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Carbonate Anion Radical Generated by the Peroxidase Activity of Copper-Zinc Superoxide Dismutase: Scavenging of Radical and Protection of Enzyme by Hypotaurine and Cysteine Sulfinic Acid

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Abstract Copper-zinc superoxide dismutase (SOD) is considered one of the most important mammalian antioxidant defenses and plays a relevant role due to its main function in catalyzing the dismutation of superoxide anion to oxygen and hydrogen peroxide. However, interaction between SOD and H_2O_2 produced a strong copper-bound oxidant (Cu(II)$^{\bullet}$OH) that seems able to contrast the self-inactivation of the enzyme or oxidize other molecules through its peroxidase activity. The bicarbonate presence enhances the peroxidase activity and produces the carbonate anion radical ($CO_3^{\bullet-}$). $CO_3^{\bullet-}$ is a freely diffusible reactive species capable of oxidizing several molecules that are unwieldy to access into the reactive site of the enzyme. Cu(II)$^{\bullet}$OH oxidizes bicarbonate to the $CO_3^{\bullet-}$, which spreads out of the binding site and oxidizes hypotaurine and cysteine sulfinic acid to the respective sulfonates through an efficient reaction. These findings suggest a defense role for sulfinates against the damage caused by $CO_3^{\bullet-}$. The effect of hypotaurine and cysteine sulfinic acid on the $CO_3^{\bullet-}$-mediated oxidation of the peroxidase probe ABTS to ABTS cation radical ($ABTS^{\bullet+}$) has been studied. Both sulfinates are able to inhibit the oxidation of ABTS mediated by $CO_3^{\bullet-}$. The effect of hypotaurine and cysteine sulfinic acid against SOD inactivation by H_2O_2 (~42% protection of enzyme activity) has also been investigated. Interestingly, hypotaurine and cysteine sulfinic

acid partially avoid the H_2O_2-mediated SOD inactivation, suggesting that the two sulfinates may have access to the SOD reactive site and preserve it by reacting with the copper-bound oxidant. In this way hypotaurine and cysteine sulfinic acid not only intercept $CO_3^{\bullet-}$which could move out from the reactive site and cause oxidative damage, but also prevents the inactivation of SOD.

Abbreviations: SOD, Cu-Zn superoxide dismutase; H_2O_2, hydrogen peroxide; $CO_3^{\bullet-}$, carbonate radical anion; HCO_3^-, bicarbonate; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic); RSO_2^-, sulfinates; $RSO_2^{\bullet}$, sulfonyl radicals; RSO_3^-, sulfonates; HTAU, hypotaurine; CSA, cysteine sulfinic acid; TAU, taurine; CA, cysteic acid

1.1 INTRODUCTION

Copper-zinc superoxide dismutase (SOD) is considered one of the most important mammalian antioxidant defenses and plays a relevant role in the knowledge of oxidative damage. The main function of SOD is to catalyze the disproportionation of superoxide anion ($O_2^{\bullet-}$) to oxygen and hydrogen peroxide thanks to its catalytic copper ion (Fridovich 1989). However, SOD also interacts with hydrogen peroxide, producing a powerful oxidant species. This can either undergoes through self-inactivation or oxidize exogenous substrates acting as a relatively nonspecific peroxidase (Hodgson and Fridovich 1975a and 1975b; Gunther et al. 2002; Yim et al. 1993). It has been reported a potential correlation between a mutated form of superoxide dismutase and the neurodegenerative disease, familial amyotropic lateral sclerosis. (Yim et al. 1996; Widedau-Pazos et al. 1996; Valentine and Hart 2003; Liochev et al. 1998).
The SOD peroxidative mechanism is due to the production of a copper(II) hydroxyl radical (Cu(II)$^{\bullet}$OH), a one-electron oxidant, at the enzyme active site (reactions 1-2).

$$\text{SOD-Cu(II)} + H_2O_2 \rightarrow \text{SOD-Cu(I)} + O_2^{\bullet-} + 2H^+ \quad (1)$$

$$\text{SOD-Cu(I)} + H_2O_2 \rightarrow \text{SOD-Cu(II)}^{\bullet}\text{OH} + OH^- \quad (2)$$

In this mechanism the inactivation of SOD (self-inactivation) is due to either the oxidative activity of the copper(II)$^{\bullet}$OH towards histidine residues which are close to the oxidant or towards other molecules that reach the catalytic site, such as small anions (Hodgson and Fridovich, 1975a; Hodg-

son and Fridovich 1975b). In this latter circumstance, the inactivation is prevented as the active site seems to be preserved. For example imidazole, urate, and formate, seems to compete with histidine residues in the active site, reacting directly with Cu(II)$^{\bullet}$OH and preventing the SOD-inactivation due to hydrogen peroxide presence (Liochev and Fridovich 2002; Goldstone et al. 2006).

It has been suggested that in vivo, where the plasma concentration of bicarbonate (HCO_3^-) is 25 mM, the peroxidase action of the SOD could be relevant (Sankarapandi and Zweier 1999; Zhang et al. 2000). When bicarbonate (or CO_2) and H_2O_2 are present, strong evidences indicate that SOD is able to produce the carbonate anion radical ($CO_3^{\bullet-}$) (Liochev and Fridovich 2002; Liochev and Fridovich 2004; Goss et al. 1999) A relatively small anion like bicarbonate can access to the SOD active site and be oxidized through one-electron mechanism by Cu(II)$^{\bullet}$OH. $CO_3^{\bullet-}$ is a freely diffusible reactive species capable of oxidizing several molecules that are unwieldy to access the enzyme reactive site (Zhang et al. 2000 and 2002; Goss et al. 1999). The copper-bound oxidant (Cu(II)$^{\bullet}$OH) oxidizes HCO_3^- to the carbonate radical anion, which diffuses out and consequently oxidizes substrates (reaction 3).

$$\text{SOD-Cu(II)}^{\bullet}\text{OH} + HCO_3^- \rightarrow \text{SOD-Cu(II)} + CO_3^{\bullet-} + H_2O \quad (3)$$

Augusto et al. (2002) reported that "$CO_3^{\bullet-}$ is a strong one-electron oxidant that oxidizes suitable electron donors via electron transfer mechanisms". Moreover, $CO_3^{\bullet-}$, formed by SOD-mediated peroxidase activity, has the property to diffuse rapidly away from the reactive site of the SOD, and promptly abstracts electrons from cellular target, such as tyrosine and/or tryptophan, and enhances the DNA damage through oxidation (Huie et al. 1991; Bonini and Augusto 2001; Yermilov et al. 1996; Shafirovich and Dourandin 2001).

Recently, it has been shown that hypotaurine and cysteine sulfinic acid are efficiently oxidized to the respective sulfonates by $CO_3^{\bullet-}$ originated by SOD through its peroxidase activity (Baseggio Conrado et al. 2014). Due to pulse radiolysis studies, the rate constants between $CO_3^{\bullet-}$ and sulfinates have been reported, with a value of 1.1 x 10^9 $M^{-1}s^{-1}$ for hypotaurine and 5.5 x 10^7 $M^{-1}s^{-1}$ for cysteine sulfinic acid, . Moreover, this study has supported the evidence of the production of sulfonyl radical ($RSO_2^{\bullet}$). In particular, $CO_3^{\bullet-}$ oxidizes, via a one-electron transfer mechanism, the sulfinic group (RSO_2^-) of hypotaurine and cysteine sulfinic acid to form the $RSO_2^{\bullet}$ radical that reacts with O_2 to lead to sulfonate formation (RSO_3^-). These

results suggest a defense action for sulfinates against the damage caused by $CO_3^{\bullet-}$ (Fontana et al. 2005 and 2006; Baseggio Conrado et al. 2014).
In order to explore the ability of sulfinates to prevent the oxidation mediated by carbonate radical anions generated by the SOD/H_2O_2/HCO_3^- system, the effect of both sulfinates on the oxidation of the peroxidase probe 2,2-azino-bis[3-ethylbenzothiazoline]-6-sulfonic acid (ABTS) to ABTS radical cation ($ABTS^{\bullet+}$) has been studied. As reported (Zhang et al. 2000), the addition of bicarbonate to SOD/H_2O_2 is required to perform the ABTS peroxidation. Due to the fact that ABTS is a large molecule and rarely reaches the SOD reactive site, it could undergo a process of oxidation with carbonate radical anion. As reported above, several small anionic molecules, such as azide, nitrite and formate, can enter into the SOD reactive site preventing the enzyme inactivation by H_2O_2, anagously hypotaurine is known to be capable of avoiding this inactivation (Pecci et al. 2000a; Liochev and Fridovich 2002; Goldstone et al. 2006). However, many of these experiments including ours were performed in bicarbonate buffer, not considering the oxidation and consequently the production of $CO_3^{\bullet-}$ from the same buffer. Consequently, we have investigated the effect sulfinates on the inactivation of SOD by H_2O_2 in more detail.

1.2 METHODS

1.2.1 Oxidation of ABTS by SOD/H_2O_2/HCO_3^-

ABTS (20 μM) with SOD (1 mg/mL, from bovine erythrocytes EC 1.15.1.1) and sodium bicarbonate (0.025 M) was incubated in buffer (0.1 M K-phosphate) at pH 7.4 plus 100 μM DTPA added to prevent metal-catalyzed reactions. Addition of 1 mM H_2O_2 started the reaction where H_2O_2 concentration was previously analyzed at 240 nm with ε = 43.6 $M^{-1}cm^{-1}$ (Hildebrant and Roots 1975). The oxidation rates of ABTS were measured at 37° C using a Cary 50 Scan spectrophotometer using ε $ABTS^{\bullet+}$ = 3.6 x 10^4 $M^{-1}cm^{-1}$ at 415 nm (Childs and Bardsley 1975).

1.2.2 Assay of SOD activity

1 mM hypotaurine (HTAU) or cysteine sulfinic acid (CSA) in the presence of 1 mg/mL of SOD plus 1 mM of H_2O_2 were incubated at 37°C for 60 min in buffer (0.1 M K-phosphate) at pH 7.4, with 100 μM DTPA. Addition of 1 mM H_2O_2 started the reaction. To stop the reaction, catalase (220

units/mL) was added. The ferri-cytochrome *c* (cyt *c*) reduction assay was used to measure the SOD activity at 550 nm. For the assay, Goss et al. (1999) method was followed with the difference in the K-phosphate buffer (0.1 M) and in the presence of DTPA (0.1 mM).

1.2.3 Oxidation of sulfinates by SOD activity and HPLC analyses

HPLC analyses were performed following the methods discussed in our previous study based on the work of Hirschberger and collaborators (Baseggio Conrado et al. 2014; Hirschberger et al. 1985).

1.2.4 Statistical analysis

The experiments performed were carried out for a minimum three separate time and each time in duplicate (mean ± SEM). GraphPad Prism 4 software was used to perform data analysis and graphics. Differences with a $P < 0.05$ are considered significant.

1.3 RESULTS

1.3.1 Effect of sulfinates on ABTS oxidation by SOD/H_2O_2/HCO_3^-

$CO_3^{\bullet-}$ is known to oxidize ABTS with a mechanism of electron transfer to the radical cation $ABTS^{\bullet+}$ (Zhang et al., 2000). To investigate sulfinate ability to prevent carbonate radical anion-mediated oxidation, their effect on ABTS oxidation were studied.

To evaluate the rate of ABTS oxidation, the increase of the absorbance was analyzed at 415 nm owing to the radical cation formation ($\varepsilon = 3.6 \times 10^4\ M^{-1}cm^{-1}$) (Childs and Bardsley 1975). Sulfinates are able to inhibit dose-dependently the formation of $ABTS^{\bullet+}$ (Figure 1). The ability of sulfinates, hypotaurine and cysteine sulfinic acid, to inhibit the ABTS oxidation rate has been investigated at pH 7.4. Cysteine sulfinic acid showed an inhibitory effect greater than hypotaurine.

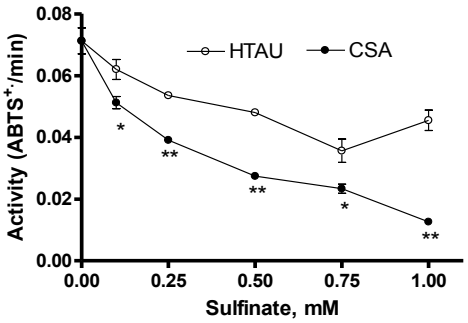


Fig. 1. Rate of ABTS formation in function of sulfinate concentration through $SOD/H_2O_2/HCO_3^-$. ABTS (20 μM) with SOD (1 mg/mL), H_2O_2 (1 mM), and $NaHCO_3$ (25 mM) in the absence (control) or in the presence of 1 mM HTAU or CSA, was incubated in buffer (0.1 M K-phosphate) at pH 7.4, plus DTPA (0.1 mM). The rate of ABTS formation was measured spectrophotometrically at 415 nm. Values are given as the mean ± SEM (n = 3). **P* < 0.05 and **P < 0.01 CSA values compared to HTAU values.

1.3.2 Effect of sulfinates on H_2O_2-mediated SOD inactivation

An enzyme copper-bound hydroxyl radical (SOD-Cu(II)$^{\bullet}$OH) is generated after the interaction of H_2O_2 with the active site of SOD. This powerful oxidant can oxidize an accessible substrate or attack amino acid residues at the active site, leading to enzyme inactivation (self-inactivation) (Hodgson and Fridovich 1975a and 1975b). To determine whether HTAU and CSA can affect the H_2O_2-mediated SOD inactivation, their effect on SOD activity was investigated.

Figure 2 shows that HTAU and CSA partially protected SOD from self-inactivation, suggesting that the two sulfinates may enter the reactive site of SOD scavenging the copper bound-$^{\bullet}$OH. This effect is similar to that exerted by bicarbonate, which is known to decrease H_2O_2-mediated SOD inactivation by reacting with the enzyme-bound oxidant (Goss et al. 1999). The extent of protection is slightly affected when sulfinates are added simultaneously to bicarbonate, suggesting that the compounds compete for binding to the active site of SOD (Figure 2).

The sulfinate protection on SOD self-inactivation were examined as a function of time (Figure 3).

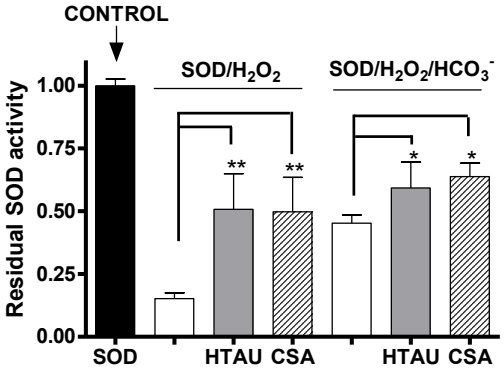


Fig. 2. Sulfinate effect on H_2O_2-mediated SOD inactivation. The cyt *c* reduction assay was used to measure the SOD activity. Enzyme activity is reported as residual SOD activity observed in the different mixtures. With the exception of the control, performed without H_2O_2, all mixtures contained SOD (1 mg/mL) with H_2O_2 (2 mM) with or without 1 mM HTAU or CSA. The "H_2O_2 + HCO_3^-" mixture also contained $NaHCO_3$ (25 mM). All mixtures were incubated for 2h at 37°C in buffer (0.1 M K-phosphate) at pH 7.4, plus DTPA (0.1 mM). Values are mean + SEM (n = 4). **P* < 0.05 and ***P* < 0.01.

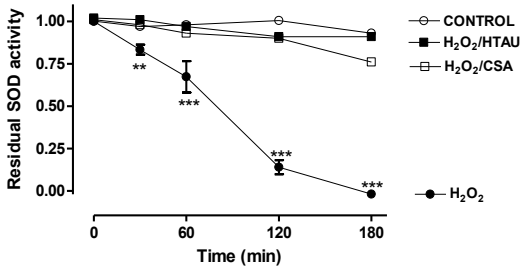


Fig. 3. Sulfinate effect on H_2O_2-mediated SOD inactivation as a function of time. The cyt *c* reduction assay was used to measure the SOD activity. Enzyme activity is reported as residual SOD activity observed. Mixtures contained SOD (1 mg/mL) with H_2O_2 (2 mM) in the absence or in the presence of 2 mM HTAU or CSA. At 0', 30', 1h, 2h and 3 h, aliquots (30 µL) of mixtures were analyzes in the cyt *c* reduction assay. Values are given as mean + SEM (n = 4). ***P* < 0.01 and ****P* < 0.001 compared to control and H_2O_2/sulfinate samples.

1.3.3 SOD peroxidase-mediated oxidation of sulfinates

We next evaluated the effect of the SOD/H_2O_2 system on the oxidation of both sulfinates, hypotaurine (HTAU) and cysteine sulfinic acid (CSA). The amount of the HTAU and CSA depletion and the formation of the corresponding sulfonates, taurine (TAU) and cysteic acid (CA) was monitored to determine the extent of sulfinate oxidation (Table 1). HPLC analysis showed that HTAU oxidation by SOD/H_2O_2 system produces mainly TAU. After 60 min incubation, 90% of depleted HTAU is recovered as TAU. On the contrary, when 1 mM CSA is reacted for 60 min in the SOD/H_2O_2 system, under the same oxidative conditions used for HTAU, 53 ± 2 µM CA is produced. By comparing the values of the CA yield with those of depleted CSA, CA formation was roughly 36% of the depleted corresponding sulfinate (CSA)..

Table 1. SOD/H_2O_2-mediated oxidation of sulfinates

Conditions*	substrate (1mM)	Sulfonate production (µM)#	Sulfinate depletion (µM)#
H_2O_2	HTAU	48 ± 2	n.d.
	CSA	34 ± 2	n.d.
SOD/H_2O_2	HTAU	140 ± 8	155 ± 6
	CSA	53 ± 2	146 ± 6

* 1 mM H_2O_2; 1 mg/mL SOD; 60 min at 37°C;
# Sulfinates and sulfonates concentrations were determined by HPLC

1.4 Discussion

Several studies have proposed sulfinates, hypotaurine (HTAU) and cysteine sulfinic acid (CSA) as antioxidant biomolecules and free radical scavengers (Fontana et al. 2004 and 2008; Baseggio Conrado et al. 2014 and 2015). The present study demonstrates that HTAU and CSA can prevent carbonate radical anion-mediated oxidation of ABTS, indicating that sulfinates can act as protective agents against the $CO_3^{\bullet-}$-induced oxidative damage. Furthermore, HTAU and CSA partially prevent the H_2O_2-mediated SOD inactivation, suggesting that the two sulfinates may reach the SOD active site , thus protecting the enzyme by reacting with the copper-bound oxidant (Cu(II)$^{\bullet}$OH).
The ABTS oxidation to ABTS radical cation (ABTS$^{\bullet+}$) requires bicarbonate as the SOD/H_2O_2 system does not oxidize ABTS to ABTS$^{\bullet+}$ when bicarbonate is not present. The addition of bicarbonate induces the oxidation of ABTS. The ABTS formation is not affected in any way by hydroxyl radical. Therefore, in the SOD/H_2O_2/bicarbonate system free hydroxyl

radicals are not produced and are not responsible for the oxidation of ABTS (Zhang et al. 2000). Instead, $ABTS^{\bullet+}$ is formed from the oxidation of ABTS by $CO_3^{\bullet-}$. $CO_3^{\bullet-}$ is a selective and strong oxidant that is able to spread out from the SOD reactive site and oxidizes ABTS to $ABTS^{\bullet+}$ by an electron transfer mechanism (Lyochev and Fridovich 1999). In this way, a large molecule like ABTS, which is unlikely to reach the active site of SOD, could still be oxidized by the peroxidase activity of SOD in the presence of added HCO_3^-. The results shown in this study indicate that both HTAU and CSA can inhibit the oxidation of ABTS mediated by carbonate radical anions. Furthermore, cysteine sulfinic acid exhibits a protective effect higher than hypotaurine. The different fate of sulfonyl radicals ($RSO_2^{\bullet}$) resulting from the sulfinate reaction with carbonate radical anions can explain this finding. Differently, CSA-derived sulfonyl radical can degrade with production of sulfite (Pecci et al. 2000b; Harman et al. 1984; Fontana et al. 2005). Sulfite ions undergo one-electron oxidation by several radicals, including carbonate radical anions (Neta and Huie 1985). Thus, the observed higher inhibitory effect of CSA on carbonate radical anion-mediated ABTS oxidation could be attributed to the concomitant sulfite formation. Similar results were shown in our previous work, where sulfinates, hypotaurine and cysteine sulfinic acid, exert a protective effect on the tyrosine dimerization mediated by the carbonate radical anion (Baseggio Conrado et al. 2014; Fontana et al. 2008). Due to the sulfinate ability to react with $CO_3^{\bullet-}$, HTAU and CSA can be included in the scavengers exerting protective effect on reactions mediated by carbonate radical anion such as ABTS oxidation and tyrosine dimerization.

As reported, H_2O_2 can react with the SOD active site with formation of a copper-bound hydroxyl radical ($Cu(II)^{\bullet}OH$), which can either attack amino acid residues at the active site, leading to enzyme inactivation, or oxidize an accessible substrate, preventing enzyme inactivation in this case. According to this, bicarbonate, which is oxidized by the copper-bound oxidant to $CO_3^{\bullet-}$ (reaction 3), inhibits SOD self-inactivation (Goss et al. 1999 and this work). The results reported here show that HTAU and CSA partially prevent SOD inactivation by H_2O_2. This finding suggests that the two sulfinates may enter into the SOD active site and protect the enzyme by reacting with copper-bound $^{\bullet}OH$ which causes the inactivation. In agreement, HTAU and CSA are oxidized, although at low level, by SOD/H_2O_2 system with formation of the sulfonates, taurine (TAU) and cysteic acid (CA), respectively. The production of taurine and cysteic acid by the SOD/H_2O_2 system reveals that the copper-bound oxidant is involved in the oxidative mechanism of sulfinates suggesting that the sulfinic group of sul-

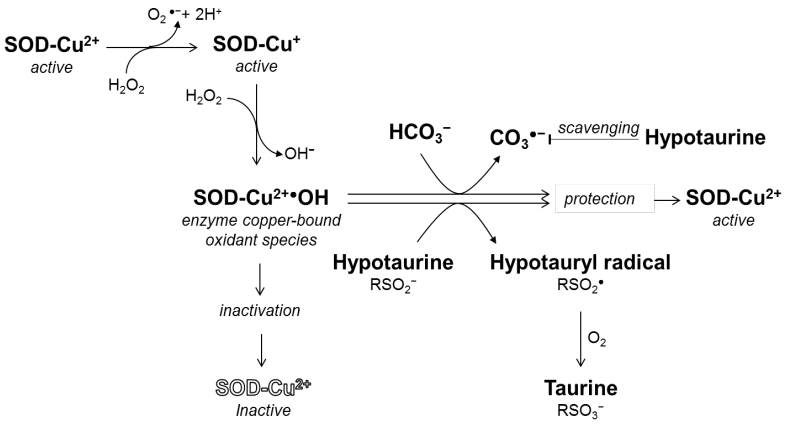
finates (RSO_2^-) rescues the enzyme in an active form (SOD-Cu(II)) and is concurrently oxidized to the sulfonyl radical ($RSO_2^{\bullet}$), as in reaction 4.

$$\text{SOD-Cu(II)}^{\bullet}\text{OH} + RSO_2^- \rightarrow \text{SOD-Cu(II)} + RSO_2^{\bullet} + OH^- \quad (4)$$

Subsequently, sulfonyl radicals ($RSO_2^{\bullet}$) trigger an oxygen-dependent radical chain reaction with sulfonates (RSO_3^-), TAU and CA, as final products. SOD copper-bound $^{\bullet}OH$ radical oxidizes HTAU and CSA to the same extent as shown by sulfinate depletion measurements, in agreement with the reaction rate constants reported in our previous work, between hydroxyl radical and hypotaurine ($k = 5.2 \times 10^9\ M^{-1}s^{-1}$) and cysteine sulfinic acid ($k = 4.5 \times 10^9\ M^{-1}s^{-1}$) (Baseggio Conrado et al. 2014). However, only 36% of depleted CSA is recovered as CA. This result can be explained, as reported above, as CSA-derived sulfonyl radical presents a higher propensity to decay (Harman et al. 1984).

1.5 CONCLUSION

Sulfinates, such as hypotaurine, not only intercept the carbonate anion radical which could move out from the active site and consequently damage oxidatively relevant biomolecules but also prevent the H_2O_2-mediated inactivation of SOD (as summarized graphically in scheme 1), which is a crucial antioxidant enzyme catalyzing the superoxide dismutation in vivo.



Scheme 1. Hypotaurine scavenger effect and its protection of SOD activity

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