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Kinetics of the Oxidation of Reduced Cu,Zn-Superoxide Dismutase by Peroxymonocarbonate

Kalina Ranguelova¹, Douglas Ganini¹, Marcelo G. Bonini³, Robert E. London², and Ronald P. Mason¹

¹Laboratory of Pharmacology and Toxicology, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina, USA

²Laboratory of Structural Biology, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina, USA

³Section of Cardiology, Department of Medicine and Department of Pharmacology, University of Illinois at Chicago, Chicago, Illinois, USA

Abstract

Kinetic evidence is reported for the role of the peroxymonocarbonate, HOOCO_2^- , as an oxidant for reduced Cu,Zn-superoxide dismutase-Cu(I) (SOD1) during the peroxidase activity of the enzyme. The formation of this reactive oxygen species results from the equilibrium between hydrogen peroxide and bicarbonate. Recently, peroxymonocarbonate has been proposed to be a key substrate for reduced SOD1 and has been shown to oxidize SOD1-Cu(I) to SOD1-Cu(II) much faster than H_2O_2 . We have reinvestigated the kinetics of the reaction between SOD1-Cu(I) and HOOCO_2^- by using conventional stopped-flow spectrophotometry and obtained a second-order rate constant of $k = 1600 \pm 100 \text{ M}^{-1}\text{s}^{-1}$ for SOD1-Cu(I) oxidation by HOOCO_2^- . Our results demonstrate that peroxymonocarbonate oxidizes SOD1-Cu(I) to SOD1-Cu(II) and is in turn reduced to the carbonate anion radical. It is proposed that the dissociation of His61 from the active site Cu(I) in SOD-Cu(I) contributes to this chemistry by facilitating the binding of larger anions, such as peroxymonocarbonate.

Introduction

It is well known that Cu,Zn-superoxide dismutase (SOD1) has additional catalytic activities other than its dismutase activity, such as peroxidase activity [1–4]. The SOD1 peroxidase cycle involves several steps. Reduction of SOD1-Cu(II) in the active site to SOD1-Cu(I) by H_2O_2 is followed by oxidation of SOD1-Cu(I) by a second molecule of H_2O_2 , thus generating a hypothetical hypervalent or hydroxyl radical-like bound oxidant at the active site. This oxidant has been proposed to involve various high energy intermediates such as SOD-Cu(II)($\cdot\text{OH}$), SOD-Cu(I)=O, or SOD1-Cu(III) [3,5]. This oxidant, produced at the active site and derived from the reaction of SOD-Cu(I) with H_2O_2 , has been demonstrated to oxidize histidines at the enzyme's active site [6,7], ultimately leading to loss of the catalytic copper ion and SOD1 inactivation [8], and in the oxidation of nitrite [1,9].

Address correspondence to R. P. Mason, National Institutes of Health/NIEHS, MD F0-02, P.O. Box 12233, Research Triangle Park, NC 27709 USA. Telephone: (919) 541-3910. Fax: (919) 541-1043. mason4@niehs.nih.gov.

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It has also been found that the addition of CO_2 causes marked increases in peroxidase activity [3–5], which led to the proposal that bicarbonate is oxidized to the diffusible $\text{CO}_3^{\bullet-}$ [1,10]. The $\text{CO}_3^{\bullet-}$ is believed to mediate the oxidations promoted by the SOD peroxidase activity [1,11–13]. Alternatively, it has been proposed that CO_2 reacts directly with the hypervalent/bound hydroxyl radical oxidant produced at the SOD active site, producing the carbon dioxide cation radical $\text{CO}_2^{\bullet+}$, which then hydrolyzes to produce $\text{CO}_3^{\bullet-}$ [3] according to Eqns. (3) and (4) or to produce $\text{CO}_3^{\bullet-}$ directly (5):

The primary difficulty with the proposed mechanism according to Eqns. (3) and (4) is that the ionization potential (IP) of CO_2 has been determined to be 14.4 V [14,15]; in general, only very high-energy radiation is capable of producing $\text{CO}_2^{\bullet+}$.

Alternative mechanisms for $\text{CO}_3^{\bullet-}$ formation from SOD/ H_2O_2 in bicarbonate buffer have been proposed by some investigators [16–21] and criticized by others [22]. We have hypothesized that peroxymonocarbonate (HCO_4^-), which is a small anionic species, can diffuse through the anionic channel to the enzyme active site and be reduced to the $\text{CO}_3^{\bullet-}$ radical by the SOD1-Cu(I) (Scheme 1) [21]. Peroxymonocarbonate ion (HOOCO_2^-) is a well characterized covalent adduct of CO_2 and hydroperoxide anion with a corresponding equilibrium constant of $K_{\text{eq}} = 0.32 \text{ M}^{-1}$ [23]. Very recent kinetic simulations were shown to be consistent with a mechanism in which CO_2 forms from bicarbonate and subsequently reacts with hydrogen peroxide via perhydration [24] (Eqn. 6):

Deprotonation of the percarbonic acid (HOOCO_2H) forms peroxymonocarbonate (Eqn. 7):

Equivalently, CO_2 can react directly with the conjugate base HOO^- via base-catalyzed perhydration (Eqn. 8):

Richardson and co-workers published an estimation of the rate constants for the forward reactions (6) and (7) at 25°C ($0.002 \text{ M}^{-1}\text{s}^{-1}$ and $280 \text{ M}^{-1}\text{s}^{-1}$, respectively) and proposed that the contributions of the HOO^- and H_2O_2 pathways are equivalent at pH 8, but above this value HOO^- is dominant in the formation of HOOCO_2^- [24].

It has been shown that peroxymonocarbonate is able to oxidize organic sulfides [23,25] and sulfur-containing biomolecules [26,27], and in biological systems the formation of HOOCO_2^- is also accelerated by the presence of carbonic anhydrase [13,24]. On the basis of recent ESR and ^{13}C -NMR studies [20], it was proposed that once SOD1-Cu(II) is reduced to SOD1-Cu(I) via the reaction shown in Eqn. (1) [3,5], the enzyme can be oxidized back to SOD1-Cu(II) by peroxymonocarbonate more efficiently than by H_2O_2 itself or HOO^- (Scheme 1):

Previous estimated rates for Eqn. (9) were derived from ESR measurements and disagreed with the data published by Medinas et al. [19]. In the present investigation, we have reexamined the rates and mechanisms of the bicarbonate-catalyzed hydrogen peroxide oxidation of SOD1-Cu(I) using a conventional stopped-flow technique. We provide kinetic evidence that peroxymonocarbonate (HOOCO_2^-) resulting from the equilibrium between carbon dioxide (CO_2) and peroxide anion (HOO^-) reacts with reduced SOD1-Cu(I).

Materials and Methods

Materials

Bovine kidney superoxide dismutase (SOD1) was purchased from Calzyme Laboratories, Inc. (San Luis Obispo, CA). Sodium phosphate, sodium bicarbonate, hydrogen peroxide (obtained as a 30% solution), ascorbic acid, and diethylenetriaminepentaacetic acid (DTPA) were from Sigma (St Louis, MO). The hydrogen peroxide concentration was determined from its absorbance at 240 nm ($\epsilon = 39.4 \text{ M}^{-1}\text{cm}^{-1}$).

Stopped-flow optical experiments and kinetics

The kinetics of the reactions of SOD1 with hydrogen peroxide, bicarbonate (HCO_3^-), and peroxymonocarbonate (HOOCO_2^-) were measured using a double-mixing stopped-flow apparatus model SF-61DX2 (HiTech Scientific, Bradford-on-Avon, UK) equipped with a rapid scanning diode array spectrophotometer and Kinet-Asyst software for data acquisition and analysis. Phosphate buffer, pH 7.4, was used with or without bicarbonate and treated with Chelex and 500 μM DTPA to protect the enzyme against H_2O_2 -induced, copper-catalyzed SOD1 non-active site fragmentation [28]. The concentration of SOD1 was determined from the band at 680 nm ($\epsilon = 300 \text{ M}^{-1}\text{cm}^{-1}$), which results from the d-d transitions of the Cu atom [29–32]. For the reactions, SOD1 was loaded into one syringe, and H_2O_2 or $\text{H}_2\text{O}_2/\text{HCO}_3^-$ was loaded into a second syringe. The drive syringes and the reaction cell were equilibrated at 25 °C, and equal volumes of reactants were mixed to initiate the reaction. Spectra were acquired from 350 to 700 nm as a function of time to follow the reduction or oxidation of SOD1. The change in absorbance was monitored at 680 nm, and the data were fit to first-order-reaction kinetics. For reduction of SOD1-Cu(II) by H_2O_2 , pseudo-first-order conditions were achieved by keeping the hydrogen peroxide concentration to at least a fivefold excess over the enzyme.

To obtain the second-order rate constant of SOD1 oxidation by peroxymonocarbonate (HOOCO_2^-), 400 μM SOD1-Cu(I) was prepared by incubating 400 μM SOD1-Cu(II) with a fivefold excess of ascorbic acid (2 mM), and the complete reduction was monitored at 680 nm with a Cary 100 spectrophotometer (Varian Inc., Palo Alto, CA) using a 500 μl quartz cuvette [33]. After the complete reduction, the ascorbate was removed by using 7 ml/9K MWCO concentrators (Pierce, Rockford, IL), and the reduced enzyme was resuspended in 100 mM phosphate buffer, pH 7.4. Concentrations of HOOCO_2^- were at least eightfold greater than SOD1-Cu(I) and were calculated from the equation $[\text{HOOCO}_2^-] = K_{\text{eq}} \times$

$[\text{HCO}_3^-] \times [\text{H}_2\text{O}_2]$, where $K_{\text{eq}} = 0.32 \text{ M}^{-1}$ is the equilibrium constant of Eqn. (8) [23]. The initial concentration of HCO_3^- was kept constant at 500 mM, but in order to preform different amounts of peroxymonocarbonate, the initial $[\text{H}_2\text{O}_2]$ was varied, being set to 20 mM, 30 mM, 40 mM, or 50 mM.

Results

Reduction of SOD1-Cu(II) by H_2O_2

When a 10-fold excess of hydrogen peroxide (2 mM final) was added to SOD1-Cu(II) (200 μM final), the absorbance maximum at 680 nm quickly disappeared as the enzyme was reduced to Cu(I). Our stopped-flow data ($k = 45 \pm 5 \text{ M}^{-1}\text{s}^{-1}$) for the reduction of SOD1-Cu(II) by H_2O_2 via Eqn. (1) (data not shown) confirmed previous results obtained by both ESR [20] and optical spectrometry [20,34]. Next, we examined mild reduction of the enzyme by using ascorbate [34]. This cellular reductant was able to reduce the enzyme, but more slowly than H_2O_2 , and the complete reduction was achieved within 40 min.

Effect of $\text{HCO}_3^-/\text{CO}_2$ on the reduction of SOD1-Cu(II) mediated by H_2O_2

To demonstrate the effect of bicarbonate on the reduction of SOD1-Cu(II), 20 mM and 500 mM HCO_3^- were equilibrated for 30 min with 4 mM H_2O_2 at pH 7.4 before mixing with the protein (1:1 volume ratio). Upon mixing, these solutions yielded approximately 12.8 μM and 320 μM peroxymonocarbonate, respectively. Figure 1 shows the results of the stopped-flow experiments in which 400 μM SOD1-Cu(II) (initial concentration) was mixed in a 1:1 volume ratio with HCO_3^- (20 mM or 500 mM)/ H_2O_2 (4 mM) at pH 7.4. As demonstrated in time trace b, 10 mM HCO_3^- (final concentration) had no effect on the initial rate of reduction of Cu(II), but it significantly decreased the yield of SOD1-Cu(I). The presence of bicarbonate decreases the net reduction of SOD1-Cu(II) by approximately 50%. This result is in agreement with previously published data [35], which showed that 10 mM HCO_3^- had no effect on the reduction rate of the enzyme by 2 mM H_2O_2 . The authors also observed the decreased formation of SOD1-Cu(I) (Fig. 3 in reference [35]), but did not discuss it.

At the higher HCO_3^- concentration, the reduction of SOD1-Cu(II) was significantly diminished upon mixing with the enzyme (Fig. 1, trace c). Apparently, because of the presence of higher HOOCO_2^- (320 μM), SOD1-Cu(I) cannot accumulate in its reduced form due to the fast oxidation of SOD1-Cu(I) back to SOD1-Cu(II) [20].

Reoxidation of SOD1-Cu(I) by peroxymonocarbonate

To demonstrate that SOD1-Cu(I) is oxidized by peroxymonocarbonate, we reduced the resting SOD1 enzyme (400 μM) with 1 mM ascorbate. After the removal of the reductant (see Materials and Methods), we used stopped-flow experiments to calculate the rate of reoxidation of SOD1-Cu(I) back to SOD1-Cu(II). To obtain the equilibrium concentrations of peroxymonocarbonate, we separately mixed 500 mM and 200 mM HCO_3^- with 20 mM, 30 mM, 40 mM, or 50 mM H_2O_2 30 min prior to the experiments. Based on Eqn. (9) and the previously published value for K_{eq} of the equilibrium represented by Eqn. (8), we estimated the steady-state initial concentration of HOOCO_2^- for the first set of data with 500 mM HCO_3^- to be 3.2 mM, 4.8 mM, 6.4 mM, and 8 mM. For the second set of data when using 200 mM HCO_3^- and the same concentrations of H_2O_2 , the equilibrium concentrations of peroxymonocarbonate were calculated as 1.28 mM, 1.92 mM, 2.56 mM, and 3.2 mM. Figures 2A and 2B show the time courses of the reaction of SOD1-Cu(I) with the two sets of peroxymonocarbonate concentrations. Under these pseudo-first-order conditions, the initial rates of the reaction of SOD1-Cu(I) with peroxymonocarbonate were measured for each particular concentration of HOOCO_2^- by using a linear fit of the first 500 msec of the time course. The initial rates were divided by the initial concentration of the reduced enzyme to

provide k_{obs} rates. The second-order rate constant for SOD1-Cu(II) formation was determined from the k_{obs} rates as a function of HOOCO_2^- concentration (Fig. 2C) and was estimated to be $1600 \pm 100 \text{ M}^{-1}\text{s}^{-1}$, which is ~10-fold more rapid than the rate previously determined based on our ESR experiments [20] but similar to the rate reported by Medinas et al. ($2.0 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$) [19]. As shown in Fig. 2C, there is a linear dependence between both sets of data. Since we varied the concentrations of hydrogen peroxide to obtain the equilibrium concentrations of HOOCO_2^- and the pseudo-first-order conditions were kept in respect to H_2O_2 , we plotted the determined k_{obs} values as a function of H_2O_2 . As shown in Fig. 2D, each set of data could be fitted linearly with H_2O_2 concentration, but **different rate constants** were obtained depending on the two different bicarbonate concentrations, which demonstrates that in the presence of bicarbonate, no reaction of SOD1-Cu(I) with hydrogen peroxide occurs on this time scale.

Control experiments of reoxidation of SOD1-Cu(I) without preequilibration

To confirm that SOD1-Cu(I) was oxidized by peroxymonocarbonate, we used stopped-flow experiments without any preequilibration. First, as a control, we mixed reduced SOD1 (200 μM final) with 250 mM bicarbonate, and the typical time trace showed no oxidation of Cu(I), as expected (Fig. 3, trace a). Next, to characterize the ability of H_2O_2 to oxidize SOD1-Cu(I), we mixed the protein with the lowest and highest concentrations of H_2O_2 (10 mM and 25 mM) that we used for the experiments with preequilibration described above. The very slow absorbance increase at 680 nm (traces b and c) indicated a negligible oxidation of Cu(I) by H_2O_2 , which is in agreement with our previously published ESR data showing that H_2O_2 is not an efficient mediator of SOD1-Cu(I) oxidation [Fig. 2, trace c in reference [20]]. To further demonstrate that SOD1-Cu(I) is oxidized by HOOCO_2^- , we prepared 400 μM SOD1-Cu(I) in 100 mM phosphate buffer containing 500 mM bicarbonate at pH 7.4 in one of the stopped-flow reactors and filled the other reactor with 50 mM H_2O_2 . Upon mixing (1:1 volume ratio), no detectable SOD1-Cu(II) was formed. During the time course of 100 s, the known inactivation of SOD by H_2O_2 with a rate of $3.1 \text{ M}^{-1}\text{s}^{-1}$ would result in the loss of >90% of the SOD activity [7]. In the initial absence of peroxymonocarbonate, the SOD1 would be rapidly destroyed by H_2O_2 , accounting for the lack of significant SOD1-Cu(II) formation in Fig. 3, trace d.

Discussion

Recently, we proposed that the SOD1 peroxidase cycle proceeds via two steps [20]. First, hydrogen peroxide reduces SOD1-Cu(II) and second, the reduced SOD1-Cu(I) is oxidized back to SOD1-Cu(II) by peroxymonocarbonate anion (Scheme 1). It has been previously proposed that peroxymonocarbonate anion, like other small anions, can diffuse through the anion channel of SOD to the catalytically active site [21]. Structural studies indicate that small anions such as azide and thiocyanate are able to bind directly to the active-site copper ion [36,37], while larger anions such as acetate, borate, formate, perchlorate, phosphate, and sulfite interact with the anion-binding channel but do not appear to bind directly to the copper [38–40]. Nevertheless, larger ions such as dithionite (in equilibrium with $\text{SO}_2^{\bullet-}$) and sulfite are able to reduce the active site SOD-Cu(II) to SOD-Cu(I) [37,41].

However, recent theoretical analyses of SOD activity indicate that the bond connecting the bridging His61 (His63 in the human enzyme) with the copper is ruptured upon formation of the SOD-Cu(I) species [42]. In this type of structure, the anion binding site becomes able to accommodate substantially larger ligands. For example, in the analogous two- Zn^{2+} structure in which the His63 imidazole does not bind directly with the active-site Zn^{2+} (PDB code: 1P1V [16]), a sulfate ion is bonded directly with the active-site Zn^{2+} ion. Thus, it seems quite possible for the peroxymonocarbonate ion to interact directly with the SOD-Cu(I) ion

in a structure lacking the Cu(I)-His61 bond. This direct interaction between the Cu(I) ion and the peroxydicarbonate may be important for the observed redox chemistry; however, it is not essential for electron transfer.

It has also been suggested that in the absence of bicarbonate buffer, SOD1-Cu(I) can be oxidized to Cu(I) peroxide complex, which probably decays to a copper-bound hydroxyl radical complex, SOD1-Cu(II)($\bullet$ OH), ultimately causing enzyme inactivation. Originally, it was assumed that in the presence of bicarbonate buffer, the hydroxyl radical-like SOD intermediate would react with HCO_3^- , leading to $\text{CO}_3^{\bullet-}$ production [1,34,43]. More recently it was proposed that CO_2 may be the substrate for the enhanced peroxidase activity of SOD1 on the basis of the carbonic anhydrase-induced inhibition of the stimulation of NADPH oxidation by SOD1 in the presence of H_2O_2 and CO_2 -saturated water [3]. However, the oxidation of SOD1-Cu(I) by peroxydicarbonate would form the $\text{CO}_3^{\bullet-}$ ($E = 1.78 \text{ V}$) [44] directly, without the need for the formation of the much higher energy hydroxyl radical ($E = 2.31 \text{ V}$) [45] or its equivalent SOD intermediate, SOD1-Cu(II)($\bullet$ OH). The substantially lower oxidation potential of the $\text{CO}_3^{\bullet-}$ radical relative to the hydroxyl radical-like species is likely reflected in a much more favored reaction [28].

^{13}C NMR experiments [19,20] have confirmed the existence of a peroxydicarbonate (HOOCO_2^-) intermediate as the product of the reaction of CO_2 with hydrogen peroxide in agreement with Richardson's studies [23–26]. The stopped-flow experiments performed by Medinas *et al.* also supported a direct reaction between peroxydicarbonate and human SOD1 [20]. Medinas *et al.* favor a related mechanism in which SOD1 reacts with HOOCO_2^- produced as a result of carbon dioxide addition to SOD1-Cu(I)(OOH $^-$) rather than the reaction of SOD1-Cu(I) with freely diffusing HOOCO_2^- which, in these experiments, is preformed outside the active site. However, our results are fully consistent with the conclusion that HOOCO_2^- increases human SOD1 turnover with production of $\text{CO}_3^{\bullet-}$ [19].

Further support for the conclusion that SOD1-Cu(I) is oxidized by HOOCO_2^- produced in the bulk solvent is derived from our nonequilibrated stopped-flow studies (Fig. 3). These studies show that very slow, if any, oxidation of native SOD1-Cu(I) by H_2O_2 or unequilibrated mixtures of H_2O_2 with HCO_3^- occurs. However, the results with preequilibrated mixtures of HCO_3^- and H_2O_2 (Fig. 2) are consistent with peroxydicarbonate being the reactive oxygen species which oxidizes SOD1-Cu(I) with a rate constant of $1600 \pm 100 \text{ M}^{-1}\text{s}^{-1}$ at 25°C , pH 7.4.

From previously published ESR data we calculated a rate constant of $150 \text{ M}^{-1}\text{s}^{-1}$ for SOD1-Cu(I) oxidation by preequilibrated $\text{H}_2\text{O}_2/\text{HCO}_3^-$ mixtures [20]. In contrast, published kinetic simulations by Medinas *et al.* [19] estimated a more than 10-fold higher second-order rate constant for human SOD1-Cu(I) oxidation by peroxydicarbonate. This discrepancy led us to reexamine the kinetic experiments, but using a stopped-flow technique to minimize any inaccuracy. By reexamining the kinetic experiments with the stopped-flow technique, we estimated the second-order rate to be $1600 \text{ M}^{-1}\text{s}^{-1}$. This rate constant is close to the rate estimated for human SOD1 [19] and is 10-fold higher than the rate previously published [20], which was apparently underestimated due to inaccuracies with the relatively slow manual freezing for ESR studies. Regardless of the previously published value or the differences in the proposed mechanism, the rate constants determined here and by Medinas *et al.* [19] indicate that the oxidation of SOD1-Cu(I) by HOOCO_2^- is more than 100-fold faster than the oxidation of SOD1-Cu(I) by H_2O_2 [34]:

With the rate constants for both reactions and the initial concentrations of the reactants, we compared both rates and estimated the ratio between Eqns. (2) and (9) according to the rate law

$$\frac{v_2}{v_9} = \frac{[\text{SOD1-Cu(I)}] \times [\text{H}_2\text{O}_2] \times k_2}{[\text{SOD1-Cu(I)}] \times [\text{HOOCO}_2^-] \times k_9}$$

from Eqns. (2) and (9), respectively. The ratio between the two rates is given by:

$$\frac{v_2}{v_9} = \frac{[\text{SOD1-Cu(I)}] \times [\text{H}_2\text{O}_2] \times k_2}{[\text{SOD1-Cu(I)}] \times [\text{HOOCO}_2^-] \times k_9} = \frac{[\text{H}_2\text{O}_2] \times k_2}{[\text{HOOCO}_2^-] \times k_9} \quad (10)$$

At the physiological concentration of bicarbonate (25 mM) and a concentration of H_2O_2 of 10 mM, the equilibrium concentration of peroxymonocarbonate is 80 μM . After substitution of the initial concentrations of the reactants and estimated rate constants, the rates can be compared according to:

$$\frac{v_2}{v_9} = \frac{[\text{H}_2\text{O}_2] \times k_2}{[\text{HOOCO}_2^-] \times k_9} = \frac{10 \times 10^{-3} \times 13}{80 \times 10^{-6} \times 1600} \approx 1,$$

which gives $v_9 \approx v_2$. The fact that the reaction rate is the same for both Eqns. (2) and (9) raises the question of whether they are, in fact, two interpretations of the reaction we presented in Eqn. (9). The rate of formation (v_2) of the SOD1-bound hydroxyl radical was determined indirectly by the oxidation of NADPH by $\text{CO}_3^{\bullet-}$ [34], whereas our data from direct measurement of SOD1-Cu(II) formation demonstrated that peroxymonocarbonate is the oxidant for SOD1-Cu(I) via Eqn. (9). Thus, our results indicate that Eqn. (9) rather than Eqns. (3) and (4) or (5) is responsible for the formation of $\text{CO}_3^{\bullet-}$. As a result, the reaction of H_2O_2 and SOD1-Cu(I) with a reported rate constant $13 \text{ M}^{-1}\text{s}^{-1}$ [22,34] had, in fact, not been measured at all and may be near zero, and, in any case, is much slower than the inactivation of SOD1 by H_2O_2 as we have found (Fig. 4). Fridovich and Liochev [22] have argued that CO_2 pulls the equilibrium of Eqn. (3) or (5) to the right by reacting with the SOD1-bound oxidant. Since we have determined the initial rates of our reaction (9), subsequent reactions such as Eqn. (3) can have no effect.

Regardless of the merit of our mechanism (Scheme 1), the oxidation of CO_2 by the SOD oxidant, Eqn. (3), formed at the active site, Eqn. (2), is thermodynamically very unfavorable [14,15]. Even assuming that the hypervalent/bound hydroxyl radical SOD oxidant is as strong as the free hydroxyl radical ($E = 2.32 \text{ V}$) [45], the oxidation of CO_2 will be essentially zero as the following calculations show (*vide infra*). As discussed in the Introduction, the ionization potential for $\text{CO}_2^{\bullet+}$ was determined by Mackay as 14.4 V [14,15]. Previous studies have correlated the ionization and oxidation potentials of many compounds [46,47]. For example, Miller et al. [48] used the equation $E = 0.89 \times (\text{IP}) - 6.04$ with a correlation coefficient of 0.95. Based on this equation, we estimated that the oxidation potential for Eqn. (10) is 6.78 V:

The sum of Eqn. (10) and (11) gives Eqn. (3), already discussed above:

Given $\Delta G_{\text{reaction}}^0 = -nF\Delta E^0 = -1 \times 23.06 \times [(-6.78 \text{ V}) - (-2.32 \text{ V})] = 102.8 \text{ kcal} = 430 \text{ kJ}$, where n is the number of electrons transferred per mole, F is the Faraday constant = 23.06 kcal/V, and $\Delta E_{\text{reaction}}^0 = E_{10}^0 - E_{11}^0$ [45], we calculated the Gibbs energy for Eqn. (3), showing that the reaction is thermodynamically very unfavorable ($\Delta G \gg 0$). Knowing the Gibbs energy for Eqn. (3) allows one to calculate the equilibrium constant as $K_{\text{eq}} = e^{-\Delta G/RT}$, which we determined to be $K_{\text{eq}} = 2.71 \times 10^{-76}$. Assuming that Eqn. (3) is reversible [3,22] and that the reverse reaction can not exceed the diffusion limit $1 \times 10^{10} \text{ M}^{-1}\text{s}^{-1}$ requires that the forward reaction can not be faster than $2.71 \times 10^{-66} \text{ M}^{-1}\text{s}^{-1}$, i.e., effectively zero.

In summary, using direct optical measurements of SOD1-Cu(II) formation, we show that SOD1-Cu(I) is oxidized by HOOCO_2^- generated in the equilibration of $\text{HCO}_3^-/\text{CO}_2$ and H_2O_2 . We propose that bicarbonate is unique among the small anions known to interact with the copper of SOD1 in that it alone is known to interact with hydrogen peroxide to form an inorganic peroxide (peroxymonocarbonate) that will be rapidly reduced by SOD1-Cu(I) to form the oxidizing carbonate anion radical.

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Highlights

Direct optical measurement of SOD1 Cu(I) oxidation by HOOCO_2^- .

HOOCO_2^- formed from CO_2 and H_2O_2 is the precursor to the carbonate anion radical.

Other mechanisms proposed for SOD1 peroxidase activity are examined.

Figure 1. Effect of bicarbonate concentration on SOD1-Cu(II) reduction

The time traces of the reduction of 200 μ M SOD1-Cu(II) by (a) 2 mM H_2O_2 in 100 mM phosphate buffer, pH 7.4; (b) 2 mM H_2O_2 preincubated for 30 min with 100 mM phosphate buffer containing 10 mM HCO_3^- , pH 7.4; and (c) 2 mM H_2O_2 premixed for 30 min with 100 mM phosphate buffer containing 250 mM HCO_3^- , pH 7.4.

Figure 2. Time traces and kinetic fits of the oxidation of SOD1-Cu(I) by HOOCO_2^- at pH 7.4
 SOD1-Cu(I) (200 μM) was rapidly mixed with HOOCO_2^- . (A) Bicarbonate buffer (500 mM, pH 7.4) and 20 mM, 30 mM, 40 mM, or 50 mM H_2O_2 were equilibrated for 30 min. The concentration of HOOCO_2^- was calculated according to $[\text{HOOCO}_2^-] = K_{\text{eq}} \times [\text{HCO}_3^-] \times [\text{H}_2\text{O}_2]$, where $K_{\text{eq}} = 0.32 \text{ M}^{-1}$. For each time trace, $[\text{HOOCO}_2^-]$ was (a) 1.6 mM, (b) 2.4 mM, (c) 3.2 mM, and (d) 4 mM. (B) Bicarbonate buffer (200 mM, pH 7.4) and the same concentrations of H_2O_2 as in panel A were mixed and equilibrated. For each time trace, $[\text{HOOCO}_2^-]$ was (a) 0.64 mM, (b) 0.96 mM, (c) 1.28 mM, and (d) 1.6 mM. (C) Determination of pseudo-first-order rate constants for oxidation of SOD1-Cu(I) by HOOCO_2^- . The black and grey circles represent pseudo-first-order rates obtained from panels (A) and (B), respectively. The second-order rate constant is calculated from the slope. The error bars represent $\pm$ SD of three independent experiments. (D) Determination of pseudo-first-order rate constants from panels (A) and (B) as a function of H_2O_2 concentration.

Figure 3. No detectable reaction of SOD1-Cu(I) with H₂O₂

Time traces of reduced SOD1 (200 μM final) mixed without equilibration with (a) 250 mM HCO₃⁻, (b) 10 mM H₂O₂, (c) 25 mM H₂O₂, and (d) SOD1-Cu(I) were mixed with 500 mM HCO₃⁻ before mixing with 25 mM H₂O₂. All the experiments were performed at pH 7.4.

Scheme 1.

Reduction of the active site of SOD1 by hydrogen peroxide and its oxidation by peroxymonocarbonate