Direct Magnetic Resonance Evidence for Peroxymonocarbonate Involvement in the Cu,Zn-Superoxide Dismutase Peroxidase Catalytic Cycle*

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Marcelo G. Bonini‡¶, Scott A. Gabel§, Kalina Ranguelova‡, Krisztian Stadler‡, Eugene F. DeRose‡, Robert E. London‡, and Ronald P. Mason†

From the ‡Laboratory of Pharmacology and the §Laboratory of Structural Biology, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina 27709

Cu,Zn-superoxide dismutase (SOD1) is a copper- and zinc-dependent enzyme. The main function of SOD1 is believed to be the scavenging and detoxification of superoxide radicals. Nevertheless, the last 30 years have seen a rapid accumulation of evidence indicating that SOD1 may also act as a peroxidase, an alternative function that was implicated in the onset and progression of familial amyotrophic lateral sclerosis. Although SOD1 peroxidase activity and its dependence on carbon dioxide have been well described, the molecular basis of the SOD1 peroxidase cycle remains obscure, because none of the proposed catalytic intermediates have so far been identified. In view of recent observations, we hypothesized that the SOD1 peroxidase cycle relies on two steps: 1) reduction of SOD-Cu(II) by hydrogen peroxide followed by 2) oxidation of SOD-Cu(I) by peroxymonocarbonate, the product of the spontaneous reaction of bicarbonate with hydrogen peroxide, to produce SOD-Cu(II) and carbonate radical anion. This hypothesis has been investigated through electron paramagnetic resonance and nuclear magnetic resonance to provide direct evidence for a peroxycarbonate-driven, SOD1-catalyzed carbonate radical production. The results gathered herein indicate that peroxymonocarbonate (HOOCO$_2$) is a key intermediate in the SOD1 peroxidase cycle and identify this species as the precursor of carbonate radical anions.

Cytosolic Cu,Zn-superoxide dismutase (SOD1) is a metal-dependent enzyme capable of accelerating the rate of spontaneous superoxide dismutation into O$_2$ and H$_2$O$_2$ through the redox cycling of its copper ion (1, 2). SOD1 is widely distributed in mammalian cells and tissues and has been demonstrated to be located in the cytosol and in the intermembrane space of the mitochondria (see Ref. 3 and references therein). Because of that, SOD1 is believed to be a major player in the first line defense against reactive oxygen species, in particular superoxide anion.

In addition to its dismutase activity, SOD1 possesses a well described but incompletely understood peroxidase activity which is dependent on hydrogen peroxide and markedly stimulated by small oxidizable anions such as nitrite and the ubiquitous carbon dioxide (3–12). The peroxidase activity of SOD1 has been proposed to impact the onset and progression of familial amyotrophic lateral sclerosis, a severely debilitating fatal disease characterized by the selective death of motor neurons (13–18). Although several reports exist in the literature indicating the formation of SOD1 aggregates and accumulation as a potential cause in the pathology progression, conflicting hypotheses are still under debate concerning the mechanisms that lead to the formation of SOD1-protein aggregates (19–21). Although some support the suggestion that free radical-induced covalent cross-links among SOD1 amino acids play a fundamental role in aggregate formation (22, 23), others support the view that metal loss from the enzyme structure leads to an unstable apo-form of SOD1 with increased capacity to form aggregates (24, 25). A detailed understanding of the SOD1 peroxidase cycle is essential to unraveling the mechanisms through which SOD1 aggregates are produced.

The SOD1 peroxidase cycle is initiated when SOD1-Cu(II) is reduced by H$_2$O$_2$ or its deprotonated form (12), the peroxide anion (HOO$^-$), to SOD1-Cu(I). This latter species is subsequently oxidized to a hypervalent intermediate (proposed to be either SOD1-Cu(III), SOD1-Cu(II)-OH, or SOD1-Cu=O) (8, 9) that remains to be characterized. The reduction of this hypervalent intermediate by small anions is supposed to close the cycle, leading to the native enzyme and diffusible highly reactive radicals derived from the anionic substrates (6, 10).

During its peroxidase cycle, a considerable fraction of SOD1 is inactivated due to the oxidation of the copper-binding histidines to oxohistidine, presumably by the hypervalent intermediate, in a process that can be prevented by the presence of reducing substrates and, in their absence, unavoidably leads to copper loss (26). Although this process is well described for the heme-dependent peroxidase cycle, current literature data (9, 27–30) and the fact that the proposed SOD1-bound hypervalent copper states (Cu(III), Cu(II)=O, and Cu(II)/OH) have never been characterized suggested to us that an alternative mechanism may take place, leading to CO$_3^{2-}$ production from HCO$_3^-$ and H$_2$O$_2$ by the enzyme, a process that does not involve copper oxidation beyond the thermodynamically stable Cu(II) form. In the presence of HCO$_3^-$, a significant fraction of H$_2$O$_2$ is promptly converted to HOOCO$_2^-$ through the perhydration of CO$_2$ (27, 28, 31, 32). The peroxo bond in peroxymonocarbonate...
Materials and Methods

Chemicals—Superoxide dismutase from bovine erythrocytes, sodium bicarbonate, and sodium dithionite were purchased from Sigma. Acetonitrile (spectroscopy grade) was purchased from Caledon Laboratories (Georgetown, Canada). Sodium phosphate was purchased from Mallinkrodt Baker Inc. (Paris, KY). Chelex 100 resin was purchased from Bio-Rad. Phosphate buffers (0.1 M) were treated with Chelex 100 resin to remove traces of transition metal ions, and 200 μM diethylentriaminepentaacetic acid (DTPA) was added to minimize the possibility of metal interference, except for the NMR studies, where cyclohexanediaminetetraacetic acid (CDTA) was used.

Electron Paramagnetic Resonance Experiments—EPR spectra were recorded on a Bruker EMX EPR spectrometer (Billerica, MA) operating at 9.81 GHz with a modulation frequency of 100 kHz and equipped with an ER 4122 SHQ cavity. All reactions were performed at room temperature and transferred to 1-ml polyethylene syringes. After the indicated incubation times, samples were frozen in liquid nitrogen, the tips of the syringes were cut off, and the frozen contents were pushed into a Dewar flask containing liquid nitrogen. EPR spectra were recorded at 77 K. All samples contained 15% acetonitrile to minimize cracking of the frozen content. Linear base-line correction of the EPR spectra was computationally performed using Bruker’s WINEPR software version 2.11. For kinetic studies, estimation of SOD-Cu(II) concentrations at each time point was performed by doubly integrating the acquired spectra after smoothing the noise using the same software. Solution pH was controlled by the use of phosphate buffer. Incubations were performed in sealed containers to avoid loss of CO₂. All incubations were recovered at the end of each experiment and had their pH double-checked in order to guarantee that the pH remained unchanged over the course of the experiment.

NMR Study—NMR data were collected using a Varian Inova 600 spectrometer (Palo Alto, CA) with a 5-mm broadband probe tuned to carbon (150.83 MHz). Bovine kidney SOD1 was purchased from Calzyme Laboratories (San Luis Obispo, CA). ¹³C-Labeled sodium hydrogen carbonate was purchased from Isotec/Sigma. Samples were placed in 5-mm NMR tubes, sealed with septum caps (Wilmed Glass, Buena, NJ), and then allowed to equilibrate for 30 min. Additions to the samples were made by injecting compounds through the septum cap using a Hamilton syringe. 10% D₂O was added to the samples for the purpose of locking and shimming. All studies were run at 25 °C.

Kinetics of SOD1-mediated H₂O₂ Consumption—H₂O₂ uptake experiments were performed using an H₂O₂ electrode attached to an Apollo 4000 free radical analyzer unit (World Precision Instruments, Sarasota, FL). Amperometric detection of H₂O₂ was carried out using a poised voltage of +400mV. The reaction was constantly stirred throughout the course of the experiment.
Spectroscopic Study of the Cu,Zn-SOD Peroxidase Cycle

Calculations—calculations were performed by fitting the initial decay or recovery of SOD1-Cu(II) EPR intensity at g ~ 2 mediated by H₂O₂ or H₂O₂/HCO₃⁻, respectively, using Sigma Plot (SYSTAT) software (version 8.0). For calculation of the pseudo-first-order rate constants, the enzyme was mixed with a 10-fold molar excess of H₂O₂ or HCO₃⁻. Concentrations of pre-formed HCO₃⁻ were estimated by using [HCO₃⁻] = 250 mM, [H₂O₂] = 10 mM, and the equilibrium constant, \( K_{eq} = [\text{HCO}_3^-]/[\text{H}_2\text{O}_2] \times pK_a = 0.32 \) M⁻¹, as previously published (27, 31).

The data were fit to a monoeponential decay or growth model using the following equations,

\[
[SOD1-Cu(II)] = A_1 \exp(-k_\text{obs} \times t) \quad \text{(Eq. 1)}
\]

(for the exponential decay) or

\[
[SOD1-Cu(II)] = A_1(1 - \exp(-k_\text{obs} \times t)) \quad \text{(Eq. 2)}
\]

(for the exponential growth), where \( A_1 \) is the initial and final concentration of the enzyme. The rate is given by Equation 3,

\[
\text{Rate} = k[SOD1-Cu(II)] [\text{reactant}] = k_{\text{obs}}[SOD1-Cu(II)]
\]

(Eq. 3)

the reactants being either H₂O₂ or HCO₃⁻. Assuming that the observed rates were linearly dependent upon the reactant concentration, the slopes of these plots give the apparent second-order rate constants \( (k) \) for the decay and recovery of SOD1-Cu(II).

Computer Simulation—The computer simulation of kinetic curves was performed using Gepasi (version 3.30) software, available to the public through the Internet (34, 35).

RESULTS

pH Dependence of SOD-Cu(II) Reduction by H₂O₂—The first step in the peroxidase catalytic cycle of SOD1 is the reduction of the active site Cu(II) cation to Cu(I), SOD1-Cu(II) has a very characteristic EPR signal at 77 K, which is lost upon the reduction of Cu(II) (23). Therefore, mixtures of SOD1 with H₂O₂ were prepared at different pH values to evaluate the rate of reduction of SOD1 by H₂O₂. Indeed, the intensity of Cu(II) (23). Therefore, mixtures of SOD1 with H₂O₂ were prepared at different pH values to evaluate the rate of reduction of SOD1 by H₂O₂. Indeed, the intensity of Cu(II) spectrum was decreased by 70% (peak-to-peak intensity), indicating that most of the enzyme had been reduced by the peroxide.

The strong dependence of SOD1-Cu(II) reduction on the pH and the much faster reduction rate observed at alkaline pH are consistent with HOO⁻ rather than molecular H₂O₂ being the species that gains access to the SOD1 anion channel to reduce the active site’s Cu(II), which is in agreement with activity experiments showing that high pH accelerates SOD1 reduction by H₂O₂ (36, 37).

In addition to studying the pH effect on the H₂O₂-mediated reduction of SOD1, experiments were performed to roughly estimate the rate of disappearance of the EPR g ~ 2 signal at physiological pH. For that purpose, the remaining SOD1-Cu(II) (100 μM) concentrations were measured by the double integration of the EPR spectra as a function of time.

From the pseudo-first-order rates of disappearance of SOD-Cu(II) (0.037 s⁻¹) and assuming that the second-order rate constants are linearly dependent upon hydrogen peroxide concentration, we estimated \( k \approx 37 \) M⁻¹ s⁻¹ for the reduction of SOD1 by H₂O₂ (Fig. 1, inset). Although this value should be taken as a rough estimate, it is in good agreement with previous reports that determined \( k = 50 \) M⁻¹ s⁻¹ by evaluating H₂O₂-induced SOD1 bleaching (38).

Effect of HCO₃⁻/CO₂ on the Yield of SOD-Cu(II) from the H₂O₂-mediated Reduction of SOD1-Cu(II)—To assess the effect of HCO₃⁻/CO₂ upon the H₂O₂-mediated reduction of SOD1-Cu(II) to SOD1-Cu(I) and its reoxidation back to SOD1-Cu(II), the experiments shown in Fig. 1 were repeated in the presence of HCO₃⁻. For most of the experiments, HCO₃⁻ was allowed to equilibrate in solution for 15 min before the addition of H₂O₂. The only exception was the pH 9.8 spectrum (Fig. 2C), where a pH jump experiment was performed in order to specifically evaluate the effects of CO₂ rather than HCO₃⁻ (predominant at pH 9.8) upon SOD-Cu(I) reoxidation, as discussed below. For that, bicarbonate dissolved in water was added immediately before hydrogen peroxide so as to attain considerably higher initial concentrations of CO₂ in solution than would be present at equilibrium at pH 9.8. Different incubation times were used because, as demonstrated in Fig. 1, the SOD1-Cu(II) reduction rate is strongly dependent on pH. Therefore, to attain appreciable reduction yields, incubations at lower pH values required longer times.

As shown in Fig. 2, HCO₃⁻/CO₂ had only a minor influence on the reduction yield of SOD1-Cu(II) at pH 5.4. At pH 5.4, both hydrogen peroxide and bicarbonate are predominantly in their protonated acidic forms (see Table 1), thus limiting the formation of HOOCO₂⁻. This is likely to have limited the effect of HCO₃⁻/CO₂ upon SOD1-Cu(I) reoxidation.

At pH 7.4, bicarbonate had a marked effect on the yield of SOD1-Cu(II) reduction by H₂O₂ after a 3-min incubation. At this pH, bicarbonate is mostly in its monoprotonated form, HCO₃⁻ (CO₂ ~ 10%, HCO₃⁻ ~ 90%, pK_a = 6.4). The fraction of the H₂O₂ that is in the anionic form is ~0.01% (pK_a 11.8). Therefore, since CO₂ and HOO⁻ coexist at pH 7.4, significant yields of HOOCO₂⁻ are produced from the nucleophilic attack of HOO⁻ on CO₂, which will then oxidize SOD1-Cu(I) back to its resting state.

At pH 9.8, CO₂ (spectrum C), but not HCO₃⁻ (spectrum D, equilibrated), strongly influenced the steady-state concentration of SOD1-Cu(I). At pH 9.8, ~1% of the H₂O₂ is in the anionic form, HOO⁻. Therefore, in the presence of CO₂ (in non-equilibrated mixtures, such as in spectrum C), both HOO⁻ and CO₂ are present in high enough concentrations to produce significant yields of HOOCO₂⁻. In the equilibrated solution, the CO₂ concentration is very limited, and, therefore, no effect of HCO₃⁻ on the reduction yield of SOD1-Cu(II) was noted.
Taken together, the studies shown in Fig. 2 indicate that the formation of \( \text{HOOCO}_2^/-/\text{HCO}_3^- \) is dependent on both \( \text{CO}_2 \) and the peroxide anion \( \text{HOO}^- \)/\( \text{H}_2\text{O}_2 \) and that it increases the steady-state SOD1-Cu(II) concentration, presumably by reoxidizing the enzyme back to its SOD1-Cu(II) state and concomitantly producing \( \text{CO}_3^/- \).

Effect of \( \text{H}_2\text{O}_2 \) and \( \text{H}_2\text{O}_2/\text{HCO}_3^-/\text{CO}_2 \) on the Reoxidation of SOD1-Cu(I) to SOD1-Cu(II)—To determine whether \( \text{HOOCO}_2^- \) oxidizes SOD1-Cu(I) more efficiently than \( \text{H}_2\text{O}_2 \) or \( \text{HOO}^- \)/\( \text{H}_2\text{O}_2 \) alone, we incubated preformed SOD1-Cu(I) with \( \text{H}_2\text{O}_2 \) in the presence and in the absence of \( \text{HCO}_3^-/\text{CO}_2 \). SOD1-Cu(I) was prepared by briefly incubating SOD1-Cu(II) with sodium dithionite at a molar ratio of 1:1.2 under anaerobic conditions (for details, see “Materials and Methods”).

As demonstrated in Fig. 3, dithionite at this concentration led to an almost complete reduction of SOD1-Cu(II); no appreciable traces of remaining \( \text{SO}_2^- \), which is in equilibrium with dithionite, were detected by EPR. The addition of \( \text{H}_2\text{O}_2 \) to prereduced SOD1 solutions had a minor effect upon the SOD1 EPR signal intensity (Fig. 3), suggesting that \( \text{H}_2\text{O}_2 \) by itself is not an efficient mediator of SOD1-Cu(I) oxidation to SOD1-Cu(II). Indeed, the rate constant for the oxidation of SOD1-Cu(I) by \( \text{H}_2\text{O}_2 \) has been estimated to be \( 13 \text{M}^{-1} \text{s}^{-1} \) (38).

The addition of \( \text{HCO}_3^-/\text{CO}_2 \) to the samples markedly increased the intensity of the SOD1-Cu(II) EPR signal in a concentration-dependent manner (Fig. 3, D and E), indicating a key role for \( \text{HOOCO}_2^- \) as the mediator of SOD1-Cu(I) reoxidation back to the native enzyme with the consequent formation of carbonate radicals. In order to estimate the efficiency of

\[ 5 \times 10^4 \]. Each spectrum is the result of four accumulations. For the inset, each point represents the average of three independent experiments whose EPR spectrum was accumulated eight times. Please note that our measurements refer to the disappearance of the g = 2 signal attributable to SOD1-Cu(II). Although we believe that this disappearance is due to the reduction of SOD1-Cu(II) to SOD1-Cu(I), it is not possible to exclude the formation of other EPR-silent copper-derived species.
TABLE 1
Reactions and constants used to simulate HOOCO$_2$$^-$ decay in mixtures of SOD1 and pre-equilibrated H$_2$O$_2$/HCO$_3$ according to the conditions used for the experiment shown in Fig. 7.

| Reaction                                                                 | Constant       | Reference |
|-------------------------------------------------------------------------|----------------|-----------|
| HOCCO$_2$$^+$ + H$^+$ $\leftrightarrow$ HOCCO$_2$ + H$_2$O          | $pK_a = 6.4^a$ | Ref. 27   |
| HOCCO$_2$$^-$ + H$_2$O $\leftrightarrow$ HOCCO$_2$                   | $K = 0.32$     | Ref. 50   |
| HOO$^-$$^+$ + H$^+$ $\leftrightarrow$ HOO$^-$                         | $pK_a = 11.75$ | This work |
| SOD1-Cu(II) + HOOCO$_2$$^-$ $\rightarrow$ SOD1-Cu(I) + O$_2$          | $k = 40 \text{ M}^{-1} \text{s}^{-1}$ | Refs. 48 and 49 |
| SOD1-Cu(II) + CO$_3$$^-$ $\rightarrow$ SOD1-Cu(I) + O$_2$ + O$_2$    | $k = 9.7 \times 10^3 \text{ M}^{-1} \text{s}^{-1}$ | Ref. 50 |
| SOD1-Cu(I) + CO$_3$$^-$ $\rightarrow$ SOD1-Cu(II) + O$_2$ + O$_2$    | $k = 1 \times 10^{10} \text{ M}^{-1} \text{s}^{-1}$ | Ref. 51 |
| SOD1-Cu(I) + HOCCO$_2$$^-$ $\rightarrow$ SOD1-Cu(II) + CO$_3$$^-$    | $k = 150 \text{ M}^{-1} \text{s}^{-1}$ | This work |
| SOD1-Cu(I) + HOCCO$_2$$^-$ $\rightarrow$ SOD1-Cu(II) + O$_2$          | $k = 13 \text{ M}^{-1} \text{s}^{-1}$ | Ref. 38 |
| HOCCO$_2$$^-$ + O$_2$ + H$_2$O $\rightarrow$ HOOCO$_2$$^-$ + O$_2$   | $k = 2.7 \times 10^{-4} \text{ M}^{-1} \text{s}^{-1}$ | Ref. 52 |
| SOD1-Cu(II) + HOCCO$_2$$^-$ $\rightarrow$ SOD1-Cu(I) + O$_2$ + O$_2$ | $k = 9 \times 10^{-3} \text{ s}^{-1}$ | Refs. 53 and 54 |
| SOD1-Cu(II) + HOCCO$_2$$^-$ $\rightarrow$ SOD1-Cu(I) + CO$_3$$^-$    | $k = 0.65 \text{ M}^{-1} \text{s}^{-1}$ | Ref. 26 |

$^a$ Equilibrium constant found in Ref. 55.
$^b$ Reaction rate constant for CO$_3$$^-$ reaction with reduced cuprous ion.
$^c$ This rate constant was estimated from reaction rate constants determined for CO$_3$$^-$ reaction with reduced Cu$^{2+}$ complexes based on cited references.
$^d$ This rate was assumed to be 0.65 M$^{-1}$ s$^{-1}$ based on the cited reference.

SOD1-Cu(I) oxidation by preformed HOOCO$_2$$^-$, samples of SOD1-Cu(I) were prepared as previously described. A preincubated mixture of H$_2$O$_2$/HCO$_3$ was added to reduced SOD1 solutions and allowed to react for different times as indicated in Fig. 3, inset. Reaction mixtures were then frozen and SOD1-Cu(II) concentrations were measured by EPR spectroscopy. From the observed rate of SOD1-Cu(II) formation extracted from Fig. 3 (inset) ($k_{obs} = 0.12$ s$^{-1}$), we roughly estimated the second-order rate of SOD1-Cu(I) oxidation by HOOCO$_2$$^-$ to be $\sim 150 \text{ M}^{-1} \text{s}^{-1}$. Importantly, the rate constant for the reaction of SOD1-Cu(I) with HOOCO$_2$$^-$ was more than 10 times higher than that determined for the reaction of SOD1-Cu(I) and H$_2$O$_2$ (13 M$^{-1}$ s$^{-1}$) (38), which is consistent with the observation that HOCCO$_2$$^-$/CO$_3$$^-$ stimulates SOD1 peroxidase activity, apparently by accelerating SOD1-Cu(I) oxidation back to the resting state with concomitant formation of CO$_3$$^-$.

Recently, we became aware of studies performed by Medinas et al. (47) with human SOD1 in which the rate constant for the reaction between SOD1-Cu(I) and HOOCO$_2$ was obtained. In this study, the authors measured a rate constant of $2 \times 10^{10} \text{ M}^{-1} \text{s}^{-1}$ for the reaction between reduced SOD1 and HOOCO$_2$$^-$ and used the same conditions and time points as in the present study. Although the reasons for this discrepancy of more than 1 order of magnitude are not clear to us at this point, this study suggests a considerably higher rate constant for the reaction between the human isomer of SOD1-Cu(I) and HOOCO$_2$$^-$.

Effect of Carbonic Anhydrase upon H$_2$O$_2$/HCO$_3$$^-$/CO$_3$$^-$-mediated Oxidation of SOD1-Cu(I) to SOD1-Cu(II)—So far, our results indicate an important role for CO$_3$$^-$ but not HCO$_3$$^-$, in the formation of HOOCO$_2$$^-$ from the reaction of HOO$^-$ with CO$_3$$^-$ to oxidize SOD1-Cu(I). To further test the potential effect of HCO$_3$$^-$, we evaluated the effect of carbonic anhydrase upon preformed SOD1-Cu(I) oxidation by H$_2$O$_2$/HCO$_3$$^-$/CO$_3$$^-$. As shown in Fig. 3, spectrum F, the addition of carbonic anhydrase to the samples increased the SOD1-Cu(II) EPR signal intensity. Carbonic anhydrase accelerated the relatively slow equilibration of HCO$_3$$^-$/CO$_3$$^-$ by several orders of magnitude, replenishing CO$_3$$^-$ as it is consumed by HOO$^-$.$^-$. The more efficient supply of CO$_3$$^-$ by carbonic anhydrase is likely to increase HOOCO$_2$$^-$ production and accelerate SOD1-Cu(I) oxidation.

Effect of HCO$_3$$^-$/CO$_3$$^-$/H$_2$O$_2$ on the Overall SOD1 Activity—To gather further information about HCO$_3$$^-$/CO$_3$$^-$/H$_2$O$_2$ effects upon SOD1 peroxidase activity, the rate of H$_2$O$_2$ decay was directly measured using an H$_2$O$_2$ electrode. As shown in Fig. 4, HCO$_3$$^-$/H$_2$O$_2$/HCO$_3$$^-$/ HOOCO$_2$$^-$ addition led to a concentration-dependent acceleration of H$_2$O$_2$ uptake by SOD1. The maximum initial rates of SOD1-mediated H$_2$O$_2$ degradation measured at 0 and 100 mM HCO$_3$ were 0.4 and 1.7 $\mu$mol min$^{-1}$, respectively, per active site.

Direct Nuclear Magnetic Resonance Demonstration of HOOCO$_2$$^-$ Interaction with Active Site Copper Cation—Taken together, the EPR experiments indicated that SOD1-Cu(II) is promptly reduced by excess H$_2$O$_2$ to SOD1-Cu(I), especially at nearly neutral to alkaline pH, suggesting a peroxide anion (HOO$^-$) requirement for copper ion reduction. Moreover, the oxidation of SOD1-Cu(I) to SOD1-Cu(II) was shown to be strongly dependent on CO$_3$$^-$ as a precursor of HOOCO$_2$$^-$.

To confirm the interaction of HOOCO$_2$$^-$ with SOD1-copper, we designed NMR experiments using $^{13}$C-labeled bicarbonate (shown in Figs. 5 and 6). For these experiments, H$^{13}$CO$_3$ was dissolved in phosphate buffer and left undisturbed for at least 15 min for equilibration. After this time, H$_2$O was added and incubated for at least an additional 10 min to allow equilibration. The yields of HOOCO$_2$$^-$ dependent on pH and the concentrations of H$_2$O$_2$ and HCO$_3$$^-$. Experimental conditions were varied so as to attain an appreciable HOO$^{13}$CO$_2$ NMR peak intensity. The conditions shown in Fig. 5 were the ones that gave good HOO$^{13}$CO$_2$ yield with significant retention of SOD1 activity over the time required to complete the analysis (Fig. 8) and were used for the NMR studies.

As shown in Fig. 5, dissolving H$^{13}$CO$_3$ into phosphate buffer led to the detection of two pronounced peaks attributable to H$^{13}$CO$_3$$^-$(160.5 ppm) and $^{13}$CO$_2$(124.8 ppm) (27, 32). The addition of H$_2$O$_2$ to bicarbonate solutions led to a pronounced diminution of the $^{13}$CO$_2$ peak and the appearance of a new peak at 158.9 ppm (as in previous observations) attributed to peroxymonocarbonate (HOO$^{13}$CO$_2$) (32). Interestingly, the addition of SOD1 to the H$^{13}$CO$_3$$^-$/H$_2$O$_2$/HCO$_3$$^-$/CO$_3$$^-$/H$_2$O$_2$ led to a nearly complete disappearance of the HOO$^{13}$CO$_2$ peak over time (5–20 min), indicating peroxy monocarbonate and, indirectly, peroxide consumption by SOD1 (Fig. 5A).

Because the NMR experiments with SOD1 were performed in the presence of CDTA, and copper is released by the action of H$_2$O$_2$ upon SOD1, control experiments using an aqueous copper-CDTA complex (Fig. 5B) were performed to confirm SOD1 activity as a fundamental requirement for HOO$^{13}$CO$_2$ con-
As shown in Fig. 5B, Cu(II)-CDTA failed to diminish the HOO\textsuperscript{13}CO\textsubscript{2}/H\textsubscript{11}O\textsubscript{2}\textsuperscript{13} peak, demonstrating that this chelated copper does not mediate HOO\textsuperscript{13}CO\textsubscript{2}/H\textsubscript{11}O\textsubscript{2}\textsuperscript{13} decomposition. In contrast to SOD1, copper addition to the H\textsuperscript{13}CO\textsubscript{3}/H\textsubscript{11}O\textsubscript{2}/H\textsubscript{2}O\textsubscript{2} led to a strong broadening of the H\textsuperscript{13}CO\textsubscript{3}/H\textsubscript{11}O\textsubscript{2}\textsuperscript{13} peak, which overlapped the HOO\textsuperscript{13}CO\textsubscript{2}/H\textsubscript{11}O\textsubscript{2}\textsuperscript{13} NMR signal (data not shown). Nevertheless, the addition of CDTA promptly narrowed the H\textsuperscript{13}CO\textsubscript{3}/H\textsubscript{11}O\textsubscript{2}\textsuperscript{13} peak and restored the HOO\textsuperscript{13}CO\textsubscript{2}/H\textsubscript{11}O\textsubscript{2}\textsuperscript{13} NMR signal to its initial levels (data not shown). Peroxycarbonate formation depends on relatively slow equilibria; therefore, these data indicate that the copper CDTA complex is not able to consume HOO\textsuperscript{13}CO\textsubscript{2}/H\textsubscript{11}O\textsubscript{2}\textsuperscript{13} at appreciable rates, as opposed to SOD1. It has been shown that at H\textsubscript{2}O\textsubscript{2}/SOD1 concentration ratios of \( \sim 10:1 \), DTPA (25–50 M) significantly inhibit SOD-bound radical formation assayed through immunospin trapping (39). This inhibition indicates that copper release is involved in protein radical formation when SOD is exposed for prolonged times (\( \sim 10 \) min) to high H\textsubscript{2}O\textsubscript{2} concentrations (\( > 100 \) M). Therefore, the relevance of Cu(II) loss by SOD induced by H\textsubscript{2}O\textsubscript{2} in vivo requires further investigation, because the rapid detoxification of H\textsubscript{2}O\textsubscript{2} in the cellular milieu is likely to limit both the attainable concentrations and lifetime of the peroxide.

Together with the data reported here, these findings support SOD1-produced CO\textsubscript{3} as a major oxidant mediating SOD1 peroxidase activity, as previously proposed (5, 10, 11). Nevertheless, in light of the relatively slow equilibria and rates measured previously (see Table 1) and in this study, it seems unlikely that the SOD1 peroxidase activity will be a relevant source of CO\textsubscript{3} in vivo.

To further explore the effects of CO\textsubscript{2} and HOO\textsuperscript{−} upon HOO\textsuperscript{13}CO\textsubscript{2}/H\textsubscript{11}O\textsubscript{2}\textsuperscript{13} formation and decay, NMR experiments were repeated at different pH values. As seen in Fig. 6, raising the pH from 6.4 to 8.4 induced a marked acceleration in the rate of HOO\textsuperscript{13}CO\textsubscript{2}/H\textsubscript{11}O\textsubscript{2}\textsuperscript{13} decay. This result further characterizes the reduction of SOD1-Cu(II) by HOO\textsuperscript{−} as the rate-limiting step in the peroxidase catalytic cycle of SOD1.

Using the available rate constants (for the reactions and rate constants, see Table 1), the NMR results were computationally simulated using Gepasi software at physiological pH values (Fig. 7). Interestingly, our simulations suggest that HOO\textsuperscript{13}CO\textsubscript{2}/H\textsubscript{11}O\textsubscript{2}\textsuperscript{13} is rapidly consumed by SOD1 and does not accumulate, because its rate of formation from H\textsubscript{2}O\textsubscript{2} and H\textsubscript{11}O\textsubscript{2}\textsuperscript{13} is too slow at this pH to efficiently replenish consumed HOO\textsuperscript{13}CO\textsubscript{2}/H\textsubscript{11}O\textsubscript{2}\textsuperscript{13}. In addition, SOD1 inactivation is much slower in the presence of HOO\textsuperscript{13}CO\textsubscript{2}/H\textsubscript{11}O\textsubscript{2}\textsuperscript{13}.
than in its absence, which is consistent with the protection exerted by HCO$_3^-$ at high molar excess upon SOD1 inactivation by H$_2$O$_2$ (10, 26). Indeed, from the data shown in Fig. 8, it is clear that SOD1 inactivation is substantially accelerated after the 10 min time point, when HOOCO$_2^-$ is considerably lower. Also relevant, the simulations demonstrate that SOD1 does not accumulate in its reduced form in the presence of HOOCO$_2^-$ under the conditions of Fig. 6, a direct consequence of its rapid oxidation by HOOCO$_2^-$ as opposed to a fairly slow reduction of SOD-Cu(II) by H$_2$O$_2$.

**DISCUSSION**

Although SOD1 peroxidase activity has been well characterized in recent years, important details about the enzyme’s catalytic cycle remain obscure. This is in part because of the assumption that SOD1 peroxidase activity relies on the formation of hypervalent states in clear analogy to those identified in the heme-peroxidase catalytic mechanism. The hypothesis that the SOD1 peroxidase cycle closely paralleled that of the conventional peroxidases led to the proposal of hypothetical SOD1 hypervalent states, such as SOD1-Cu(III), SOD1-Cu(II)/O, or SOD1-Cu(II)/OH, which have never been characterized. Recent literature has reported several developments that have been important in unraveling the SOD1 peroxidase cycle: first, the demonstration that SOD1 peroxidase activity is greatly stimulated by HCO$_3^-$ (5, 6) and dependent on CO$_2$ (8); second, the characterization by Richardson’s group (27) of HOOCO$_2^-$ as a product of the spontaneous reaction of HCO$_3^-$ with H$_2$O$_2$; and third, Valentine and co-workers’ (29) molecular models of the SOD1 active site’s interaction with HOOCO$_2^-$.

These contributions led to the proposal in 2002 that HOOCO$_2^-$ might be an important biological oxidant precursor of the carbonate radical anion CO$_3^-$ (30, 33). This idea has inspired continued investigation (30, 31, 39–41). In the case of SOD1, it is hypothesized that the SOD1 peroxidase cycle is unique and depends on the reduction of SOD1-Cu(II) to SOD-Cu(I) followed by HOOCO$_2^-$-mediated oxidation of SOD1-Cu(I) to SOD1-Cu(II) with the formation of the carbonate radical in the second step of the catalytic cycle.
This hypothesis is based on previous reports that metal ion-catalyzed heterolysis of HOOCO$_2^-$ might lead to CO$_3^-$ radical production (30, 33). Through EPR investigations, we have unequivocally demonstrated that HOO$^-$ mediates SOD1-Cu(II) reduction (Fig. 1) at a fairly slow rate at physiological pH values ($k = 40-50$ M$^{-1}$ s$^{-1}$) (Ref. 38 and this work).

In addition, our results clearly demonstrated that HOO$^-$ minimizes HOOCO$_2^-$-mediated SOD1-Cu(II) reduction yield (Fig. 2), probably because of the formation of HOOCO$_2^-$, which induces SOD1-Cu(I) oxidation back to SOD-Cu(II). Indeed, using pre-equilibrated H$_2$O$_2$/HCO$_3^-$ mixtures (at concentrations high enough to attain sufficient HOOCO$_2^-$) and previously reduced SOD1, we estimated the second-order rate constant for SOD1-Cu(I) oxidation by HOOCO$_2^-$ to be 150 M$^{-1}$ s$^{-1}$, which is 10-fold higher than the rate constant for SOD1-Cu(I) oxidation by H$_2$O$_2$ (13 M$^{-1}$ s$^{-1}$) (38). This result certainly establishes HOOCO$_2^-$ as a key substrate for SOD1-Cu(I), once more suggesting the intermediacy of HOOCO$_2^-$ in closing the HCO$_3^-$-dependent peroxidase cycle (see Scheme 1) and as a precursor of CO$_3^-$.

Direct proof for the consumption of HOOCO$_2^-$ by SOD1 came from NMR studies in which the addition of SOD1 to the reaction mixtures caused the disappearance of HOOCO$_2^-$.

All experiments reported in this study were performed using adequate concentrations of the metal chelators, DTPA or CDTA, which excludes the possibility of HOOCO$_2^-$ consumption mediated by released aqueous copper. From the SOD1 inactivation data (Fig. 8) and the NMR data at physiological pH (Fig. 6, middle), it was possible to computationally simulate the SOD1 peroxidase cycle. Interestingly, the simulation indicated that SOD1 inactivation in the presence of HOOCO$_2^-$ is negligible, thus furnishing a molecular basis for the HCO$_3^-$-mediated protection of SOD1 activity in the presence of H$_2$O$_2$. In addition, the simulation indicated that uptake of pre-equilibrated HOOCO$_2^-$ is much faster than its uncatalyzed formation, which suggests that HOOCO$_2^-$ would not accumulate in the presence of SOD1.

Thermodynamically, the proposed mechanism, which involves heterolysis of HOOCO$_2^-$ by reduced copper produced at the SOD1 active site at the expense of H$_2$O$_2$, does not rest on the assumption that high energy intermediates, such as Cu(III), Cu(II)=O, or Cu(II)/OH, are formed to oxidize the thermodynamically stable HCO$_3^-$ or CO$_3^-$ in addition, the HOOCO$_2^-$ reduction produces H$_2$O and CO$_3^-$ ($E' = 1.7$ V), which is at least 0.4 V less oxidizing than *OH ($E' = 2.1$ V), the product of H$_2$O$_2$ reduction. The fact that CO$_3^-$ and not *OH radicals are produced in the active site may be the reason behind the protection afforded by HCO$_3^-$ against H$_2$O$_2$-induced SOD1 inactivation (10, 26), because *OH reacts at the site of formation to oxidize the distal histidines, whereas part of the CO$_3^-$ diffuses away from the active site. The anionic character of HOOCO$_2^-$ should also contribute to facilitating the access of HOOCO$_2^-$ to the active site, making the proposed mechanism feasible not only from...
the thermodynamic but also the kinetic and structural points of view.

Besides being an intriguing biochemical problem, the fact that SOD1 peroxidase activity was invoked as a possible factor leading to the development of amyotrophic lateral sclerosis, a severely debilitating disease (42, 43), demands that the SOD1 peroxidase cycle be characterized in detail so as to shed light onto which biochemical processes are physiologically relevant and thus serve as a molecular basis for the development of new therapeutic approaches. Our results indicate that the reduction of SOD1-Cu(II) by HOO\textsuperscript{−}/H\textsubscript{2}O\textsubscript{2} followed by the oxidation of SOD1-Cu(I) by HOOCO\textsubscript{2}/H\textsubscript{2}O\textsubscript{2} is too slow of a process to account for significant carbonate radical production under physiological conditions, supporting the current view that SOD1 peroxidase activity is not a main contributor to the development of amyotrophic lateral sclerosis (24, 44–46).

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Further references and citations are not visible in the provided image.