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Sulfite oxidase activity of cytochrome c: Role of hydrogen peroxide

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ABSTRACT

In humans, sulfite is generated endogenously by the metabolism of sulfur containing amino acids such as methionine and cysteine. Sulfite is also formed from exposure to sulfur dioxide, one of the major environmental pollutants. Sulfite is used as an antioxidant and preservative in dried fruits, vegetables, and beverages such as wine. Sulfite is also used as a stabilizer in many drugs. Sulfite toxicity has been associated with allergic reactions characterized by sulfite sensitivity, asthma, and anaphylactic shock. Sulfite is also toxic to neurons and cardiovascular cells. Recent studies suggest that the cytotoxicity of sulfite is mediated by free radicals; however, molecular mechanisms involved in sulfite toxicity are not fully understood. Cytochrome c (cyt c) is known to participate in mitochondrial respiration and has antioxidant and peroxidase activities. Studies were performed to understand the related mechanism of oxidation of sulfite and radical generation by ferric cytochrome c (Fe^{3+} cyt c) in the absence and presence of H_2O_2 . Electron paramagnetic resonance (EPR) spin trapping studies using 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) were performed with sulfite, Fe^{3+} cyt c, and H_2O_2 . An EPR spectrum corresponding to the sulfite radical adducts of DMPO (DMPO-SO_3^-) was obtained. The amount of DMPO-SO_3^- formed from the oxidation of sulfite by the Fe^{3+} cyt c increased with sulfite concentration. In addition, the amount of DMPO-SO_3^- formed by the peroxidase activity of Fe^{3+} cyt c also increased with sulfite and H_2O_2 concentration. From these results, we propose a mechanism in which the Fe^{3+} cyt c and its peroxidase activity oxidizes sulfite to sulfite radical. Our results suggest that Fe^{3+} cyt c could have a novel role in the deleterious effects of sulfite in biological systems due to increased production of sulfite radical. It also shows that the increased production of sulfite radical may be responsible for neurotoxicity and some of the injuries which occur to humans born with molybdenum cofactor and sulfite oxidase deficiencies.

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1. Introduction

In humans, sulfite is generated endogenously by the metabolism of sulfur containing amino acids such as methionine and cysteine [1]. Sulfite is also formed from exposure to sulfur dioxide, one of the major environmental pollutants [2]. Sulfite is used as an antioxidant and preservative in dried fruits, vegetables, pickled onion, and beverages such as fruit juice, grape juice, beer, and wine to prevent or reduce spoilage [2–4]. Sulfite is also used as a stabilizer in many drugs and cosmetics [2,5,6]. For the majority of people, exposure to sulfites occurs during consumption of foods and drinks that contain sulfite preservative [2]. Sulfite toxicity has been associated with allergic reactions characterized by sulfite sensitivity, asthma, chronic airway diseases, dermatitis,

anaphylactic shock, and early death [2,7,8]. The most frequently reported physiological response for those sensitive to sulfite is difficulty in breathing due to bronchoconstriction [2]. Steroid-dependent asthmatics and children with chronic asthma are especially vulnerable to such toxicity [2]. Sulfite is also toxic to neurons and cardiovascular system [9–14]. The level of sulfite in serum was found to be unregulated in several disease conditions, such as pneumonia and end-stage renal failure [15,16]. Studies have suggested that the cytotoxicity of sulfite is mediated by free radicals [9,17,18]. There is no specific treatment for sulfite toxicity, and the molecular mechanisms of the potentially toxic reactions of sulfite are poorly understood.

In humans, sulfite is detoxified in the liver and lung to sulfate by sulfite oxidase (SO), a molybdenum dependent mitochondrial enzyme [19]. SO ensures that intracellular levels of the sulfite ion remain at acceptably low levels. In cells, SO is localized in the intermembrane space of the mitochondria. Sulfite oxidation is the final step in the metabolism of sulfur derived from sulfur

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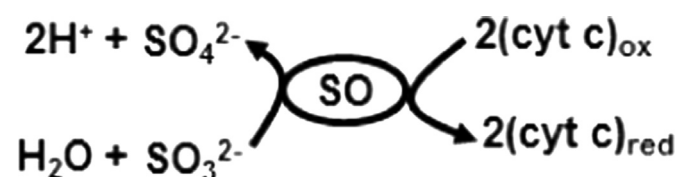
containing amino acids. SO catalyzes the oxidation of endogenous or exogenous sulfite to sulfate, which is excreted in to the urine [20]. In humans, SO deficiency is one of the most accepted causes of sulfite hypersensitivity and toxicity [21]. A congenital deficiency of SO can cause an excessive accumulation of sulfite and lead to early death in infancy (usually between 2 and 6 years of age), or in neonatal cases, neurological abnormalities, mental retardation, intractable seizures, and ocular lens dislocation [8,20–22]. Molybdenum cofactor deficiency, which would compromise SO activity, results in profound mental retardation, brain damage, microcephaly, and spasticity [23]. It has also been suggested that hypoxic-ischemic encephalopathy is due to molybdenum cofactor deficiency [21,24]. Importantly, in SO and molybdenum cofactor deficiency cases, the level of sulfite is increased in plasma and urine and also accumulates within the body [8,21,22,24–27]. Despite great advances in understanding the pathophysiology of SO and molybdenum cofactor deficiencies [22,23], there are no available therapies to reduce mortality or to improve quality of life in survivors. Thus, a greater understanding of the mechanisms by which excess sulfite leads to pathophysiological complications could lead to the development of more effective therapies.

Under normal physiological conditions, SO catalyzes the oxidation of sulfite to sulfate with cytochrome c (cyt c) as oxidizing substrate as shown in Scheme 1 [28,29]. Mammalian cytochrome c (cyt c) is a small, globular protein that exists in high concentrations (0.5–5 mM) in the inner membrane of mitochondria [30,31]. At least 15% of cyt c is tightly bound to the inner membrane and the remainder is loosely attached to the inner membrane and can be readily mobilized [32]. Under physiological conditions, cyt c mediates electron shuttling between cytochrome c reductase (complex III) and cytochrome c oxidase (complex IV) during mitochondrial respiration [32]. The loosely associate cyt c also mediates superoxide removal, and prevents oxidative stress [32–34], whereas the tightly bound cyt c accounts for the peroxidase activity [35–39]. The peroxidase activity of cyt c increases under conditions of oxidative and nitrosative stress [31,40,41]. Release of cyt c from the inner mitochondrial membrane into the cytosol is a pro-apoptotic factor [42,43]. Early in apoptosis, the redox function of cyt c in the respiratory chain switches to a peroxidase function [44,45]. The increased peroxidase activity of cyt c is implicated in various neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis (ALS) [46]. To gain a better understanding of the role of oxidized cytochrome c (Fe^{3+} cyt c) in oxidative sulfite toxicity, we have employed the powerful, sensitive, and specific technique of electron paramagnetic resonance (EPR) spin trapping technique to investigate the oxidation of sulfite and generation of free radicals by the Fe^{3+} cyt c in the absence and presence of H_2O_2 .

2. Materials and methods

2.1. Materials

Oxidized cytochrome c (Fe^{3+} cyt c, from horse heart), hydrogen peroxide (H_2O_2), and sodium sulfite (Na_2SO_3) were purchased



Scheme 1. Sulfite oxidase catalyzes the oxidation of sulfite to sulfate and reduces oxidized cytochrome c.

from Sigma. Diethylenetriaminepentaacetic acid (DTPA) and 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) were obtained from Aldrich. Purified 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was purchased from Dojindo laboratories, Kumamoto, Japan.

2.2. Electron Paramagnetic Resonance (EPR) measurements

EPR spectra were recorded using a quartz flat cell at room temperature with a Bruker ESP 300E spectrometer operating at X-band with 100 kHz modulation frequency and a TM_{110} cavity. The instrument settings were as follows: microwave frequency of 9.779 GHz, modulation amplitude of 0.5 G, microwave power of 20 mW, scan time of 30 s, time constant of 82 ms, and a single scan. EPR spectral recording began two minutes after the addition of H_2O_2 . All the experiments were carried out in phosphate buffer (50 mM and pH 7.4) containing 0.1 mM DTPA. Reactions were initiated by the addition of H_2O_2 . Quantitation of the observed free radical signals was performed by computer simulation of the spectra with comparison of the double integral of the observed signal to that of a TEMPO standard (1 μM) measured under the identical conditions [47].

3. Results

3.1. EPR spin trapping studies of the oxidation of sulfite by Fe^{3+} cyt c

It has been demonstrated that Fe^{3+} cyt c can oxidize various thiol compounds and superoxide radical [48–50]. To gain insight into the molecular mechanisms involved in the process of oxidation associated with sulfite toxicity, we studied the oxidation of sulfite and free radical formation by Fe^{3+} cyt c. EPR spin trapping is a powerful technique to measure formation of free radical intermediates. EPR spin trapping studies using the spin trap DMPO were carried out to investigate the oxidation of sulfite by Fe^{3+} cyt c. EPR spectra were recorded from the reaction mixture containing DMPO (0.1 M), Fe^{3+} cyt c (0.1 mM), and sulfite (1 mM) in the presence of DTPA (0.1 mM). A prominent EPR signal was seen, corresponding to the sulfite radical adduct of DMPO (DMPO-SO_3^-), as shown in Fig. 1A. From the EPR spectrum, the calculated isotropic hyperfine coupling constants are $a_N = 14.57$ G and $a_H = 16.09$ G, which are in agreement with previous reports [51]. In the absence of Fe^{3+} cyt c, a trace level of DMPO-SO_3^- signal was obtained, as shown in Fig. 1B. No EPR signal was obtained in the absence of sulfite, as shown in Fig. 1C. These results show that Fe^{3+} cyt c oxidizes sulfite to form the sulfite radical.

The level of sulfite is increased under various pathological conditions, including environmental exposure [2,3,25]. EPR spin trapping studies were carried out with varying concentrations of sulfite. The sulfite concentration dependence of sulfite radical formation is shown in Fig. 2. The EPR signal intensity increases with increasing sulfite concentration (Fig. 2).

3.2. EPR spin trapping studies of the oxidation of sulfite by Fe^{3+} cyt c in the presence of H_2O_2

In mitochondria, ~1–2% of the oxygen consumed undergoes partial reduction to form superoxide radical and hydrogen peroxide under physiological conditions [52]. It has been demonstrated that Fe^{3+} cyt c acts as a peroxidase and is involved in the detoxification of H_2O_2 [31,38]. During peroxidase activity, Fe^{3+} cyt c reacts with H_2O_2 to form the peroxidase Compound I-type intermediate, as shown in Scheme 2. The peroxidase activity of Fe^{3+} cyt c oxidizes various endogenous antioxidants/molecules such as GSH, ascorbate, and NADH in the presence of H_2O_2 , as shown in Scheme 2 [31,49,53].

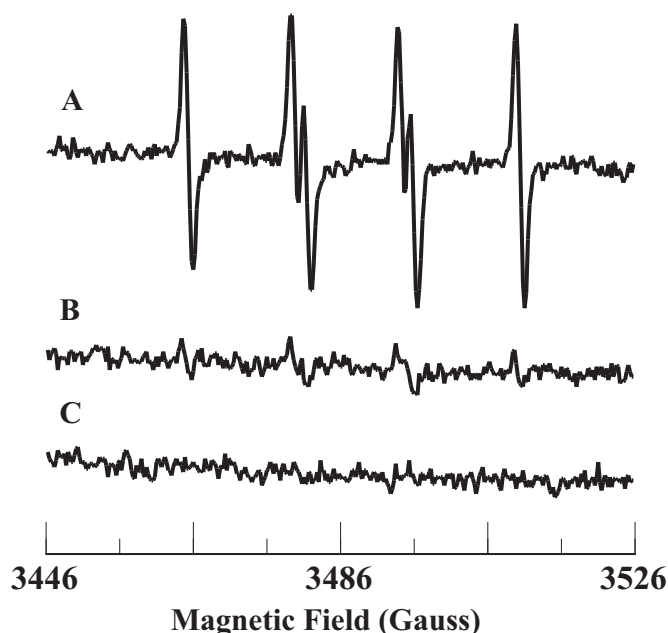


Fig. 1. Room temperature EPR spectra of the sulfite radical adduct of DMPO, DMPO-SO₃⁻. All the reactions were performed in 50 mM phosphate buffer (pH=7.4) containing 0.1 mM DTPA. Spectrum A: DMPO (0.1 M), Fe³⁺cyt c (0.1 mM), and sulfite (1 mM). Spectrum B: DMPO (0.1 M) and sulfite (1 mM). Spectrum C: DMPO (0.1 M) and Fe³⁺cyt c (0.1 mM). EPR instrument parameters used were as described in the Materials and Methods section. EPR spectra are sum of 10 scans.

To gain further insight into the molecular mechanisms involved in sulfite toxicity, we studied the oxidation of sulfite and free radical generation by the Fe³⁺cyt c in the presence of H₂O₂. EPR spin trapping studies using the spin trap DMPO were carried out to investigate the oxidation of sulfite by Fe³⁺cyt c and H₂O₂. EPR spectra were recorded from the reaction mixture containing DMPO (0.1 M), Fe³⁺cyt c (0.1 mM), H₂O₂ (0.1 mM), and sulfite (0.5 mM) in the presence of DTPA (0.1 mM). A prominent EPR signal was seen corresponding to the sulfite radical adduct of DMPO (DMPO-SO₃⁻), as shown in Fig. 3A. In the absence of Fe³⁺cyt c, a weak signal of DMPO-SO₃⁻ was obtained, as shown in Fig. 3B. A trace level of the DMPO-SO₃⁻ signal was obtained in the absence of H₂O₂, as shown in Fig. 3C. No EPR signal was obtained in the absence of Fe³⁺cyt c and H₂O₂, as shown in Fig. 3D. These results show that H₂O₂ alone oxidizes sulfite to the sulfite radical, but the oxidation of sulfite to sulfite radical is greatly increased by the combination of Fe³⁺cyt c and H₂O₂.

The level of sulfite is increased due to consumption of food and drink containing sulfite preservatives and under pathological conditions such as SO deficiency [2,21]. EPR spin trapping studies were carried out with varying concentrations of sulfite and H₂O₂. The sulfite concentration dependence of sulfite radical formation is shown in Fig. 4. The EPR signal intensity increases with increasing sulfite concentration (Fig. 4, Top). A plot of the initial rate of formation of DMPO-SO₃⁻ vs concentration of sulfite is linear up to 0.25 mM and decreases at higher concentration of sulfite (0.5 mM) as shown in Fig. 4, (Bottom).

The level of reactive oxygen species (ROS) is increased under various pathological conditions such as ischemia/reperfusion and diabetes [54–56]. The H₂O₂ concentration dependence of sulfite radical formation is shown in Fig. 5. The EPR signal intensity increases with increasing H₂O₂ concentration (Fig. 5, Top). A plot of the initial rate of formation of DMPO-SO₃⁻ vs concentration of H₂O₂ is linear up to 0.1 mM as shown in Fig. 5 (Bottom). These results show that formation of sulfite radical increases with increasing level of H₂O₂.

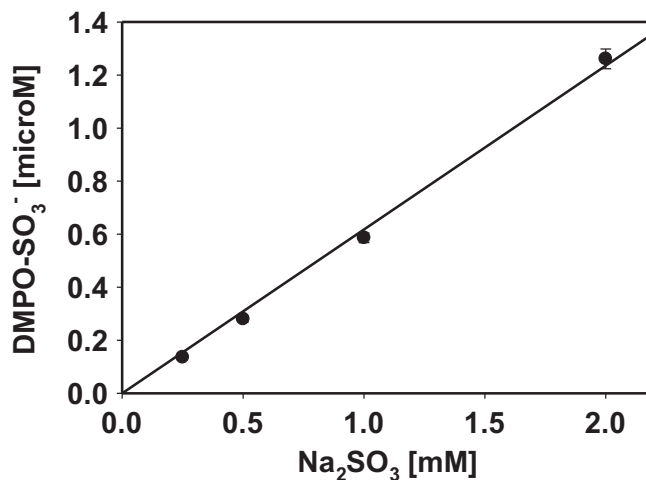
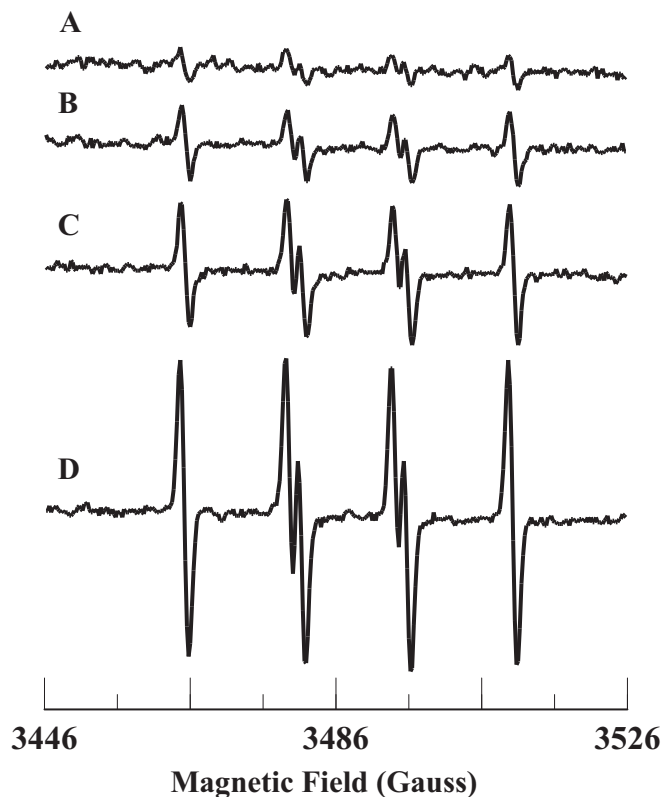
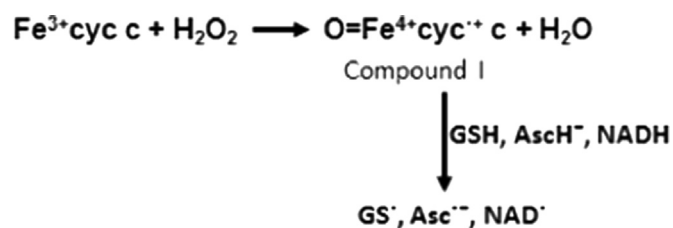


Fig. 2. Top: room temperature EPR spectra of the sulfite radical adduct of DMPO, DMPO-SO₃⁻. All the reactions were performed in 50 mM phosphate buffer (pH=7.4) containing 0.1 mM DTPA. Spectrum A: DMPO (0.1 M), Fe³⁺cyt c (0.1 mM), and sulfite (0.25 mM). Spectrum B: DMPO (0.1 M), Fe³⁺cyt c (0.1 mM), and sulfite (0.5 mM). Spectrum C: DMPO (0.1 M), Fe³⁺cyt c (0.1 mM), and sulfite (1 mM). Spectrum D: DMPO (0.1 M), Fe³⁺cyt c (0.1 mM), and sulfite (2 mM). EPR instrument parameters used were as described in the Materials and Methods section. EPR spectra are sum of 10 scans. Bottom: Plot of the concentration of DMPO-SO₃⁻ vs Na₂SO₃. EPR spectra from the top panel were quantified by computer simulation and comparison of the double integral of the observed signal with that of a TEMPO standard (1 μM) measured under identical conditions. Data represent mean ± SE (n=3).

4. Discussion

In this study, EPR spin trapping experiments were performed to understand the molecular mechanisms involved in the sulfite toxicity. Cyt c is found in high concentrations (0.5–5 mM) in the inner membrane of mitochondria [30]. The results in Fig. 1 show



Scheme 2. Mechanism of activation of Fe^{3+} cyt c to a peroxidase Compound I-type intermediate by H_2O_2 and its oxidation of substrates.



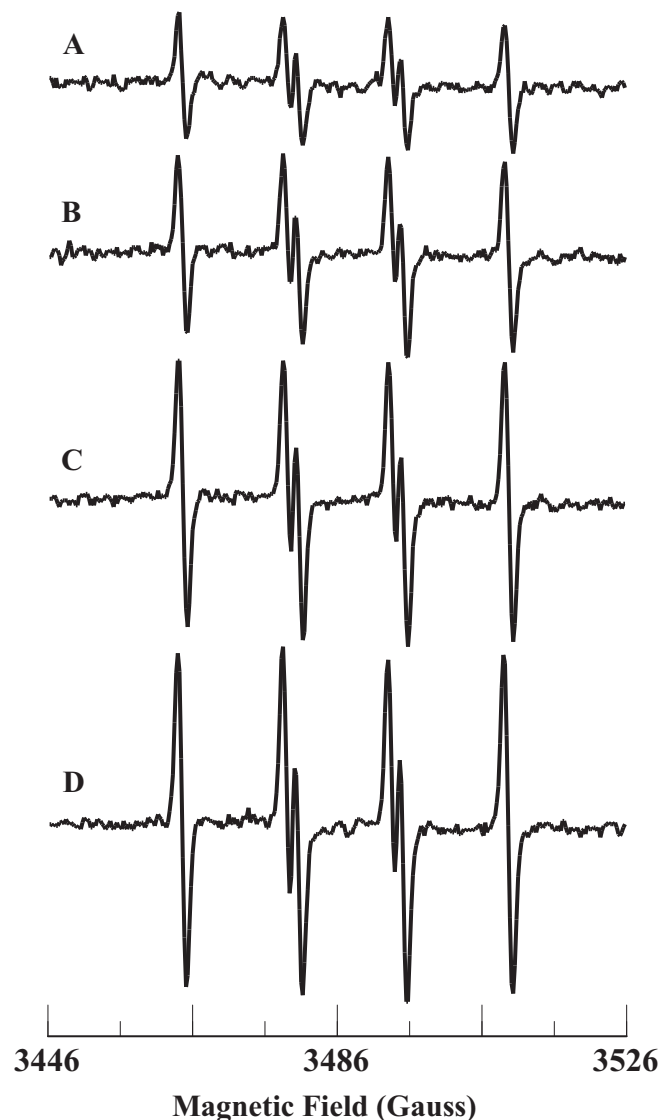
Fig. 3. Room temperature EPR spectra of the sulfite radical adduct of DMPO, $\text{DMPO}\text{-SO}_3^-$. All the reactions were performed in 50 mM phosphate buffer (pH=7.4) containing 0.1 mM DTPA. Spectrum A: DMPO (0.1 M), Fe^{3+} cyt c (0.1 mM), sulfite (0.5 mM), and H_2O_2 (0.1 mM). Spectrum B: DMPO (0.1 mM), sulfite (0.5 mM), and H_2O_2 (0.1 mM). Spectrum C: DMPO (0.1 M), Fe^{3+} cyt c (0.1 mM), and sulfite (0.5 mM). Spectrum D: DMPO (0.1 M) and sulfite (0.5 mM). EPR instrument parameters used were as described in the Materials and Methods section. Each EPR spectrum is a single scan.

that Fe^{3+} cyt c oxidizes sulfite to the sulfite radical. In addition, the EPR spectra in Fig. 2 show that oxidation of sulfite to sulfite radical by Fe^{3+} cyt c increases with an increasing concentration of sulfite. Studies have demonstrated that the sulfite radical further reacts with oxygen to form peroxyxymonosulfate and sulfate radicals,

Fig. 4. Top: room temperature EPR spectra of the sulfite radical adduct of DMPO, $\text{DMPO}\text{-SO}_3^-$. All the reactions were performed in 50 mM phosphate buffer (pH=7.4) containing 0.1 mM DTPA. Spectrum A: DMPO (0.1 M), Fe^{3+} cyt c (0.1 mM), sulfite (0.05 mM), and H_2O_2 (0.1 mM). Spectrum B: DMPO (0.1 M), Fe^{3+} cyt c (0.1 mM), sulfite (0.1 mM), and H_2O_2 (0.1 mM). Spectrum C: DMPO (0.1 M), Fe^{3+} cyt c (0.1 mM), sulfite (0.25 mM), and H_2O_2 (0.1 mM). Spectrum D: DMPO (0.1 M), Fe^{3+} cyt c (0.1 mM), sulfite (0.5 mM), and H_2O_2 (0.1 mM). EPR instrument parameters used were as described in the Materials and Methods section. Each EPR spectrum is a single scan. Bottom: Plot of initial rate of formation of $\text{DMPO}\text{-SO}_3^-$ versus sulfite concentration. Rates were obtained from the initial slope of the formation of $\text{DMPO}\text{-SO}_3^-$. EPR spectra were quantified by computer simulation of the spectra and comparison of the double integral of the observed signal with that of a TEMPO standard (1 μM) measured under the identical conditions. Data represent means \pm SE ($n=3$).

which are powerful oxidants, Scheme 3 [51]. However, the spin trap DMPO (100 mM) competes with oxygen and scavenges sulfite radical [51].

In cells, reactive oxygen species such as H_2O_2 are produced by the mitochondria under physiological and pathophysiological conditions [52]. In the mitochondrial intermembrane space, var-



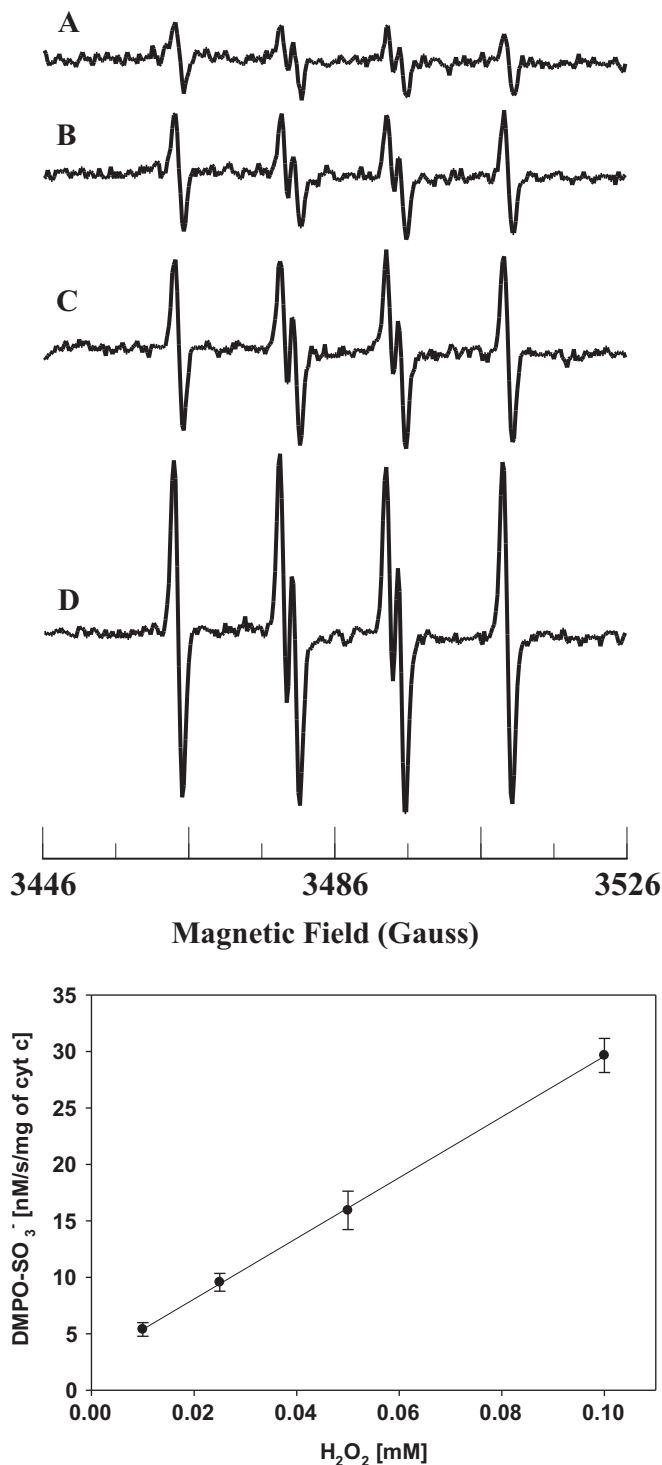
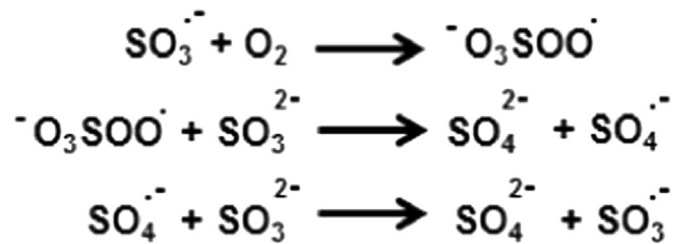


Fig. 5. Top: room temperature EPR spectra of the sulfite radical adduct of DMPO, DMPO-SO₃. All the reactions were performed in 50 mM phosphate buffer (pH=7.4) containing 0.1 mM DTPA. Spectrum A: DMPO (0.1 M), Fe³⁺cyt c (0.1 mM), sulfite (0.5 mM), and H₂O₂ (0.01 mM). Spectrum B: DMPO (0.1 M), Fe³⁺cyt c (0.1 mM), sulfite (0.5 mM), and H₂O₂ (0.025 mM). Spectrum C: DMPO (0.1 M), Fe³⁺cyt c (0.1 mM), sulfite (0.5 mM), and H₂O₂ (0.05 mM). Spectrum D: DMPO (0.1 M), Fe³⁺cyt c (0.1 mM), sulfite (0.5 mM), and H₂O₂ (0.1 mM). EPR instrument parameters used were as described in the Materials and Methods section. Each EPR spectrum is a single scan. Bottom: Plot of initial rate of formation of DMPO-SO₃ vs H₂O₂ concentration. Rates were obtained from the initial slope of the formation of DMPO-SO₃. EPR spectra were quantified by computer simulation of the spectra and comparison of the double integral of the observed signal with that of a TEMPO standard (1 μM) measured under the identical conditions. Data represent mean ± SE (n=3).



Scheme 3. Reactions of oxidation of sulfite radical to peroxymonosulfate and sulfate radicals through chain propagation.

ious enzymes are also involved in the generation of ROS [57,58]. Our EPR spin trapping experiments demonstrate the oxidation of sulfite to sulfite radical by H₂O₂ (Fig. 3). The direct oxidation of sulfite into sulfite radical by H₂O₂ occurs and can cause oxidative damage to proteins, lipids, RNA, and DNA. Further, the oxidation of sulfite to sulfite radical by Fe³⁺cyt c increases in the presence of H₂O₂. The EPR results in Fig. 4 (Top) show that the oxidation of sulfite by Fe³⁺cyt c in the presence of H₂O₂ increases with increasing concentration of sulfite. A plot of the initial rate of formation of DMPO-SO₃ as a function of sulfite concentration is linear up to 0.5 mM (Fig. 4, Bottom). The EPR spectra in Fig. 5 (Top) show that the oxidation of sulfite by Fe³⁺cyt c increasing with an increasing concentration of H₂O₂. A plot of the initial rate of formation of DMPO-SO₃ as function of H₂O₂ is linear (Fig. 5, Bottom), indicating a first-order dependence on H₂O₂. These EPR spin trapping studies show that the increased production of ROS (H₂O₂) increases the formation of the Compound I intermediate of Fe³⁺cyt c which oxidizes sulfite to sulfite radical. These results show that the increased production of H₂O₂ induces increased production of sulfite radical.

Under normal physiological conditions, sulfur containing amino acids such as methionine and cysteine are metabolized to sulfite [59]. In addition, cysteine is also metabolized to H₂S, which functions as a cell signaling molecule in biology [59]. Under physiological conditions, mitochondria also rapidly oxidize H₂S to thiosulfate and subsequently to sulfite and sulfate [59–61]. In humans, SO is an essential protein residing in the mitochondrial intermembrane space which catalyzes the essential oxidation/degradation of endogenous or exogenous sulfite to sulfate, which is excreted into the urine [20]. Simultaneously, the reduced SO reduces Fe³⁺cyt c to Fe²⁺cyt c, Scheme 1. In the mitochondrial electron transport chain (ETC), Fe²⁺cyt c donates an electron to cytochrome c oxidase (complex IV), which reduces molecular oxygen to water [62]. Thus, SO and Fe³⁺cyt c play an important role in protecting the mitochondria/cells/tissues from sulfite toxicity.

In wine, sulfite is used as a preservative and can reach the concentration of 6 mM [3,4,63]. In asthmatic patients, asthma symptoms are sometimes worsened after alcohol consumption [64]. A cohort study has reported that sulfite in wine triggers asthmatic reactions [65]. Very little is known about the mechanisms involved in these reactions. EPR spin trapping studies show that oxidation of sulfite to the sulfite radical by Fe³⁺cyt c in the absence/presence of H₂O₂ increases with increasing concentration of sulfite. This shows that this reaction may play an important role in the wine-induced asthmatic responses. This also suggests that Fe³⁺cyt c could be involved in oxidative damage and tissue injury in sulfite-exacerbated allergic reactions.

Sulfur dioxide is one of the major air pollutants near large cities [66]. In the nasal passage and lung, sulfur dioxide is hydrated rapidly into bisulfite and sulfite [67]. In aqueous medium, bisulfite and sulfite are in equilibrium [67]. At physiological pH, sulfite predominates over bisulfite [67]. Exposure to sulfur dioxide induces accumulation of neutrophils into the airways [68]. Sulfite

can also stimulate a respiratory burst and ROS production by neutrophils [69]. It has been demonstrated that sulfite is oxidized to the sulfite radical by guinea pig lung microsomes [67]. In humans, SO is expressed in the alveoli and tissues of lung and liver [70,71]. However, the expression and activity of SO is very low in human lung tissue (135-fold lower than liver) [70]. It has been suggested that various peroxidase enzymes are involved in the oxidation of sulfite to sulfite radical in sulfite toxicity [67]. Very recently, it has been proposed that the peroxidase activity of Fe^{3+} cyt c is involved in the oxidation of phospholipids in the lungs exposed to air pollutants [72]. Highly reactive sulfite radical can oxidize lipids. Our EPR spin trapping experiments show that oxidation of sulfite by Fe^{3+} cyt c in the presence of H_2O_2 increases the generation of sulfite radical. This study shows that Fe^{3+} cyt c may play a major role in sulfite toxicity in lungs exposed to environmental air pollutants such as sulfur dioxide.

Sulfite is one of the few sulfating agents approved by the Food and Drug Administration as a food preservative and antioxidant to prevent or reduce spoilage [3]. It is also used as an ingredient of many medications, such as antibiotics, analgesics, and anesthetics [2,5,6]. It has been suggested that the presence of sulfite in dexamethasone preparations has increased its neurotoxicity of the preparation [73]. However, the molecular mechanisms involved in this neurotoxicity are not known. The increased peroxidase activity of Fe^{3+} cyt c is implicated in various neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, and ALS [46]. Exposure of rat neuronal cells to sulfite increases cell death by apoptosis [18]. The release of cyt c is an early step in apoptosis [42,43]. Moreover, the peroxidase activity of cyt c increases during apoptosis [74,75]. Exposure to sulfite results in DNA fragmentation, characteristic of apoptosis [18]. It has been shown that sulfite radical can damage DNA [76,77]. However, the source of the formation of sulfite radical in neuronal cell death is not known. Based on our EPR spin trapping results, we propose that increased production of sulfite radical due to the oxidation of sulfite by Fe^{3+} cyt c may be responsible for DNA fragmentation, apoptosis, increased neurotoxicity, and neuronal cell death.

In humans, SO is expressed in liver, kidney, skeletal muscle, heart, placenta, and brain [78]. The loss of SO is fatal in infancy or early childhood [21]. In SO-deficient and functional loss of SO patients, the main clinical symptoms are severe progressive neurologic damage, attenuated brain growth, mental retardation and alterations in muscle tone [79,80]. SO deficiency is also one of the most accepted causes of sulfite hypersensitivity and toxicity [21]. In cases of SO deficiency and molybdenum cofactor deficiency, the levels of sulfite and sulfo derivatives of amino acids, proteins, and various compounds are increased in urine [21,24]. SO deficiency also results in the accumulation of sulfite in various tissues, especially in the brain [81]. However, the molecular mechanisms involved are not fully understood. In clinical studies of SO and molybdenum cofactor deficiencies, it was proposed that neuronal toxicity was due to decreased ATP or energy deficit/failure [82,83]. It has also been suggested that excess sulfite might damage mitochondrial function *via* disruption of membrane integrity [9,84]. In rat brain, mitochondrial sulfite induced a decrease in ATP synthesis and disturbance of mitochondrial energy homeostasis [9,81]. In neurons and human fetal liver cells, it has also been shown that ATP is depleted due to sulfite toxicity [9]. Similar observations were made in rat kidney mitochondria and other non-neuronal cells [85]. In addition, it has been shown that sulfite inhibits mitochondrial glutamate dehydrogenase activity [9]. However, the molecular mechanisms involved in the inactivation of mitochondria and its enzymes are still unclear.

Mitochondrial dysfunction contributes to the pathophysiology of neurologic disorders and neurodegenerative diseases [86]. Accumulation of damaged mitochondria is associated with neurodegenerative

diseases [87]. The disorders of mitochondrial oxidative phosphorylation are associated with neurodegenerative diseases [88]. Mitochondrial precursor proteins (~99%) are synthesized in cytosol [89]. Precursor proteins targeted to mitochondria are imported in to the mitochondrial matrix by the protein transport machinery localized in the outer and inner membrane of mitochondria [89,90]. Similarly, RNAs and heme are imported into mitochondria by the PNPASE protein and translocator protein (TSPO) respectively, residing in the mitochondrial intermembrane space [91–93].

During mitochondrial fission, various nuclear encoded proteins and transcription factors are transported into the preexisting mitochondria and imported before fission and subsequent incorporation into the mitochondrial network [86,94]. Abnormalities in mitochondrial fission have been identified in several neurodegenerative diseases [86]. It has been demonstrated that sulfite toxicity is due to the increased production of ROS in the mitochondria and inactivation of mitochondrial proteins [9]. Studies have also shown that sulfite radical is capable of damaging DNA/RNA, lipids, and proteins [51,76,77, 95–97]. The increased production of oxidants can damage the mitochondrial proteins, lipids, DNA/RNA and this is implicated in several neurological diseases [86,91,98].

Our EPR spin trapping studies show that H_2O_2 can directly oxidize sulfite to the sulfite radical, and that Fe^{3+} cyt c, a mitochondrial protein, oxidizes sulfite to the sulfite radical in the absence or presence of H_2O_2 . The increased production of sulfite radical in the mitochondrial intermembrane space can damage the proteins, lipids, DNA/RNA and proteins involved in various transport machinery/processes. Moreover, the increased production of oxidants increases the oxidation of mitochondrial innermembrane cardiolipin [31,99]. Oxidized cardiolipin translocates to the mitochondrial outer membrane and enhances apoptosis [86]. In rats, it has been shown that sulfite disrupts brain mitochondrial energy homeostasis, increases swelling, and induces mitochondrial permeability transition (MPT) pore opening [81]. MPT pore opening inhibits the ETC complex I activity, releases cyt c, and increases apoptosis [81,100]. In the arterial system, sympathetic neurons dictate the distribution of blood flow and oxygen transport, dependent on need [101]. Defective sympathetic neurons lead to cerebral hypoperfusion [101]. Hence, the increased production of highly reactive sulfite/sulfate radical by the Fe^{3+} cyt c and H_2O_2 can alter the physiological functions and dynamics of mitochondria and increase apoptosis and necrosis, which results in increased neurotoxicity, hypoperfusion, and neuronal cell death. Therefore, mitochondria are important targets for neuroprotective interventions. Mitochondria have been targeted by many experimental neuroprotective interventions [86]. Our EPR results suggest that radical metabolism may be central to the pathogenesis of SO and molybdenum cofactor deficiencies.

Based on our studies and reported literature, we propose that Fe^{3+} cyt c in the absence and presence of H_2O_2 is involved in the oxidation of sulfite to the sulfite radical and facilitation of electron flow in the ETC as depicted in Fig. 6.

In conclusion, under physiological conditions, SO and Fe^{3+} cyt c play a protective role in detoxifying sulfite in the mitochondrial intermembrane space. However, with elevated levels of sulfite and pathophysiological conditions accompanied by oxidative stress, the oxidation of sulfite by Fe^{3+} cyt c and/or H_2O_2 causes potentially toxic reactions between the sulfite radical intermediate and biologically important molecules such as proteins, lipids, and DNA. The highly reactive sulfite/sulfate radical can damage the transport machinery involved in the transport of proteins, RNAs, heme, and various other biomolecules in to mitochondria and disrupts mitochondrial energy homeostasis. The oxidation of sulfite into sulfite radical by Fe^{3+} cyt c and/or H_2O_2 in mitochondria may be responsible for some of the damage which occurs in humans born

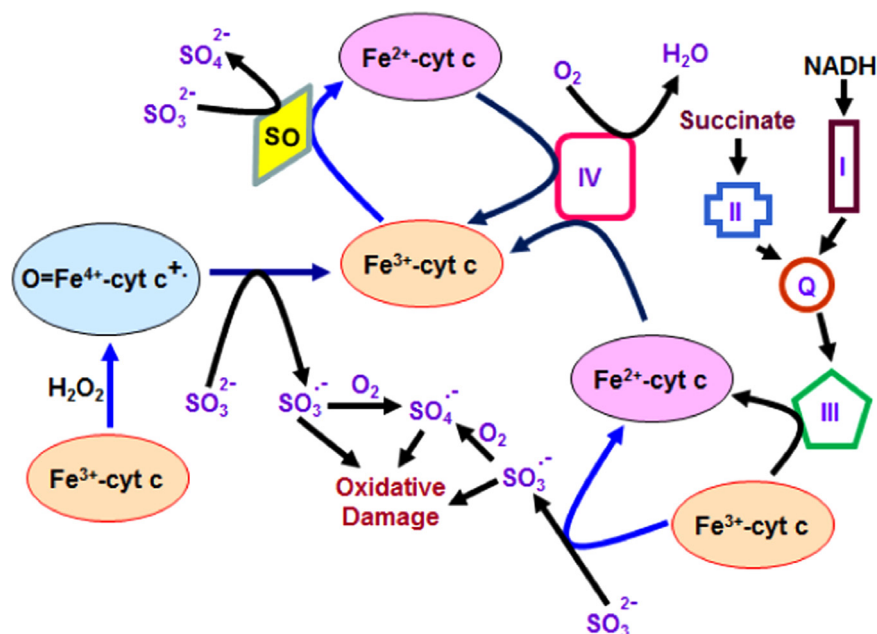


Fig. 6. Proposed model of cytochrome c-mediated oxidation of sulfite to sulfite radical and alternative electron transfer pathway in mitochondria. The oxidation of sulfite by sulfite oxidase and cytochrome c are two and one electron processes respectively.

with SO and/or molybdenum cofactor deficiencies. Targeted clearance and replacement of damaged organelles has been identified as a neuroprotective strategy against acute neural injury [102]. Hence, sulfite-mediated mitochondrial injury may be of key importance in sulfite mediated neurological and systemic pathology.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bbrep.2015.11.025>.

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