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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.



## 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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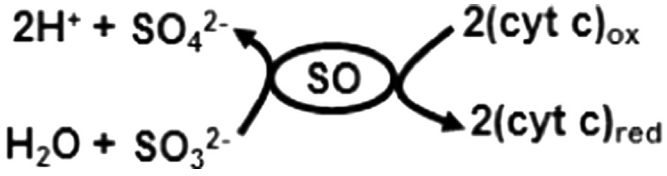
containing amino acids. SO catalyzes the oxidation of endogenous or exogenous sulfite to sulfate, which is excreted in to the urine [20]. In humans, SO deficiency is one of the most accepted causes of sulfite hypersensitivity and toxicity [21]. A congenital deficiency of SO can cause an excessive accumulation 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 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.




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

### 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].