Direct Probing of Copper Active Site and Free Radical Formed during Bicarbonate-dependent Peroxidase Activity of Bovine and Human Copper,Zinc-superoxide Dismutases

LOW-TEMPERATURE ELECTRON PARAMAGNETIC RESONANCE AND ELECTRON NUCLEAR DOUBLE RESONANCE STUDIES*

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Using X-band electron paramagnetic resonance (EPR) and electron nuclear double resonance (ENDOR) spectroscopy at liquid helium temperatures, the Cu(II) coordination geometry at the active site of bovine and human copper,zinc-superoxide dismutases (bSOD1 and hSOD1) treated with H₂O₂ and bicarbonate (HCO₃⁻) was examined. The time course EPR of wild type human SOD1 (WT hSOD1), W32F hSOD1 mutant (tryptophan 32 substituted with phenylalanine), and bSOD1 treated with H₂O₂ and HCO₃⁻ shows an initial reduction of active site Cu(II) to Cu(I) followed by its oxidation back to Cu(II) in the presence of H₂O₂. However, HCO₃⁻ induced a Trp-32-derived radical from WT hSOD1 but not from bSOD1. The mutation of Trp-32 by phenylalanine totally eliminated the Trp-32 radical signal generated from W32F hSOD1 treated with HCO₃⁻ and H₂O₂. Further characterization of the free radical was performed by UV irradiation of WT hSOD1 and bSOD1 that generated tryptophan and tyrosyl radicals. Both proton (¹H) and nitrogen (¹⁴N) ENDOR studies of bSOD1 and hSOD1 in the presence of H₂O₂ revealed a change in the geometry of His-46 (or His-44) and His-48 (or His-46) coordinated to Cu(II) at the active site of WT hSOD1 and bSOD1, respectively. However, in the presence of HCO₃⁻ and H₂O₂, both ¹H and ¹⁴N ENDOR spectra were almost identical to those derived from native bSOD1. We conclude that HCO₃⁻-derived oxidant does not alter significantly the Cu(II) active site geometry and histidine coordination to Cu(II) in SOD1 as does H₂O₂ alone; however, the oxidant derived from HCO₃⁻ (i.e. carbonate anion radical) reacts with surface-associated Trp-32 in hSOD1 to form the corresponding radical.

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‡ The abbreviations used are: bSOD1, bovine copper,zinc-superoxide dismutase; HCO₃⁻, bicarbonate anion; HCO₃, carbonate anion radical; EPR, electron paramagnetic resonance; ENDOR, electron nuclear double resonance; WT hSOD1, wild type human SOD1; W32F hSOD1, hSOD1 mutant with Phe-32 substituted for Trp-32; DTPA, diethylene-triaminepentaacetic acid; W, watt.

Pioneering research from Fridovich and co-workers (1–4) has demonstrated a unique peroxidase activity from bovine SOD1 (bSOD1)¹ that was dependent on bicarbonate levels in phosphate buffers. The peroxidase activity was proposed to result as shown in Equations 1 and 2.

\[
\text{E-Cu(II) + H₂O₂ → E-Cu(I) + O}_2^- + 2\text{H}^+ \quad (\text{Eq. 1})
\]

\[
\text{E-Cu(I) + H₂O₂ → E-Cu(II) - OH + OH}^- \quad (\text{Eq. 2})
\]

The oxidant, E-Cu(II)-OH, derived from bSOD1 is able to oxidize several small anionic ligands, viz. azide, formate, and others (3–8), that gain access to the active site through a narrow channel as shown in Fig. 1. It has been proposed that the bicarbonate (HCO₃⁻), a ubiquitous anion present in high concentrations (~25 mM) in biological systems, may enter the active site and be oxidized to form the carbonate anion radical, CO₃²⁻, which can diffuse out and oxidize other substrates in the bulk solution (3, 11–13) as shown in Equation 3.

\[
\text{E-Cu(II) - OH + HCO₃⁻ → E-Cu(II) + CO₃²⁻ + H₂O} \quad (\text{Eq. 3})
\]

Previously, we reported that HCO₃⁻ enhances the peroxidase activity of human Cu,Zn-SOD (hSOD1) in a concentration-dependent manner, causing protein aggregation through Trp-32-derived oxidation products (14).

Recently, Elam et al. (15) proposed an alternative mechanism for HCO₃⁻-mediated peroxidase activity and inactivation of SOD1 via an enzyme-associated peroxy carbonate (HCO₄⁻) intermediate, which apparently oxidized the histidine coordinated to Cu(II) at the active site causing its inactivation (15). It was proposed that the active species (HCO₃⁻ or CO₃²⁻) formed during the peroxidase activity is enzyme-associated and non-diffusible (15). The changes in geometry and coordination that occur at the Cu(II) site during the HCO₃⁻-dependent SOD1 peroxidase activity have not yet been investigated directly. To obtain a better understanding of the interaction between the histidine ligands and the oxidant generated at the active Cu(II) site during SOD1 peroxidase activity, we used the low temperature EPR and ENDOR techniques that can directly probe the paramagnetic metal ion at the active site, the coordinating ligands containing the NMR active nuclei, and the enzyme-derived free radical.

Although EPR is used to detect and characterize unambiguously paramagnetic metal ions at the active site in metalloenzymes, the EPR spectra are, in general, inhomogeneously broadened due to unresolved g-anisotropy and hyperfine cou-
plunging from the metal ion and the ligand nuclei (e.g. nitrogen) linked to the metal ion. In such cases, electron nuclear double resonance (ENDOR) can be used to probe the NMR-active nuclei that are coordinated to the paramagnetic center (16–21). Low temperature ENDOR has been used previously to determine the copper-histidine coordination in Cu,Zn-SOD (18, 21).

In this study, using the low temperature X-band EPR and ENDOR spectroscopy, we investigated the geometry and coordination of the Cu(II) active site as well as the structure of the free radical generated during SOD1 peroxidase activity. Results from this study show that H2O2 drastically alters the copper-histidine coordination at the active site and is inhibited partially by HCO3−. During HCO3−-dependent peroxidase activity, the Trp-32 located on the surface of hSOD1 was oxidized to the corresponding Trp-32 radical. The copper-bound histidines His-44 and His-46 in bSOD1 and His-46 and His-48 in WT hSOD1 are affected predominantly due to oxidation by H2O2 only in the absence of HCO3−. These findings are relevant to understanding oxidant-mediated site-specific damage, oxidative inactivation of the enzyme, and the coherent aggregation of WT hSOD1 and amyotrophic lateral sclerosis SOD1 mutants.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine Cu,Zn-superoxide dismutase was obtained from Roche Diagnostics. Tyrosine, tryptophan, hydrogen peroxide, sodium bicarbonate, sodium hydrogen phosphate, and DTPA were purchased from Sigma.

**Expression and Purification of Human SOD1**—The wild type human and W32F mutant Cu,Zn-SOD (Cu(II) 97%; Zn(II) 108%) were expressed and purified as described previously (14). The SOD1 mutations were created by a two-cycle polymerase chain reaction-based mutagenesis protocol described by Zhao et al. (22) using mutagenic primers to introduce the desired mutants and cloned subsequently into a pET-3d expression system (Novagen, Madison, WI).

**EPR Measurements**—A typical reaction mixture (200 μl) for EPR measurements consisted of SOD1 (30 μM), H2O2 (1 mM), and HCO3− (25 mM) in a phosphate buffer (100 mM) containing DTPA (100 μM). The X-band EPR of Cu(II) and free radical were recorded at liquid helium and liquid nitrogen temperatures on a Bruker E500 ELEXYS spectrometer with a 100-kHz field modulation equipped with an Oxford Instruments ESR-9 helium flow cryostat and a DM 0101 cavity. Incubation mixtures (200 μl) containing SOD1 and H2O2 were transferred to a 4-mm quartz EPR tube (Wilmad) and frozen immediately in liquid nitrogen for EPR analysis. EPR spectral parameters were extracted from the spectra using the QPowa program (23) based on the successful fitting to the other orthorhombic spin Hamiltonian as shown in Equation 4 (16),

\[
H = \beta g_S S_B + g_S S_B + A S_I + A S_I + A S_I (\text{Eq. 4})
\]

where \(\beta\) is the Bohr magneton; \(g_S\), \(g_B\), and \(A\), \(A\), and \(A\) are the components with respect to the principal axes of the \(g\)-tensor and the hyperfine tensor. Spectrometer conditions were as follows: microwave frequency, 9.63 GHz; modulation frequency, 100 kHz; modulation amplitude, 5 G; receiver gain, 85 dB; time constant, 0.01 s; conversion time, 0.08 s; sweep time, 83.9 s; and microwave power, 100 μW.

For UV irradiation studies, bovine or human SOD1 (30 μM) in a phosphate buffer (100 mM, pH 7.4) containing DTPA (0.1 mM) was irradiated at indicated time intervals with UV light using an Eclipse VIX 300 UV 300 X Xenon arc source and water as filter.

**ENDOR Measurements—X-band ENDOR spectra were recorded on a Bruker E500 ELEXYS spectrometer using an ENDOR/triple accessory (Bruker) cavity. ENDOR spectra were recorded by fixing the magnetic field at an EPR resonance and by applying saturating microwave power partially while sweeping the NMR transition with a radio frequency source.**

\[H = \beta B e S + \sum_{i=1}^{A} -g_S B e I + S A I + i Q I (\text{Eq. 5})\]

where \(\beta\) is the external magnetic field and \(A\) and \(Q\) correspond to the hyperfine and quadrupole tensors, respectively, of nucleus \(i\). \(A\) is described by its three principal values \((A_x, A_y, A_z)\) that transform its principal axis system \((x', y', z')\) to that of \((x, y, z)\). To simulate the resulting spectra, each nitrogen spectrum was multiplied by a factor chosen such that their sum gave the best fit to the experimental data (25). For protons with a spin of \(I = \frac{1}{2}\), the ENDOR transition frequencies are given to first order by Equation 6 (16),

\[v_H = \left| v_H \pm A_H^I \right| (\text{Eq. 6})\]

where \(A^I\) is the orientation-dependent nuclear hyperfine coupling constant and \(v_H\) is the nuclear Larmor frequency. If \(v_H > A_H^I\) as is usually the case for protons in biological systems, the ENDOR spectrum consists of a hyperfine–quadrupole doublet centered around the Larmor frequency. For \(v_H < A_H^I\) as is the case for nuclei with small nuclear Larmor frequencies, the ENDOR spectrum consists of a Larmor–split doublet centered at \(A_H^I\). For a quadrupolar nucleus such as \(^{14}\text{N}\) \((I = 1)\) where the ligand nuclei have a spin greater than \(\frac{1}{2}\), the ENDOR pattern is further split into 21 lines given by the first order resonance frequencies as shown in Equation 7 (16),

\[v_\gamma(m) = |v_N \pm A_H^I + 3P(m \cdot 2m - 1)| (\text{Eq. 7})\]

where \(m\) is the spin state of the lower two energy levels, which are involved in the transition given by \(-1 \leq m \leq I\). This hyperfine splitting is governed by \(P^2\), the orientation-dependent quadrupole coupling constant (26).

**RESULTS**

The Effect of H2O2 and Bicarbonate on the EPR Spectra of the Active Site Cu(II) in Human and Bovine SOD1—The EPR spectra of bovine SOD1, WT hSOD1, and mutant W32F hSOD1 along with the computer simulations (dotted lines) are shown in Fig. 2. Based on the \(g\)-tensor \((g_x = 2.030, g_y = 2.090, g_z = 2.268)\) and the Cu(II) hyperfine splitting \((A = -395\, \text{MHz})\) parameters (27, 28), it is evident that the Cu(II) active site geometry is similar in all cases. The precise determination of hyperfine tensor parameters, \(A_x\), was hindered due to spectral broadening in this region as a result of close \(g\) values...
and lower hyperfine coupling. The Euler angles of $\alpha = 85^\circ$ and $\beta = 20^\circ$ that describe the relative orientations of $g$- and $A$-tensors were obtained from simulating EPR spectra of W32F hSOD1, WT hSOD1, and bSOD1. Generally, the non-coincidence of $g$- and $A$-tensors and low anisotropy of $A_{z}$-tensor element of all SOD1 indicates the presence of low symmetry at the Cu(II) active site (29, 30). Large $g$- and $A$-strains indicating the distribution of $g$- and $A$-tensors due to the conformational heterogeneity of SOD1 were required to simulate the observed spectra.}

The time-dependent EPR spectra obtained from incubations containing the WT hSOD1, W32F hSOD1, and bSOD1 and $H_{2}O_{2}$ in the absence (left) and presence of $HCO_{3}^{-}$ (right) along with computer simulations (dotted lines) are shown in Fig. 2. Although it is probable that a mixture of several species is formed during the reaction with $H_{2}O_{2}$, we have simulated the EPR spectra based on the spin Hamiltonian parameters of two predominating species with one corresponding to the native SOD1 and the other corresponding to the Cu(II) species, characterized by less rhombic parameters ($g_{x} = 2.047, g_{y} = 2.055, g_{z} = 2.268, A_{z} = -420$ MHz). In the presence of $HCO_{3}^{-}$, the native enzyme parameters were found to be dominant, whereas in the absence of $HCO_{3}^{-}$, the less rhombic parameters were predominant for simulations. In the presence of $H_{2}O_{2}$, the Cu(II) signal intensity of WT hSOD1 slowly decreased up to 15 min because of the reduction of Cu(II) to Cu(I) and then increased in intensity because of reoxidation of Cu(I) to Cu(II) (Fig. 2). The Cu(II) hyperfine lines are also broadened possibly due to oxidation of His-46 (or His-44) and His-48 (or His-46) by the enzyme-derived oxidant, $E$-Cu(II)-$OH$. However in the presence of $HCO_{3}^{-}$, the EPR signal intensity of Cu(II) decreased significantly at 1 min and later increased until 30 min. The Cu(II) hyperfine signals reappeared much like the native enzyme, indicating that the Cu(II) active site geometry remained
more intact in the presence of HCO$_3^-$ as compared with the Cu(II) active site geometry in the presence of H$_2$O$_2$ alone.

In the presence of H$_2$O$_2$ and HCO$_3^-$, a new EPR signal appeared in the free radical region (Fig. 2, indicated by an arrow), which grew in intensity until 30 min and then decreased. The $P_v$ values were calculated to be $-0.072$ mW at 30 K and 0.32 mW at 77 K, suggesting that the tryptophan-derived radical saturates at a relatively low microwave power. As the EPR spectra obtained in the presence of 95% N$_2$ and 5% CO$_2$ were similar (not shown), we conclude that the free radical formed probably is carbon-centered and not oxygen-centered (31).

As with WT hSOD1, the Cu(II) signal intensity of W32F hSOD1 decreased initially but grew with time because of rapid oxidation of Cu(I) by H$_2$O$_2$. In the presence of HCO$_3^-$, the EPR signal intensity decreased but not to the same extent as did WT-SOD1. However, no free radical signal was detected from W32F SOD1 (Fig. 2, right panel). This confirms that the free radical detected from incubations containing WT hSOD1, H$_2$O$_2$, and HCO$_3^-$ was derived, most probably, from Trp-32.

**Effect of UV on WT and W32F Human SOD1 and Bovine SOD1**—Irradiation of WT hSOD1 and bSOD1 with UV light induced tryptophanyl and tyrosyl radicals. Typical EPR spectra obtained from UV-irradiated hSOD1 and bSOD1 and measured at 8 K are shown in Fig. 3. The UV irradiation of W32F hSOD1 under similar conditions did not induce the free radical signal (Fig. 3, bottom panel). This finding suggests that the free radical probably is derived from Trp. Upon expanding the free radical region, hyperfine features were resolved (Fig. 3, insert).

To identify the nature and structure of the free radical, we used the literature values (32–35). Computer simulations (shown as dotted lines) were performed using the EPR parameters reported for Trp and Tyr radicals obtained through ENDOR and high frequency EPR studies (32–35) and $\beta$-methylene hydrogen hyperfine coupling constants (vide infra). Thus, the experimental spectra could be fitted successfully using the literature values (32–35). Based on the EPR parameters shown in Tables I and II, we conclude that the doublet signal is the result of a Trp-32-derived radical in WT hSOD1. Unlike hSOD1, which has Trp-32, bSOD1 has a tyrosine residue. The corresponding signal (Fig. 3) is attributed to the tyrosyl radical.

**Probing of Copper-Histidine Coordination by ENDOR during SOD1 Peroxidase Activity**—The histidyl protons at the active Cu(II) site in SOD1 show $v_\mu$ and $v_\nu$ absorptions centered around $v_{\mu}$ that are split by their hyperfine coupling constants (18). Fig. 4A shows the time course of proton (1$^H$) ENDOR spectra from histidines coordinated to Cu(II) at the active site of bSOD1 in the presence of H$_2$O$_2$ and/or HCO$_3^-$ obtained at the field positions I, II, and III indicated in Fig. 4A (insert). The proton signal (marked $n$ in Fig. 4A) from His-44 is absent in the presence of H$_2$O$_2$ alone (18). However, the proton ENDOR spectra of bovine SOD1 treated with H$_2$O$_2$ in the presence of HCO$_3^-$ remained virtually unchanged (Fig. 4A).

The time course of the $^{14}$N ENDOR spectra from histidines liganded to Cu(II) of bovine SOD1 with H$_2$O$_2$ and/or HCO$_3^-$ is shown in Fig. 4B. The spectra for the field position I were simulated (Fig. 4B, dotted line) using the published parameters for native bSOD1 by Reinhard et al. (18) based on the superposition of the $A_x$ and $A_y$ features. As shown previously (18), the following two features became evident. (i) For the $A_x$ direction, there are two types of nitrogen signals, $N_{(1)}$ (His-46(44) and His-48(46)) and $N_{(2)}$ (His-63(61) and His-120(118)). However, the two values are overlapping at this orientation to give only a single set of lines, $N_{(1,2)}$ (Fig. 4B at field position II). (ii) For the $A_y$ direction, two sets of $^{14}$N signals, i.e., $N_{(1)}$ and $N_{(2)}$ were used for simulation as shown in Fig. 4B at field position III. In the presence of HCO$_3^-$ and H$_2$O$_2$, there was an excellent fit between the experimental ENDOR and the computer simulations using the values obtained by Reinhard et al. (18). From these findings, we conclude that the copper-bound His is not affected greatly in the presence of HCO$_3^-$ and H$_2$O$_2$. However, in the presence of H$_2$O$_2$ alone, the triplet signal in the low frequency region was absent (Fig. 4B at field position I). The triplet signal has been assigned previously to the His-46(44) and His-48(46) nitrogens (18). Thus, the two coordinated nitrogens of His are altered with H$_2$O$_2$ treatment, and the geometry is relaxed slightly, resulting in a lower Cu(II) hyperfine coupling (an $A_{\mu}$ value of 420 MHz) compared with the pure tetragonal axial symmetric systems, viz., copper tetraphenylporphyrin and copper phthalocyanin with $A_{\mu}$ values of 631 and 637 MHz, respectively (36). However, in the presence of HCO$_3^-$, there was only a minimal change in the spectra, indicating that the active site geometry was not affected greatly under these conditions. The lower Cu(II) hyperfine $A_{\mu}$ value coupled with an unusually large value for the $^{14}$N quadrupole tensor measured in the presence of H$_2$O$_2$
EPR/ENDOR of HCO$_3^-$-dependent Peroxidase Activity of Cu,Zn-SOD

**TABLE I**

| System (Tryptophanyl radical) | Tensor elements | g | A value $(\rho_1^H, \rho_2^H)$ |
|------------------------------|----------------|---|------------------|
| hSOD$^{a,b}$                  | X              | 2.0035 | $(-1.80, 4.88)$, $(-1.80, 4.88)$, $3.54$, $40.08$ |
|                              | Y              | 2.0024 | $(-2.80, 4.48)$, $(-2.80, 4.48)$, $3.54$, $40.08$ |
| Trp-177$^{a}$                | X              | 2.0035 | $(-2.80, 4.48)$, $(-2.80, 4.48)$, $3.54$, $40.08$ |
| Trp-111$^{c}$                | X              | 2.0025 | $(-2.80, 4.48)$, $(-2.80, 4.48)$, $3.54$, $40.08$ |
| Trp-111 in E. coli, Y122F$^{d}$ | X           | 2.0031 | $(-2.80, 4.48)$, $(-2.80, 4.48)$, $3.54$, $40.08$ |

$^{a}$ Line widths (in MHz) are 3.8 (for X), 3.8 (for Y), and 4.8 (for Z).
$^{b}$ This study.
$^{c}$ Ref. 32.
$^{d}$ Ref. 33.

**DISCUSSION**

Using EPR and ENDOR spectroscopy, we investigated the copper coordination geometry of human and bovine SOD1 treated with H$_2$O$_2$ and HCO$_3^-$. The results indicate that, in the presence of H$_2$O$_2$, the copper coordination geometry became less rhombic presumably because of oxidative modifications of two coordinated histidines. However, in the presence of H$_2$O$_2$ and HCO$_3^-$, the ENDOR pattern was identical to that of native SOD1, suggesting that the active site copper geometry was affected minimally. In the presence of HCO$_3^-$ and H$_2$O$_2$, a free radical signal attributable to Trp-32 was induced from WT hSOD1.

**TABLE II**

| System (Tyr radical) | Tensor elements | g | A value $(\rho_1^H, \rho_2^H)$ |
|----------------------|----------------|---|------------------|
| hSOD$^{a,b}$         | X              | 2.0068 | $(-1.80, 4.88)$, $(-1.80, 4.88)$, $3.54$, $40.08$ |
| Tyr                   | Y              | 2.0047 | $(-1.80, 4.88)$, $(-1.80, 4.88)$, $3.54$, $40.08$ |
| Tyr-Pro$^{a}$        | X              | 2.0021 | $(-1.80, 4.88)$, $(-1.80, 4.88)$, $3.54$, $40.08$ |
| Tyr-177$^{a}$        | X              | 2.0021 | $(-1.80, 4.88)$, $(-1.80, 4.88)$, $3.54$, $40.08$ |

$^{a}$ Line widths (in MHz) are 5.7 (for X), 28.3 (for Y), and 5.2 (for Z).
$^{b}$ This study.
$^{c}$ Ref. 34.
$^{d}$ Ref. 35.
$^{e}$ Ref. 32.

...
SOD1 (10) (from Protein Data Bank accession code 2SOD) were obtained from the x-ray crystal structure data. Using these θ values, the $A_{\perp\parallel}$ and $A_{\parallel\parallel}$ were calculated and used for the simulation. The best fit anisotropic $g$ and the hyperfine couplings of Trp and Tyr radicals in human SOD1 and bovine SOD1 are given in Tables I and II.

**Direct Probing of the Cu(II) Active Site by ENDOR**—As shown in Fig. 2, the time-dependent EPR spectra of the SOD1/H$_2$O$_2$/HCO$_3^-$ are broadened inhomogeneously, making it impossible to obtain hyperfine parameters from the coordinating histidine ligands. In contrast, the ENDOR spectroscopic technique enabled us to probe the Cu(II) active site and obtain additional hyperfine information from the coordinating histidines. Elam et al. (15) propose that the enzyme-associated non-diffusible oxidant, viz. HCO$_3^-$/CO$_3^-\cdot$ formed at the active site reacts rapidly with the copper-bound histidines, resulting in HCO$_3^-$-enhanced enzyme inactivation during peroxidase activity (15). However, the present ENDOR data show that the coupled proton signals and the triplet signal due to Cu(II)-bound His in the $^1$H and $^{14}$N ENDOR spectra are present in the presence of HCO$_3^-$ and H$_2$O$_2$ (Fig. 4, A and B). In the absence of HCO$_3^-$, the ENDOR spectra obtained from solutions containing Cu-Zn-SOD and H$_2$O$_2$ show drastic changes with regard to histidine at the active site geometry (Fig. 4, A and B). The ENDOR/EPR results reveal the following. (i) HCO$_3^-$ preserves the copper coordination to histidine that is damaged by H$_2$O$_2$.

(ii) The oxidant formed at the active site in the presence of HCO$_3^-$/H$_2$O$_2$ diffuses out of the active site and oxidizes Trp-32 to the Trp radical.

In conclusion, using the low temperature EPR and ENDOR, we showed that H$_2$O$_2$ drastically alters the copper-histidine coordination at the active site that was partially inhibited by HCO$_3^-$. The observation of a Trp radical formed at the surface by EPR from the site-specific oxidation of Trp suggests that the active oxidant formed from HCO$_3^-$ is diffusible, contrary to what had been proposed earlier (15), and clearly rules out the generation of “free hydroxyl radical” or an “enzyme-bound HCO$_3^-$” reactive intermediate in the active site. The proposed mechanism based on a site-specific inhibition of the damage of copper-bound histidine, CO$_3^-\cdot$ radical generation, and its scavenging by antioxidants may lead to a better understanding of the oxidative hypothesis proposed in amyotrophic lateral sclerosis.

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