Published in final edited form as:

Free Radic Biol Med. 2009 July 15; 47(2): 128–134. doi:10.1016/j.freeradbiomed.2009.04.006.

New Insights into the Detection of Sulfur Trioxide Anion Radical by Spin Trapping: Radical Trapping versus Nucleophilic Addition

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Abstract

It has recently been proposed that (bi)sulfite (hydrated sulfur dioxide) reacts with 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) in biological systems via a nonradical, nucleophilic reaction, implying that the radical adduct (DMPO/*SO₃[−]) formation in these systems is an artifact and not the result of spin trapping of sulfur trioxide anion radical (*SO₃[−]). Here, the one-electron oxidation of (bi)sulfite catalyzed by horseradish peroxidase/H₂O₂ has been re-investigated by ESR spin trapping with DMPO and oxygen uptake studies in order to obtain further evidence for the radical reaction mechanism. In the case of ESR experiments, the signal of DMPO/*SO₃[−] radical adduct was detected, and the initial rate of its formation was calculated. Support for the radical pathway via *SO₃[−] was obtained from the stoichiometry between the amount of consumed molecular oxygen and the amount of (bi)sulfite oxidized to sulfate (SO₄^{2−}). When DMPO was incubated with (bi)sulfite, oxygen consumption was completely inhibited due to the efficiency of DMPO trapping. In the absence of DMPO, the initial rate of oxygen and H₂O₂ consumption was determined to be half of the initial rate of DMPO/*SO₃[−] radical adduct formation as determined by ESR, demonstrating that DMPO forms the radical adduct by trapping the *SO₃[−] exclusively. We conclude that DMPO is not susceptible to artifacts arising from nonradical chemistry (nucleophilic addition) except when both (bi)sulfite and DMPO concentrations are at nonphysiological levels of at least 0.1 M and the incubations are for longer time periods.

Keywords

ESR spin trapping; DMPO; sulfur-derived radicals; kinetics

Introduction

Sulfur dioxide, one of the major atmospheric pollutants, is water-soluble and, in aqueous solution at neutral pH, exists primarily as sulfite (SO₃^{2−}) and (bi)sulfite (HSO₃[−]) (pK_a = 7.2) [1–5]. Due to its antioxidant and antimicrobial properties, (bi)sulfite is used extensively as a preservative in beverages and foods [6], but it has also been reported that (bi)sulfite is toxic at high concentrations [7]. (Bi)sulfite has been studied for decades, and it is believed that detoxification in vivo occurs primarily as a result of the function of sulfite oxidase, a

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mitochondrial enzyme which oxidizes (bi)sulfite to sulfate via one two-electron oxidation step without any radical formation [7–9].

In addition to the sulfite oxidase route, (bi)sulfite can be oxidized to sulfate by trace transition metal ions via free radicals. This metal-catalyzed autooxidation generates sulfur trioxide anion radical ($\bullet\text{SO}_3^-$) in an initiating one-electron oxidation step through an oxygen-consuming chain reaction [3,5,10,11]. It has also been shown that the one-electron oxidation of (bi)sulfite to sulfate is catalyzed enzymatically by prostaglandin H synthase (hydroperoxide) [12], horseradish peroxidase [13,14] and xanthine oxidase [15–17] with formation of $\bullet\text{SO}_3^-$. This predominantly sulfur-centered radical [18] reacts with molecular oxygen by forming peroxymonosulfate anion radical ($^-\text{O}_3\text{SOO}^\bullet$), which upon reaction with excess (bi)sulfite forms the sulfate anion radical ($\text{SO}_4^{\bullet-}$).

Two of these free radicals were characterized in biological systems with the ESR spin-trapping technique using the spin traps 5,5-dimethyl-1-pyrroline *N*-oxide (**1**, DMPO) [11,12,17,19,20] and 5-(diethoxy-phosphoryl)-5-methyl-pyrrolidine *N*-oxide (DEPMPO) [21–23]. ($\bullet\text{SO}_3^-$ was also detected by direct ESR [14].) The ESR spin-trapping experiments demonstrated formation of nitroxide radical adduct **2** (DMPO/ $\bullet\text{SO}_3^-$), which has been reported to occur through the radical addition across the double bond of the spin trap. However, Potapenko et al. have recently reported that DMPO and DEPMPO undergo nucleophilic addition of sulfite with the formation of the corresponding hydroxylamine adduct **3** which, upon mild one-electron oxidation, could form radical adduct **2** [24,25]. They caution that a nonradical mechanism may be the actual origin of the paramagnetic adduct and suggest that more careful analysis and additional experiments are necessary to prove the radical mechanism.

The suggestion of Potapenko et al. is a special case of a more general issue that the formation of radical adducts may occur by nonradical nucleophilic reactions [26–28]. In fact, Eberson and coworkers described several cases with nitron spin traps such as DMPO and α -phenyl-*N*-tert-butyl nitron (PBN) where the detection of radical adduct does not necessarily involve radical attack upon the spin trap (Eqn. (1)) [29,30]. The two phenomena leading to artifacts in spin trapping are “inverted spin trapping” [31,32] and the Forrester-Hepburn mechanism [33](Eqn. (2)):

(1)

(2)

where Ox = $\text{K}_3\text{Fe}(\text{CN})_6$ [24] or $\text{Na}_2\text{Cr}_2\text{O}_7$ [25].

The mechanism suggested by Forrester and Hepburn is initiated by addition of a nucleophile such as $\text{HSO}_3^-/\text{SO}_3^{2-}$ to spin trap **1**. The resulting hydroxylamine adduct **3** can easily be oxidized by oxidants to give a radical adduct identical to **2**. "Inverted spin trapping" is applicable under strongly oxidizing conditions that allow oxidation of the spin trap to its radical cation ($\text{DMPO}^{\bullet+}$), which can further react with a nucleophile and give radical adduct **2**. However, DMPO is very stable and can be oxidized only at very high potential ($E_{\text{DMPO}^{\bullet+}/\text{DMPO}} = 1.63 \text{ V}$) [34]. This limits the reaction to situations employing photochemical oxidation of the spin trap or the use of strong oxidants such as tris(4-bromophenyl)ammonium ion ($\text{TBPA}^{\bullet+}$) [31], followed by further reaction of the cation radical with nucleophiles (hydrogendiacetate, tetramethylsuccinimide, triethylphosphite) [32]. Bhattacharjee et al. have shown the formation of DMPO cation radical upon ionizing radiation in dry Freon matrices at 77°K [35]. On melting in the absence of water, the $\text{DMPO}^{\bullet+}$ was found to be very stable, but in the presence of water in very low concentrations, the radical cations were converted into $\text{DMPO}/\bullet\text{OH}$ adducts.

Herein, we have re-investigated the reactions of sulfur trioxide anion radical ($\bullet\text{SO}_3^-$) formed from the one-electron oxidation of (bi)sulfite in the horseradish peroxidase/ H_2O_2 system using ESR spin trapping and oxygen and H_2O_2 uptake experiments. We have compared the initial rate of formation of the spin-trap radical adduct with the rate of oxygen and H_2O_2 consumption in the absence of the spin trap. It was found that the formation of $\text{DMPO}/\bullet\text{SO}_3^-$ is twice as fast as the consumption of oxygen and H_2O_2 , in agreement with the radical mechanism. From these data, we conclude that the paramagnetic species $\text{DMPO}/\bullet\text{SO}_3^-$ is the product of a reaction between the spin trap and the free anion radical, and there is no possibility for nonradical reactions between (bi)sulfite and the spin trap under our experimental conditions or the conditions normally used in biochemical systems.

Materials and methods

Chemicals

Sodium sulfite (Na_2SO_3), hydrogen peroxide (30 %), TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl), diethylenetriaminepentaacetic acid (DTPA) and horseradish peroxidase (Type VI) were from Sigma Chemical Co. (St. Louis, MO). The hydrogen peroxide concentration was determined from the absorbance at 240 nm ($\epsilon = 39.4 \text{ M}^{-1}\text{cm}^{-1}$). DMPO (high purity, $\geq 99\%$) from Alexis Biochemicals (San Diego, CA) was sublimed twice under vacuum at room temperature and stored under argon atmosphere at -80°C before use. Chelex-100 resin was purchased from Bio-Rad Laboratories (Hercules, CA).

ESR spectroscopy

ESR spectra and their time courses were obtained with a Bruker EMX spectrometer. The instrument was equipped with an ER 4122 SHQ cavity operating at 9.78 GHz at room temperature. For quantitative ESR experiments, a standard curve of double-integrated intensity of the spin standard 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPOL) was used. The concentration of TEMPOL was determined in distilled water at 429 nm ($\epsilon = 13.4 \text{ M}^{-1}\text{cm}^{-1}$) [36]. Starting with 100 mM stock, TEMPOL solutions were diluted to 300 μM , 200 μM , 100 μM , 75 μM , 50 μM , 25 μM , 12.5 μM and 1 μM . The double integral of the ESR spectra from each dilution was calculated using WinEPR software. The concentrations of $\text{DMPO}/\text{SO}_3^-$ were determined from the plots of double integrals of samples on the standard curve for TEMPOL. Typically, sodium sulfite (Na_2SO_3) was mixed with DMPO (100 mM) and H_2O_2 , and horseradish peroxidase was added to initiate the enzymatic reaction. The mixture was transferred to a flat cell immediately after horseradish peroxidase addition, and the recording of spectra was initiated within 1 min of starting the reaction. The samples were prepared in phosphate buffer (100 mM) stored over Chelex-100 for 24 h, followed by the addition of 100 μM DTPA to suppress possible transition metal-catalyzed reactions. The ESR spectrometer settings were as follows: modulation frequency, 100 kHz; modulation amplitude, 1.0 G; scan range, 80 G; microwave power, 20 mW; receiver gain, 5×10^5 ; time constant, 2.56 ms; and sweep time, 2.62 s. The double integral values are an average of 64 scans. Data presented are the means $\pm$ SD from three independent determinations using freshly prepared solutions.

The kinetic studies were performed using an aspiration of samples for analysis of *in situ* radicals by ESR [37]. The incubations in the flat cell were replaced while the spectrometer was still in the operate mode to avoid any change in the tuning and the position of the cell. The incubation mixture (3.6 mL) contained various concentrations of sodium sulfite, DMPO (100 mM), H_2O_2 (0.5 μM), and horseradish peroxidase (0.5 μM) as a radical initiator and was aspirated into the flat cell through stainless steel needle tubing. The elapsed time from the initiation of the reaction in a vortexed sample until the sample was within the microwave cavity was 3 sec. The spectrometer settings for the time-sweep spectra were as follows: modulation frequency, 100 kHz; modulation amplitude, 2.0 G; field position, 3468 G; microwave power, 20 mW; receiver gain, 5×10^5 ; time constant, 328 ms; and sweep time, 336 s.

Oxygen uptake

Typically, sodium sulfite and H_2O_2 were mixed in a 2-mL chamber equipped with a Clark electrode and a stirrer. The reaction mixture (1.8 mL) was initiated by horseradish peroxidase (equimolar to H_2O_2), and the oxygen uptake curves were obtained at room temperature with a Yellow Springs Instrument Company oxygen monitor (model 53). For quantitative analysis, the oxygen polarographic electrode was calibrated by the depletion of oxygen by the oxidation of hypoxanthine in the presence of xanthine oxidase and catalase [38]. The oxygen solubility was also calculated from Henry's law using the atmospheric pressure value taken from the Internet for the nearest location. Data presented are the means $\pm$ SD from three independent determinations using fresh preparations of all reaction components.

The computer simulation of kinetic curves was performed using Gepasi (3.30) software that is available to the public through the Internet (<http://www.gepasi.org>) [39].

Hydrogen peroxide-selective electrode experiments

Hydrogen peroxide measurements were carried out using an H_2O_2 -selective electrode (APOLLO 4000 Free Radical Analyzer; World Precision Instruments, Sarasota, FL). Experiments were performed at 25 $^\circ\text{C}$ by immersing the electrode in 3 mL of 100 mM phosphate buffer, pH 7.2. Hydrogen peroxide at a concentration of 0.5 μM was added to a

continuously stirred buffer solution containing various concentrations of sodium sulfite. Where indicated, horseradish peroxidase (0.5 μM final) was added to the reaction mixture, and the rise and decay of H_2O_2 concentration were monitored. Data presented are the means $\pm$ SD from three independent determinations using fresh preparations of all reaction components.

Results

ESR spin trapping of sulfur trioxide anion radical ($\text{SO}_3^{\cdot-}$) by DMPO

When sodium sulfite (50 mM) was mixed with H_2O_2 (10 μM) and the reaction was initiated by adding horseradish peroxidase (10 μM), it produced a sulfur trioxide anion radical ($\text{SO}_3^{\cdot-}$) which was trapped with 100 mM DMPO (Fig. 1A, spectrum a). The hyperfine coupling constants of the assigned radical adduct, $\text{DMPO}/\text{SO}_3^{\cdot-}$ ($a_{\beta}^{\text{H}} = 16.0$ G and $a^{\text{N}} = 14.7$ G), are consistent with previous reports [12,19]. Control experiments with horseradish peroxidase, (bi)sulfite, or DMPO omitted resulted in no radical adduct formation (Fig. 1A, spectra b, e, and f, respectively). In the absence of H_2O_2 , only the control reaction contained a relatively small amount of the radical adduct (Fig. 1A, spectrum c). This peroxidase activity could be explained by a small amount of contaminating peroxide formed by autoxidation of (bi)sulfite; it was inhibited by catalase (Fig. 1A, spectrum d). The double integral of spectrum a is also presented as an example of quantitation by the integration of the ESR absorption of the sample (see below).

The proposed mechanism of enzymatic oxidation of (bi)sulfite to the $\text{SO}_3^{\cdot-}$ radical by the horseradish peroxidase/ H_2O_2 system occurs by two sequential one-electron reduction reactions of compound I by (bi)sulfite [12,14,19,40]:

The net equation is:

The reaction rate of $\text{SO}_3^{\cdot-}$ formation in equation (6) can be represented by:

$$-\frac{d}{dt}[\text{H}_2\text{O}_2] = -\frac{1}{2}\frac{d}{dt}[\text{SO}_3^{2-}] = \frac{1}{2}\frac{d}{dt}[\text{SO}_3^{\cdot-}] = \frac{1}{2}\frac{d}{dt}[\text{DMPO}/\text{SO}_3^{\cdot-}] \quad (7)$$

To confirm the stoichiometry in eq. (6), we measured the yield of the radical adduct $\text{DMPO}/\text{SO}_3^{\cdot-}$ relative to the concentration of H_2O_2 using quantitative spin-trapping ESR analysis. Samples containing different amounts of (bi)sulfite were incubated with 100 mM DMPO and 10 μM H_2O_2 , and then the reaction was initiated with 10 μM horseradish peroxidase. The double-integrated intensity of each ESR spectrum (Fig. 1A) was plotted on a standard curve for a nitroxide, TEMPOL, to calculate the concentration of $\text{DMPO}/\text{SO}_3^{\cdot-}$ that

was present in the flat cell. Fig. 1B shows that the calculated concentrations of the radical adduct were proportional to the concentrations of (bi)sulfite, and the data were fitted to an exponential growth. After extrapolating the plateau value to the Y-axis, we obtained a maximum yield of DMPO/ $\bullet\text{SO}_3^-$ adduct of $19.5 \pm 0.3 \mu\text{M}$. This 2:1 stoichiometry ($2\text{DMPO}/\bullet\text{SO}_3^- : 1\text{H}_2\text{O}_2$, $10 \mu\text{M}$) is in excellent quantitative agreement with the calculated stoichiometry in equation (6).

Oxygen consumption during (bi)sulfite oxidation in the presence or absence of DMPO – quantitative and kinetic analysis

To provide a more complete understanding of the radical mechanism of sulfur-derived radicals in the horseradish peroxidase/ H_2O_2 system, we extended the oxygen uptake experiments previously reported in the model with this system [12,14]. In this sense, molecular oxygen is a spin trap not unlike DMPO. In agreement with these reports, when millimolar (bi)sulfite (3–30 mM range) was incubated with $10 \mu\text{M}$ H_2O_2 and initiated with $10 \mu\text{M}$ horseradish peroxidase, a very fast oxygen consumption was observed; control experiments confirmed that all the components had to be present in order to observe oxygen consumption (data not shown). The oxygen consumption is a result of the formation of peroxymonosulfate radical, $^-\text{O}_3\text{SOO}^\bullet$, in the following free radical chain mechanism [3,19,41]:

The reaction rate of oxygen consumption in equation (12) can be represented by:

$$-\frac{d}{dt}[\text{O}_2] = -\frac{1}{2} \frac{d}{dt}[\text{SO}_3^{2-}] = \frac{1}{2} \frac{d}{dt}[\text{SO}_4^{2-}], \quad (13)$$

The initial rate of H_2O_2 consumption was also predicted to be one half of the rate at which (bi)sulfite disappears and one half the rate at which the radical adduct DMPO/ $\bullet\text{SO}_3^-$ appears according to eqn. (7). Therefore, we obtain for the initial rate equation:

$$\text{Rate} = -\frac{d}{dt}[\text{O}_2] = -\frac{d}{dt}[\text{H}_2\text{O}_2] = \frac{1}{2} \frac{d}{dt}[\text{DMPO}/\bullet\text{SO}_3^-] \quad (14)$$

This mechanism has been accepted and supported by various experiments [19,43,44]. In order to confirm the “purity” of the radical mechanism between the DMPO and SO_3^- discussed above, we measured the stoichiometric molar ratio between the oxygen and (bi)sulfite from the equation (12) and calculated the initial rate of oxygen consumption.

Figure 2A shows the simulations [39] of the kinetic system (bi)sulfite/horseradish peroxidase/ H_2O_2 based on equations (9)-(11) and the previously published rate constants [42]. The initial concentration of oxygen determined in phosphate buffer, pH 7.4, was 245 μM , and this value was used in the simulation program (see Materials and Methods). The concentration of (bi)sulfite was 300 μM ; the initiating concentration of the SO_3^- anion radical was chosen to match the experimental curve and was determined to be only 1.4×10^{-7} μM . This result supports the literature in that SO_3^- formation is dominated by the radical chain reaction. As expected, a complete conversion of (bi)sulfite to sulfate was calculated, and one-half as much oxygen (~ 147 μM) was predicted to be consumed (Fig. 2A), which is in agreement with the mechanism discussed above.

Based on this simulation, in the next set of oxygen uptake experiments, 0–500 μM (bi)sulfite was mixed with 10 μM H_2O_2 , and the reaction was initiated with 10 μM horseradish peroxidase. Figure 2B demonstrates the dependence of oxygen consumption on the (bi)sulfite concentration. A very good agreement between the calculated and experimental oxygen consumption was observed for spectrum d in Fig. 2B, where the (bi)sulfite concentration (300 μM) was the same as in the simulated data. The experimental oxygen consumption was $148.0 \mu\text{M} \pm 2.6$, in good agreement with the theoretical stoichiometric value of 150 μM and the simulated amount of consumed oxygen (Fig. 2A).

Next we evaluated the DMPO concentration dependence of oxygen uptake experiments using 300 μM (bi)sulfite, 10 μM H_2O_2 , and 10 μM horseradish peroxidase (Fig. 3). In the presence of only 100 μM DMPO (spectrum d), oxygen uptake was inhibited by more than 50%. Incubation of (bi)sulfite with higher DMPO concentrations (1–100 mM) showed that the inhibition of oxygen consumption positively correlated with the spin trap concentration. Interestingly, at the concentrations of DMPO usually used in ESR experiments (100 mM), oxygen consumption was inhibited almost completely (Fig. 3, spectrum a). This result demonstrates again the very high efficiency of DMPO trapping of the SO_3^- anion radical formed either during the horseradish peroxidase-catalyzed oxidation of (bi)sulfite or the subsequent chain reaction. The effect of DMPO was simulated for spectrum d using the rate constant for the reaction between SO_3^- anion radical and the spin trap ($k = 1.2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ [45]) (data not shown).

Quantitative and kinetic ESR analysis. Calculation of the initial rates of oxygen and H_2O_2 consumption and formation of DMPO/ SO_3^- radical adduct

To provide further insight into the radical mechanism of (bi)sulfite oxidation, kinetic ESR spin-trapping experiments were performed. Concentrations of the reactants were adjusted to give a reaction rate slow enough for conventional ESR instruments. Figure 4A shows a time course plot of the first peak (at 3468 G and $\nu = 9.766$ GHz) of the DMPO/ SO_3^- signal. The samples of micromolar (bi)sulfite (75–500 μM) were mixed with 100 mM DMPO and 0.5 μM H_2O_2 . The reaction was initiated with 0.5 μM horseradish peroxidase and immediately aspirated into the flat cell (see Materials and Methods). In this incubation, the radical adduct formation increased linearly for the first 25–30 sec. At higher initial concentrations of (bi)sulfite (400 μM and 500 μM), the maximum accumulation of DMPO/ SO_3^- adduct was observed at ~ 100 –150 sec after horseradish peroxidase initiation, followed by decay. This maximum could be explained by the absence of sufficient H_2O_2 to continue SO_3^- generation in the presence of 100 mM DMPO where the chain reaction is blocked. The initial rates of the reaction of SO_3^- with DMPO were measured for each particular concentration of (bi)sulfite oxidized

by 0.5 μM H_2O_2 /0.5 μM horseradish peroxidase by using a linear fit of the first 30 sec of the time course (see below).

Oxygen uptake experiments were performed using the same conditions (in the absence of DMPO) as for the kinetic ESR spin-trapping experiments described above. When 75–500 μM (bi)sulfite was mixed with 0.5 μM H_2O_2 and 0.5 μM horseradish peroxidase as initiator, the dependence of oxygen consumption on (bi)sulfite concentration was measured (Fig. 4B). In order to test whether the formation of the sulfur trioxide anion radical is a result of the one-electron oxidation step of (bi)sulfite rather than from the nucleophilic addition of (bi)sulfite to the spin trap DMPO followed by the oxidation of the resulting hydroxylamine adduct, we compared the initial rate of the $\text{DMPO}/\cdot\text{SO}_3^-$ radical adduct formation for the ESR spin trapping with the initial rate of oxygen consumption under the same conditions. Figure 5A represents the time course of both techniques using 300 μM (bi)sulfite and 0.5 μM H_2O_2 /0.5 μM horseradish peroxidase. The first 30 sec after initiation of the reaction were fitted using linear regression (the dotted lines), and the values obtained for the initial rates were $1.49 \pm 0.02 \text{ nM sec}^{-1}$ and $2.83 \pm 0.05 \text{ nM sec}^{-1}$ for oxygen uptake and the ESR time courses, respectively. These values are in very good agreement with equation (14) where the rate of $\text{DMPO}/\cdot\text{SO}_3^-$ radical adduct formation is twice that of the oxygen consumption. This result suggests that the $\cdot\text{SO}_3^-$ radical formation is entirely responsible for the oxygen consumption and that the $\text{DMPO}/\cdot\text{SO}_3^-$ radical adduct is a true product of $\cdot\text{SO}_3^-$.

For comparison, the above experiments were repeated (in the absence of DMPO) using an H_2O_2 -selective electrode to determine the initial rate of H_2O_2 consumption. As a control, horseradish peroxidase (0.5 μM) was added to a continuously stirred H_2O_2 (0.5 μM) buffer solution, where it catalyzed a very slow consumption of peroxide due to catalase activity (Fig. 5B, trace b). This background reaction was not taken into account for the calculation of the initial rate (see below). When the same experiments were repeated by adding increasing amounts of (bi)sulfite, the rate of H_2O_2 consumption was significantly increased (traces c–f), indicating that (bi)sulfite has a role in accelerating this process. The time course corresponding to the experiments shown in Fig. 5A is presented as trace d in Fig. 5B. The initial rate of H_2O_2 consumption was calculated by fitting the first 30 sec, and the value obtained was $1.38 \pm 0.09 \text{ nM sec}^{-1}$, which is almost equal to the oxygen consumption rate and approximately 50% of the initial rates of $\text{DMPO}/\cdot\text{SO}_3^-$ radical adduct formation calculated above. From the rate equations (7), (13) and (14), the ratio between the rates of H_2O_2 consumption and sulfur trioxide anion radical formation should be 1:2 and, in fact, this was observed.

Discussion

The present data confirm that the enzymatic oxidation of (bi)sulfite by horseradish peroxidase and H_2O_2 proceeds via a radical mechanism as demonstrated using quantitative oxygen and H_2O_2 uptake and ESR spin-trapping experiments. It has recently been reported that the reaction between DMPO and 100 mM (bi)sulfite in the presence of potassium ferricyanide as an oxidant represents a typical case of the Forrester-Hepburn mechanism (Eqn. (2)), i.e., a reversible addition of the nucleophile ($\text{HSO}_3^-/\text{SO}_3^{2-}$) to spin trap **1** to give a hydroxylamine derivative **3**, followed by oxidation of the latter [24,33].

Our ESR spin-trapping data (Fig. 4A) show that mixing DMPO with micromolar (bi)sulfite followed by immediate oxidation by horseradish peroxidase/ H_2O_2 does form the $\text{DMPO}/\cdot\text{SO}_3^-$ radical adduct. Previous work from Potapenko and co-workers on the reaction mechanism of (bi)sulfite oxidation in the presence of DMPO or DEPMPO suggested that a non-radical addition of (bi)sulfite with DMPO was responsible for the ESR detection of the radical adduct [24,25]. A reversible nucleophilic addition of (bi)sulfite anion to the double bond of the spin traps was proposed, resulting in the formation of the hydroxylamine which,

upon oxidation by ferricyanide or sodium dichromate, formed an ESR-detected DMPO/ $\bullet\text{SO}_3^-$ complex. According to the authors, this radical adduct formation is an example of a detectable paramagnetic species that did not occur from radical chemistry.

Our demonstration of kinetically coupled ESR and oxygen consumption experiments on the DMPO/ $\bullet\text{SO}_3^-$ formation in the current work directly tested this hypothesis. The reported DMPO-sulfite hydroxylamine adduct was detected after pre-incubation of 100 mM (bi)sulfite with DMPO [24,25]. In short, high concentrations of both (bi)sulfite and DMPO were used for long incubation times. These experimental conditions facilitate the relatively slow nucleophilic addition of (bi)sulfite to the double bond of the nitron spin trap, resulting in the formation of NMR-detected hydroxylamine with a calculated equilibrium constant $K_{\text{eq}} \approx 181 \text{ mol}^{-1}$. It is very unlikely that the low concentration of H_2O_2 and the immediate recording of the ESR spectra (within 3 min) allow the corresponding DMPO-sulfite hydroxylamine to accumulate and be further oxidized as is described by Potapenko et al. [24,25]. Furthermore, these prior studies, in which the radical adduct formation was reported to form by oxidation of the hydroxylamine by millimolar concentrations of ferricyanide or sodium dichromate [24,25], neglected the fact that Fe(III) and Cr(VI) are known to catalyze the autoxidation of (bi)sulfite via $\bullet\text{SO}_3^-$ [11,46–51]:

where $M = \text{Fe(III)}$ or CrO_4^{2-} .

Our results showed an evident effect of correlation between the calculated rates of DMPO/ $\bullet\text{SO}_3^-$ ESR formation and both oxygen and H_2O_2 consumption, which suggests that if reversible nucleophilic addition exists under our experimental conditions, it affects neither the quantitative analysis of the data nor the conclusion about the biochemical origin of the DMPO/ $\bullet\text{SO}_3^-$ adduct.

The oxygen consumption observed during the oxidation of (bi)sulfite by horseradish peroxidase/ H_2O_2 can be explained by equations (8)–(12). Sulfur trioxide anion radical ($\bullet\text{SO}_3^-$) reacts with oxygen at a diffusion-controlled rate to form $^-\text{O}_3\text{SOO}^\bullet$ which then reacts with SO_3^{2-} to produce $\text{SO}_4^{\bullet-}$. The reaction between $\text{SO}_4^{\bullet-}$ and SO_3^{2-} will readily form $\bullet\text{SO}_3^-$, which will consume another molecule of oxygen [12]. Equations (8)–(12) constitute a chain reaction by which (bi)sulfite can be oxidized via a radical mechanism once $\bullet\text{SO}_3^-$ formation is initiated. At higher concentrations of DMPO (100 mM), we observed a negligible consumption of oxygen (Fig. 3). This is likely due to the effective trapping of $\bullet\text{SO}_3^-$ and/or other radicals in the chain reaction. The second-order rate constants for the reactions between HRP Compounds I and II with (bi)sulfite at neutral pH are $7.6 \pm 0.8 \times 10 \text{ M}^{-1} \text{ s}^{-1}$ and $1.8 \pm 0.06 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$, respectively [40]. Although these reactions are relatively slow, only a low concentration of $\bullet\text{SO}_3^-$ is necessary for spin-trapping experiments and to initiate the radical chain reaction. The oxygen uptake experiments show that the spin trap and oxygen compete to react with sulfur trioxide anion radicals.

Quantitative oxygen uptake experiments (in the absence of DMPO) are in agreement with the stoichiometry of the radical mechanism according to equations (8)–(12). Our results indicate that consumption of oxygen strongly depends on the (bi)sulfite concentration in a molar ratio 1:2 as in Equation (12); this is also confirmed by our simulation data (Fig. 2A). In addition to the chain reaction, the $\bullet\text{SO}_3^-$ radicals can undergo disproportionation (Eq. (16)) and/or dimerization (Eq. (18)) [12,14,52], but these reactions are second-order in $\bullet\text{SO}_3^-$ concentration and are consequently slow at low radical concentrations even if the reaction is diffusion limited.

and/or

Apparently, it seems that the reaction with oxygen is the major pathway for the metabolism of $^{\bullet}SO_3^{-}$ radical since there is an excellent agreement between the experimentally consumed oxygen and the theoretical prediction.

We have demonstrated that, under our experimental conditions, the ESR-detected DMPO/ $^{\bullet}SO_3^{-}$ radical adduct is formed upon addition of the $^{\bullet}SO_3^{-}$ radical across the double bond of DMPO, not indirectly by nucleophilic addition of (bi)sulfite to the spin trap. The calculations from quantitative ESR experiments show that there is a strong correlation between the rate of formation of DMPO/ $^{\bullet}SO_3^{-}$ radical adduct and the rates of oxygen and H_2O_2 uptake. It has been shown that the consumption of oxygen can proceed via radical chain chemistry of $^{\bullet}SO_3^{-}$ and oxygen. Therefore, it would appear that DMPO is not susceptible to artifacts arising from nonradical chemistry (nucleophilic addition) except when both sulfite and DMPO concentrations are at nonphysiological levels of at least 0.1 M and the incubations are for longer time periods.

Acknowledgments

We are grateful to Mary Mason and Dr. Ann Motten for their editing of the manuscript. Also, we would like to acknowledge Jean Corbett for the purification of DMPO. This work was supported by the Intramural Research Program of the National Institutes of Health and the National Institute of Environmental Health Sciences.

Abbreviations

ESR	electron spin resonance
H_2O_2	hydrogen peroxide
HRP	horseradish peroxidase
Na_2SO_3	sodium sulfite
DMPO	5,5-dimethyl-1-pyrroline <i>N</i> -oxide
DEPMPO	5-(diethoxy-phosphoryl)-5-methyl-pyrrolidine <i>N</i> -oxide
PBN	

α -phenyl-*N*-*tert*-butylnitron

References

- Huss A, Eckert CA. Equilibria and ion activities in aqueous sulfur dioxide solutions. *J Phys Chem* 1977;81:2268–2270.
- Huss A, Lim PK, Eckert CA. Oxidation of aqueous sulfur dioxide. 1. Homogeneous manganese(II) and iron(II) catalysis at low pH. *J Phys Chem* 1982;86:4224–4228.
- Hayon E, Treinin A, Wilf J. Electronic spectra, photochemistry, and autoxidation mechanism of the sulfite-bisulfite-pyrosulfite systems. SO_2^- , SO_3^- , SO_4^- , and SO_5^- - radicals. *J Am Chem Soc* 1972;94:47–57.
- Connick RE, Tam TM, von Deuster E. Equilibrium constant for the dimerization of bisulfite ion to form $\text{S}_2\text{O}_5^{2-}$. *Inorg Chem* 1982;21:103–107.
- Neta P, Huie RE. Free-radical chemistry of sulfite. *Environ Health Persp* 1985;64:209–217.
- Danilewicz JC. Review of reaction mechanisms of oxygen and proposed intermediate reduction products in wine: central role of iron and copper. *Am J Enol Vitic* 2003;54:73–85.
- Gunnison AF. Sulphite toxicity: a critical review of *in vitro* and *in vivo* data. *Fd Cosmet Toxicol* 1981;19:667–682.
- Cohen HJ, Fridovich I. Hepatic sulfite oxidase. Purification and properties. *J Biol Chem* 1971;246:359–366. [PubMed: 5542006]
- Johnson JL, Rajagopalan KV. Tryptic cleavage of rat liver sulfite oxidase. Isolation and characterization of molybdenum and heme domains. *J Biol Chem* 1977;252:2017–2025. [PubMed: 14956]
- McCord JM, Fridovich I. The utility of superoxide dismutase in studying free radical reactions. I. Radicals generated by the interaction of sulfite, dimethyl sulfoxide, and oxygen. *J Biol Chem* 1969;244:6056–6063. [PubMed: 4981789]
- Shi X. Generation of $\text{SO}_3^{\bullet-}$ and OH radicals in SO_3^{2-} reactions with inorganic environmental pollutants and its implications to SO_3^{2-} toxicity. *J Inorg Biochem* 1994;56:155–165. [PubMed: 7798899]
- Mottley C, Mason RP, Chignell CF, Sivarajah K, Eling TE. The formation of sulfur trioxide radical anion during the prostaglandin hydroperoxidase-catalyzed oxidation of bisulfite (hydrated sulfur dioxide). *J Biol Chem* 1982;257:5050–5055. [PubMed: 6279657]
- Araiso T, Miyoshi K, Yamazaki I. Mechanisms of electron transfer from sulfite to horseradish peroxidase-hydroperoxide compounds. *Biochemistry* 1976;15:3059–3063. [PubMed: 8081]
- Mottley C, Trice TB, Mason RP. Direct detection of the sulfur trioxide radical anion during the horseradish peroxidase-hydrogen peroxide oxidation of sulfite (aqueous sulfur dioxide). *Mol Pharmacol* 1982;22:732–737. [PubMed: 6296662]
- Fridovich I, Handler P. Xanthine oxidase. III. Sulfite oxidation as an ultra sensitive assay. *J Biol Chem* 1958;233:1578–1580. [PubMed: 13610876]
- Fridovich I, Handler P. Detection of free radicals generated during enzymic oxidations by the initiation of sulfite oxidation. *J Biol Chem* 1961;236:1836–1840. [PubMed: 13701912]
- Sun X, Shi X, Dalal NS. Xanthine oxidase/hydrogen peroxide generates sulfur trioxide anion radical ($\text{SO}_3^{\bullet-}$) from sulfite (SO_3^{2-}). *FEBS Lett* 1992;303:213–216. [PubMed: 1318848]
- Chantry GW, Horsfield A, Morton JR, Rowlands JR, Whiffen DH. The optical and electron spin resonance spectra of SO_3^- . *Mol Phys* 1962;5:233–239.
- Mottley C, Mason RP. Sulfate anion free radical formation by the peroxidation of (Bi)sulfite and its reaction with hydroxyl radical scavengers. *Arch Biochem Biophys* 1988;267:681–689. [PubMed: 2850769]
- Constantin D, Bini A, Meletti E, Moldeus P, Monti D, Tomasi A. Age-related differences in the metabolism of sulphite to sulphate and in the identification of sulphur trioxide radical in human polymorphonuclear leukocytes. *Mech Ageing Dev* 1996;88:95–109. [PubMed: 8803926]
- Karoui H, Hogg N, Frejaville C, Tordo P, Kalyanaraman B. Characterization of sulfur-centered radical intermediates formed during the oxidation of thiols and sulfite by peroxynitrite. ESR-spin trapping and oxygen uptake studies. *J Biol Chem* 1996;271:6000–6009. [PubMed: 8626383]

22. Chamulitrat W. Activation of the superoxide-generating NADPH oxidase of intestinal lymphocytes produces highly reactive free radicals from sulfite. *Free Radical Biol Med* 1999;27:411–421. [PubMed: 10468216]
23. Liu KJ, Miyake M, Panz T, Swartz H. Evaluation of DEPMPO as a spin trapping agent in biological systems. *Free Radical Biol Med* 1999;26:714–721. [PubMed: 10218661]
24. Potapenko DI, Bagryanskaya EG, Reznikov VV, Clanton TL, Khramtsov VV. NMR and EPR studies of the reaction of nucleophilic addition of (bi)sulfite to the nitron spin trap DMPO. *Magn Reson Chem* 2003;41:603–608.
25. Potapenko DI, Clanton TL, Bagryanskaya EG, Gritsan NP, Reznikov VA, Khramtsov VV. Nonradical mechanism of (bi)sulfite reaction with DEPMPO: cautionary note for $\text{SO}_3^{\bullet-}$ radical spin trapping. *Free Radical Biol Med* 2003;34:196–206. [PubMed: 12521601]
26. Happ JW, Janzen EG. Electron spin resonance studies of radical formation in nucleophilic addition reactions. II. Cyanide ion addition to electrophilic compounds. The *N*-methyl-9-cyanoacridanyl radical. *J Org Chem* 1970;35:96–102.
27. Janzen EG, Pickett JB, Happ JW, DeAngelis W. Electron spin resonance studies of radical formation in nucleophilic addition reactions. I. Radical formation and chemiluminescence in the hydroxide ion addition to *N,N'*-dimethyl-9,9'-biacridinium dinitrate (lucigenin). *J Org Chem* 1970;35:88–95.
28. Happ JW, Janzen EG, Rudy BC. Electron spin resonance studies of radical formation in nucleophilic addition reactions. III. On the mechanism of radical formation and chemiluminescence in the cyanide addition and oxygenation of *N*-methylacridinium chloride. *J Org Chem* 1970;35:3382–3389.
29. Ebersson L. Formation of hydroxyl spin adducts via nucleophilic addition-oxidation to 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO). *Acta Chem Scand* 1999;53:584–593.
30. Alberti A, Carloni P, Ebersson L, Greci L, Stipa P. New insights into *N*-tert-butyl- α -phenylnitron (PBN) as a spin trap. Part 2. The reactivity of PBN and 5,5-dimethyl-4,5-dihydropyrrole *N*-oxide (DMPO) toward *N*-heteroaromatic bases. *J Chem Soc, Perkin Trans* 1997;2:887–892.
31. Ebersson L. 'Inverted Spin Trapping'. Reactions between the radical cation of α -phenyl-*N*-tert-butyl nitron and ionic and neutral nucleophiles. *J Chem Soc, Perkin Trans* 1992;2:1807–1813.
32. Ebersson L. Inverted Spin Trapping. Part III. Further studies on the chemical and photochemical oxidation of spin traps in the presence of nucleophiles. *J Chem Soc Perkin Trans* 1994;2:171–176.
33. Forrester AR, Hepburn SP. Spin traps. A cautionary note. *J Chem Soc C* 1971:701–703.
34. McIntire GL, Blount HN, Stronks HJ, Shetty RV, Janzen EG. Spin trapping in electrochemistry. 2. Aqueous and nonaqueous electrochemical characterizations of spin traps. *J Phys Chem* 1980;84:916–921.
35. Bhattacharjee S, Khan MN, Chandra H, Symons MCR. Radical cations from nitron spin-traps: reaction with water to give OH adducts. *J Chem Soc, Perkin Trans* 1996;2:2631–2634.
36. Kooser RG, Kirchman E, Matkov T. Measurements of spin concentration in electron paramagnetic resonance spectroscopy. *Concepts in Magn Reson* 1992;4:145–152.
37. Mason RP. Assay of in situ radicals by electron spin resonance. *Meth Enzymol* 1984;105:416–422. [PubMed: 6328195]
38. Holtzman JL. Calibration of the oxygen polarograph by the depletion of oxygen with hypoxanthine-xanthine oxidase-catalase. *Anal Chem* 1976;48:229–230. [PubMed: 1244763]
39. Mendes P, Kell DB. Non-linear optimization of biochemical pathways: applications to metabolic engineering and parameter estimation. *Bioinformatics* 1998;14:869–883. [PubMed: 9927716]
40. Roman R, Dunford HB. Studies on horseradish peroxidase. XII. A kinetic study of the oxidation of sulfite and nitrite by compounds I and II. *Can J Chem* 1973;51:588–596.
41. Reed GA, Curtis JF, Mottley C, Eling TE, Mason RP. Epoxidation of (+/-)-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene during (bi)sulfite autooxidation: activation of a procarcinogen by a cocarcinogen. *Proc Natl Acad Sci USA* 1986;83:7499–7502. [PubMed: 3463979]
42. Neta P, Huie RE, Ross AB. Rate constants for reactions of inorganic radicals in aqueous solution. *J Phys Chem Ref Data* 1988;17:1027–1284.
43. McElroy WJ. The aqueous oxidation of SO_2 by OH radicals. *Atmos Environ* 1986;20:323–330.

44. Erben-Russ M, Michel C, Bors W, Saran M. Determination of sulfite radical ($\text{SO}_3^{\bullet-}$) reaction rate constants by means of competition kinetics. *Radiat Environ Biophys* 1987;26:289–294. [PubMed: 3685252]
45. Taniguchi H, Madden KP. An in situ radiolysis time-resolved ESR study of the kinetics of spin trapping by 5,5-dimethyl-1-pyrroline-*N*-oxide. *J Am Chem Soc* 1999;121:11875–11879.
46. Bal Reddy K, Coichev N, van Eldik R. Redox cycling of iron in atmospheric water: the important role of sulphite. *J Chem Soc, Chem Commun* 1991:481–483.
47. Berglund J, Fronaeus S, Elding LI. Kinetics and mechanism for manganese-catalyzed oxidation of sulfur(IV) by oxygen in aqueous solution. *Inorg Chem* 1993;32:4527–4538.
48. Shi X, Mao Y. 8-Hydroxy-2'-deoxyguanosine formation and DNA damage induced by sulfur trioxide anion radicals. *Biochem Biophys Res Commun* 1994;205:141–147. [PubMed: 7999014]
49. Brandt C, Fabian I, van Eldik R. Kinetics and mechanism of the iron(III)-catalyzed autoxidation of sulfur(IV) oxides in aqueous solution. Evidence for the redox cycling of iron in the presence of oxygen and modeling of the overall reaction mechanism. *Inorg Chem* 1994;33:687–701.
50. Brandt C, Elding LI. Role of chromium and vanadium in the atmospheric oxidation of sulfur(IV). *Atmos Environ* 1998;32:797–800.
51. Lima S, Bonifacio RL, Azzellini GC, Coichev N. Ruthenium(II) tris(bipyridyl) ion as a luminescent probe for oxygen uptake on the catalyzed oxidation of HSO_3^- . *Talanta* 2002;56:547–556. [PubMed: 18968527]
52. Eriksen TE. pH Effects on the pulse radiolysis of deoxygenated aqueous solutions of sulphur dioxide. *J Chem Soc Faraday Trans I* 1974;70:208–215.

Fig. 1.

(A) Formation of radical adduct in reaction between sodium sulfite (Na_2SO_3) and H_2O_2 /horseradish peroxidase (HRP) in the presence of DMPO. (Spectrum a) Reaction mixture containing Na_2SO_3 (50 mM), DMPO (100 mM) and H_2O_2 (10 μM) in 100 mM phosphate buffer, pH 7.4. After initiation with HRP (10 μM), the mixture was placed immediately into the flat cell. The double integral of the spectrum is proportional to the concentration of DMPO/ SO_3^- radical adduct. (Spectrum b) Same as in (spectrum a) without HRP. (Spectrum c) Same as in (spectrum a) without H_2O_2 . (Spectrum d) Same as in (spectrum c) with 30,000 units of catalase/mL of incubation. (Spectrum e) Same as in (spectrum a), except the Na_2SO_3 was not added. (Spectrum f) Same as in (spectrum a) without the spin trap. (Spectrum

g) Same as in (spectrum b) without H₂O₂. The instrument parameters were as follows: microwave frequency, 9.78 GHz; microwave power, 20 mW; modulation amplitude, 1.0 G; field sweep, 80 G; receiver gain, 5×10^5 ; time constant, 2.56 ms; and sweep time, 2.62 s. The double integral values are an average of 64 scans. (B) Dependence of the concentration of DMPO/ SO_3^- radical adduct on the (bi)sulfite concentrations in the presence of 10 μM H₂O₂. Reaction mixtures containing various concentrations of sodium sulfite (0.075, 0.150, 0.200, 0.300, 0.400, 0.500, 1, 5, 25 and 50 mM), DMPO (100 mM) and 10 μM H₂O₂ were initiated with 10 μM HRP in 100 mM phosphate buffer, pH 7.4. The concentration of the paramagnetic species was calculated from the double integrals of the ESR spectra. The estimated maximum concentration of DMPO/ SO_3^- radical adduct obtained from the extrapolated nonlinear fit is $19.5 \pm 0.3 \mu\text{M}$. Error bars represent the standard deviation of three independent experiments.

Fig. 2.

(A) Computer simulation of the concentration changes of reactants as a function of time. The initial concentrations were: $[\text{O}_2]_0 = 245 \mu\text{M}$, $[\text{SO}_3^{2-}]_0 = 300 \mu\text{M}$, $[\text{SO}_3^{\cdot-}]_0 = 1.4 \times 10^{-7} \mu\text{M}$, and $[\text{SO}_4^{2-}]_0 = 0 \mu\text{M}$. (B) Oxygen uptake curves as a function of (bi)sulfite concentration. Sodium sulfite (Na_2SO_3) was placed in a chamber with $10 \mu\text{M}$ H_2O_2 in 100 mM phosphate buffer, pH 7.4, and the reaction was initiated with $10 \mu\text{M}$ horseradish peroxidase (HRP) at the times indicated. The concentration of (bi)sulfite for each curve was as follows: (a) $0 \mu\text{M}$, (b) $150 \mu\text{M}$, (c) $225 \mu\text{M}$, (d) $300 \mu\text{M}$ and (e) $500 \mu\text{M}$.

Fig. 3.

Oxygen uptake curves as a function of DMPO concentration. DMPO was placed in a chamber with sodium sulfite (300 μM) and H₂O₂ (10 μM) in 100 mM phosphate buffer, pH 7.4, and the reaction was initiated with 10 μM horseradish peroxidase (HRP) at the times indicated. The concentration of DMPO for each curve was as follows: (a) 100 mM, (b) 10 mM, (c) 1 mM, (d) 0.1 mM and (e) 0 mM.

Fig. 4.

(A) Time-sweep ESR kinetics formation of DMPO•SO₃⁻ radical adduct. Reaction mixtures containing sodium sulfite as indicated, DMPO (100 mM) and H₂O₂ (0.5 μM) were initiated with 0.5 μM horseradish peroxidase (HRP) in 100 mM phosphate buffer, pH 7.4. The instrument parameters were as follows: microwave frequency, 9.78 GHz; microwave power, 20 mW; modulation amplitude, 2.0 G; field position, 3468 G; receiver gain, 5 × 10⁵; time constant, 328 ms; sweep time, 336 s. (B) Oxygen uptake curves using identical conditions as in Fig. 4A, except without spin trap DMPO. The concentration of sodium sulfite for each curve was as follows: (a) 0 μM, (b) 75 μM, (c) 150 μM, (d) 300 μM, (e) 400 μM and (f) 500 μM.

Fig. 5.

(A) ESR time course of $\text{DMPO} \cdot \text{SO}_3^-$ adduct formation in comparison with oxygen uptake curve. The solutions for both instruments contained $300 \mu\text{M}$ Na_2SO_3 , $0.5 \mu\text{M}$ H_2O_2 , $0.5 \mu\text{M}$ horseradish peroxidase (HRP) as initiator and 100 mM DMPO (only for ESR spin trapping of SO_3^- radical). The instrumental conditions were the same as those described in the legend of Figure 4A for ESR. (B) Hydrogen peroxide consumption by HRP in the presence of sodium sulfite as a function of time. Na_2SO_3 was placed in a chamber with $0.5 \mu\text{M}$ H_2O_2 in 100 mM phosphate buffer, pH 7.4, and the reaction was initiated with $0.5 \mu\text{M}$ HRP at the times indicated. (a) H_2O_2 alone. The concentration of sodium sulfite for each curve was as follows: (b) $0 \mu\text{M}$, (c) $100 \mu\text{M}$, (d) $300 \mu\text{M}$, (e) $400 \mu\text{M}$ and (f) $500 \mu\text{M}$.