Nitric oxide protects Cu,Zn-superoxide dismutase from hydrogen peroxide-induced inactivation

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Abstract Reaction of Cu,Zn-superoxide dismutase (SOD1) and hydrogen peroxide generates a putative oxidant SOD-Cu^{2+}-$^{\bullet}OH$ that can inactivate the enzyme and oxidize 5,5′-dimethyl-1-pyrroline-*N*-oxide (DMPO) to DMPO-$^{\bullet}OH$. In the presence of nitric oxide ($^{\bullet}NO$), the SOD1/H_2O_2 system is known to produce peroxynitrite ($ONOO^-$). In contrast to the proposed cytotoxicity of $^{\bullet}NO$ conferred by $ONOO^-$, we report here a protective role of $^{\bullet}NO$ in the H_2O_2-induced inactivation of SOD1. In a dose-dependent manner, $^{\bullet}NO$ suppressed formation of DMPO-$^{\bullet}OH$ and inactivation of the enzyme. Fragmentation of the enzyme was not affected by $^{\bullet}NO$. Bicarbonate retarded formation of $ONOO^-$, suggesting that $^{\bullet}NO$ competes with bicarbonate for the oxidant SOD-Cu^{2+}-$^{\bullet}OH$. We propose that $^{\bullet}NO$ protects SOD1 from H_2O_2-induced inactivation by reducing SOD-Cu^{2+}-$^{\bullet}OH$ to the active SOD-Cu^{2+} with concomitant production of NO^+ which reacts with H_2O_2 to give $ONOO^-$.

Key words: Cu,Zn-superoxide dismutase; Hydrogen peroxide; Nitric oxide

1. Introduction

The biological role of superoxide dismutase is detoxification of superoxide radical ($O_2^{\bullet -}$) by converting it to H_2O_2 and O_2. However, it has been known for long [1] that Cu,Zn-superoxide dismutase (SOD1) can function as a peroxidase in the presence of H_2O_2, leading to inactivation of the enzyme. Using 5,5′-dimethyl-1-pyrroline-*N*-oxide (DMPO) as an electron paramagnetic resonance (EPR) spin trap, Yim et al. [2] observed a spectrum of DMPO-$^{\bullet}OH$ and suggested that free hydroxyl radical ($^{\bullet}OH$) was produced by the reaction. Later it was found that the DMPO-$^{\bullet}OH$ signal could be detected only in the presence of HCO_3^- at neutral pH. This led Sankarapandi and Zweier [3,4] to propose that HCO_3^- facilitated the cleavage of H_2O_2. The view has been modified to a currently accepted one [5–8] in which HCO_3^- is oxidized by SOD-Cu^{2+}-$^{\bullet}OH$ to CO_3^- radical which in turn oxidizes and hydroxylates DMPO to generate DMPO-$^{\bullet}OH$, as summarized in Reactions 1–3. DH_2, in Reaction 3, can be an added substrate (e.g. DMPO or HCO_3^-) or a histidine residue in the active site whose oxidation is responsible for the enzyme inactivation.

$$\mathrm{SOD-Cu^{2+} + H_2O_2 \rightarrow SOD-Cu^{1+} + O_2^{\bullet -} + 2\,H^+} \quad (1)$$

$$\mathrm{SOD-Cu^{1+} + H_2O_2 \rightarrow SOD-Cu^{2+} - {}^{\bullet}OH + OH^-} \quad (2)$$

$$\mathrm{SOD-Cu^{2+} - {}^{\bullet}OH + DH_2 \rightarrow SOD-Cu^{2+} + {}^{-}DH + H_2O} \quad (3)$$

Nitric oxide may interfere with the peroxidatic reaction of SOD1 since Reactions 1 and 2 involve SOD-Cu^{1+} and $O_2^{\bullet -}$ that can react with $^{\bullet}NO$ to produce SOD-Cu^{1+}-$^{\bullet}NO$ and peroxynitrite ($ONOO^-$), respectively. The former will protect the enzyme from oxidative inactivation whereas the latter can nitrate a tyrosine residue. According to the only report on this matter published recently by McBride et al. [9], the peroxidatic reaction in the presence of $^{\bullet}NO$ resulted in oxidation of dihydrorhodamine-1,2,3 (DHR123) and nitration of phenol, both of which were taken as evidence for the formation of $ONOO^-$. They proposed that $O_2^{\bullet -}$ (in Reaction 1) reacted with $^{\bullet}NO$ to form $ONOO^-$. In this case, $^{\bullet}NO$ should not affect the formation of SOD-Cu^{1+} which, upon reacting with H_2O_2, yields the oxidant SOD-Cu^{2+}-$^{\bullet}OH$. They did not, however, study the effect of $^{\bullet}NO$ on the formation of SOD-Cu^{2+}-$^{\bullet}OH$ and activity of SOD1 after H_2O_2 treatment.

In this work, we found that $^{\bullet}NO$ suppressed formation of DMPO-$^{\bullet}OH$ and protected SOD1 from H_2O_2-induced inactivation. We propose a mechanism in which SOD-Cu^{2+}-$^{\bullet}OH$ oxidizes $^{\bullet}NO$ and the resulting NO^+ reacts with H_2O_2 to form $ONOO^-$.

2. Materials and methods

2.1. Materials

Bovine erythrocyte SOD1 and bovine liver catalase were purchased from Roche Biochemicals (Mannheim, Germany) and used without further purification. DMPO was from Aldrich (Milwaukee, WI, USA) and purified by two cycles of vacuum distillation. *S*-Nitroso-*N*-acetylpenicillamine (SNAP) was purchased from Biomol (Plymouth Meeting, PA, USA). All other chemicals including DHR123, horse cytochrome *c* (type VI) and xanthine oxidase (grade III) were obtained from Sigma (St. Louis, MO, USA). The KPi/HCO_3^- buffer was prepared by dissolving $NaHCO_3$ (23.5 mM) in the KPi buffer (100 mM potassium phosphate, pH 7.4) and readjusting the pH to 7.4. Measurements were carried out in the KPi/HCO_3^- buffer unless otherwise stated. Solutions were treated with Chelex to remove trace metal ions. Gaseous $^{\bullet}NO$, produced by adding acid to $NaNO_2$, was bubbled through anaerobic water to obtain $^{\bullet}NO$-saturated solution [10]. Concentration of $^{\bullet}NO$ was determined by the method based on the conversion of oxyhemoglobin to methemoglobin [10]. The same method was used to measure the rate of $^{\bullet}NO$ release by SNAP.

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Abbreviations: DHR123, dihydrorhodamine-1,2,3; DMPO, 5,5′-dimethyl-1-pyrroline-*N*-oxide; DTPA, diethylenetriaminepentaacetic acid; EPR, electron paramagnetic resonance; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SOD1, Cu,Zn-superoxide dismutase

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2.2. EPR spin trapping

SOD1 (16 μM) was dissolved in the KPi/HCO_3^- buffer containing 1 mM diethylenetriaminepentaacetic acid (DTPA) and 100 mM DMPO. H_2O_2 (10 mM) was mixed in to initiate the reaction and the reaction mixture was rapidly transferred into a flat quartz EPR cell. The EPR spectra were obtained at 25°C on a Bruker ER200 X-band spectrometer (microwave frequency, 9.77 GHz; modulation frequency, 100 kHz).

2.3. Activity measurements and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

The SOD1 activity was measured by a method based on the ferricytochrome *c* reduction. The enzyme (16 μM) was allowed to react with H_2O_2 (10 mM) for a given period of time in the presence or absence of SNAP (2 mM) and an aliquot (5 μl) was withdrawn and diluted in the KPi/HCO_3^- buffer (1 ml) containing catalase (1000 U/ml). The diluted solution was allowed to stand for ~30 min before activity measurement in order to decompose unreacted H_2O_2 and SNAP although their concentrations were very low due to dilution. Xanthine (0.5 mM), xanthine oxidase (0.05 U/ml) and DTPA (100 μM) were added and the reduction of ferricytochrome *c* (20 μM) was monitored by the absorbance at 550 nm with a Hewlett-Packard 8483 diode array spectrophotometer. An aliquot was also removed for SDS–PAGE.

2.4. Fluorometric measurements of the DHR123 oxidation

To SOD1 (16 μM) in the KPi or KPi/HCO_3^- buffer containing 100 μM DTPA were added DHR123 (50 μM) and H_2O_2 (200 μM). When necessary, NO-saturated buffer was added to a final concentration of 5 μM. Oxidation of DHR123 was followed by the fluorescence at 536 nm with an excitation at 500 nm on an SLM-Aminco AB-2 luminescence spectrophotometer.

3. Results

Fig. 1a shows the EPR spectrum of DMPO-•OH radical ($a_N = a_H = 14.9$ G) generated by the reaction of SOD1 and H_2O_2 in the presence of DMPO. As previously reported [3,4,8], exclusion of HCO_3^- from the buffer almost completely abolished the signal. Due to limited accessibility to the active site of SOD1, bulky DMPO cannot be oxidized by SOD-Cu^{2+}-•OH. On the other hand, HCO_3^- can be oxidized by SOD-Cu^{2+}-•OH in the active site and the resulting CO_3^- diffuses out to subsequently oxidize and hydroxylate DMPO to produce DMPO-•OH [8]. Time-dependence of the DMPO-•OH intensities varies with the concentrations of H_2O_2. When incubated with 10 mM H_2O_2, the intensity of DMPO-•OH increased with time, reached a maximum at ~20 min and slowly decayed thereafter (Fig. 1c, open circles). At 30 mM H_2O_2, the intensity was largest at the beginning and monotonously decayed (not shown).

The DMPO-•OH signal was weakened by •NO in a dose-dependent manner. In the presence of 2 mM SNAP, which released •NO at a rate of 0.5 μM/s, the DMPO-•OH signal was reduced by ~70% at 15 min of incubation (Fig. 1a,b). This •NO-dependent attenuation of the DMPO-•OH signal was also observed with lower concentrations of H_2O_2 although the sensitivity of the EPR spectrometer did not allow the measurements below 200 μM H_2O_2 (not shown). Upon releasing •NO, SNAP liberates thiyl radicals that may interfere with the reaction. However, thiyl radicals in general are oxidants rather than reductants so that we should have observed an increase in DMPO-•OH in the presence of SNAP if the thiyl radicals oxidized DMPO. Moreover, we were not able to detect any DMPO-trapped thiyl radicals. This excludes possible involvement of the thiyl radicals in the reaction. We repeated the measurements with •NO-saturated buffer to find

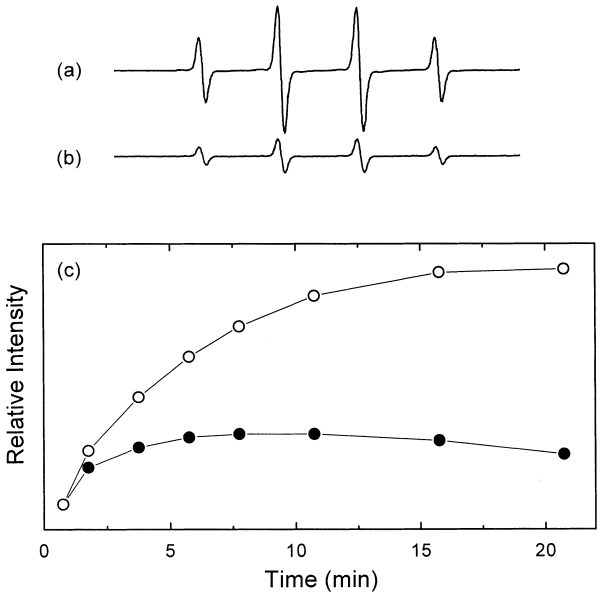

Fig. 1. Effects of •NO on the EPR intensity of DMPO-•OH. SOD1 (16 μM) was dissolved in 100 mM potassium phosphate buffer (pH 7.4) containing 23.5 mM HCO_3^-, 100 μM DTPA and 100 mM DMPO. The peroxidatic reaction was initiated by adding 10 mM H_2O_2 and the EPR spectra of DMPO-•OH were obtained as a function of time. The EPR spectra of DMPO-•OH produced after 15 min of incubation in the absence and presence of 2 mM SNAP are shown in (a) and (b), respectively. In (c), relative intensities of the second lowest field line are plotted as a function of incubation time in the absence (open symbols) and presence (closed symbols) of 2 mM SNAP.

that the formation of DMPO-•OH was suppressed as well (not shown). We had to add a relatively high concentration of •NO, however, which might react with O_2 to generate $•NO_2$, complicating the reaction system. In order to avoid such complications, we chose SNAP which supplied a low level of •NO continuously.

According to Reaction 3, SOD-Cu^{2+}-•OH is responsible for the generation of DMPO-•OH and inactivation of the enzyme. Then a weaker intensity of DMPO-•OH in the presence of •NO suggests that •NO may protect the enzyme from H_2O_2-induced inactivation by lowering the concentration of SOD-Cu^{2+}-•OH. This turned out to be true. As shown in Fig. 2, incubation of SOD1 with 10 mM H_2O_2 resulted in a significant inactivation of the enzyme even at the early stages (open circles). As expected, the extent of inactivation increased with the incubation time (open squares and triangles). •NO effectively slowed down the inactivation process regardless of the incubation period (closed symbols). Addition of •NO-saturated buffer was also protective and the potency depended on the concentration of SNAP or •NO.

The reaction of SOD1 and H_2O_2 caused fragmentation of the enzyme as previously reported by others [11,12]. Incubation with 10 mM H_2O_2 resulted in random fragmentation of the enzyme as shown in the inset of Fig. 2. SNAP at 2 mM, however, did not make any difference, suggesting that •NO did not slow down fragmentation, a process that could be caused by released copper [12]. Since, under the same conditions, •NO suppressed formation of DMPO-•OH and protected the enzyme activity, the random fragmentation appeared not to be correlated with the H_2O_2-induced inactivation.

Assuming that $ONOO^-$ oxidizes DHR123 to fluorescent rhodamine123, McBride et al. [9] fluorometrically followed oxidation of DHR123 by the SOD1/H_2O_2/NO system in KPi buffer and argued that $O_2^{\bullet-}$ generated by Reaction 1 reacted with $^\bullet NO$ to form $ONOO^-$. We repeated their experiment and, in addition, extended the measurements in the KPi/HCO_3^- buffer which provided valuable information on the mechanism of H_2O_2-induced inactivation of SOD1. As shown in Fig. 3, oxidation of DHR123 by the SOD1/H_2O_2 system in the KPi buffer was slow in the absence of $^\bullet NO$ (open squares) due to the limited access of bulky DHR123 to the active site SOD-Cu^{2+}-$^\bullet OH$. Without SOD1, H_2O_2 alone or in combination with $^\bullet NO$ oxidized DHR123 at even a lower rate (not shown). A remarkable increase in DHR123 oxidation was observed when HCO_3^- was included in the buffer (open circles). The result indicates that $CO_3^{\bullet-}$, produced by the reaction of HCO_3^- and SOD-Cu^{2+}-$^\bullet OH$, can also oxidize DHR123. Enhancement of the EPR intensity of DMPO-$^\bullet OH$ has been explained by a similar mechanism in which $CO_3^{\bullet-}$ oxidizes DMPO to DMPO-$^\bullet OH$ [8]. Addition of 5 μM $^\bullet NO$ greatly accelerated the DHR123 oxidation both in the absence (closed squares) and presence (closed circles) of HCO_3^-. The reaction product appeared to be $ONOO^-$ because, in the presence of phenol instead of DHR123, the absorption of nitrophenol at 400 nm increased when $^\bullet NO$ was added (Fig. 3, inset b). The same result was obtained by McBride et al. [9] and the nitration of phenol is often taken as evidence for the $ONOO^-$ formation. Addition of $^\bullet NO$ in the absence of SOD1 also produced nitrophenol at a slower rate (inset a). As pointed out by Halliwell [13], $^\bullet NO_2$ generated by the reaction of $^\bullet NO$ and O_2 probably nitrated phenol without producing $ONOO^-$.

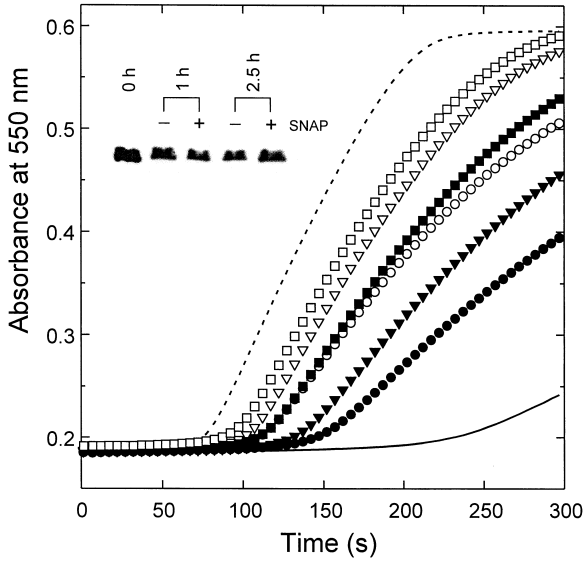

Fig. 2. Effects of $^\bullet NO$ on the activity of H_2O_2-treated SOD1. SOD1 (16 μM) was allowed to react with H_2O_2 (10 mM) for 0.5 (circles), 1 (triangles) and 2.5 h (squares) in the presence (closed symbols) and absence (open symbols) of SNAP (2 mM) and an aliquot (5 μl) was withdrawn and diluted in the KPi/HCO_3^- buffer (1 ml) containing catalase (1000 U/ml). After adding xanthine (0.5 mM), xanthine oxidase (0.05 U/ml) and DTPA (100 μM), reduction of ferricytochrome *c* (20 μM) was monitored by the absorbance at 550 nm. The absorbance changes corresponding to the full activity (solid line) and no activity (dotted line) were obtained by omitting H_2O_2 and SOD1, respectively, from the reaction mixture. In the inset, SDS–PAGE of the same samples in the presence and absence of SNAP is presented to show the enzyme fragmentation.

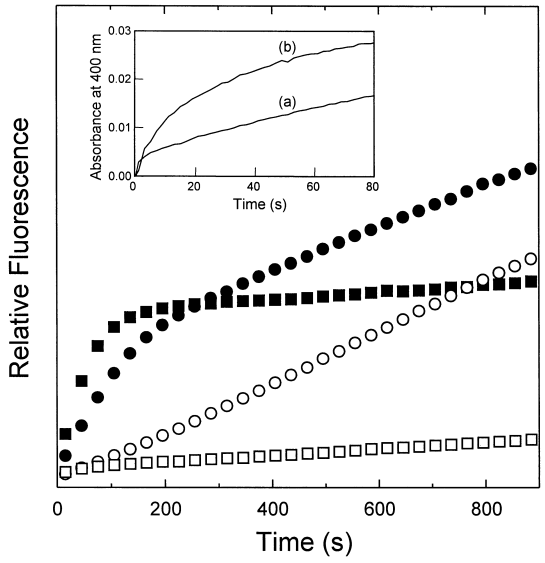

Fig. 3. Oxidation of DHR123 by the peroxidatic reaction of SOD1. SOD1 (16 μM), DHR123 (50 μM) and DTPA (100 μM) were dissolved in 100 mM potassium phosphate buffer (pH 7.4) with (circles) and without (squares) 23.5 mM HCO_3^-. After the initiation by 200 μM H_2O_2, oxidation of DHR123 in the absence (open symbols) and presence (closed symbols) of 5 μM $^\bullet NO$ was followed by the fluorescence at 536 nm upon excitation at 500 nm. Inset: phenol (5 mM) was added to the same reaction mixture in 100 mM KPi without DHR123 and the formation of nitrophenol was monitored by the absorbance at ~400 nm. Phenol was nitrated even in the absence of SOD1 (a) but in the presence SOD1 (b) the rate of nitration was faster due to the formation of $ONOO^-$ (see text).

After the added $^\bullet NO$ was exhausted, the rates of DHR123 oxidation returned to those in the absence of $^\bullet NO$. The fact that the rate of DHR123 oxidation in the presence of $^\bullet NO$ (the fast phase) is greater in KPi than in the KPi/HCO_3^- strongly suggests that $^\bullet NO$ competes with HCO_3^- for the oxidant SOD-Cu^{2+}-$^\bullet OH$. In other words, $^\bullet NO$, like HCO_3^-, directly reduced SOD-Cu^{2+}-$^\bullet OH$ to SOD-Cu^{2+}, providing a protection against the oxidative inactivation of SOD1.

4. Discussion

Inactivation of SOD1 by H_2O_2 has attracted much attention in relation to the cause of amyotrophic lateral sclerosis [14,15]. The reaction generates a strong oxidant SOD-Cu^{2+}-$^\bullet OH$ which is responsible for the formation of DMPO-$^\bullet OH$ and oxidative inactivation of the enzyme. As previously reported [8], direct oxidation of DMPO or DHR123 is slow due to a limited access of these large molecules to the active site. HCO_3^-, which has free access to the active site, enhanced the EPR intensity of DMPO-$^\bullet OH$ and the oxidation rate of DHR123: HCO_3^- is first oxidized by the putative oxidant SOD-Cu^{2+}-$^\bullet OH$ and the resulting $CO_3^{\bullet-}$ radical oxidizes DMPO or DHR123.

The first step of the peroxidatic reaction of SOD1 (Reaction 1) involves reduction of Cu^{2+} to Cu^{1+} and oxidation of H_2O_2 to $O_2^{\bullet-}$. Since $^\bullet NO$ often binds to Cu^{1+} [16] or reacts rapidly with $O_2^{\bullet-}$ [17], the peroxidatic reaction of SOD1 may be influenced by $^\bullet NO$. McBride et al. [9] indeed observed formation of $ONOO^-$ and proposed that $O_2^{\bullet-}$ generated by Reac-

tion 1 reacted with $^{\bullet}NO$ to produce $ONOO^-$. Depletion of $O_2^{\bullet-}$ in Reaction 1 by NO, however, will not suppress the formation of SOD-Cu^{2+}-$^{\bullet}OH$. This is inconsistent with our results in which $^{\bullet}NO$ retarded inactivation of the enzyme. Moreover, Jewett et al. [12] recently suggested that the first step (Reaction 1) generates O_2 rather than $O_2^{\bullet-}$ according to the equation: $2\,SOD-Cu^{2+}+H_2O_2 \rightarrow 2\,SOD-Cu^{1+}+O_2+2\,H^+$.

Another intermediate that can react with $^{\bullet}NO$ is SOD-Cu^{1+} in Reaction 1. The resulting SOD-Cu^{1+}-$^{\bullet}NO$ will not react with H_2O_2 preventing formation of SOD-Cu^{2+}-$^{\bullet}OH$, which explains the observed protection by NO. Since SOD-Cu^{1+}-NO can be written as SOD-Cu^{2+}-NO^-, its reaction with O_2 may produce $ONOO^-$ [18] returning the enzyme to the active form (SOD-Cu^{2+}). However, we can exclude this possibility because the $ONOO^-$ formation is competitively inhibited by HCO_3^- which does not bind to SOD-Cu^{1+}. Moreover, we were not able to detect the EPR active SOD-Cu^{1+}-$^{\bullet}NO$ species even under anaerobic conditions. This leaves SOD-Cu^{2+}-$^{\bullet}OH$ as the only candidate that reacts with $^{\bullet}NO$.

Attenuation of the DMPO-$^{\bullet}OH$ formation and suppression of the enzyme inactivation can be readily explained by a direct reaction of $^{\bullet}NO$ and SOD-Cu^{2+}-$^{\bullet}OH$: $^{\bullet}NO$ is oxidized to NO^+ by SOD-Cu^{2+}-$^{\bullet}OH$ and NO^+ subsequently reacts with H_2O_2 to form $ONOO^-$ (Reactions 4 and 5 below). NO^+ itself may oxidize DHR123 but we assume that it reacts further with H_2O_2 producing $ONOO^-$ because the SOD1/H_2O_2/NO system nitrates phenol [9]. The reaction of NO^+ (or acidified HNO_2) and H_2O_2 is a commonly used method for the preparation of $ONOO^-$. The standard reduction potentials of $CO_3^{\bullet-}/HCO_3^-$ and NO^+/NO are 1.59 V [19] and 1.21 V [20], respectively. Therefore if SOD-Cu^{2+}-$^{\bullet}OH$ oxidizes HCO_3^- to $CO_3^{\bullet-}$ as suggested by others [5–8], it may as well oxidize $^{\bullet}NO$ to NO^+.

A direct evidence that $^{\bullet}NO$ reacts with SOD-Cu^{2+}-$^{\bullet}OH$ comes from the fact that $^{\bullet}NO$ competes with HCO_3^- for the active site. Since HCO_3^-, unlike $^{\bullet}NO$, does not bind to SOD-Cu^{1+}, competitive inhibition by HCO_3^- of the $ONOO^-$ formation means that both $^{\bullet}NO$ and HCO_3^- react with a common reactant, SOD-Cu^{2+}-$^{\bullet}OH$. Decrease in the DMPO-$^{\bullet}OH$ signal can be explained alternatively by the reaction of $CO_3^{\bullet-}$ and $^{\bullet}NO$, i.e. $^{\bullet}NO+CO_3^{\bullet-}+H^+ \rightarrow HCO_3^-+NO^+$, which lowers the concentration of $CO_3^{\bullet-}$, the oxidant leading to the formation of DMPO-$^{\bullet}OH$. The reaction is not feasible, however, because the standard free energy change is small (0.38 V) and the concentration of HCO_3^- is very large (23.5 mM). The above arguments lead us to propose the following mechanism (Reactions 4 and 5).

$$SOD-Cu^{2+}-{}^{\bullet}OH+H^++{}^{\bullet}NO \rightarrow SOD-Cu^{2+}+H_2O+NO^+ \quad (4)$$

$$NO^+ + H_2O_2 \rightarrow ONOO^- + 2\,H^+ \quad (5)$$

As pointed out by Liochev and Fridovich [7], the reaction may assume importance only when cells are under oxidative stress. Pecci et al. [21] reported that Reaction 1 is very slow because H_2O_2 must be deprotonated before it reacts with Cu^{2+} and that is why a high concentration of H_2O_2 is required at neutral pH. The reaction may, however, be facilitated at a lower concentration of H_2O_2 if the reduction of Cu^{2+} can be achieved by a reductant (e.g. ascorbate) other than H_2O_2.

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