Bicarbonate Is Required for the Peroxidase Function of Cu,Zn-Superoxide Dismutase at Physiological pH*

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Cu,Zn-superoxide dismutase (SOD1) acts as a peroxidase in the presence of H₂O₂ at high pH (pH > 9). The high pH species of H₂O₂, HO₂⁻, was previously implicated as the reactive species. However, recent EPR studies of the enzyme performed in the physiological pH range 7.4–7.8 with the spin trap 5,5′-dimethyl-1-pyrolline-N-oxide attributed the intense EPR signal of 5,5′-dimethyl-1-pyrolline-N-oxide-OH obtained from SOD1 and H₂O₂ to the peroxidase activity of the enzyme. The present study establishes that this intense signal is obtained only in the presence of bicarbonate. To explore the critical role of HCO₃⁻, a comprehensive EPR investigation of the radical production and redox state of the active site copper was performed. The results indicate that HCO₃⁻ competes with other anions for the anion-binding site of SOD1 (Arg¹⁴¹) but does not bind directly to the copper. Structurally different anions that bind to Arg¹⁴¹ did not stimulate, but rather blocked, peroxidase function, ruling out an effect due to mere anion binding. However, the structurally similar anions HSO₃⁻ and HSO₄⁻ mimic HCO₃⁻ in stimulating peroxidase function. These data suggest that HCO₃⁻ bound to Arg¹⁴¹ anchors the neutral H₂O₂ molecule at the active site copper, enabling its redox cleavage. Thus, SOD1 acquires peroxidase activity at physiological pH only in the presence of HCO₃⁻ or structurally similar anions. Alterations in pH that shift the HCO₃⁻/CO₂ equilibrium as occur in disease processes such as ischemia, sepsis, or shock would modulate the peroxidase function of SOD1.

Superoxide dismutases (SODs; EC 1.15.1.1) are a ubiquitous family of metalloenzymes that catalyze the dismutation of superoxide anion, O₂⁻, to form O₂ and H₂O₂. These enzymes are highly efficient in catalyzing the dismutation at a rate close to the diffusion limit, over the entire pH range from 5 to 10 (1, 2). Three distinct isozymes are present in mammalian cells: the cytosolic, homodimeric Cu,Zn-enzyme (Cu,Zn-SOD or SOD1), the manganese-containing mitochondrial SOD (Mn-SOD or SOD2) and the extracellular form of CuZn-SOD (or SOD3) (2). Several lines of evidence indicate that CuZn-SOD, but not Mn-SOD, undergoes free radical damage by its own product H₂O₂ (3), resulting in inactivation (4–7) and fragmentation of the enzyme (8, 9) and production of the highly reactive oxidant •OH (5, 9–12). The inactivation of CuZn-SOD is reported to be caused by the oxidation of the active site histidine, His¹¹⁸ (4–6, 10), but the mechanism of this reaction is not fully understood. Hodgson and Fridovich (5, 14) proposed a mechanism in which H₂O₂ first reduces the CuII and then reacts with the CuI to give a potent oxidant, most likely a Cu²⁺-bound •OH, which can attack an adjacent histidine and destroy the integrity of the catalytic site. Alternately, exogenous reductants such as xanthine, urate, formate, and azide can protect the enzyme when they serve as sacrificial substances and spare the essential histidines (5, 14, 15). Thus, Cu-Zn-SOD, in addition to its SOD activity, can exert a peroxidase function toward these exogenous reductants at rates competitive with its own oxidative inactivation (5, 14).

Prior studies reported that inactivation of CuZn-SOD by H₂O₂ proceeds rapidly only at pH values above 9.0 (5–8, 12, 14, 16–18). HO₂⁻, rather than H₂O₂, was implicated as the active species, due to its resemblance to O₂⁻ in structure and charge (6–8, 12, 16–18). Although H₂O₂ was able to reduce the active site Cu²⁺ to Cu⁺ at high concentrations, the reaction of H₂O₂ to form the bound •OH was favored only when the pH was raised above 8, since the pKₐ for H₂O₂ is 11.9 (12). However, more recently, EPR spin trapping studies demonstrated that CuZn-SOD and H₂O₂ at normal physiological pH values in the presence of the spin trap, DMPO, yield large amounts of the hydroxyl adduct, DMPO-OH, which was attributed to free •OH (10, 11). Subsequently, two related spin trapping studies (19, 20), also performed at pH 7.4, implicated this phenomenon in the gain-of-function of CuZn-SOD mutants associated with the familial form of amyotrophic lateral sclerosis, a progressive degenerative disorder of motor neurons leading to paralysis. In general, it is difficult to explain these results based on the alkaline-based HO₂⁻-mediated reaction mechanism. While H₂O₂ with its pKₐ of 11.9 gives rise to traces amounts of HO₂⁻ at pH 7.4 and this might react with Cu⁺ at the active site, the rate constant of this reaction is quite low at neutral pH (kₐobs < 0.1 M⁻¹ s⁻¹) (12). Therefore, one would expect little if any detectable DMPO-OH generation at pH 7.4. Hence, the high magnitude EPR signals obtained (10, 11, 20) cannot be accounted for by this reaction at neutral pH.

While most of the in vitro spin trapping studies in the physiological pH range are carried out in a variety of buffers, all of the above spin trapping studies with CuZn-SOD (10, 19, 20) and H₂O₂ were performed only in bicarbonate buffer. However, there has been no prior explanation for the importance of HCO₃⁻ in this system. Therefore, in the present study, we evaluate the role of HCO₃⁻ in the peroxidase function of CuZn-

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‡ The abbreviations used are: SOD, superoxide dismutase; SOD1 or CuZn-SOD, Cu,Zn-superoxide dismutase; O₂⁻, superoxide; DMPO, 5,5′-dimethyl-1-pyrolline-N-oxide; TEMPO, 2,2,6,6-tetramethyl-1-piperidinyloxy (nitrotriacetate); CAPS, 3-(cyclohexylamino)-propanesulfonic acid; ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid.)
SOD. We observe that large magnitude DMPO-OH generation occurs only in bicarbonate buffer with no significant signal obtained in other buffers. The critical role of HCO$_3^-$ in producing these signals is characterized. Structural changes of the active site copper in the presence of HCO$_3^-$ are studied by EPR measurements of the Cu(II). These studies suggest that at physiological pH, HCO$_3^-$ binds to the anion-binding site of CuZn-SOD and facilitates the approach of H$_2$O$_2$ to the active site, enabling its redox cleavage.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine erythrocyte CuZn-SOD was purchased from Sigma (98% enzyme, 4000–5000 units/mg) or Worthington (100% enzyme, 2273 units/mg). Activity was assayed by the method of Beauchamp and Fridovich (21). Human CuZn-SOD, Mn-SOD, sodium formate, sodium azide, sodium bicarbonate, sodium bisulfite, sodium hydrogen selenite, hydrogen peroxide (30% w/w), Hepes, Tris, sodium hydrogen phosphate, disodium hydrogen phosphate, CAPS, potassium thiocyanate, potassium sulfate, sodium nitrate, borate, 2,2,6,6-tetramethyl-1-piperidinyl-1-oxide (TEMPO), diethylenetriaminepentaacetic acid (DTPA), Me$_3$SO, and ethanol were obtained from Sigma. Deferoxamine mesylate was obtained from Ciba Pharmaceuticals, Inc., and Fe$^{3+}$-(nitrilotriacetate)$_2$ (Fe-NTA) was prepared as described previously (22). 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) was obtained from Boehringer Mannheim. Purified DMPO was purchased from Oklahoma Medical Research Foundation Spin Trap Source. Phosphatase buffer (pH 7.0), containing 100 mM DTPA and purged with dry nitrogen gas, was used to prepare stock solutions of DMPO.

**EPR and Optical Spectroscopy**—EPR spectra were recorded in quartz flat cells at room temperature with a Bruker ER 300 or ESP 300E spectrometer operating at X-band with 100-kHz modulation frequency and a TM 110 cavity. The microwave frequency and magnetic field were precisely measured using an EIP 575 microwave frequency counter and a Bruker ER035MR NMR gaussmeter. EPR spectral simulations were performed using computer programs as described previously (23). Quantitation of the free radical signals was performed by computer simulation of the spectra and by comparison of the double integral of the observed signal with that of a TEMPO standard (10 mM) measured under identical conditions (24). EPR spectra of the active site Cu$^{2+}$ of CuZn-SOD were recorded in 3-mm quartz tubes at 77 K using a liquid nitrogen dewar. A Perkin-Elmer Lambda-6 UV-VIS spectrophotometer was used for optical studies.

**RESULTS**

Either human or bovine CuZn-SOD (1.25 μM), dissolved in 23.5 mM bicarbonate buffer (pH 7.4) equilibrated with 5% CO$_2$ and 95% N$_2$, gave rise to a large EPR signal when treated with H$_2$O$_2$ (1 mM) in the presence of DMPO (50 mM) (Fig. 1A). The quartet signal with typical intensity ratio, 1:2:2:1, and hyperfine couplings, $a_H = a_N = 14.9$ G, confirmed by computer simulation, corresponds to DMPO-OH (24, 25). The intense signal was only obtained in bicarbonate buffer and was largely insensitive to changes in pH in the range 7.0–8.0. With similar conditions of enzyme and H$_2$O$_2$ concentrations but in other buffers including Hepes, PBS, and Tris (Fig. 1B), no signal was observed. Previously, the reaction between H$_2$O$_2$ and the reduced copper of the active site was shown to increase at high pH, due to the ionization of H$_2$O$_2$ to HO$_2$ ($pK_a = 11.9$) (6, 12). Such an enhanced interaction was suggested to promote the formation of the copper-bound ‘OH and rapid inactivation of the enzyme (6, 12). Anticipating a parallel increase in the formation of the copper-bound ‘OH and rapid inactivation of the enzyme. EPR spectra were measured every 5 min for more than 1 h following the addition of H$_2$O$_2$ (1 mM) to CuZn-SOD (1.25 μM) and H$_2$O$_2$ (1 mM) in the presence of DMPO-OH formation, experiments were carried out at pH 10.5. While no signal was observed in CAPS buffer (Fig. 1C), a small DMPO-OH signal was obtained in Na$_2$CO$_3$ buffer (Fig. 1D). Thus, the presence of HCO$_3^-$ rather than a high pH is critical for the DMPO-OH formation. To exclude the possibility of trapping some bicarbonate-derived radicals obtained from a reaction between ‘OH and HCO$_3^-$, a Fenton ‘OH generating system of Fe-NTA (10 μM) and H$_2$O$_2$ (1 mM) was carried out in phosphate buffer (pH 7.0) with DMPO (50 mM) in the presence or absence of HCO$_3^-$ (20 mM). Except for the first 5 min, the DMPO-OH signal declined in the presence of HCO$_3^-$ compared with that observed in the absence of HCO$_3^-$ (Fig. 2). In similar experiments with the Fenton ‘OH generating system at pH 10.5, similar DMPO-OH spectra were observed, demonstrating that the decrease in signal from SOD at pH 10.5 shown in Fig. 1D was not simply due to a loss of efficacy of the trap.

The vital role played by HCO$_3^-$ in the peroxidative function of CuZn-SOD toward DMPO in producing DMPO-OH was further investigated in the context of the enzyme’s affinity for anions (2, 26). The substrate for SOD itself is an anion, and several investigations have reported the effects of anion binding to CuZn-SOD with some of them inhibiting the enzymatic activity (2, 26–35). Production of DMPO-OH by CuZn-SOD and H$_2$O$_2$ was examined in the presence of a variety of anions, such as azide, nitrate, sulfate, borate, thiocyanate and formate. These anions were reported to have affinity for the anion site of the enzyme. EPR spectra were measured every 5 min for more than 1 h following the addition of H$_2$O$_2$ (1 mM) to CuZn-SOD (1.25 μM), DMPO (50 mM), and the anion (20 mM) at pH 7.0 (Fig. 3). As shown in Fig. 3, these anions did not cause significant DMPO-OH generation in comparison with HCO$_3^-$.$^\text{1}$ However, these anions when taken together with HCO$_3^-$, diminished the signal normally observed with HCO$_3^-$.$^\text{1}$ As an example, the effect of NaH$_2$PO$_4$ and NaCl are shown in Fig. 4, A and B. The decrease in the signal ranged from 20 to 90%, depending on the type of anion and its concentration.

In the past, x-ray crystallography and EPR investigations of CuZn-SOD indicated the proximity of anion binding residues to the copper site (2, 27–30, 33–37). Only certain anions that directly coordinate the copper cause significant changes in the EPR spectrum of the Cu$^{2+}$ (2, 27, 29–31, 36). Thiocyanate ($K_d$
200 mM) and azide (Kd 10–16 mM) are typical examples that coordinate the Cu2+ and induce distinct changes in the copper hyperfine splitting parameters (27–30). We compared the effects of thiocyanate (200 mM), azide (20 mM), and bicarbonate (200 mM) at pH 7.0 on the Cu2+-EPR at 77K of CuZn-SOD (0.5 mM) (Fig. 5). The anisotropic hyperfine coupling constants (A∥) of SCN-SOD (148 G; Fig. 5B) and N3-SOD (156 G; Fig. 5C) were considerably larger than that of the native enzyme (135 G, Fig. 5A), in agreement with the previous reports (27–30). In contrast, the A∥ value for CuZn-SOD with HCO3− (Fig. 5D) remained the same as the native enzyme, indicating that HCO3− does not bind directly to the copper.

To further understand the unique mechanism in which HCO3− facilitates the access of the active site by the neutral H2O2, several anions were studied based on their structural similarity to HCO3−. Indeed, bisulfite (HSO3−) and biselenite (HSeO3−), which are isostructural to HCO3−, effected the generation of DMPO-OH in a similar fashion as HCO3−. While the sample containing 1.25 mM CuZn-SOD, and 1 mM H2O2, in the presence of DMPO did not give rise to any signal in the absence of these anions or HCO3− (Fig. 6A), it generated intense EPR signals when supplemented with 20 mM HCO3− (Fig. 6B), HSO3− (Fig. 6C), or HSeO3− (Fig. 6D). The signal obtained with HSO3− (Fig. 6C) actually corresponds to the zSO3− radical adduct of DMPO, DMPO-zSO3−, as confirmed by the characteristic hyperfine splittings, aH = 16.0 G and aN = 14.7 G (38). Prior studies reported that zSO3− radicals might also be generated by the autooxidation of the bisulfite anion, which could be prevented by 1 mM DTPA (38). In addition, H2O2 has been reported to react directly with bisulfite to produce zSO3− (38). Hence, we included 1 mM DTPA in our experiments to prevent spontaneous zSO3− generation, and control measurements in the absence of CuZn-SOD were performed to subtract the contribution from the direct reaction of bisulfite with H2O2 for the signal of DMPO-zSO3− in Fig. 6C.

Two distinct experiments were performed to assess the effect of concentrations of H2O2 and HCO3− on the DMPO-OH signal.
Superoxide, a normal by-product of aerobic metabolism, is generated in diverse physiological and pathological processes such as oxidative phosphorylation and the respiratory burst of activated phagocytes (15, 40). \( \text{O}_2^- \) has a very short half-life and can lead to the formation of various reactive oxygen species including ‘OH, \( \text{H}_2\text{O}_2 \), and peroxynitrite (ONOO\(^-\)), which can damage cellular macromolecules and contribute to tissue injury (15). The fundamental necessity for SOD as a first defense against \( \text{O}_2^- \) is documented by its presence in all aerobic organisms, and its deficiency has been reported to cause abnormalities in various organisms (15). SOD has been intensively studied as a therapeutic agent in pathological conditions related to oxidative stress, tissue damage, and inflammation (40, 41). Contrary to this, there have been reports implicating CuZn-SOD in the pathogenesis of certain disorders through impaired function or overexpression (42, 43). For instance, overexpression of the SOD1 gene on chromosome 21 of Down syndrome patients was reported to cause oxidative damage to biomacromolecules (42). Recent studies demonstrated point mutations in the SOD1 gene in some cases of familial ALS (44, 45). Initial studies of the familial amyotrophic lateral sclerosis-associated mutants predicted that the mutations destabilize the protein structure, leading to a less active enzyme (45). Subsequent studies with transgenic mice overexpressing familial amyotrophic lateral sclerosis-linked mutations suggested that motor neuron degeneration was caused by some toxic function gained by the mutant SOD1 (45). The nature of this cytotoxic gain-of-function is yet to be identified, and current studies of the mechanisms have focused on the non-SOD activity of the en-

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**FIG. 5.** EPR spectra of the active site Cu\(^{2+}\) of SOD. Spectra were observed from 0.5 mM CuZn-SOD in phosphate buffer (50 mM, pH 7.4) at 77 K. A, control with no anions; B, with 200 mM KSCN; C, with 20 mM NaNO\(_2\); D, with 200 mM NaHCO\(_3\). Spectra were recorded at a microwave frequency of 9.35 GHz, a microwave power of 20 milliwatts, and a modulation amplitude of 4 G.

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**DISCUSSION**

types of measurements, it was observed that the peroxidase activity as evidenced by the formation of the radical cation is bicarbonate-dependent.

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**FIG. 6.** Mimicking of bicarbonate-mediated DMPO-OH generation by isostructural anions. EPR spectra were observed from CuZn-SOD (1.25 \( \mu \)M) and \( \text{H}_2\text{O}_2 \) (1 mM), DTPA (1 mM) in Hepes buffer (50 mM, pH 7.4) containing no anions (A), 20 mM NaHCO\(_3\) (B), 20 mM NaHSO\(_3\) (C), or 20 mM NaHSO\(_4\) (D). EPR spectral parameters were as described in the legend to Fig. 1. Each spectrum consists of the sum of 10 30-s scans recorded immediately after the addition of \( \text{H}_2\text{O}_2 \).
zyme involving H$_2$O$_2$ (19, 20, 45). Hodgson and Fridovich (5, 14) showed that in addition to the major SOD activity, CuZn-SOD possesses a peroxidase activity that utilizes its own dismutation product, H$_2$O$_2$, as a substrate according to the following mechanism.

\[
\begin{align*}
\text{SOD-Cu}^2+ + \text{H}_2\text{O}_2 &\Leftrightarrow \text{SOD-Cu}^3+ + \cdot\text{O}_2^- + 2\text{H}^+ \\
\text{SOD-Cu}^2+ + \text{H}_2\text{O}_2 &\rightarrow \text{SOD-Cu}^3-\text{OH} + \cdot\text{OH} \\
\text{SOD-Cu}^2-\text{OH} + \text{ImH} &\rightarrow \text{SOD-Cu}^3+ + \text{Im} + \text{H}_2\text{O}
\end{align*}
\]

**REACTIONS** 1–3

The oxidant ·OH generated in Reaction 2 was assumed to remain bound to the copper because it did not react with known scavengers for free ·OH like alcohols or benzoate. This oxidant attacks the imidazole (ImH) of an adjacent histidine (at the active site) as in Reaction 3 and inactivates the enzyme (5). Exogenous electron donors such as formate, azide, and urate were found to serve as sacrificial substrates and prevent the inactivation (5, 14).

However, early studies reported that the interaction between CuZn-SOD and H$_2$O$_2$ was an affinity reaction increasing with increasing pH, with rapid enzyme inactivation at pH > 9.0 (5–8, 12, 14, 16–18). Blech and Borders (6) proposed that the reactive species was HO$_2^-$. The pK$_a$ for H$_2$O$_2$ is 11.9, so an increase in pH would lead to an increase in the concentration of HO$_2^-$. It was proposed that HO$_2^-$ coordinated directly to the copper to form the reactive complex (6). They proposed a kinetic model for the inactivation process as follows (6).

\[
\begin{align*}
\text{H}_2\text{O}_2 &\Leftrightarrow \text{HO}_2^- + \text{H}^+ \\
k_1 &\quad \text{SOD-HO}_2^- \rightarrow \text{SOD}_{\text{inact}}
\end{align*}
\]

**REACTION** 4

The apparent dissociation constant for the enzyme-peroxide complex, decreased progressively with increasing pH, from 15.5 mM at pH 9.0 to 1.11 mM at pH 11.5 (6). This was justified in view of the enzyme's high electrostatic affinity for anionic substances including its substrate, O$_2^-$ (12). The x-ray structure of CuZn-SOD illustrated the existence of a positively charged channel with amino acid residues, Lys120 and Lys134 at the top and Arg141 inside the channel positioned close (4–5 Å) to the active site copper (37). This channel is responsible for the electrostatic guidance of the anionic substrate to the active site (37). Arg$^{141}$ with a pK$_a$ of 12 has been frequently implicated as essential in anchoring the superoxide anion in a suitable position (2, 37). Other small anions such as cyanide, azide, halides, phosphate, borate, formate, cyanate, and thiocyanate were also known to have easy access to the channel and bind to the active site copper or Arg$^{141}$ (26, 28). Since HO$_2^-$ is a small negatively charged species, differing from O$_2^-$ by only a hydrogen atom, the electrostatic effects are presumed to be similar (12). This ex-
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The generation of the ABTS radical cation (Fig. 8, bottom panel) and changes in the optical absorption spectrum of ABTS (Fig. 8, top panel) confirm that the bicarbonate-induced peroxidase function of Cu,Zn-SOD at physiological pH is a generalized phenomenon occurring with substrates other than DMPO.

Experiments performed with different anions that bind to the anion site demonstrate that mere binding of any anion was not sufficient for DMPO-OH generation (Fig. 3). However, the ability of these anions to decrease the signal amplitude observed with HCO$_3^-$ indicates that HCO$_3^-$ indeed binds to the anion site (Arg$^{141}$) for which other anions compete (Fig. 4). In the past, techniques including X-ray crystallography (28), NMR (46), and EPR (27, 29) have been used to identify anions that bind directly to the copper. For instance, the crystal structure of azide-inhibited CuZn-SOD suggested that azide mimicked O$_2^-$ binding, with a direct coordination to the Cu$^{2+}$ to Cu$^{2+}$ reaction (18).

In the absence of O$_2$, the first report (18) demonstrated that enzyme inactivation by H$_2$O$_2$ occurred only in the presence of O$_2$ (37 °C). The second report (8) indicated that CuZn-SOD inactivation and oxidative protein degradation in red blood cells was greater when cells were exposed to a flux of O$_2$ and H$_2$O$_2$ than a bolus of H$_2$O$_2$ alone. Both reports implicate the role of H$_2$O$_2$.

Therefore, it is clear that the large amounts of DMPO-OH generated by CuZn-SOD and H$_2$O$_2$ at pH 7.4 in the present study as well as prior reports cannot be explained by a simple interaction between the enzyme and H$_2$O$_2$. It is important to note that all these spin-trapping studies were performed only in bicarbonate buffer. In the present study, we observed that no significant DMPO-OH was obtained in other buffers. Hence, the aim of the present work was to explain the interaction between the enzyme and H$_2$O$_2$ in the generation of DMPO-OH at physiological pH. This report delineates the critical role played by HCO$_3^-$ in the peroxidase function of CuZn-SOD at physiological pH, and in turn, it establishes that the HCO$_3^-$/CO$_2$ equilibrium may influence free radical metabolism in normal pathophysiology and disease.

We demonstrate that the large amounts of DMPO-OH obtained from CuZn-SOD, H$_2$O$_2$, and DMPO are formed by a rapid bicarbonate-assisted one-electron oxidation and hydroxylation of DMPO (Fig. 1). The fact that high pH alone could not trigger this signal generation indicates that this mechanism is quite distinct from that of high pH inactivation by H$_2$O$_2$. The large signal decrease observed in the Na$_2$CO$_3$ buffer at pH 10.5 (Fig. 1D) can be explained by a combination of factors. First, based on the pK of free HCO$_3^-$ of 10.25, its concentration would be more than 2-fold decreased at pH 10.5. Furthermore, the pK of the HCO$_3^-$ bound to the arginine of the anion-binding site, Arg$^{141}$, might be further shifted downward. In addition, compared with pH 7.4, some deprotonation of Arg$^{141}$ would occur at pH 10.5 (6). It has also been reported that at high pH, OH$^-$ competes for the anion site (2). At this highly alkaline pH, changes in protein conformation could also occur, altering the structure of the enzyme and the relationship of the anion and copper sites. All of these factors would contribute to decreasing bicarbonate binding and explain the decrease in bicarbonate-dependent peroxidase function at high pH. However, even at pH 10.5, a small signal was seen as shown in Fig. 1D in contrast to the absence of signal in CAPS buffer at this pH (Fig. 1C).

Other probable reactions such as trapping of a fast decaying HCO$_3^-$ radical intermediate obtained from 'OH or improvement of the spin trapping efficiency in bicarbonate buffer was ruled out by the control experiments with the Fenton system, where the HCO$_3^-$ greatly decreased the DMPO-OH formation (Fig. 2). In the Fenton system HCO$_3^-$ (23.5 mM) resulted in more than a 5-fold decrease in the DMPO-OH signal, while in the CuZn-SOD system HCO$_3^-$ induced more than a 100-fold increase in DMPO-OH. Further evidence that adduct formation occurs at the active site of the enzyme is provided by the fact that DMPO alkyl adducts are not observed even in the presence of Me$_3$SO or ethanol (10).

The velocity equation for this system is given by the following expression (47).
According to this equation, at a constant $[S]_0$, increasing $[A]_0$ will result in an increasing rate up to the point where all of the $S$ is converted to SA (this will happen when $[S]_0 = [A]_0$ when $K_0$ is very small compared with $[A]_0$) (47). Increasing $[A]_0$ further will decrease the rate, as $A$ competes with $S$ for the enzyme (47). Fig. 7 reflects a similar phenomenon, suggesting the validity of the Scheme I for this system. It would be interesting to ascertain the precise arrangement of these molecules in the active site by structural methods. Unfortunately, the reaction between CuZn-SOD and H$_2$O$_2$ in the presence of HCO$_3^-$ proceeds rapidly, suggesting that the ternary complex is quite labile.

Interestingly, there is a precedent for an obligatory role of HCO$_3^-$ binding for metalloprotein function. Over 20 years ago, it was shown that metal binding to the iron transport protein transferrin is HCO$_3^-$-dependent, and subsequently arginine was identified as the anion binding ligand (48–50). However, in transferrin, HCO$_3^-$ also binds to the metal. Thus, there are both similarities and differences in the process of anion binding by transferrin and CuZn-SOD.

It is possible that the peroxidase function of CuZn-SOD is a mechanism by which the enzyme degrades H$_2$O$_2$ released at the active site following superoxide dismutation. This could serve the important biological role of preventing H$_2$O$_2$-mediated injury under conditions where other peroxidases are not present. Identifying intrinsic substrates, which are as potent as DMPO in accepting one electron, would be important in determining the role of this process in normal physiology and disease. Similarly, understanding the role of HCO$_3^-$ in this system is very important for elucidating the larger physiological implications of the effects of alterations in pH and the HCO$_3^-$/CO$_2$ equilibrium on this process. In a number of important disease processes such as ischemia, sepsis, or shock, marked metabolic acidosis occurs with decreases in tissue pH to values as low as 5.5 (51). Under these conditions, HCO$_3^-$ concentration would decrease by more than 1 order of magnitude, resulting in decreased CuZn-SOD peroxidase function.

In summary, CuZn-SOD acquires a peroxidase function at physiological pH in the presence of HCO$_3^-$, and this activity is responsible for the decomposition of H$_2$O$_2$ and the oxidation and hydroxylation of DMPO. This function is distinct from the high pH peroxidase activity and inactivation of the enzyme. Bicarbonate does not bind directly to the active site copper but binds to the adjacent anion-binding site of the enzyme. These data support the existence of a peroxidase mechanism in which HCO$_3^-$ bound at the anion binding site anchors the neutral H$_2$O$_2$ molecule at the active site copper, enabling its redox cleavage. Bicarbonate anion thus plays a critical role in this peroxidase activity. Therefore, alterations in pH and the HCO$_3^-$/CO$_2$ equilibrium that occur in a number of disease processes could modulate the peroxidase function of the enzyme.

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