(Bi)sulfite Oxidation by Copper,Zinc-Superoxide Dismutase: Sulfite-Derived, Radical-Initiated Protein Radical Formation

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**BACKGROUND:** Sulfur dioxide, formed during the combustion of fossil fuels, is a major air pollutant near large cities. Its two ionic forms in aqueous solution, sulfate and (bi)sulfite, are widely used as antioxidants and preservatives in beverages and foods (Danilewicz 2003). However, the prevalence of sulfate toxicity is relatively high, and it has been associated with allergic reactions characterized by sulfate sensitivity, asthma, and anaphylactic shock (Komarnisky et al. 2003). Sensitive individuals can experience such adverse reactions when they consume sulfites, with asthma being particularly vulnerable to such toxicity.

Sulfite is detoxified in the liver and lung to sulfate by sulfate oxidase, a molybdenum-dependent mitochondrial enzyme (Cohen and Fridovich 1971); sulfate oxidase deficiency is one of the most accepted causes of sulfite hypersensitivity and toxicity. This enzymatically catalyzed oxidation has been shown to proceed via a two-electron oxidation without the formation of any detectable radical intermediates. In contrast, recent studies suggest that the cytotoxicity of (bi)sulfite is mediated by free radicals, because (bi)sulfite increases reactive oxygen species formation, and antioxidants and free radical scavengers prevent its toxicity (Niknahad and O’Brien 2008). In addition, transition metals catalyze the autoxidation of (bi)sulfite via sulfur trioxide anion radical (SO3·−) formation.

\[ M^{2+} + SO_3^{2−} → M^{(n−1)+} + SO_3^{−} \]  

where \( M \) may be copper (Cu2+), iron (Fe3+), oxovanadium anion (VO2−), manganese (Mn2+), nickel (Ni2+), or chromate anion (CrO42−) (Alipazaga et al. 2004; Berglund et al. 1993; Brandt and Elding 1998; Lima et al. 2002; Shi 1994), but this reaction requires higher concentrations of (bi)sulfite to permit effective propagation of the chain reaction. In a recent study, Alipazaga et al. (2009) reported oxidative DNA damage induced by (bi)sulfite solutions in the presence of Cu(II) peptide complexes. It has also been shown that free radicals have been produced by enzymatic initiation of the oxidation of (bi)sulfite by prostatic linum H synthase (Mottley et al. 1982a) and horseradish peroxidase (HRP) (Araso et al. 1976; Mottley et al. 1982b), with formation of “SO5=O”. This predominantly sulfur-centered radical (Chantry et al. 1962) reacts with molecular oxygen by forming the peroxysulfonase anion radical (“O3,SOO=O”), which is a precursor of the sulfur anion radical (SO3·−) (Neta et al. 1988):

\[ SO_3^{−} + O_2 → O_3,SOO=O, \quad k = 1.5 × 10^{9} \text{M}^{−1}\text{sec}^{−1} \]  

[2]

\[ O_3,SOO=O + SO_3^{2−} → SO_4^{−} + SO_4^{2−}, \quad k = 1.3 × 10^{7} \text{M}^{−1}\text{sec}^{−1} \]  

[3]

\[ SO_4^{2−} + SO_3^{2−} → SO_4^{−} + SO_4^{2−}, \quad k > 2 × 10^{8} \text{M}^{−3}\text{sec}^{−1} \]  

[4]

\( SO_4^{2−} \) is a very strong oxidant, nearly as strong as the hydroxyl radical, and it is very likely to oxidize other biomolecules by one-electron oxidation.

It is possible that bisulfite may also lead to further reactive sulfur species via the peroxysulfonase activity of enzymes such as copper-zinc-superoxide dismutase (Cu,Zn-SOD), a metalloenzyme that catalyzes the dismutation of the superoxide anion into O2 and hydrogen peroxide (H2O2). At pH 7.4, Cu,Zn-SOD exhibits peroxidase activity, with the initial step of the peroxidase cycle being a reduction of SOD-Cu(II) by H2O2 or its deprotonated form, HO2−, to SOD-Cu(I) (Bonini et al. 2009; Fuchs and Borden 1983; Hodgson and Fridovich 1973). At neutral pH, the peroxysulfonase activity of Cu,Zn-SOD is stimulated in the presence of bicarbonate (HCO3−) buffer (Bonini et al. 2004; Liochev and Fridovich 2004; Zhang et al. 2000). It has been proposed that at pH 7.4, anions structurally similar to HCO3−, such as (bi)sulfite (HSO3−) and (bi)selenite (HSeO3−), may also stimulate the peroxysulfonase activity of Cu,Zn-SOD in the presence of millimolar H2O2 (Sankarapandi and Zweier 1999).

In the present study, we evaluated the role of Cu,Zn-SOD in (bi)sulfite oxidation and found that, under our experimental conditions, SOD1-Cu(I) is slowly reduced to SOD1-Cu(II) by (bi)sulfite. We used optical spectroscopy, electron spin resonance (ESR), and oxygen uptake experiments to demonstrate that (bi)sulfite (as Na2SO3) was a one-electron donor substrate for Cu,Zn-SOD, leading to the generation of reactive sulfur radicals via Equations 2–4. We also applied immuno-spin trapping with 5,5-dimethyl-1-pyrroline N-oxide (DMPO) to investigate oxidation of target proteins [e.g., human serum albumin (HSA) at plasma levels] to protein radicals (Figure 1). We found that (bi)sulfite oxidation mediated by Cu,Zn-SOD generated...
the formation of HSA radicals, which might be responsible for the tissue injury in allergic reactions to (bi)sulfite.

**Materials and Methods**

**Chemicals.** We purchased bovine kidney superoxide dismutase (SOD) from Calzyme Laboratories Inc. (San Luis Obispo, CA). HSA (99.99% purity), diethylenetriaminepentaacetic acid (DTPA), sodium sulfite, thio-
cyanate, azide, cyanide, and H$_2$O$_2$ (obtained as a 30% solution) were from Sigma Chemical Co. (St. Louis, MO). We determined the H$_2$O$_2$ concentration from its absorbance at 240 nm ($\varepsilon = 39.4$ M$^{-1}$ cm$^{-1}$). DMPO (high purity; 99%) from Alexis Biochemicals (San Diego, CA) was sublimed twice under vacuum at room temperature and stored under an argon atmosphere at ~80°C before use. Chelex-100 resin was from Bio-Rad Laboratories (Hercules, CA).

**ESR spectroscopy.** We obtained ESR spin-trapping data at room temperature using a Bruker EMX spectrometer with 100 kHz modulation frequency and equipped with an ER 4122 SHQ cavity (Bruker BioSpin Corp., Billerica, MA). We placed samples in a 10-mm flat cell (200 µL final volume) and initiated recording of the spectra within 1 min of the start of the reaction.

We recorded low-temperature ESR data at 77 K after the indicated incubation times. Initially, we mixed SOD with (bi)sulfite at room temperature; after incubation, we transferred the reaction mixtures into 1-mL polystyrene syringes and froze them in liquid nitrogen. We added glycerol (10%) to the samples before freezing to prevent cracking of the frozen texture.

**Oxygen uptake.** For oxygen uptake measurements, we added 500 µL sodium sulfite to a chamber equipped with a Clark electrode and a stirrer. We initiated the reaction (1.8 mL) by SOD, and the oxygen uptake curves were obtained at room temperature with an oxygen monitor (model 53; Yellow Springs Instrument Co., Yellow Springs, OH).

**Chemical reactions.** Typically, we carried out reactions of 600 µM HSA, 500 µM Na$_2$SO$_3$, and 50 µM Cu,Zn-SOD in the presence or absence of 5 mM DMPO in 100 mM phosphate buffer (Chelex-treated with 25 µM DTPA) at pH 7.4 in a total volume of 30 µL. After 1 hr of incubation at 37°C, we stopped the reaction with 5 mM reduced glutathione and then diluted the samples with deionized H$_2$O for electrophoresis and immuno-spin trapping analyses.

**Coomassie blue stain, Western blot, and ELISA (enzyme-linked immunosorbent assay).** We electrophoresed the reaction mixtures under reducing conditions through duplicate 4–12% BisTris NuPage acrylamide gels (Invitrogen, Carlsbad, CA). We performed Western blotting and ELISA analysis as previously described (Detweiler et al. 2002) with minor changes (we used fish gelatin instead of casein to prevent the nonspecific binding sites).

**Optical spectroscopy.** We recorded optical data on a Cary 100 spectrophotometer (Varian Inc., Palo Alto, CA) using a 500 µM quartz cuvette. We determined Cu,Zn-SOD concentration from the broad band at 680 nm ($\varepsilon = 300$ M$^{-1}$ cm$^{-1}$ in the bovine enzyme), which results from the d-d transitions of the Cu atom (Foti et al. 1997). We carried out reactions in 100 mM phosphate buffer at pH 7.4. Cu,Zn-SOD (1 mM) was added first, followed by 20 mM (bi)sulfite, and each scan was recorded every 3 min for 30 min.

**Results**

**(Bi)sulfite oxidation by Cu,Zn-SOD detected by optical spectroscopy, ESR, and oxygen uptake.** When we added a 20-fold excess of (bi)sulfite to 1 mM Cu,Zn-SOD, the absorption band at 680 nm characteristic of the active site of SOD1-Cu(II) decreased slowly, then completely disappeared as the wild-type protein was reduced to Cu(I) (Figure 2A). We recorded the optical spectra every 3 min, and within < 30 min we observed a full reduction of Cu(II) to Cu(I) by (bi)sulfite. However,
lower concentrations of protein (50 µM) and (bi)sulfite (500 µM) were sufficient for low-temperature ESR spectra to detect the reduction of Cu(II) in Cu,Zn-SOD (Figure 2B). ESR data showed that the addition of a 10-fold excess of (bi)sulfite to Cu,Zn-SOD followed by a 1-hr incubation resulted in an approximately 40% decrease in ESR intensity compared with the untreated protein (Figure 2B). The anisotropic hyperfine coupling constant (A_H = 135 G) remained unchanged during the incubation time, indicating that (bi)sulfite does not bind directly to the active site Cu(II) (Strothkamp and Lippard 1981).

To determine whether (bi)sulfite is oxidized by Cu(II) in Cu,Zn-SOD, we also performed room-temperature ESR spin-trapping experiments. When we mixed Cu,Zn-SOD (50 µM) with (bi)sulfite (500 µM) in the presence of the spin trap DMPO (100 mM), it generated an intense ESR signal (Figure 3A, spectrum a) corresponding to the assigned ESR spectrum of DMPO/•SO_2^- (g = 2.00; A_H = 16.0 G and A_N = 14.7 G) (Mottley and Mason 1988; Mottley et al. 1982a, 1982b). Previous studies have shown that (bi)sulfite stimulates the peroxidase function of Cu,Zn-SOD and that •SO_2^- is formed when the protein is treated with 1 mM H_2O_2 in the presence of 20 mM (bi)sulfite (Sankarapandi and Zweier 1999). According to the authors, control experiments in the absence of Cu,Zn-SOD confirmed that the •SO_2^- signal was not due to direct oxidation of (bi)sulfite by H_2O_2, which is known from the literature (Flockhart et al. 1971; Mottley et al. 1982a) to proceed nonenzymatically at high concentrations of H_2O_2 via the following reaction:

\[
\text{H}_2\text{O}_2 + \text{SO}_3^{2-} \rightarrow \text{•SO}_2^- + \text{OH}^- + \text{H}_2\text{O}
\]

To determine the effect of low and nontoxic concentration of H_2O_2 (100 µM) and to confirm that •SO_2^- is generated because of the enzymatic oxidation of (bi)sulfite, we performed control experiments in the presence and absence of H_2O_2. Contrary to expectation (Sankarapandi and Zweier 1999), addition of 100 µM H_2O_2 had almost no effect on the ESR intensity of DMPO/•SO_2^- (Figure 3A, spectra a and b), and omission of (bi)sulfite (Na_2SO_3) or Cu,Zn-SOD resulted in no radical adduct formation (Figure 3A, spectra c and d, respectively). Control experiments confirmed that the reaction is insensitive to catalase, implying that H_2O_2 is not involved (data not shown).

The proposed mechanism of enzymatic oxidation of (bi)sulfite to •SO_2^- by the active Cu(II) site of Cu,Zn-SOD proceeds in a one-electron reduction reaction of Cu(II) by (bi)sulfite, similar to the oxidation of (bi)sulfite by HRP and prostaglandin H synthase (Mottley and Mason 1988; Mottley 1982a, 1982b; Roman and Dunford 1973). The resulting •SO_2^- is known to react further with molecular oxygen to form •O_2 SOO^- and •SO_2^- in the free radical chain mechanism previously reported (Hayon et al. 1972; Mottley and Mason 1988; Reed et al. 1986). To confirm our hypothesis, we next investigated the consumption of oxygen by 500 µM (bi)sulfite, with the reaction initiated by 0–500 µM Cu,Zn-SOD. When •SO_2^- reacted with oxygen in the absence of spin trap, we observed oxygen consumption strongly dependent on the Cu,Zn-SOD concentration (Figure 3B).

Addition of 500 µM Cu,Zn-SOD resulted in approximately 30% oxygen consumption after 15 min. When we examined the effect of the spin trap DMPO using 500 µM Na_2SO_3 and 500 µM Cu,Zn-SOD as the initiator, prior or later additions of 100 mM DMPO (the same amount used for the spin-trapping ESR data) almost completely prevented oxygen uptake (Figure 3B), that is, no radical chain reactions ended in the formation of •O_2 SOO^- and •SO_2^-.

To characterize the importance of Cu redox cycling at the enzyme-active site upon the generation of •SO_2^-, we mixed 500 µM Na_2SO_3 with selected inhibitors and initiated the reactions by 50 µM Cu,Zn-SOD in the presence of 100 mM DMPO (Figure 4). The ESR intensity of the spectra showed that addition of 500 µM thiocyanate, azide, or cyanide in the presence of an equimolar amount of (bi)sulfite significantly inhibited •SO_2^- production. These results strongly suggest that because these anions link directly to the Cu with high affinity, the enzymatic activity of Cu,Zn-SOD is inhibited, and no further oxidation of (bi)sulfite to sulfite-derived radicals is possible.

**Formation of HSA-DMPO nitroso adducts induced by the Cu,Zn-SOD-(bi)sulfite system as determined by immuno-spin trapping**

The optical and ESR data showed that (bi)sulfite is oxidized by Cu,Zn-SOD to •SO_2^- which will initiate the radical chain reaction with formation of •O_2 SOO^- and •SO_2^- via Equations 2–4. To characterize the ability of these radicals to oxidize amino acid(s) in target proteins, we incubated HSA with the enzyme...
and (bi)sulfite in the presence of DMPO and analyzed the reaction products by Western blotting using an anti-DMPO polyclonal antibody (Detweiler et al. 2002). We chose a concentration of DMPO that was much less than the 100 mM used for the ESR and oxygen uptake, so as to not inhibit the chain reaction yet be sufficient for the protein radicals to react with DMPO for detection by anti-DMPO antibody. We achieved the overall high yield of protein DMPO nitroso adducts by decreasing the DMPO concentration to 5 mM in the presence of the plasma concentration of HSA (600 µM). We mixed samples containing 600 µM HSA with 500 µM Na2SO3 in the presence of 5 mM DMPO and initiated the reactions with 10, 20, 30, 40, and 50 µM Cu,Zn-SOD. Coomassie blue staining of the gel verified the amount of HSA present in all treatments and showed the presence of a single band at approximately 60 kDa, which corresponds to the size of albumin, together with a small amount of HSA dimer at approximately 120 kDa (Figure 5A). We also detected a very weak band at approximately 15 kDa at a Cu,Zn-SOD concentration of 50 µM, corresponding to its monomer. We performed immunochemical detection of HSA–DMPO nitroso adducts using Western blotting and ELISA in parallel with SDS-PAGE. As shown in Figure 5B, samples lacking Cu,Zn-SOD, DMPO, or Na2SO3 contained negligible anti-DMPO cross-reacting material. Incubation of HSA with > 10 µM Cu,Zn-SOD resulted in a significant increase in HSA-DMPO-derived nitroso adducts as assessed by ELISA (Figure 5C). This result, together with the oxygen uptake experiments, demonstrated that 5 mM DMPO, because it did not trap the entire primary SO3•−, allowed the radical chemistry in Equations 2–4 to proceed with the formation of the damaging radical intermediates.

HSA-derived nitroso adducts also depended on the (bi)sulfite concentration (Figure 6A). Omission of HSA, DMPO, (bi)sulfite, or Cu,Zn-SOD resulted in no immunostaining above the background level. When 0.1 mM (bi)sulfite and 600 µM albumin were oxidized in the presence of 5 mM DMPO and 50 µM Cu,Zn-SOD, we detected a faint band of DMPO–nitroso adducts. Western blotting performed on reactions containing 0.25–3 mM (bi)sulfite showed increased production of DMPO-HSA radical-derived nitroso adducts and very weak bands of DMPO-HSA dimer at the higher (bi)sulfite concentrations.

We also determined the effect of time on the formation of HSA radical-derived nitroso adducts (Figure 6B,C). In the presence of 5 mM DMPO, 500 µM Na2SO3, and 50 µM Cu,Zn-SOD, Western blotting showed that DMPO-HSA radical-derived nitroso adduct production increased with reaction time, reaching saturation at about 1 hr. ELISA data paralleled those from Western blotting (Figure 6C).

Discussion

The present data confirm that the enzymatic oxidation of (bi)sulfite by Cu,Zn-SOD proceeds via a radical mechanism as demonstrated using optical spectroscopy, oxygen uptake, and ESR experiments. Similar results have been reported for some peroxidases (e.g., HRP, prostaglandin H synthase) (Arario et al. 1976; Mottley et al. 1982a, 1982b). Once the (bi)sulfite is oxidized by Cu(II) in Cu,Zn-SOD and SO3•− is formed, it reacts very rapidly with oxygen and generates ‘O2(SO3•−) and SO2•− (Hayon et al. 1972), which—as very powerful oxidants (E°O2(SO3•−)/O2= 1.1 V, E°SO2•−/SO3•− = 2.43 V)—can attack target proteins (e.g., HSA in plasma) (Neta et al. 1988; Steele and Appelman 1982) (Figure 1). Previous work on the oxidation of (bi)sulfite by the HRP–H2O2 system and ESR spin-trapping experiments showed that there is a strong competition between the spin trap DMPO and oxygen for ‘SO3•− (Rangelova and Mason 2009). In fact, in the latter system, the formation of the oxygen-derived radicals ‘O2(SO3•−) and SO2•− was almost prevented by high DMPO concentrations (100 mM) (Figure 3B), and a decrease of the spin-trap concentration to ≤3 mM was required to trap protein radicals formed by ‘O2(SO3•−) and SO2•− (Mottley and Mason 1988). The very slow consumption of oxygen observed even in the presence of 100 mM DMPO is likely due to the rapid reaction of ‘SO3•− with oxygen at a diffusion-controlled rate to form ‘O2(SO3•−), which then reacts with SO2•− to produce SO2•− (Figure 1).

(bi)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 (Gunnison 1981). However, sulfites have been associated with adverse allergic and asthmatic reactions experienced by sulfite-hypersensitive individuals. The most frequent sulfite-reaction symptoms are difficult in breathing, food intolerance symptoms, asthma, and occasionally anaphylactic shock. There is no specific treatment for sulfite toxicity, and in general, to our knowledge, the mechanisms of the potentially toxic reactions of (bi)sulfite are poorly understood.

One reason for the toxic potential of (bi)sulfite is a deficiency of sulfite oxidase, the molybdenum-containing enzyme that oxidizes sulfite to sulfate (SO$_4^{2-}$), and it is noteworthy in cases of sulfite oxidase deficiency, the concentration of sulfate in plasma is abnormally high (> 1 mM) (Acosta et al. 1989; Johnson et al. 1980). The capacity of sulfate oxidase for sulfite oxidation is extremely high, with the reaction proceeding via a one-step, two-electron oxidation to sulfate with no free radical intermediates (Cohen and Fridovich 1971). However, Yokoyama et al. (1971) showed that inhaled sulfur dioxide does reach the blood plasma, where the dissolved SO$_2$ [(bi)sulfite] forms oxidation products other than sulfate, such as S-sulfonates (Bechthold et al. 1993); this indicates the presence of another mechanism of (bi)sulfite oxidation besides the well-known sulfate oxidase route. Another radical mechanism of xanthine-dependent aerobic oxidation of (bi)sulfite in the presence of xanthine oxidase has been proposed by McCord and Fridovich (1968). The authors concluded that xanthine oxidase, when catalyzing the aerobic oxidation of xanthine, generated a superoxide anion, which then initiated to initiate the (bi)sulfite chain reaction. A previous report from our laboratory (Mottley et al. 1982b) demonstrated that incubation of (bi)sulfite with HRP and H$_2$O$_2$ is not sensitive to the presence of SOD, confirming that the peroxidase-catalyzed pathway does not involve a superoxide chain reaction.

In the present study we used Cu,Zn-SOD–(bi)sulfite as a source for generation of oxidants (‘$\cdot$SO$_2$O’ and ‘$\cdot$SO$_4^{2-}$’) that are diffusible and radicals themselves to show their capability to oxidize the most abundant plasma protein (albumin) to protein radicals (Figure 1). Our Western blot experiments showed that in the presence of DMPO, the Cu,Zn-SOD–(bi)sulfite system produced sulfite-derived radicals that oxidized albumin to produce protein-centered radicals trapped by the nitrore spin-trap DMPO and detected as DMPO-HSA nitroxide adducts. When DMPO or any of the other system components were eliminated, no immuno-staining appeared above the background signal levels, confirming that all of the reactants are needed for detection of radicals. The extent of immuno-spin trapping increased with spin-trap concentrations up to 10 mM and then decreased (data not shown). These results are consistent with the oxygen uptake experiments discussed above and with the ESR data for SO$_4^{2-}$ (Mottley and Mason 1988), showing that lower concentrations of the spin trap must be used so that all the primary radicals are not trapped but have a chance for further reaction. Moreover, recent studies have confirmed the ability of DMPO to trap different protein radicals from the same system by varying its concentration (Bhattacharjee et al. 2007). Production of HSA nitrooxide adducts was also dependent on (bi)sulfite and Cu,Zn-SOD concentrations; only 500 μM (bi)sulfite was sufficient to detect positive results on the anti-DMPO Western blots, whereas (bi)sulfite concentration in wines, where it is used as a preservative, is 6 mM (Gunnison 1981).

In summary, our study showed that Cu,Zn-SOD–(bi)sulfite provides an enzymatic pathway to generate the reactive intermediate ‘$\cdot$O$_2$SO’ and ‘$\cdot$SO$_4^{2-}$’, which oxidizes HSA residues to protein radicals. We also propose that Cu,Zn-SOD may contribute to oxidative damage and tissue injury in (bi)sulfite (sulfur dioxide)–exacerbated allergic reactions. Our results suggest that SOD-dependent, sulfite-mediated oxidation of albumin residues is likely to occur in vivo, particularly at sites where Cu,Zn-SOD concentration is higher. Further studies are necessary to clarify whether alterations in Cu,Zn-SOD activity affect (bi)sulfite toxicity.

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