Bicarbonate Enhances the Hydroxylation, Nitration, and Peroxidation Reactions Catalyzed by Copper, Zinc Superoxide Dismutase

INTERMEDIACY OF CARBONATE ANION RADICAL

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The effect of bicarbonate anion (HCO$_3^-$) on the peroxidase activity of copper, zinc superoxide dismutase (SOD1) was investigated using three structurally different probes: 5,5'-dimethyl-1-pyrroline N-oxide (DMPO), tyrosine, and 2,2'-azino-bis-(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS). Results indicate that HCO$_3^-$ enhanced SOD1 activity (9).1 HCO$_3^-$ stimulates the peroxidase activity of the extra-cellular SOD1 activity, which was attributed to an increased formation of hydroxyl radicals (9). Hydroxyl radicals formed from the reaction between SOD1 and H$_2$O$_2$ were suggested to be responsible for the increased cytotoxicity of SOD1 (10).

In the present work, we show that HCO$_3^-$ enhances the hydroxylation of nitroprusside trap, DMPO, (ii) oxidation and nitration of tyrosine, and (iii) oxidation of the peroxidase probe ABTS to the ABTS radical cation in the presence of SOD1 and H$_2$O$_2$. We propose that the carbonate anion radical (CO$_3^{2-}$) formed from oxidation of HCO$_3^-$ at the active site of SOD1 by the copper, zinc SOD-bound hydroxyl radical (SOD1-CuOH) is responsible for hydroxylation of DMPO, oxidation and nitration of tyrosine, and oxidation of ABTS. The proposed mechanism offers a new and different perspective on the peroxidative reactions catalyzed by the enzyme SOD1.

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Bicarbonate anion (HCO$_3^-$) is abundantly present in biological systems (1). In vitro studies have shown that HCO$_3^-$ can dramatically alter the nitrating and oxidizing ability of reactive nitrogen and oxygen species (2–4). Recently, HCO$_3^-$ was shown to exacerbate the peroxidase activity of the enzyme, copper, zinc superoxide dismutase (SOD1) (5).1 HCO$_3^-$ enhanced both the hydroxylation of 5,5'-dimethyl-1-pyrroline N-oxide (DMPO) to DMPO-OH and oxidation of ABTS chromophore to the ABTS radical cation in the presence of SOD1 and H$_2$O$_2$ (5). HCO$_3^-$ was reported to increase the peroxidase activity of SOD1 by facilitating the redox cleavage of H$_2$O$_2$ (5).

1 The abbreviations used are: SOD1, copper, zinc superoxide dismutase; DTPA, diethylenetriaminepentaacetic acid; DMPO, 5,5'-dimethyl-1-pyrroline N-oxide-hydroxyl adduct; ABTS, 2,2'-azino-bis-[3-ethylbenzothiazoline]-6-sulfonic acid; ESR, electron spin resonance; HPLC, high pressure liquid chromatography.

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4 The abbreviations used are: SOD1, copper, zinc superoxide dismutase; DTPA, diethylenetriaminepentaacetic acid; DMPO, 5,5'-dimethyl-1-pyrroline N-oxide; DMPO-OH, 5,5'-dimethyl-1-pyrroline N-oxide-hydroxyl adduct; ABTS, 2,2'-azino-bis-[3-ethylbenzothiazoline]-6-sulfonic acid; ESR, electron spin resonance; HPLC, high pressure liquid chromatography.

Experimental Procedures

SOD1 (bovine) was purchased from Roche Molecular Biochemicals. Tyrosine, hydrogen peroxide, sodium bicarbonate, sodium nitrite, and 3-nitrotyrosine were purchased from Sigma. Bio-gel P-2 was obtained from Bio-Rad. DMPO was obtained from Sigma and double distilled to remove the paramagnetic impurity. $^{17}$O-Labeled water (45%) was obtained from ICON Isotopes (Summit, NJ).

Synthesis of 3,3'-Dityrosine—Dityrosine was synthesized according to the published literature (11). Briefly, 500 ml of tyrosine (5 mM) in borate buffer (0.1 M, pH 9.1) was mixed with 10 ml of horseradish peroxidase (1 mg/ml) and 1.42 ml of H$_2$O$_2$ (3%) at 37 °C for 3 h. The reaction mixture was then mixed with 175 μl of β-mercaptoethanol and lyophilized to dryness. The lyophilized powder was dissolved in distilled water, and dityrosine was separated from other by-products through a series of chromatography: DEAE-cellulose, Bio-Gel P-2 at neutral pH, and Bio-Gel P-2 at low pH (≤3). Fractions containing dityrosine were pooled, lyophilized, and stored at −20 °C. Dityrosine was identified by comparing its spectral properties with those reported in the literature (11). The purity of dityrosine was verified by both UV and fluorescence HPLC.

Oxidation and Nitration of Tyrosine by SOD1 Peroxidase Activity—Tyrosine (1 mM) was incubated at 37 °C with SOD1 (1 mg/ml), H$_2$O$_2$ (1 mM), and NaHCO$_3$ (25 mM) in sodium phosphate buffer (0.1 M, pH 7.4) containing DTPA (100 μM). After incubating for 30 min, samples were centrifuged (10,000 rpm) in an ultracentrifuge (molecular weight cut-off, 10,000) at 1 °C for 5 min to remove SOD1 and subsequently used for HPLC analysis. All reagents were prepared in double-distilled and deionized water. The pH of the incubation mixture remained at 7.40 ± 0.02 before and after the addition of NaHCO$_3$. SOD1 activity was measured using the ferricytochrome c reduction assay as described previously (12).

HPLC Analysis—Nitrotyrosine, nitrotyrosine, and dityrosine were
separated on an HPLC system equipped with fluorescence and UV
detectors. The mobile phase was methanol:phosphate buffer (50 mM, pH
3.0) (4:96) for 30 min. The stationary phase was a C<sub>18</sub> reverse phase
column (Partisil ODS-3 250 × 4.6 mm, Alltech). UV detection at 280 nm
was used to monitor nitrotyrosine, and fluorescence detection at 284 nm
(excitation) and 410 nm (emission) was used to monitor dityrosine and
higher oxidation products of tyrosine (13, 14). The authentic standards,
tyrosine, dityrosine, 3-nitrotyrosine, and nitrodityrosine were eluted at
7.5, 9.5, 17.5, and 20 min, respectively.

Fig. 1. Bicarbonate induces hydroxylation of DMPO in the
presence of SOD1 and H<sub>2</sub>O<sub>2</sub>. DMPO (50 mM) was incubated with
SOD1 (1 mg/ml), H<sub>2</sub>O<sub>2</sub> (1 mM), and various amounts of NaHCO<sub>3</sub> in
sodium phosphate buffer (0.1 M, pH 7.4) at room temperature. Spectra
were obtained at different HCO<sub>3</sub> concentrations (A–E). Note that DMPO-OH formation is negligible in the
absence of NaHCO<sub>3</sub>.

Fig. 2. The effect of H<sup>17</sup>O-labeled H<sub>2</sub>O
on SOD1/H<sub>2</sub>O<sub>2</sub>-dependent DMPO-OH
formation. DMPO (50 mM) was incu-
bated in sodium phosphate buffer (0.1 M,
PH 7.4) with DTPA (100 μM) at room tem-
perature. A, the reaction was carried out in
H<sub>2</sub>H<sup>16</sup>O (100%). B, the reaction was car-
ried out in H<sub>2</sub>H<sup>17</sup>O (45%) and H<sub>2</sub>H<sup>16</sup>O (55%).
Line positions from H<sup>16</sup>O (○) and H<sup>17</sup>O (●)
couplings in DMPO-OH are shown. The
difference between H<sup>16</sup>O and H<sup>17</sup>O atoms is that the nuclear quantum number (I)
equals zero for the [16O] atom and 5/2 for
the [17O] atom. As a result, each line in
the DMPO-17OH adduct is split into six
lines. Because there is 55% H<sub>2</sub>H<sup>16</sup>O in
the solution, there is approximately 55% con-
tribution from the DMPO-16OH. To con-
serve H<sup>17</sup>O-labeled H<sub>2</sub>O, experiments were
performed using a loop-gap resonator.
The experimental spectrum shown in B
was simulated (dashed line), assuming
contributions from DMPO-16OH (56.6%)
and DMPO-17OH (43.4%). ESR param-
ters used in the simulation were as fol-
loows: α<sub>N</sub> = 14.9 G, α<sub>H</sub> = 14.9 G for
DMPO-16OH and α<sub>N</sub> = 14.9 G, α<sub>H</sub> = 14.9 G, and
α<sub>17O</sub> = 4.6 G for the DMPO-17OH. The
correlation coefficient for spectral simula-
tion was 0.977.

Fig. 3. Tyrosine inhibits SOD1/H<sub>2</sub>O<sub>2</sub>/HCO<sub>3</sub>-
dependent forma-
tion of DMPO-OH. A, DMPO (50 mM) was incubated with SOD1 (1
mg/ml), H<sub>2</sub>O<sub>2</sub> (1 mM), and NaHCO<sub>3</sub> (25 mM) in sodium phosphate buffer
(0.1 M, PH 7.4) with DTPA (100 μM). B—E, same as in A but containing
0.5, 1.0, 2.0, and 5.0 mM tyrosine, respectively.
ESR Spin Trapping—ESR spectra were recorded at room temperature on a Varian E-109 spectrometer operating at 9.5 GHz and with a 100 KHz field modulation equipped with a TE 102 cavity or a loop-gap resonator (15, 16). Reactions were initiated by the addition of H2O2 to the incubation mixtures containing SOD1 (1 mg/ml), DMPO (50 mM), and DTPA (100 mM). A typical reaction mixture for ESR analysis in a TE102 cavity consisted of 20 ml of SOD1 (5 mg/ml), 10 ml of DMPO (500 mM), 5 ml of NaHCO3 (250 mM), 1 ml of DTPA (200 mM), and 50 ml of phosphate buffer (0.2 M, pH 7.4) containing DTPA (200 mM) in a total volume of 100 ml. A typical reaction mixture for ESR analysis in a loop-gap resonator consisted of 2 ml of SOD1 (5 mg/ml), 1 ml of DMPO (500 mM), 1 ml of NaHCO3 (250 mM), 1 ml of H2O2 (10 mM), and 5 ml of phosphate buffer (0.2 M, pH 7.4) containing DTPA (200 mM) in a total volume of 10 ml. For experiments with [17O]H2O, all reagents were
dissolved in [17O]H2O such that the percent of [17O]H2O in the total volume remained the same (i.e. 45% [17O]H2O). The sample was transferred to a capillary tube (0.64-mm inner diameter x 0.84-mm outer diameter and 100-mm long) that was sealed with miniseal (Baxter Scientific Products, McGraw Park, IL) and placed into the loop-gap resonator. Spectrum conditions were as follows: modulation amplitude, 1 G; time constant, 64 s; scan time, 2 min; microwave power, 1 mW.

RESULTS

Bicarbonate Induces Hydroxylation of DMPO in the Presence of SOD1 and H2O2—In the absence of added HCO3− anion, incubation mixtures containing SOD1, DMPO, H2O2, and DTPA in phosphate buffer yielded very little DMPO-OH (Fig. 1E). However, the signal intensity of the DMPO-OH adduct (αN = αH = 14.9 G) increased with the addition of HCO3− in these incubation mixtures (Fig. 1, A–D). The pH level was measured immediately before and after the addition of 25 mM HCO3− to ensure that the HCO3−-mediated enhancement was not due to a change in the pH of the reaction mixture. The addition of Me3SO, a commonly used hydroxyl radical scavenger, had no effect on the ESR signal intensity of DMPO-OH (data not shown). In contrast, when Me3SO was added to a mixture containing H2O2 (1%) and DMPO followed by irradiation with UV light, the spectrum caused by the DMPO-OH adduct was replaced by that of the DMPO-methyl radical adduct (αN = 16.4 G, αH = 23.4 G) (data not shown). From these results, we conclude that free hydroxyl radicals are not responsible for the hydroxylation of DMPO by SOD1/H2O2/HCO3−. However, unequivocal proof that H2O2 (the precursor of hydroxyl radical) was not responsible for DMPO-OH formation came from studies using 17O-labeled water experiments.

To investigate the origin of the oxygen atom in the DMPO-OH adduct, spin trapping experiments were performed in buffers prepared with 17O-labeled water. The commercially available [17O]H2O consists of 45% [17O]H2O and 55% [16O]H2O. The experiments were performed in a loop-gap resonator, which enabled the use of exceedingly small volumes of [17O]-labeled materials. Fig. 2B shows the ESR spectrum of the DMPO-OH adduct obtained by adding SOD1 (1 mg/ml), H2O2 (1 mM), DMPO (50 μM), and DTPA (100 μM) to phosphate buffers (prepared in [17O]-labeled water) and bicarbonate (25 mM). Computer simulation of the ESR spectrum corresponding to DMPO-OH (dashed line spectrum in Fig. 2B) indicated a 43% contribution from DMPO-17OH to DMPO-16OH formed in this system (Fig. 2A). The relative ratios of DMPO-17OH to DMPO-16OH formed in this system reflect the ratios of H2O2 to H2O present in the buffer. This result proves that SOD1/H2O2/HCO3−-induced hydroxylation of DMPO (Fig. 2A) involves the incorporation of the oxygen atom from H2O and not from H2O2.

The Effect of Tyrosine on Hydroxylation of DMPO—Spin trapping data suggest that an oxidant formed from the oxidation of HCO3− by SOD1 and H2O2 is responsible for the hydroxylation of DMPO. We hypothesized that this oxidant is the carbonate radical anion (CO3−) (17). It is well known that CO3− reacts rapidly with phenolic substrates such as tyrosine (18). Thus, we wished to determine whether tyrosine can compete with DMPO in its ability to react with this putative oxidant. The effect of tyrosine on DMPO-OH formation is shown in Fig. 3 (B–E). A dose-dependent inhibition in DMPO-OH formation was observed with increasing addition of tyrosine to incubations containing SOD1, H2O2, DMPO, and HCO3− in phosphate buffer. The DMPO-OH signal intensity was almost completely inhibited in the presence of tyrosine (5 mM) (Fig. 3E). This result suggests that tyrosine is able to effectively compete with DMPO for the reaction with HCO3−-derived oxidant.

Bicarbonate Enhances Oxidation and Nitration Mediated by the Peroxidase Activity of SOD1—Fig. 4 shows the HPLC profile for dityrosine formation in incubations containing SOD1, tyrosine, H2O2, and DTPA in the presence and absence of HCO3−. In the absence of HCO3−, no dityrosine was detected (Fig. 4C). In the presence of added HCO3−, dityrosine was detected (Fig. 4B), the level of which increased with increasing HCO3− (Fig. 4, inset). In addition to dityrosine, two minor products (peaks I and II) (Fig. 4B) were identified. Based on the literature report (14), peaks I and II were tentatively attributed to higher oxidation products of tyrosine (e.g. tri- and tetratyrrosine).

Next we investigated the effect of HCO3− on SOD1/H2O2/nitrite-mediated nitration of tyrosine. In the absence of HCO3−, addition of SOD1 to the incubation mixture containing nitrite, H2O2, and DTPA in phosphate buffer yielded nitrotyrosine, which was measured 2 min after starting the reaction (Fig. 5, bottom trace) (19). In the presence of HCO3− (25 mM), there was a significant increase in nitrotyrosine formation (Fig. 5, middle trace).
trace). Fig. 5 (inset) shows the formation of nitrotyrosine as a function of HCO₃⁻ concentration. Fig. 6 shows that nitrotyrosine is further oxidized with time in this incubation mixture (Fig. 6A). Nitrotyrosine levels decreased with time, along with a concomitant increase in nitrodityrosine formation (the satellite peak detected at 20 min). After 60 min, a significant fraction of nitrotyrosine was converted to nitrodityrosine.

We assigned the peak appearing at 20 min to nitrodityrosine (Fig. 6) based on the following results. Authentic nitrodityrosine prepared by the addition of peroxynitrite to dityrosine (Fig. 6B, bottom trace) gave a peak that eluted at 20 min, as did incubations containing SOD1, H₂O₂, nitrite, and dityrosine (Fig. 6B, top trace). Nitrodityrosine can therefore be used as a unique diagnostic marker product of both oxidation and nitration of tyrosine.

Bicarbonate Enhances the Oxidation of ABTS to ABTS·⁺ in the Presence of SOD1 —The oxidation of ABTS to ABTS·⁺ is conveniently monitored at 415 nm, and this optical change has been used to assay the peroxidase activity of heme proteins (20). As reported previously (5), we confirmed that bicarbonate is needed for peroxidation of ABTS to ABTS·⁺ in the presence of SOD1. Fig. 7A shows the optical changes occurring during SOD1/H₂O₂/HCO₃⁻-mediated oxidation of ABTS. The increase in the absorbance of ABTS·⁺ is detected at 415 nm (Fig. 7A, inset). A time-dependent increase in the absorbance at 415 nm was monitored as a function of HCO₃⁻ concentration (Fig. 7B). In the absence of HCO₃⁻, no increase in the absorbance because of ABTS·⁺ was noticed. Fig. 7C shows the effect of H₂O₂ on the rate of formation of ABTS·⁺. This indicates that HCO₃⁻-induced SOD1 peroxidase activity could be detected over a range of H₂O₂ concentrations (50–500 μM).

DISCUSSION

Bicarbonate-mediated Hydroxylation of DMPO —Most studies on SOD1 and SOD1 familial amyotrophic lateral sclerosis mutant-induced hydroxylation of DMPO were carried out in bicarbonate buffers (6–8, 16). To our knowledge, Sato et al. (21) were the first to report the finding that SOD1/H₂O₂/HCO₃⁻ caused a dramatic increase in the hydroxylation of DMPO. The role of bicarbonate anion in these studies was largely ignored. It was suggested that DMPO was oxidized at the active site of SOD1 by a copper-bound hydroxyl radical (i.e. SOD1-Cu²⁺-OH) to DMPO·OH (21). As shown in this and other investigations (5, 21, 22), HCO₃⁻ is absolutely necessary for SOD1/H₂O₂/HCO₃⁻-induced hydroxylation of DMPO. It was proposed that CO₃⁻ formed from a one-electron oxidation of HCO₃⁻ at the active site of SOD1 could diffuse out of the active site and cause oxidation of substrates in solution (17, 22, 23). Based on these results, we put forth the following mechanisms for hydroxylation of DMPO (Scheme 1). The proposed mechanisms are consistent with those proposed previously for the hydroxylation of a related nitrotrone trap by the sulfate radical anion (24). In the present
study, experiments using $^{17}$O-labeled H$_2$O unambiguously demonstrated that nearly all of the DMPO-OH originated from the addition of water to the DMPO radical intermediate. Although this estimate is significantly higher than that reported previously (16), the reasons for the observed differences, however, are not understood.

As shown in Fig. 2, the incorporation of the $^{17}$O atom into the DMPO-OH adducts gives rise to the additional lines. The incorporation of an $^{17}$O atom into DMPO-OH increases the number of ESR lines from 4 to 15 because of the $^{17}$O coupling. Fig. 8 shows a typical ESR spectrum resulting from a 100% contribution from DMPO-$^{17}$OH. However, the commercially available [17O]H$_2$O consists of 45% [17O]H$_2$O and 55% [16O]H$_2$O. Thus, according to the proposed mechanism (Scheme 1), one would expect spectral contributions from both DMPO-$^{16}$OH and DMPO-$^{17}$OH adducts. Because there is no $^{16}$O and $^{17}$O atom exchange between DMPO-$^{17}$OH and $^{16}$O$_2$, the theoretical ratio of DMPO-$^{17}$OH and DMPO-$^{16}$OH is obtained from the computer simulation (Fig. 2).

To verify the electron transfer mechanism, the nitrone-derived cation radical must be directly detected. However, the DMPO cation radical is too unstable to be detected by direct ESR (25). In this regard, it is important to note that only the azulenyl nitrone possesses the necessary redox and structural characteristics to form a persistent cation radical (26, 27).

**Fig. 8. A stick diagram of the ESR spectrum of DMPO-$^{17}$OH.** The incorporation of an $^{17}$O atom in DMPO-OH increases the number of ESR lines from 4 to 15 because of the $^{17}$O coupling.
by SOD1 and \( \text{H}_2\text{O}_2 \)—As shown in Fig. 4, \( \text{HCO}_3^- \) is required for the peroxidatic oxidation of tyrosine by SOD1. Previously, we reported that \( \text{CO}_3^- \) formed at the active site of SOD1 was able to oxidize \( \alpha \)-tocopherol to \( \alpha \)-tocopheryl quinone (17). \( \text{CO}_3^- \) reacts rapidly with tyrosine \( (k = 10^7 \text{ to } 10^8 \text{ M}^{-1} \text{ s}^{-1}) \) to form the tyrosyl radical that subsequently dimerizes to form dityrosine (Scheme 2). Dityrosine can be oxidized by \( \text{CO}_3^- \) to form the dityrosyl radical, which will react with the tyrosyl radical to form trityrosine. Similarly, one can visualize the formation of other higher oxidative marker products. Based on the literature report (14), we attribute the minor HPLC peaks eluted at longer retention times (Fig. 4) to the tri- and tetratyrosines.

Bicarbonate stimulated nitrotyrosine formation in incubations containing nitrite anion (\( \text{NO}_2^- \)), SOD1, and \( \text{H}_2\text{O}_2 \). We attribute this as a fast electron transfer reaction between \( \text{CO}_3^- \) and \( \text{NO}_2^- \) \( (k = 4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}) \) that results in the formation of the nitrogen dioxide radical (\( \text{NO}_2^z \)), a potent nitrat ing agent (28, 29). \( \text{NO}_2^z \) can abstract the phenolic hydrogen atom from tyrosine to form the corresponding tyrosyl radical, which then recombines with \( \text{NO}_2^- \) to form the nitrotyrosine. Our results indicated that nitrotyrosine decayed and a new peak appeared with time (Fig. 6A). This new peak was assigned to nitrotyrosine, the formation of which can be explained as follows:

\[
\begin{align*}
\text{CO}_3^- + \text{NO}_2^- + \text{Tyr} \rightarrow \text{CO}_3^- + \text{NO}_2^- + \text{Tyr} \\
\text{CO}_3^- + \text{Tyr} \rightarrow \text{CO}_3^- + \text{Tyr} \\
\text{NO}_2^- + \text{Tyr} + \text{Tyr} \rightarrow \text{NO}_2^- + \text{DiTyr}
\end{align*}
\]

Thus, nitrotyrosine may be used a diagnostic marker product of both oxidation and nitration reaction in biological systems.

**Scheme 2.** Bicarbonate-mediated oxidation/nitration of tyrosine. This scheme describes the major reaction pathways leading to the formation of dityrosine, nitrotyrosine, nitrotrityrosine, and other higher oxidation products of tyrosine formed during SOD1/\( \text{H}_2\text{O}_2/\text{HCO}_3^- \)-induced oxidation of tyrosine in the presence and absence of nitrite.

**Scheme 3.** Oxidation of bicarbonate to the carbonate anion radical by the copper-bound hydroxyl radical at the active site of SOD1. The \( \text{CO}_3^- \) radical, a potent oxidant formed at the active site of SOD1, is able to diffuse out of the active site and oxidizes several structurally different molecules including DMPO, ABTS, tyrosine, and nitrite anion in the bulk solution.

**REACTIONS 1–3**

A Common Mechanism for Oxidation and Hydroxylation of Peroxidase Substrates by SOD1/\( \text{H}_2\text{O}_2/\text{HCO}_3^- \)—The x-ray crystal structure of SOD1 indicates that access to the active site of copper via a narrow channel is restricted for large molecules (30) (Scheme 3). However, a relatively small anion such as \( \text{HCO}_3^- \) could reach the active site of SOD1 where it can be oxidized to \( \text{CO}_3^- \) by the enzyme-bound hydroxyl radical (22). \( \text{CO}_3^- \) is a diffusible oxidant that could leave the active site and cause the oxidation of various compounds outside the active site. The one-electron oxidation potential for the \( \text{CO}_3^-/\text{CO}_3^- \) couple is +1.59 V (31), which makes oxidation of \( \text{HCO}_3^- \) by SOD1-Cu\(^{2+}\)/\( \text{OH}^- \) TO \( \text{CO}_3^- \) thermodynamically feasible. \( \text{HCO}_3^- \) can thus effectively export oxidation from the sterically hindered active site to large molecules in bulk solution (22). This model was originally proposed by Hodgson and Fridovich (32, 33). They proposed that the “copper-bound hydroxyl radical,” which is generated in the reaction between SOD1 and \( \text{H}_2\text{O}_2 \), oxidizes several small molecular weight anion ligands such as azide, formate, and nitrite anions (32, 33). The oxidizing potential of the putative SOD1-Cu\(^{2+}\)/\( \text{OH}^- \) species was suggested to be similar to that of “free” hydroxyl radical, which is considerably higher than the potentials associated with conventional...
peroxidases (17, 19). The earlier studies were performed in bicarbonate buffers, but the possibility of oxidation of HCO$_3^-$ to CO$_3^-$ was not realized at that time.

The present data and the published reports (17, 22, 23) strongly implicate a role for CO$_3^-$ in the HCO$_3^-$-dependent peroxidative mechanism of SOD1. This new perspective clearly rules out a mechanism involving direct oxidation/hydroxylation of DMPO, ABTS, or tyrosine by either free or bound hydroxyl radical (7, 8, 16, 34, 35). HCO$_3^-$, however, facilitates oxidation/hydroxylation of substrates through formation of the CO$_3^-$ intermediate. Thus, irrespective of the structure of peroxidase substrates, SOD1/H$_2$O$_2$/HCO$_3^-$ induces their oxidation, hydroxylation, or nitration via a common oxidizing intermediate (Scheme 3).

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