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Copper,zinc superoxide dismutase catalyzes hydroxyl radical production from hydrogen peroxide

(electron paramagnetic resonance/spin trapping/oxidative damage)

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ABSTRACT **Cu,Zn superoxide dismutase (Cu,Zn-SOD; EC 1.15.1.1) is known to be inhibited slowly by H_2O_2. Using EPR and the spin traps 5,5-dimethyl-1-pyrroline 1-oxide (DMPO) and *N-tert*-butyl-α-phenylnitrone (PBN), we have shown that Cu,Zn-SOD catalyzes the formation of "free" ·OH radicals from H_2O_2 in pH 7.6 bicarbonate buffer. Supporting evidence includes the following: (*i*) H_2O_2 and active Cu,Zn-SOD are required to yield significant signals from spin-trap–·OH adducts. (*ii*) With O_2^-, Cu,Zn-SOD causes the appearance of intense resonance signals due to DMPO–OH adducts. These signals were inhibited strongly by catalase. (*iii*) With H_2O_2, Cu,Zn-SOD, and DMPO, radical scavengers formate and azide, but not ethanol, decrease DMPO–OH signals while causing new intense signals due to their corresponding DMPO-radical adducts. Failure of ethanol to quench DMPO–OH signals is discussed in light of the positively charged active channel of the enzyme. (*iv*) With PBN as a spin trap, ethanol quenches ·OH radical signals and yields PBN-trapped hydroxyethyl radical signals. (*v*) Mn-SOD does not catalyze "free" ·OH radical formation and it also exerts no effect on the signals of DMPO–OH adducts when added together with the Cu,Zn-SOD. The capacity of Cu,Zn-SOD to generate "free" ·OH radicals from H_2O_2 may in part explain the biological damage associated with elevated intracellular SOD activity.**

Superoxide dismutase (SOD; EC 1.15.1.1) catalyzes the reaction $2\ O_2^- + 2\ H^+ \rightarrow H_2O_2 + O_2$ (ref. 1). The reaction of H_2O_2 with various SODs has been studied extensively (2–13). Both copper,zinc SOD (Cu,Zn-SOD) and iron SOD (Fe-SOD) are inactivated by H_2O_2, whereas manganese SOD (Mn-SOD) is unaffected. The inactivation of the Cu,Zn-SOD appears to be caused by the modification of one histidine at the active site of the enzyme (6, 7). Some radical scavengers can protect the enzyme against inactivation by H_2O_2, but others, especially alcohols, are ineffective (6–11). These observations suggest that the "free" ·OH radical is probably not the reactive oxidant responsible for inactivation of the enzyme (8–10). A reaction scheme has been proposed for the H_2O_2-inactivation process (8):

$$\text{Enz-Cu}^{2+} + H_2O_2 \rightleftarrows \text{Enz-Cu}^{+} + O_2^- + 2\ H^+ \quad [1]$$

$$\text{Enz-Cu}^{+} + H_2O_2 \rightleftarrows \text{Enz-Cu}^{2+}\text{-}\dot{O}H + OH^- \quad [2]$$

$$\text{Enz-Cu}^{2+}\text{-}\dot{O}H + \text{ImH} \rightarrow \text{Enz-Cu}^{2+} + \text{Im}\cdot + H_2O \quad [3]$$

where Enz is the enzyme and ImH is the imidazole moiety of a histidine residue at the active site. In this scheme, an enzyme-metal-bound ·OH is postulated as the reactive species that causes the inactivation.

There are reports that elevated intracellular SOD activity induces cell killing (14, 15), increases lipid peroxidation (16, 17), interferes with the transport of biogenic amines (16), and causes hemolysis (18). In Down syndrome, which is associated with the presence of one extra copy of chromosome 21, excessive oxidative damage to biologically important molecules has been proposed and attributed to the overexpression of the Cu,Zn-SOD gene (16, 19–21). All of these observations are archetypical results inducible by oxygen radicals. Since SOD will disproportionate O_2^- radicals, a likely candidate for this toxicity is "free" ·OH radicals present away from active-site metal ions so that they can exert oxidative damage to other macromolecules in the cell. We envisioned that, in addition to the enzyme-metal-bound ·OH, another reactive species may be produced during the H_2O_2 inactivation of SOD.

We present here evidence for the production of "free" ·OH radicals catalyzed by Cu,Zn-SOD during the inactivation by H_2O_2 and their subsequent escape from the active channel. In this study a spin-trapping method and electron paramagnetic resonance (EPR) spectroscopy were used.

MATERIALS AND METHODS

The spin-trapping technique (22), a general method to convert transient free radicals to stable free radicals, has been widely used for studies involving reactive free radicals. Two spin traps, 5,5-dimethyl-1-pyrroline 1-oxide (DMPO) and *N-tert*-butyl-α-phenylnitrone (PBN), were employed in this study. The reaction between a spin trap (ST) and a free radical (A·) follows Eq. **4**:

$$\text{ST} + \text{A}\cdot \rightarrow \text{ST–A}\cdot \quad [4]$$

The hyperfine coupling (hfc) constants that depend on the nature of the trapped free radical can be measured by EPR spectroscopy.

DMPO and PBN purchased from Aldrich were purified by treatment with activated charcoal under an N_2 atmosphere and protected from light. The nitroxide spin label 3-carbamoylproxyl and potassium superoxide were also obtained from Aldrich. Catalase from beef liver and Cu,Zn-SOD from bovine erythrocytes were obtained from Boehringer Mannheim, and Mn-SOD from *Escherichia coli* was purchased from Sigma. The unit of SOD activity used here was defined by McCord and Fridovich (1). Calcium hydride and Chelex 100 resin (sodium form) were obtained from Fluka and Bio-Rad, respectively. Dimethyl sulfoxide (Me_2SO) was dried with calcium hydride under N_2 atmosphere. All buffers were treated with Chelex 100 resin.

Superoxide radicals were prepared immediately before use in Me_2SO by introducing an excess of potassium superoxide, stirring, and centrifuging under N_2 atmosphere. The concen-

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Abbreviations: SOD, superoxide dismutase; Cu,Zn-SOD, copper, zinc SOD; Fe-SOD, iron SOD; Mn-SOD, manganese SOD; DMPO, 5,5-dimethyl-1-pyrroline 1-oxide; PBN, *N-tert*-butyl-α-phenylnitrone; hfc, hyperfine coupling; Me_2SO, dimethyl sulfoxide.

tration of superoxide radicals in KO_2-saturated Me_2SO was taken as 5.1 mM as reported (23). The inactivation of SOD was carried out by immersion of reaction vessels containing 0.2 mg of SOD in 0.2 ml of buffer in a boiling water bath for 30 min. The vessels were used directly for further additions of reactants to prepare the reaction mixtures of control experiments. The reaction vessels, thus, contained copper and zinc ions released from the denatured enzyme.

The EPR spectrometer and sample transfer were described previously (24). Spectral acquisitions began 40 sec after initiation of the reaction by an injection of superoxide radicals in Me_2SO or H_2O_2. The conditions for the acquisition of spectral data were as follows: Temperature, 25°C; microwave power, 20 mW; modulation amplitude, 1 G (0.1 mT); conversion time, 10.24 msec; time constant, 82 msec; sweep time, 21 sec; accumulation, two times; sweep width, 100 G with 2048-point resolution.

The 3-carbamoylproxyl spin label was used as a standard to estimate the concentration of the DMPO–OH adduct. The double-integration value of the first-derivative EPR spectrum originating from the DMPO–OH adduct was compared with the one obtained from the spin label freshly prepared in the identical buffer and at identical spectrometer settings.

RESULTS

Formation of Hydroxyl Radicals in the Solution Containing H_2O_2, SOD, and DMPO. The first-derivative EPR spectra in Fig. 1 were obtained with solutions containing 100 mM DMPO, 30 mM H_2O_2, and 1.25 μM (600 units) Cu,Zn-SOD in various buffers. The four hyperfine splitting lines (Fig. 1, spectrum A) were observed with $NaHCO_3/CO_2$ buffer at pH 7.6 (23.5 mM sodium bicarbonate saturated with 5% CO_2/95% N_2 mixture). These lines are attributed to DMPO–OH adduct on the basis of hfc constants of 14.9 G due to couplings by one nitrogen and one hydrogen. In the presence of Mn-SOD (data not shown) or heat-denatured SOD (Fig. 1, spectrum B), these resonances appeared with much reduced amplitudes. In addition, there was no significant difference in the signal intensity of DMPO–OH adducts generated with Cu,Zn-SOD in the presence or absence of Mn-SOD (data not shown). The formation of free radicals was greatly affected by various buffer systems. In 25 mM borate at pH 8.1 or 25 mM phosphate at pH 7.3 (Fig. 1, spectrum C), the amplitude of DMPO–OH signals was reduced dramatically. Similar results have also been observed in our previous investigations on the H_2O_2 dismutation and amino acid oxidation by Mn(II) ions (24–26).

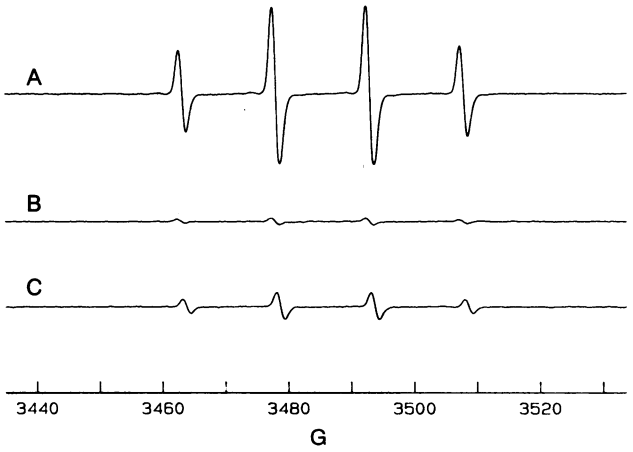

FIG. 1. Effect of Cu,Zn-SOD on the formation of DMPO–OH from H_2O_2. First-derivative EPR spectra of DMPO–OH radical adducts in reaction mixtures containing 30 mM H_2O_2 and 100 mM DMPO in various buffer systems in the presence of 1.25 μM Cu,Zn-SOD (600 units) or the equal amount of boiled Cu,Zn-SOD. Spectra: A, 23.5 mM $NaHCO_3$ bubbled with 5% CO_2/95% N_2 gas mixture and active Cu,Zn-SOD (pH 7.6); B, same as A except that enzyme is boiled Cu,Zn-SOD; C, same as A except the buffer is either 25 mM N_2-bubbled borate (pH 8.1) or phosphate (pH 7.3). The spectra were recorded at 6 min. Other experimental conditions and spectrometer settings were as described in *Materials and Methods*.

In $NaHCO_3/CO_2$ buffer, the amplitude of DMPO–OH signals increased with time and reached its maximum in 6 min. The concentration at this time, generated with 1.25 μM enzyme, was estimated to be 20 μM by comparing the double-integration value of the spectrum with that of a standard spin label, 3-carbamoylproxyl. This result suggests that production of DMPO–OH by Cu,Zn-SOD is a catalytic reaction.

DMPO–OH adduct can be formed by the trapping of "free" ·OH radical (Eq. **5**) or through the breakdown of DMPO–OOH adduct, which is formed by the reaction of superoxide radical with DMPO according to Eq. **6** (27):

$$\text{DMPO} + \cdot\text{OH} \xrightarrow{k_5} \text{DMPO–OH} \qquad [5]$$

$$\text{DMPO} + O_2^{\cdot-} \rightarrow \text{DMPO–OOH} \begin{cases} \xrightarrow{\cdot\text{OH},\ \text{DMPO}} \text{DMPO–OH} \\ \rightarrow \text{nonradical species} \end{cases} \qquad [6]$$

Since we were unable to observe resonance lines from DMPO–OOH adducts in this reaction, the DMPO–OH signals are most likely due to trapping of a free ·OH by DMPO according to Eq. **5**, suggesting a direct generation of ·OH by the catalytic action of Cu,Zn-SOD on H_2O_2. To confirm this supposition further, we have carried out other experiments in the presence of chemically generated $O_2^{\cdot-}$ and in the presence of radical scavengers.

Radical Adduct Formation in the Solution of KO_2/Me_2SO, DMPO, and SOD. Superoxide radicals were prepared chemically by dissolving KO_2 in dried Me_2SO as described in *Materials and Methods*. Fig. 2 shows EPR spectra obtained from a solution containing 0.2 mM superoxide radicals, 0.56 M Me_2SO, and 100 mM DMPO in $NaHCO_3/CO_2$ buffer at pH 7.6. Spectrum A in Fig. 2, recorded at 1.5 min after injection of KO_2/Me_2SO, shows resonance lines of DMPO–OOH radicals (marked by asterisks) and those of DMPO–OH adducts. The spectrum changes with time, and at 5.5 min

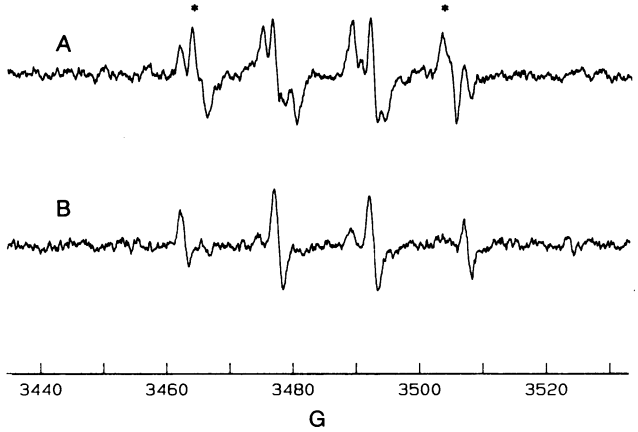

FIG. 2. First-derivative EPR spectra of DMPO–radical adducts formed in the presence of $O_2^{\cdot-}$ and the absence of SOD. The sample contained 0.2 mM $O_2^{\cdot-}$, 560 mM Me_2SO, and 100 mM DMPO in $NaHCO_3/CO_2$ buffer (pH 7.6). The reaction was initiated by an injection of KO_2/Me_2SO solution. Spectrum A was recorded at 1.5 min. Spectrum B was taken at 5.5 min. The resonance lines marked with asterisks belong to the spectrum of DMPO–OOH radical adducts.

(Fig. 2, spectrum B) signals due to DMPO–OOH adduct almost vanished, while those of DMPO–OH remained. This is understandable, since DMPO–OOH adduct has been reported to have a half-life of 60 sec in neutral aqueous solution (28). The resonance lines of DMPO–OH adducts observed here must come from the decomposition product of DMPO–OOH according to Eq. **6**.

Fig. 3 shows the effect of Cu,Zn-SOD and catalase on the formation of DMPO–OH adduct in solutions containing KO_2/Me_2SO recorded at 6 min after mixing. With Cu,Zn-SOD in the reaction mixture, resonance lines of DMPO–OH adduct with large amplitudes (Fig. 3, spectrum A) were observed, while those of DMPO–OOH were not detected throughout the period of spectral acquisition, which started at 40 sec after KO_2/Me_2SO addition, due to rapid dismutation catalyzed by the enzyme. Much-reduced amplitudes of the DMPO–OH signals were observed in the presence of boiled SOD (Fig. 3, spectrum B) or in the absence of SOD (Fig. 3, spectrum C). Additions of both catalase and SOD (Fig. 3, spectrum D) inhibited the formation of the DMPO–OH adducts. These observations, together with the fact that Mn-SOD does not have any effect on the signal intensity of DMPO–OH generated with Cu,Zn-SOD, indicate that the DMPO–OH adduct formed in the presence of Cu,Zn-SOD originates mainly from "free" ·OH produced from H_2O_2 by the catalytic action of Cu,Zn-SOD (Eq. **5**), rather than from O_2^- (Eq. **6**). This result also indicates that the observed DMPO–OH adducts in the solution containing H_2O_2 and SOD are not due to the formation of O_2^- from the reversal of the O_2^- dismutation reaction.

Effect of Scavengers on Radical Formation in the Solution of H_2O_2, SOD, and DMPO. Fig. 4 depicts EPR spectra from the solutions containing H_2O_2, SOD, and DMPO, and also in the presence of various radical scavengers: formate (Fig. 4, spectrum A), azide (Fig. 4, spectrum B), and ethanol (Fig. 4, spectrum C). In the presence of formate and azide anions, resonance lines of DMPO–CO_2^- (hfc constants A^N = 15.9 G and A^H = 19.3 G) (29, 30) and DMPO–N_3· ($A^N = A^H$ = 14.7 G and $A^N_{N_3}$ = 3.2 G) (31), respectively, were observed, whereas signal amplitudes due to DMPO–OH were reduced. With ethanol, however, the formation of DMPO–hydroxyethyl radical adducts was insignificant and the resonance amplitudes due to DMPO–OH were unaffected. When free ·OH radicals are produced in the reaction, the radical will undergo competition reactions between Eq. **5** and Eq. **7**:

$$\cdot OH + CH_3CH_2OH \xrightarrow{k_7} CH_3\dot{C}HOH + H_2O \qquad [7]$$

Since k_5 = 2.1 to 5.7 × 10^9 $M^{-1}\cdot sec^{-1}$ (30, 32) and k_7 = 1.8 × 10^9 $M^{-1}\cdot sec^{-1}$ (33), we should be able to observe a significant reduction in the resonance amplitudes of the DMPO–OH adducts and concomitant appearance of rather intense resonance lines of the DMPO–hydroxyethyl adducts. Spectrum C in Fig. 4 clearly shows that ethanol is not an effective radical scavenger in this case, in contrast to its acknowledged property.

The failure to detect DMPO–hydroxyethyl radical adducts in the presence of ethanol appears to be inconsistent with the observation that DMPO–OH adducts were derived from the reaction of DMPO and free ·OH. To resolve this dilemma, experiments using a different spin trap, PBN, were undertaken.

Radical Adducts Formed in the Solution of H_2O_2, SOD, and PBN. Fig. 5 shows EPR spectra of PBN–free radical adducts generated from 45 mM PBN and 12 mM H_2O_2 in $NaHCO_3$/CO_2 solution at pH 7.6. In the presence of 1.25 μM SOD, the six-line spectrum of PBN–OH adduct with hfc constants A^N = 15.5 G and A^H = 2.68 G (34, 35) was observed as shown in spectrum A. The four lines (marked with asterisks) were due to *tert*-butylhydronitroxide, a decomposition product of PBN (35, 36). No resonance lines were observed in the absence of SOD (Fig. 5, spectrum B) or in the presence of boiled SOD (Fig. 5, spectrum C). These results indicate that SOD is required for the generation of PBN–free radical adducts as it is in the case with DMPO.

When the reaction was carried out in the presence of ethanol, another set of hfc lines (indicated by **e**) appeared in addition to those of PBN–OH adduct (Fig. 5, spectrum D). The spectrum varied with time as shown in spectra A and B of Fig. 6, recorded at 1.5 and 9 min after beginning the reaction, respectively. The concentration of the radical species **e** increased with time and dominated at 9 min (Fig. 6, spectrum B). This species was identified as PBN–hydroxyethyl radical adduct on the basis of hfc constants, A^N = 16.1 G and A^H = 3.30 G, identical to values reported in ref. 37. By subtracting spectrum B from spectrum A after amplitude

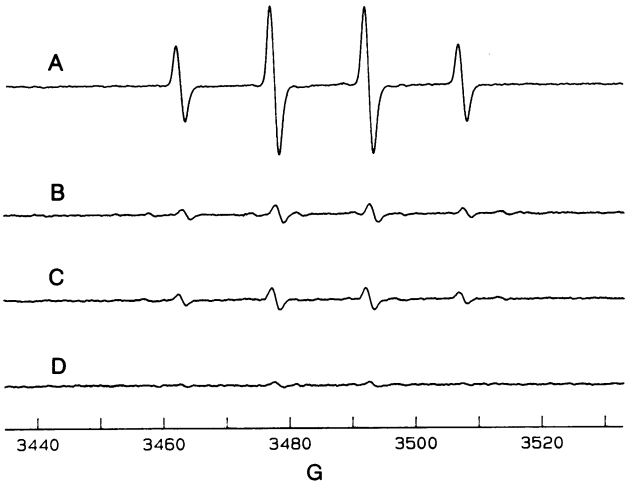

FIG. 3. SOD-induced DMPO–OH generation in the presence of O_2^- and its inhibition by catalase. First-derivative EPR spectra of DMPO–OH adducts formed in reaction mixtures containing 0.2 mM O_2^-, 560 mM Me_2SO, and 100 mM DMPO in $NaHCO_3/CO_2$ buffer (pH 7.6). In addition, the reaction mixtures include, respectively, 1.25 μM Cu,Zn-SOD (600 units) (spectrum A); 1.25 μM boiled Cu,Zn-SOD (spectrum B); no SOD (spectrum C); or 1.25 μM Cu,Zn-SOD and 3200 units of catalase (spectrum D). All spectra were taken at 6 min. The amplitudes of the resonance lines in spectrum A further increased slowly with time. The lines in the other spectra reached their maxima at this time.

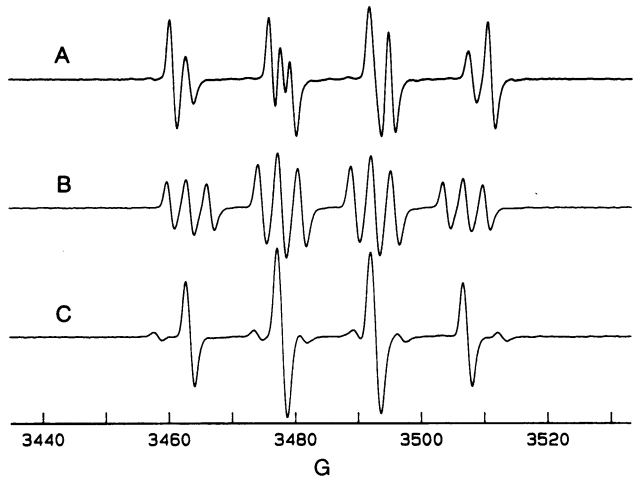

FIG. 4. Effect of radical scavengers on SOD-dependent DMPO–OH formation. First-derivative EPR spectra of DMPO–radical adducts formed in reaction mixtures containing 30 mM H_2O_2, 100 mM DMPO, 1.25 μM Cu,Zn-SOD, and various radical scavengers in $NaHCO_3/CO_2$ buffer (pH 7.6). Spectra: A, 0.8 M sodium formate; B, 0.8 M sodium azide; C, 3 M ethanol.

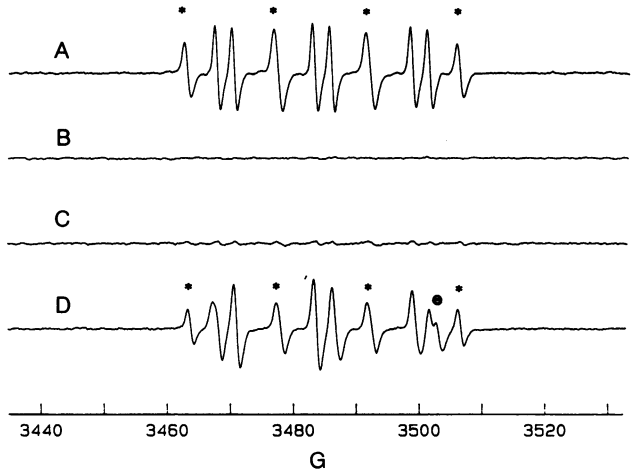

FIG. 5. Ethanol as ·OH radical scavenger detected with PBN as a spin trap. First-derivative EPR spectra of PBN–free radical adducts observed in reaction mixtures containing 12 mM H_2O_2 and 45 mM PBN in $NaHCO_3/CO_2$ buffer (pH 7.6) in the presence of 1.25 μM Cu,Zn-SOD (spectrum A); no SOD (spectrum B); 1.25 μM boiled Cu,Zn-SOD (spectrum C); or 1.25 μM Cu,Zn-SOD and 3 M ethanol (spectrum D). Resonance lines marked with asterisks belong to *tert*-butylhydronitroxide. The lines marked with e belong to PBN–$CH(OH)CH_3$ radical adducts (see text).

normalization, spectrum C was obtained. This resultant spectrum is identical to that of the PBN–OH adduct shown in spectrum D, which corresponds to spectrum A in Fig. 5 with an amplitude reduction of 50%. On the basis of the normalization factor used for the subtraction, we estimated a 2.5-fold increase in the concentration of PBN–hydroxyethyl radical adducts within 7.5 min. Using PBN as a spin trap, we demonstrated that free ·OH radicals generated from H_2O_2 catalyzed by Cu,Zn-SOD can be scavenged by ethanol.

DISCUSSION

We have shown by spin-trap experiments that formation of DMPO–OH and PBN–OH adducts from H_2O_2 is catalyzed by Cu,Zn-SOD. That both H_2O_2 and active Cu,Zn-SOD are the essential components for the generation of these radical adducts was demonstrated by (*i*) with boiled SOD, the signals from the radical adducts were greatly reduced (see Fig. 1, spectrum B, and Fig. 5, spectrum C); and (*ii*) with $O_2^{\cdot-}$, the formation of radical adducts was inhibited in the presence of both SOD and catalase, but SOD alone produced much-enhanced signals of the radical adducts (see Fig. 3). These observations suggest strongly the production of free ·OH radicals from H_2O_2 is catalyzed by Cu,Zn-SOD. The results obtained with azide and formate anions as radical scavengers are also in agreement with this observation. However, in the presence of ethanol as a scavenger and DMPO as a spin trap, no significant formation of hydroxyethyl radical adducts was detected. In contrast, by using a different spin trap, PBN, we observed PBN–hydroxyethyl radical adducts as well as PBN–OH adducts. These seemingly contradicting results may be explained by taking into consideration a unique feature of the active site of Cu,Zn-SOD.

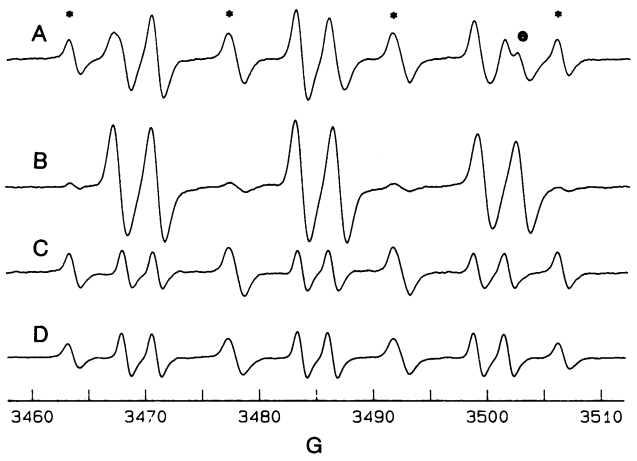

FIG. 6. Time-dependent changes in EPR spectra of PBN–free radical adducts in the presence of SOD and H_2O_2. First-derivative EPR spectra of PBN–free radical adducts produced in a reaction mixture containing 12 mM H_2O_2, 45 mM PBN, 1.25 μM Cu,Zn-SOD, and 3 M ethanol in $NaHCO_3/CO_2$ buffer (pH 7.6). Spectrum A was recorded at 1.5 min and spectrum B was taken at 9 min. Spectrum C resulted from subtraction of B from A after normalization. Spectrum D is the expanded and intensity-reduced (by 50%) spectrum of PBN–OH shown in spectrum A of Fig. 5 for comparison of line positions. Resonance lines marked with asterisks belong to *tert*-butylhydronitroxide, while those marked with e belong to PBN–$CH(OH)CH_3$ adducts.

X-ray structure of Cu,Zn-SOD (38) reveals the existence of a channel with positively charged amino acid residues, Lys-120 and Lys-134 at the top and Arg-141 inside the channel, positioned at 13, 12, and 5 Å, respectively, from the active-site Cu ion. This positively charged channel provides effective long- and short-range electrostatic guidance to the active site for its substrate $O_2^{\cdot-}$ (38–41). Other small ions can also be guided into this positively charged channel. The presence of this channel is invoked to explain the unusually rapid rate (2×10^9 $M^{-1}\cdot s^{-1}$) of $O_2^{\cdot-}$ dismutation catalyzed by this enzyme (39–42). Cyanide and azide anions have also been shown to gain access inside the channel and bind to the metal ion with association constants 1.8×10^5 M^{-1} and 87.2 M^{-1}, respectively (43). These small anions were found to protect Cu,Zn-SOD from H_2O_2 inactivation, whereas alcohols and benzoate anion did not (8).

Accordingly, in reaction mixtures studied here, azide and formate anions will also gain access inside the active channel and ethanol will remain outside. In addition, to account for all of our results, reasonable assumptions were made that PBN, having an aromatic ring, like benzoate, could not gain access inside the active channel, whereas the less hydrophobic and smaller DMPO molecules could. The reaction of H_2O_2 with the active-site metal ion produces reactive oxidants that attack specifically a histidine residue liganded to the Cu ion (6, 7). Because of this specificity, we concur with the suggestion that metal-coordinating ·OH (8) or probably caged ·OH as suggested previously (24) is the primary oxidant for the inactivation of the enzyme *itself*. However, some free ·OH radicals must be released from the metal sites and subsequently be trapped by spin traps. Thus, on one hand, ethanol and PBN, both being located outside the active channel, will react competitively with the released free ·OH radicals according to Eqs. **5** and **7** to produce PBN–hydroxyethyl and PBN–OH adducts. On the other hand, DMPO molecules, which can penetrate inside the channel, are in position to intercept the ·OH radicals released from the active sites, and they result in significant decrease of ·OH radicals available to react with ethanol to form DMPO–hydroxyethyl radical adducts in the bulk solution.

The rate of enzyme inactivation by H_2O_2 is relatively slow, 6.7 $M^{-1}\cdot sec^{-1}$ at pH 10.0 and 25°C (8). This rate is expected to be even slower at physiological pH because the affinity of SOD for H_2O_2 decreases with pH (44). The low rate of inactivation, in part, is due to the low affinity for H_2O_2, which is in accord with the observation that formation of DMPO–OH adduct in a solution containing both $O_2^{\cdot-}$ and SOD is inhibited by catalase (see Fig. 3). In that case, the majority of the H_2O_2 generated in the dismutation reaction of $O_2^{\cdot-}$ was released into the bulk solution and removed by catalase. Without catalase, the H_2O_2 could be converted to ·OH

radicals in a reaction catalyzed by SOD. As described in *Results*, the amount of ·OH radicals released within 6 min from the active site is at least severalfold higher than the concentration of the enzyme. Therefore, it is essential for living cells to possess a proper distribution of catalase, peroxidase, and other peroxide-scavenging systems so that H_2O_2 can be removed in the manner of coupled reactions. When this balance is perturbed, such as by elevation of SOD activity, it may cause serious physiological problems that may outweigh its beneficial effects.

The finding that SOD is capable of catalyzing "free" ·OH radical formation may in part explain the following observations: (*i*) Elevated intracellular SOD activity, mostly Fe-SOD, in *Escherichia coli* enhances bacterial sensitivity to O_2^- and radiation-induced mortality, whereas SOD-deficient mutants show normal or decreased sensitivity (14, 15). (*ii*) Cu,Zn-SOD activity in rat PC12 cells (16), human HeLa cells (17), and mouse-cell clones expressing elevated human Cu,Zn-SOD gene (17) caused a large degree of lipid peroxidation, and also the expression of a human Cu,Zn-SOD gene in PC12 cells interferes with transport of biogenic amines into chromaffin granules (16). (*iii*) Erythrocytes of favism patients with acute hemolytic crisis have markedly more SOD and less glutathione peroxidase (18). (*iv*) Down syndrome patients possess an extra copy of chromosome 21, which contains the Cu,Zn-SOD gene (19). Excessive oxidative damage of biomacromolecules has also been reported in these patients (16, 17, 20, 21). In addition, this observation indicates that it is unnecessary to invoke exogenous metal ions for the production of "free" ·OH radicals from the dismutation product H_2O_2 to account for the biological observations.

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