Biochemical and Biophysical Research Communications **274,** 57–60 (2000)
doi:10.1006/bbrc.2000.3092, available online at http://www.idealibrary.com on IDEAL®

Anion Protection of CuZnSOD during Peroxidative Activity with H_2O_2

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Received June 15, 2000

The "peroxidase" activity of the copper-zinc superoxide dismutase is a poorly sustained activity because of the competing inactivation of the enzyme. New evidence suggests that the bound oxidant may be partitioning between oxidizing the enzyme or oxidizing small anions. At constant peroxide, nitrite and azide only partially protect the enzyme (50%) against loss of copper(I) and inactivation up to one anion per copper. Beyond that level, there is no further protection. Bicarbonate ion also protects, but larger amounts are required. These data suggest that there is significant oxidation of the enzyme even in the presence of the small anions and therefore the formation of the bound oxidant cannot be sustained in a true catalytic process.

Key Words: **superoxide dismutase; hydrogen peroxide; oxidation; copper(I); 2-oxo-histidine; peptide fragmentation; carbonate radical; peroxidase activity; anion; bicarbonate ion; nitrite ion; nitrotyrosine.**

The ability of the copper-zinc superoxide dismutase to stimulate oxidation of substrates in the presence of hydrogen peroxide was first reported by Hodgson and Fridovich (1). Recent work from the laboratory of Kalyanaraman has documented the formation of carbonate (2) and nitro radicals (3) which carry the oxidizing power out of the active site, a process that accounts for the "peroxidase" activity of the dismutase. The formation of the copper oxidant at the active site has been examined by Jewett *et al.* (4) in terms of the sequence of events leading to inactivation through 2-oxo-histidine formation, copper(I) loss, site-specific and random peptide fragmentation, and inactivation:

$$2\,Cu^{+} + H_2O_2 \rightleftarrows 2\,Cu^{+} + O_2 + 2\,[H^{+}]$$

$$Cu^{+} + H_2O_2 \rightarrow (CuOH)^{2+} + OH^{-}$$

$$(CuOH)^{2+} + HCO_3^{-} \rightarrow Cu^{2+} + HCO_3^{\cdot} + H_2O$$

$\downarrow$

2-oxo-histidine formation
copper(I) loss
site-specific peptide fragmentation
random peptide fragmentation

This new study reports that the dismutase is protected against copper(I) loss and inactivation by the small anions, azide, nitrite, and carbonate. This result is in contrast to the lack of protection by the larger noncharged molecule, 5,5′-dimethyl-pyrolline-*N*-oxide (DMPO) (4). The active site of the dismutase is severely restricted to the approach of large molecules as shown by the work of Schultz *et al.* (5) so that the lack of protection by the DMPO was not unexpected.

MATERIALS AND METHODS

Bovine liver copper-zinc superoxide dismutase (CuZnSOD) was obtained from OXIS International, epinephrine, hydrogen peroxide, sodium nitrite, sodium azide, sodium bicarbonate, and common chemicals for enzyme assays and for nitrite analyses by the Greiss reagents were purchased from Sigma. Electrophoresis chemicals were obtained from BioRad. Water was deionized having a resistence of greater than 2 MΩ. The experimental design, standardization of hydrogen peroxide, characterization of the dismutase, superoxide dismutase assays, copper analyses, and native polyacrylamide gel electrophoresis are described in Jewett *et al.* (4). Anions were added to reaction mixtures before the addition of peroxide. Nitrite was quantified using the Greiss reagents (6) and nitrotyrosinate and nitrotyrosine were detected spectrophotometrically at pH 11 and pH 5, respectively (7).

RESULTS AND DISCUSSION

At early times of reaction at pH 7.4 with 10 H_2O_2 per Cu^{2+}, nitrite (and azide (data not shown)) significantly protect the copper-zinc superoxide dismutase from loss of copper(I) in the reaction with hydrogen peroxide as shown in Fig. 1. Bicarbonate also protects but higher levels are required even with the lower 5 H_2O_2 per Cu^{2+} (Figs. 2 and 3). For data collected at the end of the reaction with 5.0 H_2O_2 per Cu^{2+}, the protection is dependent upon the level of the anions, with a maximum of 50% protection occurring with about one azide and

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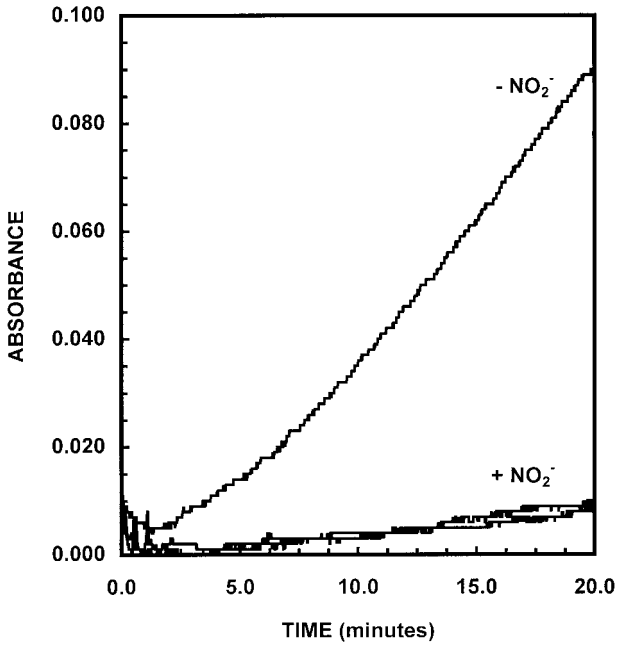


FIG. 1. Protection against the loss of copper(I) at early times in the reaction of 0.081 mM SOD with 10 H_2O_2 per Cu^{2+} in 0.050 M TRIS at pH 7.4 (μ = 0.10 M, NaCl) with 1 mM and 10 mM nitrite. The copper(I) was measured at 485 nm using bathocuproine disulfonate.

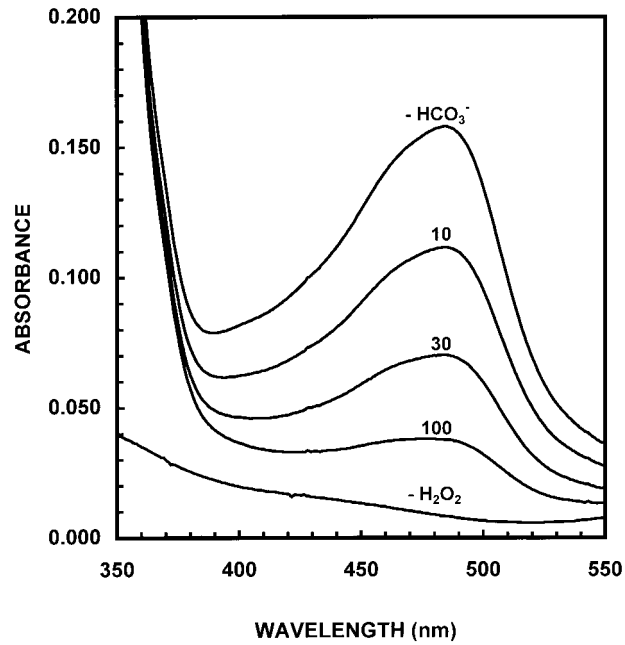


FIG. 3. Release of copper(I) from the oxidation of 0.080 mM SOD by 5.0 H_2O_2 per Cu^{2+} for 48 hours at 4°C at pH 7.4 in 0.050 M TRIS at pH 7.4 (μ = 0.10 M, NaCl). Bicarbonate was added above the copper(II) at the levels indicated. Copper(I) was measured at 485 nm with bathocuproine disulfonate.

nitrite per copper(II) (Fig. 4). This level of protection is less than the level reported by Hodgson and Fridovich (1) who used both higher peroxide and higher anion concentrations. This 50% protection is also seen in the native electrophoretic pattern (and activity measurements) in which there is an increase in the proportion of the native enzyme band I (and activity) remaining at the end of the reaction with peroxide in the presence of the anions (Fig. 4). Liochev and Fridovich (8) recently reported that bicarbonate does not protect against the loss of activity using 3 μM SOD with 10,000 μM H_2O_2 (1700 H_2O_2 per Cu^{2+}) and 1700 HCO_3^- per Cu^{2+}. This excessive peroxide would be expected to rapidly oxidize the enzyme, diminishing any protection that could be possible with this anion.

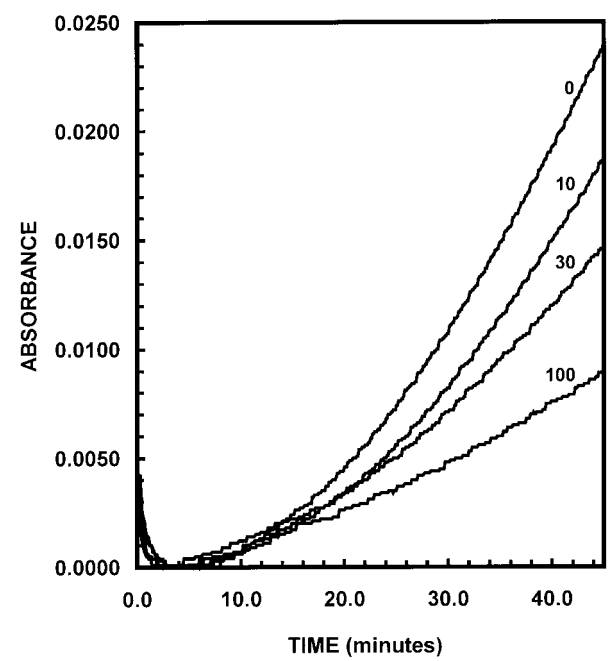


FIG. 2. Effect of bicarbonate on the loss of copper(I) at early times in the reaction of 0.080 mM SOD with 5.0 H_2O_2 per Cu^{2+} in 0.05 M TRIS at pH 7.45 ± 0.05 (μ = 0.10 M, NaCl). HCO_3^- was added at the levels indicated over the copper(II). Copper(I) was measured at 485 nm using bathocuproine disulfonate.

Nitrite analyses using the Greiss reagents indicated a small loss of nitrite ion with constant 10 NO_2^- per Cu^{2+} and varying peroxide (Fig. 5). The nitrite loss is accompanied by a comparable amount of nitrotyrosine which was identified at pH 5 and 11 (Fig. 6). A model shown in Scheme 1 was developed to account for the loss of copper(I) and for the formation of nitrotyrosine (10). For each reaction cycle, this model assumes 6% loss of copper(I) with 3% of the oxidized nitrite ion forming nitrotyrosine. The summary of the analyses is presented in Table 1 for 5 H_2O_2 per Cu^{2+} with 2 NO_2^- per Cu^{2+}. This analysis indicates that only four reaction cycles are required to consume all the peroxide and to account for the loss of copper(I) and formation of nitrotyrosine.

The data reported here indicate that the copper-zinc superoxide dismutase is not completely protected by nitrite, azide, or bicarbonate, against the oxidation by hydrogen peroxide even at low ratios of peroxide. The

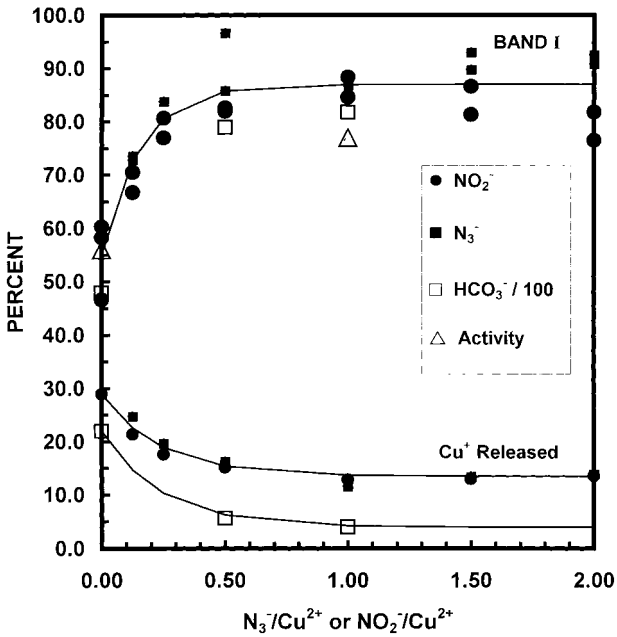


FIG. 4. Protection by anions against loss of Cu^+, band I, and activity (for NO_2^-) in the 24-h reaction at room temperature of 0.080 mM SOD with 5.0 H_2O_2 per Cu^{2+} at pH 7.4 in 0.050 M TRIS (μ = 0.10 M, NaCl). The protection by HCO_3^- requires 100-fold higher anion concentration.

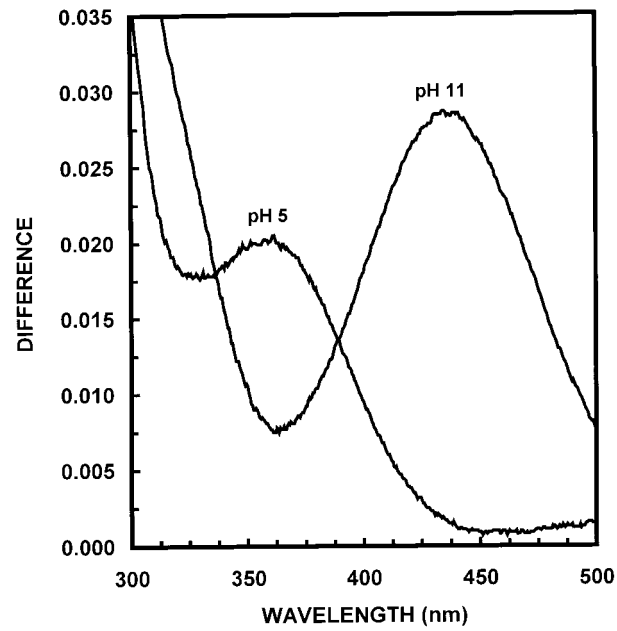


FIG. 6. Difference spectra showing the formation of nitrotyrosine in the reaction of 80 mM SOD with 5.0 H_2O_2 per Cu^{2+} and 2.0 NO_2^- per Cu^{2+}. Samples were diluted 38% in achieving the pH values shown.

less than quantitative protection can be explained by the partitioning of the bound oxidant between oxidizing a bound anion or a histidine ligand (or the site responsible for site-specific peptide fragmentation). An alternative interpretation is that the anions may be partly blocking access of hydrogen peroxide to the reduced enzyme by binding at the arginine near the active copper. Leone *et al.* (9) have shown using FT-IR that azide binds to copper only in the oxidized state of the bovine enzyme not in the reduced state. These investigators suggest that the Arg 141 of the CuZn-SOD, and not copper(I), is responsible for binding azide in the reduced state. Because nitrotyrosine is formed in the dismutase protected with nitrite, and because azide, nitrite, and bicarbonate facilitate the oxidation of other substrates that cannot enter the active site (2, 3), the small anions are also being oxidized at the bound oxidant and, to that extent, are protecting the enzyme against oxidation.

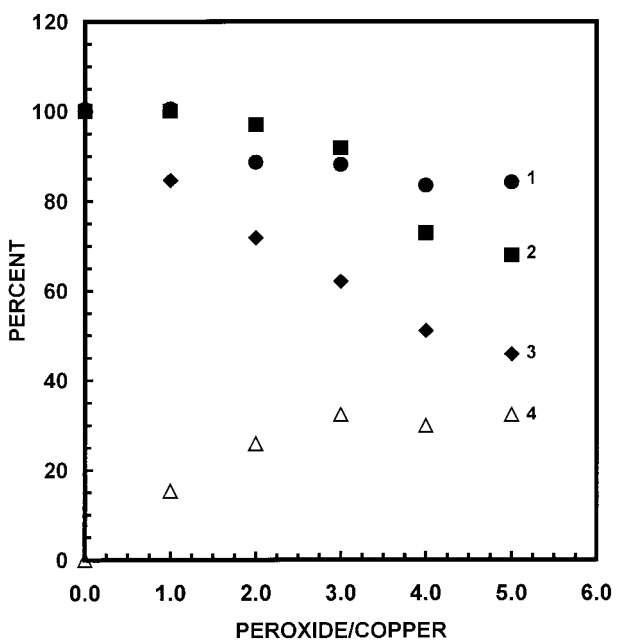
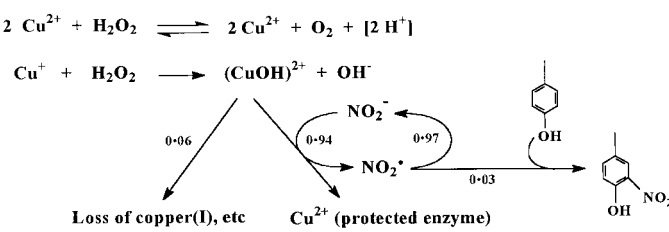


FIG. 5. Loss of nitrite ion (●1) and the major native electrophoretic band I (◆3) in the reaction of SOD with H_2O_2 for 24 h at room temperature at pH 7.4 in 0.050 M TRIS (μ = 0.10 M, NaCl). Samples protected with 10 NO_2^- per Cu^{2+} (■2) at showed a gain in Band I (△4).

The use of the term "peroxidase" activity for the CuZnSOD, while convenient and useful, should clearly be understood as transient activity. With each cycle of formation of the bound oxidant, even in the presence of the small anions, there is a finite probability that the dismutase will be inactivated so that the formation of



SCHEME 1

TABLE 1

Comparison of Calculated and Measured Amounts of Copper(I) Lost and Nitrotyrosine Formed in the Reaction of CuZnSOD with H_2O_2[1]

Cycle	Copper(I) lost[2] (μM)	Percent of Total Cu	Nitrotyrosine formed[3] (μM)	Percent of Total Cu
1	9.60	6.00	4.51	2.82
2	9.02	5.64	4.24	2.65
3	8.48	5.3	3.99	2.49
4	3.35	1.76	1.32	0.83
Theoretical Total	30.5	18.7	14.4	7.79
Experimental Total	30.0	18.7	13.2	8.23

[1] Experimental conditions were 80 μM SOD, 800 μM H_2O_2, and 320 μM NO_2^- (5 H_2O_2 per Cu^{2+} and 2 NO_2^- per Cu^{2+}) for 24 h at pH 7.4 in 0.050 M TRIS (μ = 0.10 M, NaCl).

[2] Assumes a 6.0% loss of copper(I) in each reaction cycle.

[3] Assumes that 3.0% of the nitrite oxidized in each reaction cycle forms nitrotyrosine.

the bound oxidant cannot be sustained in a true catalytic process. Evidence for the transient nature of this activity is apparent in the earliest data of Hodgson and Fridovich in that luminescence due to oxidation of compounds such as xanthine and imidazole quickly reaches a maximum with time and diminishes (1). We suggest that the ability of the copper-zinc superoxide dismutase to act as a prooxidant in the presence of hydrogen peroxide be referred to as the "peroxidative" or "prooxidant" activity in order not to confuse this activity with a true enzymatic process.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health Minority Biomedical Research Support Grant 5S06GM48680-06S1, and by Graduate Studies, the Research and Grants Committee, and the College of Science and Mathematics of California State University, Northridge.

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