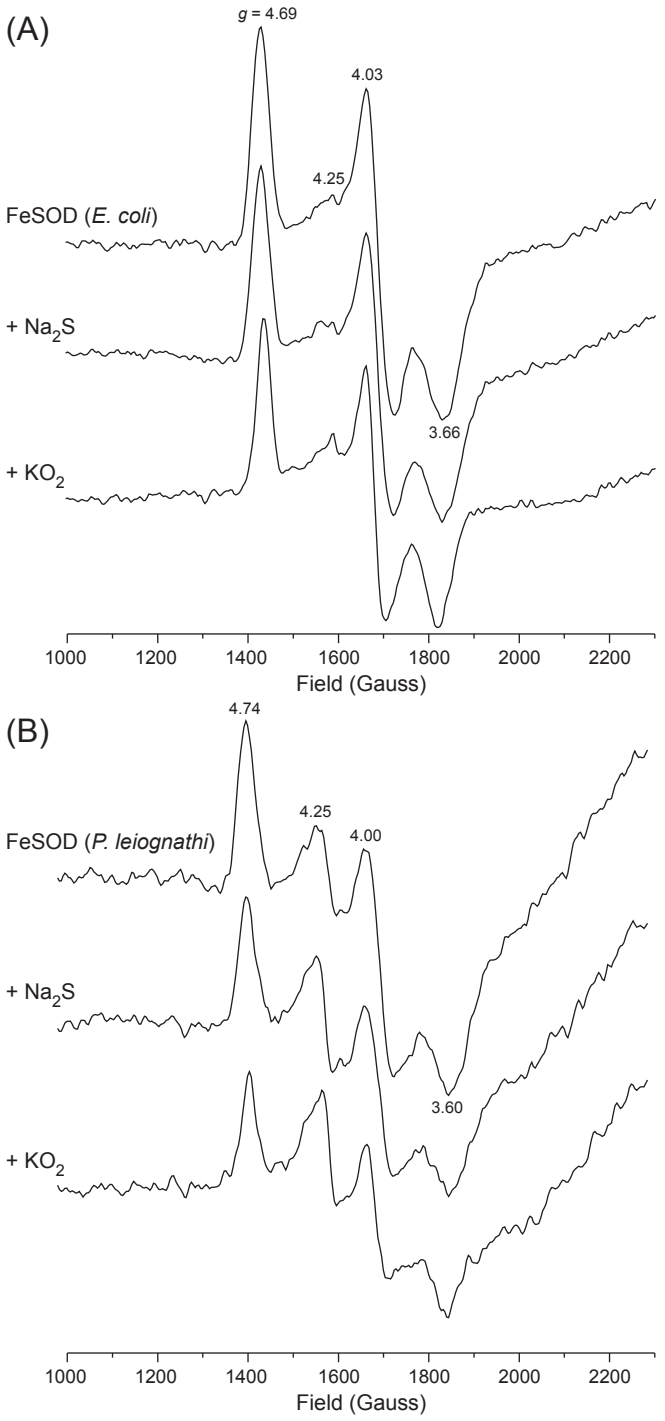

Fig. 1. 20 K EPR spectra of FeSOD. (A) FeSOD from *E. coli*; (B) FeSOD from *P. leiognathi*. The concentration of enzyme was 100 μM in HEPES buffer (50 mM, pH = 7.4). The concentrations of Na_2S and KO_2 were 2 mM and 1 mM. Signal of non-specifically bound Fe^{3+} is at $g = 4.25$.