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Accelerated CuZn-SOD-mediated oxidation and reduction in the presence of hydrogen peroxide

Michael A. Johnson*, Timothy L. Macdonald

Department of Chemistry, University of Virginia, McCormick Road, Charlottesville, VA 22904, United States

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Abstract

Copper, zinc-superoxide dismutase (CuZn-SOD) is a cytosolic, antioxidant enzyme that scavenges potentially damaging superoxide radical ($^{\bullet}O_2^-$). Under the proper conditions, CuZn-SOD also catalyzes the oxidation and reduction of certain small molecules. Here, we demonstrate that increased exposure to hydrogen peroxide (H_2O_2), a by-product of the $^{\bullet}O_2^-$ scavenging reaction, dramatically increases the ability of CuZn-SOD to oxidize melatonin and reduce *S*-nitrosoglutathione (GSNO). After a 15 min in vitro incubation with CuZn-SOD and 1 mM H_2O_2, 76% of the melatonin was oxidized, compared to 52% with 0.25 mM H_2O_2, and just 9% without H_2O_2. Pre-incubation with 1 mM H_2O_2 resulted in a 100% increase in the rate of GSNO breakdown by CuZn-SOD in the presence of glutathione (GSH) compared to untreated CuZn-SOD. Collectively, these data suggest that even small increases in intracellular H_2O_2 levels may result in the oxidation and/or reduction of small molecules critical for proper cellular function.

Keywords: Copper; Zinc-superoxide dismutase; Melatonin; Glutathione; Hydrogen peroxide; *S*-Nitrosoglutathione

Copper, zinc-superoxide dismutase (CuZn-SOD) is a key cytosolic anti-oxidant enzyme that scavenges potentially damaging superoxide radical ($^{\bullet}O_2^-$) via the dismutation reaction: $2^{\bullet}O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$ [1]. The rate of this reaction is nearly diffusion-limited, having a rate constant of $2 \times 10^9\ M^{-1}\ s^{-1}$ [2]. CuZn-SOD is particularly abundant in oxygen-rich cells, such as neurons [3] and erythrocytes [4], where a significant amount of $^{\bullet}O_2^-$ is produced. Given the high rate of catalysis of CuZn-SOD in converting $^{\bullet}O_2^-$ to hydrogen peroxide (H_2O_2) and oxygen (O_2), it is not surprising that a significant amount of H_2O_2 is produced in these highly oxygenated environments. Much of the H_2O_2 produced is scavenged by other anti-oxidant enzymes such as catalase [5] and glutathione peroxidase [6]; however, H_2O_2 not removed by these anti-oxidant systems is available to react with the copper active site of CuZn-SOD. Upon reaction with H_2O_2, highly reactive hydroxyl radical ($^{\bullet}OH$) associated with the copper active site of CuZn-SOD is formed, resulting in the oxidation of a nearby histidine residue of the protein and disruption of the active site [7,8].

Due to the reactive nature of the copper active site, CuZn-SOD catalyzes reactions in addition to the dismutation of $^{\bullet}O_2^-$. These include peroxidation, which results in the oxidation of small molecules, and reduction, which results in the breaking of labile bonds. First identified in 1975 by Hodgson and Fridovich, the peroxidase activity causes the oxidation of small molecules by reaction with copper-associated $^{\bullet}OH$ [8,9]. The oxidized product then diffuses from the active site to bulk solution. This activity has been associated with the oxidation of a number of small molecules, including uric acid, xanthine, histidine, azide [9], and the spin trap

* Corresponding author. Present address: Department of Chemistry, CB# 3290, Venable and Kenan Laboratories, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-3290, United States. Fax: +1 919 962 2388.
E-mail address: maj5j@email.unc.edu (M.A. Johnson).

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5,5′-dimethyl-1-pyrroline N-oxide [10]. Because the small molecule takes the "oxidative hit," it has been suggested that certain molecules are effective in protecting CuZn-SOD from damage associated with peroxidation [8].

The reduction of small molecules by CuZn-SOD has also been reported [11]. During the dismutation of $^{\bullet}O_2^-$, the active site copper of CuZn-SOD cycles between the +1 and +2 oxidation states. Under reducing conditions, such as those present in the cytosol, the +1 oxidation state tends to predominate [11]. This enables CuZn-SOD to donate an electron to a small molecule able to access the active site, resulting in the reduction of the molecule. Here, we will use the term "reductase" to describe this activity. An example of this reductase activity is the CuZn-SOD-mediated degradation of GSNO (*S*-nitrosoglutathione), which is a nitric oxide ($^{\bullet}NO$) conjugate of glutathione and is thought to serve a number of biological functions [12–20]. Upon exposure to reduced glutathione, CuZn-SOD catalyzes the release of $^{\bullet}NO$ from GSNO [11].

To our knowledge, the effects of H_2O_2 on the peroxidase and reductase functions of CuZn-SOD have not been addressed. To explore this area, we exposed CuZn-SOD to various concentrations of H_2O_2 and conducted functional assays to assess changes in activity. We found that the peroxidase and reductase activities of CuZn-SOD were increased following incubation with high concentrations of H_2O_2 and that this increase in activity was not a result of the removal of copper from the enzyme active site. This suggests that elevated H_2O_2 levels in the cell alter the structure of CuZn-SOD, potentially increasing the oxidation and/or reduction of small molecules critical to proper cellular function.

Materials and methods

Chemicals. All chemicals were of the highest grade and purity available. Melatonin (*N*-acetyl-5-methoxytryptamine) was purchased from TCI Chemicals (Tokyo, Japan). Deuterated melatonin (α, α, β, β-d4-*N*-acetyl-5-methoxytryptamine; d4-melatonin) was purchased from CDN Isotopes (Pointe-Claire, Quebec). High performance liquid chromatography solvents were purchased from Fisher Scientific (Fair Lawn, New Jersey). All enzymes and all other chemicals were purchased from Sigma (St. Louis, MO).

Melatonin oxidation experiments. All incubations were conducted in 23.5 mM sodium bicarbonate buffer, pH 7.4 at 37 °C. For copper loss experiments, 10 μM CuZn-SOD was incubated with various concentrations of H_2O_2 in a total volume of 200 μL. The reaction was quenched after 15 min by adding 50 μL catalase (0.25 mg/mL) to remove H_2O_2. The proteins were then filtered out using Amicon Microcon-10 concentrators (Millipore, Bedford, MA). The filtrate of each sample was then incubated with 2.5 μM melatonin and 1 mM H_2O_2 for 20 min, and the substrate remaining was quantitated using LC-MS. Controls were also conducted using the same procedures except that various concentrations of $CuSO_4$ (used as a source of Cu^{2+}) were substituted for CuZn-SOD.

For the melatonin timecourse experiments, 2.5 μM melatonin was incubated with 10 μM human CuZn-SOD and 1 mM H_2O_2 at 37 °C in 23.5 mM sodium bicarbonate ($NaHCO_3$) buffer, pH 7.4. The total incubation volume was 40 μL. Control incubations were conducted using either 20 μM $CuSO_4$ in place of CuZn-SOD or in the absence of CuZn-SOD or $CuSO_4$. Incubations were quenched at various times by the addition of 20 μL catalase (0.25 mg/mL).

Liquid chromatography–mass spectrometry (LC–MS). Fifteen microliters of the quenched incubation mix was injected onto a Waters Symmetry C18 column (2.1 × 150 mm; Milford, MA) and the sample was isocratically eluted at 200 μL/min using 65% formic acid (0.1%) and 35% methanol. The column flow was split so that the solvent flowing at a rate of 50 μL/min was directed into an LCQ quadrupole ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) fitted with an electrospray ionization (ESI) source. The remainder of the solvent flow was diverted to waste. The mass spectrometer was configured to monitor positive ions at $m/z = 233 \pm 1$ (melatonin) and $m/z = 237 \pm 1$ (d4-melatonin). To quantitate melatonin, 20 μL of d4-melatonin (10 μM) was added as an internal standard, and the ratio of the peak areas of melatonin (at $m/z = 233$) and d4-melatonin (at $m/z = 237$) was compared to the peak area ratios from an independently generated calibration curve.

GSNO reduction experiments. All GSNO experiments were conducted in 23.5 mM sodium bicarbonate buffer, pH 7.4, at 37 °C. CuZn-SOD (50 μM) was incubated with various concentrations of H_2O_2 in a total volume of 300 μL. After a 15-min incubation period, H_2O_2 was scavenged by the addition of 100 μL catalase (0.25 mg/mL). The ability of the treated CuZn-SOD samples to catalyze GSNO breakdown at 37 °C was monitored by ultraviolet (UV) absorbance at 340 nm over the course of 2 min. The cuvet contained 30 μM of the hydrogen peroxide-treated CuZn-SOD, concentrations of H_2O_2 ranging from 0 to 10 mM, 500 μM GSNO, and 500 μM glutathione. The total volume of solution in the cuvette was 500 μL. GSH reduces the copper atom in the CuZn-SOD active site from Cu^{2+} to the redox-active Cu^{1+} oxidation state. Cu^{1+} reduces the S–N bond of GSNO, releasing free $NO^{\bullet}$.

Results and discussion

Peroxidase function of CuZn-SOD

Experiments to assess the effects of H_2O_2 exposure on copper loss were conducted. CuZn-SOD (10 μM) was pre-treated with various concentrations of H_2O_2, the remaining H_2O_2 was scavenged with catalase, and the protein was filtered out by centrifugation. The filtrate was then incubated with 2.5 μM melatonin and 1 mM H_2O_2. The filtrates from all four incubations oxidized melatonin with similar efficiency (Fig. 1, left four bars; pairwise comparisons, $p > 0.05$). Next, different concentrations of free copper (Cu^{2+}) were passed through the same filter, and incubations were conducted as described above (Fig. 1, right four bars). The degree of melatonin oxidation was significantly increased, as indicated by the decrease in melatonin remaining, in the presence of Cu^{2+} (pairwise comparison to 0.0 μM: 1.0 μM, $p < 0.05$; 10 μM, $p < 0.01$; and 25 μM, $p < 0.001$). This demonstrates that Cu^{2+} passes freely through the filter in amounts great enough to catalyze melatonin oxidation. Melatonin oxidation was significantly greater when 10 μM ($p < 0.05$) and 25 μM ($p < 0.001$) $CuSO_4$ were used than when CuZn-SOD, pretreated with 1.0 mM H_2O_2, was used, suggesting that incubation with 1 mM

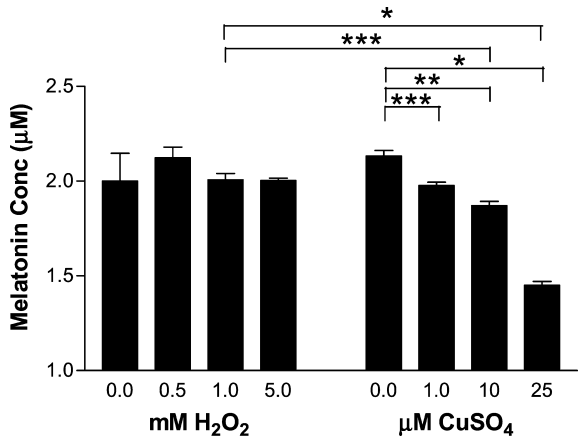

Fig. 1. Bar graph of melatonin remaining after incubation with pretreated CuZn-SOD or free Cu^{2+}. CuZn-SOD (10 μM), pretreated with different concentrations of H_2O_2, was incubated with 2.5 μM melatonin and 1 mM H_2O_2 in 23.5 mM sodium bicarbonate buffer, pH 7.4, at 37 °C for 20 min. The proteins were then filtered from the reaction mixture. Different concentrations of Cu^{2+} were treated with catalase and filtered in a manner identical to the CuZn-SOD incubations. Significance: $*p < 0.001$, $**p < 0.01$, and $***p < 0.05$.

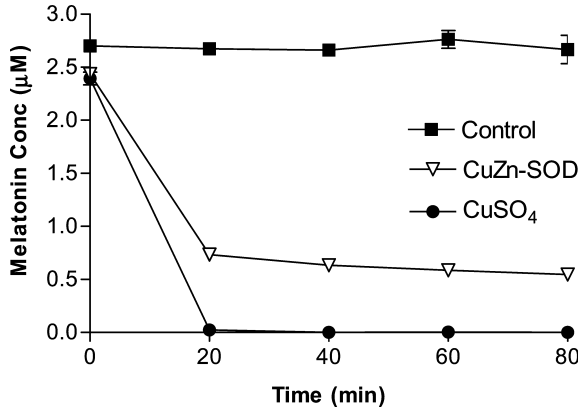

Fig. 2. Melatonin oxidation by CuZn-SOD diminishes over time. Melatonin (2.5 μM) was incubated with either 10 μM CuZn-SOD or 20 μM $CuSO_4$ in the presence of 1 mM H_2O_2 in 23.5 mM bicarbonate buffer at pH 7.4 and 37 °C. All points represent triplicate measurements and have error bars that represent means ± SEM. Symbols cover error bars at points with sufficiently small SEMs. Significance: $p < 0.001$, CuZn-SOD versus $CuSO_4$ for all points except at 0.0 min.

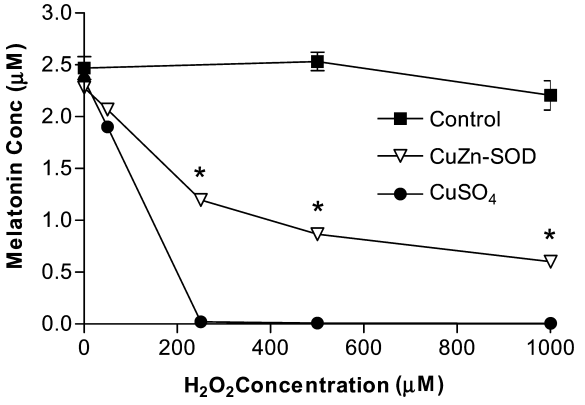

Fig. 3. Increases in H_2O_2 concentration enhance the rate of melatonin oxidation. Melatonin (2.5 μM) was incubated with either 10 μM CuZn-SOD or 20 μM $CuSO_4$ in the presence of various H_2O_2 concentrations in 23.5 mM bicarbonate buffer at pH 7.4 and 37 °C. For the control incubation, no CuZn-SOD or $CuSO_4$ is present in the mixture. All points represent triplicate measurements and have error bars that represent means ± SEM. Symbols cover error bars at points with sufficiently small SEMs. Significance: $*p < 0.001$, CuZn-SOD versus $CuSO_4$.

H_2O_2, as used in our experiments, does not cause CuZn-SOD to release copper at levels high enough to catalyze significant melatonin oxidation. Taken together, these data suggest that even high concentrations of H_2O_2 do not damage CuZn-SOD sufficiently to cause the loss of a significant amount of copper from the active site. This result is supported further by mass spectrometric studies which demonstrate that, even in the presence of 0.5 mM H_2O_2, oxidation was minimal [21]. Therefore, we concluded that melatonin oxidation was catalyzed by reaction of H_2O_2 with enzyme-bound copper.

Free copper (Cu^{2+}) in solution readily catalyzes the homolytic cleavage of the O–O bond of H_2O_2 to form two molecules of ·OH, which will oxidize the first molecule it contacts. In the case of CuZn-SOD, the surrounding protein should hinder the approach of small molecules to newly formed ·OH. To test this idea, we incubated melatonin in the presence of H_2O_2 and either copper sulfate ($CuSO_4$) or CuZn-SOD (Fig. 2). After 20 min, greater than 99% of the melatonin was oxidized in the presence of $CuSO_4$, whereas 70% of the melatonin was oxidized in the presence of CuZn-SOD ($p < 0.001$). This suggests that the protein hinders access to the active site. Moreover, the rate of melatonin oxidation in the presence of CuZn-SOD decreased dramatically after 20 min. One explanation for this decrease is that the three-dimensional structure of the active site pocket is altered over time by ·OH-mediated damage. This may further restrict the access of melatonin to the copper active site.

Next, we measured the effects of different H_2O_2 concentrations on the peroxidase activity of CuZn-SOD (Fig. 3). The reaction was quenched with catalase after 15 min. Below 250 μM H_2O_2, the concentration of H_2O_2 has a profound effect on the rate of melatonin oxidation. However, at concentrations greater than 250 μM, further increases have a diminished effect. When CuZn-SOD is replaced by free copper, these data change dramatically. Melatonin is almost completely oxidized (>99%) upon incubation with 250 μM H_2O_2. The linear portion of the CuZn-SOD plot suggests that catalytic efficiency is directly affected by H_2O_2 available for reaction at the copper active site at low H_2O_2 concentrations. Increases above 250 μM have a diminished effect on increasing the peroxidase activity of CuZn-SOD. These observations demonstrate that even small

increases in H_2O_2 concentration have a significant impact on the ability of CuZn-SOD to function as a peroxidase. Therefore, increases in intracellular oxidative stress, in the form of increased levels of H_2O_2, may result in large increases in CuZn-SOD-mediated oxidation of small molecules.

Reductase function of CuZn-SOD

To study the effects of H_2O_2 exposure on the ability of CuZn-SOD to catalyze bond reduction, we incubated 10 μM CuZn-SOD with various concentrations of H_2O_2 for 20 min and then quenched the mixture with catalase (Fig. 4A). Using UV absorbance at 340 nm, we then measured the rate at which CuZn-SOD from each incubation broke down GSNO, a nitrosylated tripeptide known to release its nitric oxide moiety upon exposure to either free Cu^{2+} or CuZn-SOD in the presence of a reductant such as GSH [11,22]. At H_2O_2 concentrations of up to 1 mM, the rate of GSNO breakdown increased linearly. Pre-treatment with greater concentrations of H_2O_2 resulted in less pronounced increases in the rate of GSNO breakdown (31% increase from 500 μM to 1 mM H_2O_2, but only a 15% increase from 1 to 2 mM H_2O_2). In the absence of CuZn-SOD and catalase, 10 mM H_2O_2 did not catalyze GSNO breakdown at a significant rate compared to incubations containing CuZn-SOD, 1 mM H_2O_2, and catalase (Fig. 4B; $p < 0.01$). Upon addition of 0.25 mg/mL catalase, the rate of GSNO degradation was negligible compared to incubations containing CuZn-SOD, 1 mM H_2O_2, and catalase ($p < 0.001$). We conclude that because we added 0.25 mg/mL catalase to scavenge only 1 mM H_2O_2, GSNO breakdown by reaction with H_2O_2 is probably not significant. These data also demonstrate a greatly accelerated rate of GSNO breakdown in the presence of even low concentrations of H_2O_2. This implies that under conditions of increased oxidative stress, the reduction of molecules critical to proper cellular function may be accelerated.

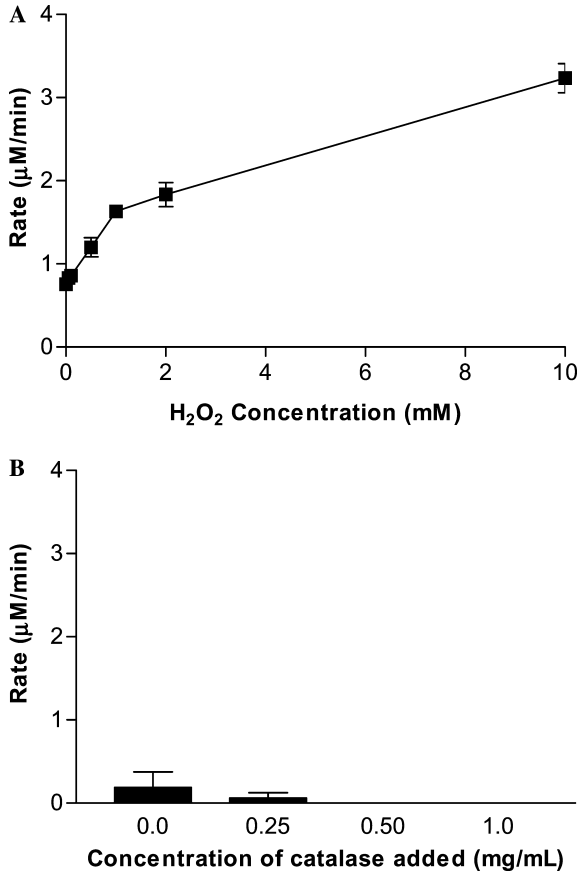

Fig. 4. Incubation with 1 mM H_2O_2 partially enhances access to the CuZn-SOD active site. (A) The initial rate of decrease in UV absorbance due to GSNO breakdown was measured for CuZn-SOD samples incubated with various concentrations of H_2O_2. (B) No-enzyme incubations were conducted in which catalase was added to 10 mM H_2O_2 and the resulting solution was assayed as in (A). Significance: for paired comparisons of all concentrations in (B), $p > 0.05$; for comparison between 0.0 mg/mL column in (B) and 1 mM point in (A), $p < 0.001$.

Here, we have described two functions of CuZn-SOD that are potentiated by increased H_2O_2 levels and the resulting damage to the enzyme itself. Our results demonstrate that in the case of both peroxidase and reductase functions, even small amounts of H_2O_2 increase the reaction rates significantly. This suggests that the copper active site becomes more exposed to the bulk solution, thereby enhancing access to small molecules. Increased oxidation and reduction of small molecules may have important implications for certain diseases. For example, it has been shown that, in the presence of H_2O_2, mutant CuZn-SODs associated with amyotrophic lateral sclerosis (ALS) preferentially oxidize DMPO, a synthetic molecule used in electron spin resonance (ESR) spectroscopic measurements [10]. This enhanced reactivity of mutant CuZn-SOD may be amplified by H_2O_2-mediated damage to the enzyme. The increased breakdown of GSNO, which is present in micromolar quantities in the brain [23] and has been shown to be neuroprotective [24,25], may also have important implications in neurodegenerative diseases in which increased oxidative stress is thought to play a role, such as ALS [10,26], Huntington's disease [27], Alzheimer's disease [28], and Parkinson's disease [29]. This study supports the view that although CuZn-SOD is a critical anti-oxidant defense, the presence of even small amounts of H_2O_2 may result in aberrant oxidative and reductive chemistry that is potentially harmful to the cell.

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