This study addresses the mechanism of covalent aggregation of human Cu,Zn-superoxide dismutase (hSOD1WT) induced by bicarbonate (HCO3-) mediated peroxidase activity. Higher molecular weight species (apparent dimers and trimers) of hSOD1WT were formed from incubation mixtures containing hSOD1WT, H2O2, and HCO3-. HCO3- dependent peroxidase activity and covalent aggregation of hSOD1WT were mimicked by UV photolysis of hSOD1WT in the presence of a [Co(NH3)6]3+ complex that generates the carbonate radical anion (CO3-). Human SOD1WT has but one aromatic residue, a tryptophan residue (Trp-32) on the surface of the protein. Substitution of Trp-32 with phenylalanine produced a mutant (hSOD1WT32F) that exhibits HCO3- dependent peroxidase activity similar to wild-type enzyme. However, unlike hSOD1WT, incubations containing hSOD1WT32F, H2O2, and HCO3- did not result in covalent aggregation of SOD1. These findings indicate that Trp-32 is crucial for CO3- induced covalent aggregation of hSOD1WT. Spin-trapping results revealed the formation of the Trp-32 radical from hSOD1 WT, but not from hSOD1WT32F. Spin traps also inhibited the covalent aggregation of hSOD1WT. Fluorescence experiments revealed that Trp-32 was further oxidized by CO3- forming kynurenine-type products in the presence of oxygen. Molecular oxygen was needed for HCO3/-H2O2-dependent aggregation of hSOD1WT, implicating a role for a Trp-32-dependent peroxidative reaction in the covalent aggregation of hSOD1WT. Taken together, these results indicate that Trp-32 oxidation is crucial for covalent aggregation of hSOD1. Implications of HCO3- dependent SOD1 peroxidase activity in amyotrophic lateral sclerosis disease are discussed.

In a pair of publications, Hodgson and Fridovich demonstrated that bovine Cu,Zn-superoxide dismutase (Cu,Zn-SOD or SOD1) exhibits a nonspecific peroxidase activity (1, 2). They provided evidence for formation of a potent oxidant in the presence of H2O2 that was ascribed to a copper-bound hydroxyl radical, at the active site of bovine SOD1. Although the rate constant for the reaction between SOD1 and H2O2 is very low (3), it was shown that the bovine SOD1 peroxidase activity could oxidize a variety of small molecular weight anionic ligands (azide, nitrite, formate, etc.) that are accessible to the active site (1, 2, 4, 5).

However, a perplexing aspect of this peroxidase activity was that even larger molecules (e.g. 2,2'-azino-bis-[3-ethylbenzothiazoline]-6-sulfonic acid, urate) that are not accessible to the active site of SOD1 were also oxidized (6). It was later discovered that the bicarbonate anion (HCO3-) that is present in the buffer was responsible for Cu,Zn-SOD peroxidase-dependent oxidations of substrates in the bulk solution (7, 8). It was proposed that the copper-bound oxidant (Cu2+-OH or CuII-OH) could oxidize the HCO3- anion (a physiologically relevant molecule) to the carbonate radical anion (CO32-), a potent oxidant that diffuses out of the active site and causes substrate oxidation (7, 8). The fact that CO32- and not hydroxyl radical, is the primary oxidant produced by SOD1-mediated peroxidase activity is physiologically significant, because CO32- has a much longer half-life (than hydroxyl radical) and can, therefore, diffuse away from the active site and oxidatively modify critical cellular targets.

Liochev et al. (7) suggested that the nitrene spin trap, 5,5'-dimethyl-1-pyrroline-N-oxide (DMPO), is oxidized and hydrolyzed by CO32-. Zhang et al. (6) provided the experimental proof for this hypothesis. Using electron spin resonance (ESR) spin trapping in the presence of oxygen-17-labeled water, the investigators showed that the oxygen atom in the DMPO-OH adduct is derived totally from water. Zhang et al. (6) also reported that HCO3- dependent peroxidase activity can be measured using a variety of methods including fluorescence and optical spectroscopy. More recently, Hink et al. (9) demonstrated that an extracellular SOD (SOD3 or eSOD) enhanced the hydroxylation of a cyclic nitrite spin trap in the presence of H2O2 and HCO3-.

In this work, we report that human Cu,Zn-superoxide dismutase (hSOD1WT) also exhibits a HCO3- dependent peroxi-
Fig. 1. Hydroxylation and oxidation of nitroprone spin traps by bicarbonate-dependent SOD1 peroxidase activity. A, SOD1 (1 mg/ml) was mixed with H₂O₂ (2 mM), HCO₃⁻ (25 mM) and various spin traps (25 mM) in a phosphate buffer (100 mM, pH 7.4) containing DTPA (0.1 mM). Similar incubations were carried out in the presence of Me₂SO (5% final concentration). Spectra were recorded within 5 min after the addition of H₂O₂. B, incubation mixtures containing FeSO₄ (200 μM), H₂O₂ (1 mM), DTPA (0.1 mM), and various spin traps (25 mM) in a phosphate buffer (100 mM, pH 7.4) in the presence and absence of Me₂SO (5% final concentration). Spectral simulations are shown by the dotted line using the ESR parameters shown in Table I.
dase activity involving the CO$_3^-$ intermediate. However, unlike bovine SOD1, hSOD1$_{WT}$-mediated HCO$_3^-$-dependent peroxi-
dase activity results in protein covalent aggregation. Results from this study show that CO$_3^-$ induces oxidation of a trypto-
phan residue located on the surface of hSOD1$_{WT}$ that leads to
intersubunit covalent bond formation and subsequent aggrega-
tion of the protein. Implications of HCO$_3^-$-dependent hSOD1$_{WT}$
peroxidase activity in the covalent aggregation of SOD1 asso-
ciated with amyotrophic lateral sclerosis (ALS) are discussed.

EXPERIMENTAL PROCEDURES

Bovine SOD1 was obtained from Roche Diagnostics. Pentammine
carbonato complex of Co(III) was synthesized according to the published
procedure (10). Briefly, 30 g of Co(NO$_3$)$_3$·6H$_2$O in 15 ml of water
was added to 45 g of ammonium carbonate dissolved in 45 ml of water,
followed by the addition of 75 ml of concentrated ammonium. Air was
bubbled through the solution for 24 h. The resulting solution was cooled in
an ice bath, and the solid product was recrystallized by dissolving it
in 55 ml of water at 90°C and then slowly cooling the solution in an ice
bath. Pure crystals were isolated and used in the experiments. Chem-
icals including glycine, SDS, ammonium persulfate, Coomassie G250
stain, and 40% acrylamide/bis were obtained from Bio-Rad. Sodium bicarbonate, hydrogen peroxide, tryptophan, tyrosine, histidine, pheny-
lalanine, and trizma base were purchased from Sigma. DMPO was also
obtained from Sigma, and the colored impurity was removed by treat-
ment with activated charcoal (11). POBN, α-phenyl-tert-butyl-N-ni-
trone, and DBNBS were obtained from Sigma and used as received.
EMPO was synthesized according to a previous report (12). Azuleny1
nitrone was a gift from Dr. David Baker (Florida International Univer-
sity, Miami, FL). DEPMPO was obtained from Dr. Paul Tordo (Univer-
site de Provence, Marseille, France).

Expression and Purification of SOD1—The wild-type human and
W32F mutant Cu/Zn-SOD (Cu$^{2+}$, 97%; Zn$^{2+}$, 108%) were expressed and
purified as described previously (13). The SOD1 mutations were created
by a two-cycle, polymerase chain reaction-based mutagenesis protocol
described elsewhere (13). SOD1 activity was determined by the cyto-
dered with activated charcoal (11). POBN, α-phenyl-tert-butyl-N-nitro-
ne, and DBNBS were obtained from Sigma and used as received.
EMPO was synthesized according to a previous report (12). Azuleny1
nitrone was a gift from Dr. David Baker (Florida International Univer-
sity, Miami, FL). DEPMPO was obtained from Dr. Paul Tordo (Univer-
site de Provence, Marseille, France).

Expression and Purification of SOD1—The wild-type human and
W32F mutant Cu/Zn-SOD (Cu$^{2+}$, 97%; Zn$^{2+}$, 108%) were expressed and
purified as described previously (13). The SOD1 mutations were created
by a two-cycle, polymerase chain reaction-based mutagenesis protocol
described elsewhere (13). SOD1 activity was determined by the cyto-
dered with activated charcoal (11). POBN, α-phenyl-tert-butyl-N-nitro-
ne, and DBNBS were obtained from Sigma and used as received.
EMPO was synthesized according to a previous report (12). Azuleny1
nitrone was a gift from Dr. David Baker (Florida International Univer-
sity, Miami, FL). DEPMPO was obtained from Dr. Paul Tordo (Univer-
site de Provence, Marseille, France).

Expression and Purification of SOD1—The wild-type human and
W32F mutant Cu/Zn-SOD (Cu$^{2+}$, 97%; Zn$^{2+}$, 108%) were expressed and
purified as described previously (13). The SOD1 mutations were created
by a two-cycle, polymerase chain reaction-based mutagenesis protocol
described elsewhere (13). SOD1 activity was determined by the cyto-
dered with activated charcoal (11). POBN, α-phenyl-tert-butyl-N-nitro-
ne, and DBNBS were obtained from Sigma and used as received.
EMPO was synthesized according to a previous report (12). Azuleny1
nitrone was a gift from Dr. David Baker (Florida International Univer-
sity, Miami, FL). DEPMPO was obtained from Dr. Paul Tordo (Univer-
site de Provence, Marseille, France).

Expression and Purification of SOD1—The wild-type human and
W32F mutant Cu/Zn-SOD (Cu$^{2+}$, 97%; Zn$^{2+}$, 108%) were expressed and
purified as described previously (13). The SOD1 mutations were created
by a two-cycle, polymerase chain reaction-based mutagenesis protocol
described elsewhere (13). SOD1 activity was determined by the cyto-
dered with activated charcoal (11). POBN, α-phenyl-tert-butyl-N-nitro-
ne, and DBNBS were obtained from Sigma and used as received.
EMPO was synthesized according to a previous report (12). Azuleny1
nitrone was a gift from Dr. David Baker (Florida International Univer-
sity, Miami, FL). DEPMPO was obtained from Dr. Paul Tordo (Univer-
site de Provence, Marseille, France).

Expression and Purification of SOD1—The wild-type human and
W32F mutant Cu/Zn-SOD (Cu$^{2+}$, 97%; Zn$^{2+}$, 108%) were expressed and
purified as described previously (13). The SOD1 mutations were created
by a two-cycle, polymerase chain reaction-based mutagenesis protocol
described elsewhere (13). SOD1 activity was determined by the cyto-
dered with activated charcoal (11). POBN, α-phenyl-tert-butyl-N-nitro-
ne, and DBNBS were obtained from Sigma and used as received.
EMPO was synthesized according to a previous report (12). Azuleny1
nitrone was a gift from Dr. David Baker (Florida International Univer-
sity, Miami, FL). DEPMPO was obtained from Dr. Paul Tordo (Univer-
site de Provence, Marseille, France).

Expression and Purification of SOD1—The wild-type human and
W32F mutant Cu/Zn-SOD (Cu$^{2+}$, 97%; Zn$^{2+}$, 108%) were expressed and
purified as described previously (13). The SOD1 mutations were created
by a two-cycle, polymerase chain reaction-based mutagenesis protocol
described elsewhere (13). SOD1 activity was determined by the cyto-
dered with activated charcoal (11). POBN, α-phenyl-tert-butyl-N-nitro-
ne, and DBNBS were obtained from Sigma and used as received.
EMPO was synthesized according to a previous report (12). Azuleny1
nitrone was a gift from Dr. David Baker (Florida International Univer-
sity, Miami, FL). DEPMPO was obtained from Dr. Paul Tordo (Univer-
site de Provence, Marseille, France).

Expression and Purification of SOD1—The wild-type human and
W32F mutant Cu/Zn-SOD (Cu$^{2+}$, 97%; Zn$^{2+}$, 108%) were expressed and
purified as described previously (13). The SOD1 mutations were created
by a two-cycle, polymerase chain reaction-based mutagenesis protocol
described elsewhere (13). SOD1 activity was determined by the cyto-
dered with activated charcoal (11). POBN, α-phenyl-tert-butyl-N-nitro-
ne, and DBNBS were obtained from Sigma and used as received.
EMPO was synthesized according to a previous report (12). Azuleny1
nitrone was a gift from Dr. David Baker (Florida International Univer-
sity, Miami, FL). DEPMPO was obtained from Dr. Paul Tordo (Univer-
site de Provence, Marseille, France).

TABLE I

| Spin adduct  | $\alpha_g$ | $\alpha_{10}$ | Other couplings |
|--------------|----------|--------------|----------------|
| DMPO-OH      | 14.9     | 14.9         |               |
| DMPO-CH$_3$  | 16.4     | 23.4         |               |
| EMPO-OH (23%)| 14.1     | 15.2         | 0.85($\alpha_{10}$) |
| EMPO-OH (77%)| 14.1     | 12.8         |               |
| EMPO-CH$_3$  | 15.4     | 22.3         |               |
| PBN-OH       | 15.5     | 2.7          |               |
| MNP-H        | 14.6     | 14.9         |               |
| PBN-CH$_3$   | 15.0     | 3.3          |               |
| PBN-CO$_3$   | 15.9     | 4.6          |               |
| POBN-OH      | 15.0     | 1.7          |               |
| POBN-CH$_3$  | 15.9     | 2.7          |               |
| POBN-CO$_3$  | 15.6     | 3.45         |               |
| DEPMPO-OH    | 14.1     | 13.2         | 47.3($\alpha_g$) |
| DEPMPO-CH$_3$| 15.2     | 22.3         | 47.7($\alpha_g$) |
| DBNBS-Trp    | 13.6     | 0.46($\alpha_g$) | 0.64, 0.76, 0.64($\alpha_{10}$) |
|              |          |              | 0.94($\alpha_{15}$) |

$^{2}$ C. Karunakaran, H. Zhang, J. P. Crow, J. S. Beckman, W. Antho-
line, and B. Kalyanaraman, unpublished data.
Spectrometer conditions were as follows: modulation amplitude, 1 G value time constant, 0.064 s; scan time, 2 min; and microwave power, 10 milliwatts. Spectral simulations were performed using the WINSIM program (NIEHS, National Institutes of Health, Research Triangle Park, NC).

**Fluorescence Measurements**—Fluorescence experiments were performed on a Shimadzu RF-5301 PC spectrofluorometer (Shimadzu Scientific Instruments Inc.). Spectra were obtained at the indicated excitation and emission wavelengths using 3–5-mm and 10–20-mm slit widths, respectively.

**RESULTS**

**Bicarbonate-induced Hydroxylation and Oxidation of Nitronate Spin Traps in the Presence of SOD1/H2O2: Intermediacy of CO3 Radical Anion and Not Hydroxyl Radical**—Previously, we reported that HCO3/H2O2 dramatically enhances hydroxylation and oxidation of the spin trap DMPO to the DMPO-hydroxyl adduct, DMPO-OH (6). Using oxygen-17-labeled water, we showed that the oxygen atom in the DMPO-OH adduct formed in the SOD1WT/H2O2/HCO3/H2O2 system is derived from water and not from H2O2 (6). We have now extended this ESR analysis to include other cyclic nitrone traps (EMPO and DEPMPO) and open-chain nitrones (e.g., POBN).

The addition of H2O2 (0.1–1.0 mM) to an incubation mixture containing SOD1 (bovine or recombinant human Cu,Zn-SOD; 31.7 μM), spin traps (25 mM of DMPO, EMPO, DEPMPO, or POBN), HCO3 (25 mM), and DTPA (0.1 mM) in a phosphate buffer (100 mM, pH 7.4) yielded the respective ESR spectra of the corresponding hydroxylated adduct (Fig. 1A). The computer-simulated ESR spectra (shown with dotted lines in Fig. 1A) were obtained using the ESR parameters (Table I). The addition of MeSO, a frequently used hydroxyl radical scavenger, had no effect on the ESR signal intensity of the hydroxylated adducts (Fig. 1A). In contrast, when MeSO was added to a mixture containing Fe2+ and hydrogen peroxide (the Fenton system), the ESR spectrum of the hydroxylated adduct was replaced by a methyl radical adduct (Fig. 1B). From these results, it can be concluded categorically that free hydroxyl radicals are not generated in the SOD1/H2O2/HCO3/H2O2 system and that ESR can be used to monitor the SOD1/H2O2/HCO3/H2O2-dependent peroxidase activity. These results indicate that MeSO can be used to differentiate between CO3 and OH formation. Other scavengers such as ethanol, azide, and formate react with both the hydroxyl radical and the carbonate radical anion and therefore cannot be used to distinguish formation of these species by ESR (Table II).

In contrast to the cyclic nitrone traps, which form persistent hydroxylated adducts, the hydroxylated adduct of the open-chain nitrone, POBN, decomposed to form a secondary radical adduct. Fig. 2 shows the ESR spectra obtained from mixtures containing human SOD1, POBN, H2O2, DTPA, and different concentrations of HCO3 in a phosphate buffer. The spectral intensity increased with increasing HCO3 concentrations. The ESR spectra consisted of two adducts corresponding to POBN-OH and the N-tert-butyl hydronitroxide, MNP-H. We propose that POBN-OH decomposed to the aldehyde and MNP-hydronitroxide, which was further oxidized by CO3 to the MNP-hydronitroxide. A similar type of radical chemistry has previously been reported for the hydroxyl adduct of α-phenyl-tert-butyl-nitroxide (16).

As reported earlier, the proposed mechanism of hydroxylation of nitrones includes a nucleophilic addition of water to
Fig. 4. The effect of spin traps on the aggregation of human Cu,Zn-SOD. A, hSOD1WT (1 mg/ml) was incubated with H₂O₂ (1 mM), HCO₃⁻ (25 mM) and various spin traps in a phosphate buffer (100 mM, pH 7.4) containing DTPA (0.1 mM) at room temperature for 45 min. Aggregation was monitored by a reducing 10% SDS-PAGE. B, densitometric analysis of SDS-PAGE gel. C, hSOD1WT (1 mg/ml) was mixed with a cobalt complex (4 mM) in the presence of spin traps, as indicated, in a phosphate buffer (100 mM, pH 7.4) containing DTPA (0.1 mM) and irradiated with UV light for 30 s. Aggregation was measured by SDS-PAGE. D, hSOD1WT (1 mg/ml) incubated with H₂O₂ (1 mM), HCO₃⁻ (25 mM), and Me₂SO (5%) in a phosphate buffer (100 mM, pH 7.4) containing DTPA (0.1 mM) at room temperature for 45 min, and aggregation was measured by SDS-PAGE.

Fig. 5. The effect of amino acids on the formation of human SOD aggregation and DMPO hydroxylation induced by a carbonate radical. A, hSOD1WT (1 mg/ml) was incubated with H₂O₂ (1 mM), HCO₃⁻ (25 mM) and various amino acids (1 mM) in a phosphate buffer (100 mM, pH 7.4) containing DTPA (0.1 mM) at room temperature for 45 min and analyzed by SDS-PAGE. B, hSOD1WT (1 mg/ml) was mixed with the cobalt complex (4 mM) and various amino acids in a phosphate buffer (100 mM, pH 7.4) containing DTPA (0.1 mM), irradiated with UV light for 30 s, and analyzed by SDS-PAGE. C, hSOD1WT (1 mg/ml) was incubated with H₂O₂ (1 mM), HCO₃⁻ (25 mM), DMPO (25 mM), and various amino acids (1 mM) in a phosphate buffer (100 mM, pH 7.4) containing DTPA (0.1 mM). The ESR spectra were recorded within 5 min. D, hSOD1WT (1 mg/ml) was mixed with a pentamine carbonate complex of Co(III) (4 mM), DMPO (25 mM), and various amino acids in a phosphate buffer (100 mM, pH 7.4) containing DTPA (0.1 mM) and irradiated with UV light for 10 s, and the ESR spectra were recorded within 5 min.
either the nitrone-carbonate radical adduct or to the radical cation intermediate (7). Independent evidence for the intermediacy of CO$_3^{2-}$ was obtained from photolysis studies using the pentamine carbonato complex of cobalt(III) (7). UV photolysis of this cobalt complex has been shown to release CO$_3^{2-}$ (17).

Irradiation of the pentammine carbonato complex of Co(III) in the presence of nitrone traps yielded ESR spectra of radical adducts that were similar to those obtained from the SOD1/H$_2$O$_2$/HCO$_3^-$/H$_2$O$_2$ system (not shown). Direct ESR detection of the CO$_3^{2-}$ radical formed from mixing concentrated solutions of ONOO$^-$ and HCO$_3^-$ was achieved using a rapid mixing technique, as reported earlier (18).

**Figure 6.** Spin-trapping of the SOD-derived radical formed from bicarbonate-dependent peroxidase activity. A (top), hSOD1$^{WT}$ (3 mg/ml) was mixed with H$_2$O$_2$ (1 mM), HCO$_3^-$ (25 mM), and spin trap DBNBS (10 mM) in a phosphate buffer (100 mM, pH 7.4) containing DTPA (0.1 mM). The mixture was transferred to a capillary tube (100 µl), and the ESR spectrum was recorded immediately. Middle, same as top but in the absence of bicarbonate. Bottom, same as top but using bovine SOD1. B (top), the sample from A was ultrafiltrated (10-kDa cutoff), and the ESR spectrum was recorded. Middle, ultrafiltrated sample was treated with Pronase (2 mg/ml) for 1 h, and the ESR spectrum was recorded. Bottom, the central line of the nitroxide adduct (middle) was expanded using a modulation amplitude of 0.1 G. C (top), bovine SOD1 (1 mg/ml) was mixed with H$_2$O$_2$ (1 mM), HCO$_3^-$ (25 mM), tryptophan (1 mM), and DBNBS (10 mM) in a phosphate buffer (100 mM, pH 7.4) containing DTPA (0.1 mM). The mixture was transferred to a capillary tube (100 µl), and the ESR spectrum was recorded immediately. Middle, same as above but in the absence of bicarbonate. Bottom, the central line of the DBNBS-Trp adduct formed from C (top) was expanded using a lower modulation amplitude. D, hSOD1$^{WT}$ (1 mg/ml) was incubated with H$_2$O$_2$ (1 mM) and HCO$_3^-$ (25 mM) in a phosphate buffer (100 mM, pH 7.4) containing DTPA (0.1 mM) in the presence and absence of DBNBS (10 mM) for 45 min and analyzed by SDS-PAGE.

**Figure 7.** Absorption and fluorescence spectra of wild-type and mutant human SOD. A, the ribbon diagram model for hSOD1$^{WT}$ generated from the Protein Data Bank. B, the ribbon diagram model of hSOD1$^{W32F}$ generated from the Protein Data Bank. C, the optical absorption spectra of hSOD1$^{WT}$ (2.5 mg/ml) and hSOD1$^{W32F}$ (2.5 mg/ml) in a phosphate buffer (50 mM, pH 7.4) containing DTPA (0.1 mM). D, the fluorescence spectra of hSOD1$^{WT}$ (2.5 mg/ml) and hSOD1$^{W32F}$ (2.5 mg/ml) in phosphate buffer (50 mM, pH 7.4).
Bicarbonate Stimulates SOD1 Peroxidase Activity

We then showed that UV photolysis of a cobalt complex that generates authentic CO$_3^-$ radicals in the presence of hSOD1$^{WT}$ caused its cross-linking (Fig. 3E). There was no apparent dimer formation cross-linking in the dark from incubations containing hSOD1$^{WT}$ and the cobalt complex (Fig. 3E). These results provide additional evidence for CO$_3^-$-induced aggregation and covalent cross-linking of hSOD1$^{WT}$.

Inhibition of hSOD1 Aggregation by Nitrone Spin Traps—
The addition of the nitrone spin traps DMPO (25 mM), α-phenyl-tert-butyl-N-nitroso (25 mM), and azulenyl nitrone (4 mM) to an incubation containing hSOD$^{WT}$, H$_2$O$_2$, DTPA, and HCO$_3^-$ in a phosphate buffer blocked apparent dimer formation, as shown in Fig. 4A. Fig. 4B shows the densitometric analysis of the dimer band obtained in the presence of spin traps. Nitrone spin traps also inhibited UV/cobalt complex-induced covalent dimerization of hSOD1$^{WT}$ (Fig. 4C). Me$_2$SO, a well known trap for hydroxyl radicals, had no effect on hSOD1$^{WT}$ dimer formation (Fig. 4D). These findings are consistent with the notion that trapping of CO$_3^-$ by nitrone inhibits hSOD1$^{WT}$ covalent dimerization.

The Effect of Amino Acids on Aggregation of SOD1 in a Human SOD$^{WT}$/H$_2$O$_2$/HCO$_3^-$ System—As shown earlier, evidence for enhanced formation of the hSOD1$^{WT}$ covalent dimer was noticeable in incubations containing hSOD1$^{WT}$, H$_2$O$_2$, and HCO$_3^-$ (Fig. 5A, lane p). Inclusion of glycine or phenylalanine had no effect on hSOD1$^{WT}$ covalent dimer formation induced in the hSOD1$^{WT}$/H$_2$O$_2$/HCO$_3^-$ system. Both tryptophan and tyrosine totally inhibited covalent dimer formation (Fig. 5A). Similar results were obtained during cobalt complex-photosensitized hSOD1$^{WT}$ covalent aggregation (Fig. 5C). These results suggest that tryptophan and tyrosine scavenged the oxidant (i.e. CO$_3^-$) responsible for hSOD1$^{WT}$ covalent aggregation. We used DMPO to measure the HCO$_3^-$-dependent hSOD1$^{WT}$ peroxidase activity. The DMPO-OH signal (Fig. 5B) was markedly inhibited in the presence of tryptophan and tyrosine (1 mM). At these concentrations, this signal intensity was not affected by phenylalanine and glycine. These data are consistent with the rapid scavenging of the carbonate anion radical by tryptophan and tyrosine (19). From these results, we conclude that either a tryptophanyl or tyrosyl residue present in hSOD1$^{WT}$ is the proximal site of interaction with CO$_3^-$ generated during HCO$_3^-$-dependent hSOD1$^{WT}$ peroxidase activity.

Spin Trapping of the Bicarbonate-mediated Protein Radical Formed in the Human SOD1/H$_2$O$_2$/System—An ESR spectrum that is characteristic of a strongly immobilized nitroxide adduct was detected from incubations containing hSOD1$^{WT}$, H$_2$O$_2$, HCO$_3^-$, and DBNBS trap (Fig. 6A, top). When the HCO$_3^-$ anion was excluded, no spectrum was obtained (Fig. 6A, middle). Under the same experimental conditions, bovine SOD1 did not form a similar nitroxide adduct (Fig. 6A, bottom). Following ultrafiltration, treatment of the nitroxide adduct (Fig. 6B, top) with the Pronase enzyme that cleaved the high molecular weight nitroxide yielded an isotropic three-line ESR spectrum (Fig. 6B, middle) with a hyperfine coupling constant of 13.6 G. Upon expansion of the center line of the ESR spectrum (Fig. 6B, middle), superhyperfine couplings were resolved (Fig. 6B, bottom). This spectrum was simulated (dotted line in Fig. 6B, bottom) using the following parameters ($\alpha_{\mathrm{N}}$, 0.37 G; $\alpha_{\mathrm{HH}}$, 0.13 G; $\alpha_{\mathrm{HG}}$, 0.95 G; $\alpha_{\mathrm{TH}}$, 0.58 G; $\alpha_{\mathrm{Hm}}$ (2), 0.92 G). In the presence of tryptophan, the incubation mixture containing bovine SOD1, H$_2$O$_2$, HCO$_3^-$, and DBNBS yielded an intense isotropic three-line ESR spectrum ($\alpha_{\mathrm{N}}$ = 13.6 G) (Fig. 6C, top). Without HCO$_3^-$, no ESR spectrum was obtained (Fig. 6C, middle). Upon expansion of the center line of the ESR spectrum (Fig. 6C, top) using a lower modulation amplitude, superhyperfine couplings could be resolved.

reproduced this result in our laboratory and observed that the half-life of this species is extremely short (~6 ms) at physiological pH values (not shown). However, using this rapid mixing technique, we could not detect the carbonate radical anion generated during HCO$_3^-$ by nitrones inhibiting hSOD1$^{WT}$ covalent aggregation (Fig. 5C). These results suggest that the subunits are covalently cross-linked via a non-disulfide type linkage. Formation of covalently linked higher molecular weight species (equivalent to dimers of 18-kDa SOD1 subunits) was initially noticeable after 15 min, and with prolonged incubation (60–120 min), fragmentation occurred. Fig. 3, C and D, depicts covalent cross-linking of hSOD1$^{WT}$ as a function of increasing H$_2$O$_2$ and hSOD1-WT concentrations.

![Fig. 8: Spin trapping of radicals formed from bicarbonate-dependent peroxidase activity of human SOD1$^{WT}$ and SOD1$^{W32F}$](image)

**Bicarbonate Enhances the Aggregation of Human SOD1$^{WT}$ in the Presence of H$_2$O$_2$**—The addition of hSOD1$^{WT}$ to solutions containing H$_2$O$_2$ (1 mM) and DTPA (0.1 mM) in a phosphate buffer (100 mM, pH 7.4) caused a concentration-dependent hSOD1$^{WT}$ peroxidase activity. The DMPO-OH signal (Fig. 5B) was markedly inhibited in the presence of tryptophan and tyrosine (1 mM). At these concentrations, this signal intensity was not affected by phenylalanine and glycine. These data are consistent with the rapid scavenging of the carbonate anion radical by tryptophan and tyrosine (19). From these results, we conclude that either a tryptophanyl or tyrosyl residue present in hSOD1$^{WT}$ is the proximal site of interaction with CO$_3^-$ generated during HCO$_3^-$-dependent hSOD1$^{WT}$ peroxidase activity.
This spectrum was simulated using contributions from a nitrogen atom, three nonequivalent protons, and the two meta-protons present in DBNBS, as reported previously (20) (dotted line in Fig. 6C, bottom) (Table I). The structure of the adduct was assigned to the DBNBS-tryptophanyl adduct (DBNBS-Trp). Based on these results, the immobilized nitroxide spectrum (Fig. 6A, top) is attributed to trapping of a radical formed from the tryptophan residue in hSOD1 WT. Concomitant with the trapping of the radical formed from the tryptophan residue, DBNBS markedly diminished HCO₃⁻/H₂O₂-mediated peroxidase-dependent covalent dimer formation from hSOD1 WT (Fig. 6D).

The Effect of Substituting Phenylalanine for Tryptophan on Bicarbonate-mediated Hydroxylation and Oxidation Reactions in the hSOD1³²F/H₂O₂ System—The wild-type human SOD1 has a single tryptophan residue (Trp-32) located on the surface of the protein, as shown in the ribbon diagram model (Fig. 7A). To assess the role of tryptophan in hSOD1 WT/H₂O₂/HCO₃⁻-mediated oxidation and aggregation reactions, a site-directed mutant of hSOD1, containing Phe in place of Trp-32, was produced (Fig. 7B). The fluorescence spectra of hSOD1 WT and hSOD1³²F confirmed the loss of tryptophan fluorescence upon substitution with phenylalanine (Fig. 7, dotted line). The copper ESR spectra of hSOD1 WT and hSOD1³²F were identical, demonstrating no change at the active copper site (not shown).

The next step was to compare the ESR spectra of the DBNBS adducts obtained from incubation mixtures containing either hSOD1 WT or hSOD1³²F, DBNBS trap, H₂O₂, and HCO₃⁻ in a phosphate buffer (100 mM, pH 7.4) containing DTPA. An intense ESR spectrum of a protein-derived radical was detected from the hSOD1 WT (Fig. 8A). No immobilized ESR spectrum was detected from the hSOD1³²F/H₂O₂/HCO₃⁻-mediated reaction (Fig. 8C). The HCO₃⁻-dependent peroxidase activity of hSOD1 WT and hSOD1³²F was monitored by DMPO hydroxylation to DMPO-OH (Fig. 8, B and D). The lack of Trp-32 in hSOD1³²F actually enhanced hSOD1³²F/H₂O₂/HCO₃⁻/DMPO-dependent DMPO hydroxylation by hSOD1 WT/H₂O₂ and HCO₃⁻. This was further verified by using free tryptophan as a substrate for SOD1/bicarbonate-mediated peroxidase activity. The spin trap DBNBS was used to trap the tryptophan-derived carbon-centered radical. The ESR spectra of DBNBS adducts obtained from incubations containing hSOD1 WT or hSOD1³²F, DBNBS, H₂O₂, and HCO₃⁻ in a phosphate buffer containing DTPA are shown in Fig. 8, A and C. In the presence of 50 μM
free tryptophan, an ESR spectrum due to the DBNBS-Trp adduct was detected from hSOD1\textsuperscript{WT}/H\textsubscript{2}O\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{−}/DBNBS system (not shown), and at 1 mM free tryptophan, the intensity of the spectrum was higher (not shown). At a lower concentration of free Trp, spectra from both DBNBS-hSOD1\textsuperscript{WT} and DBNBS-Trp were detected; however, at 1 mM free Trp, the ESR spectrum was solely due to the DBNBS-Trp adduct and was almost the same as that observed from hSOD1\textsuperscript{W32F} (not shown). These results unequivocally demonstrate that hSOD1\textsuperscript{WT} and hSOD1\textsuperscript{W32F} exhibit the same extent of bicarbonate-mediated peroxidase activity, and the lack of ESR spectrum from hSOD1\textsuperscript{W32F} suggests that CO\textsubscript{3}\textsuperscript{−} reacts with the tryptophan residue to form a tryptophan-derived carbon-centered radical that was trapped by DBNBS.

It was then of interest to find out whether substituting Trp-32 with Phe-32 has any effect on the aggregation and covalent cross-linking of the protein. We compared the covalent aggregations of hSOD1\textsuperscript{WT} and hSOD1\textsuperscript{W32F} in the presence of H\textsubscript{2}O\textsubscript{2} and HCO\textsubscript{3}\textsuperscript{−}. Fig. 8\textit{E} shows that covalent dimerization of hSOD1\textsuperscript{W32F} did not occur under the same experimental conditions (Fig. 8A) that resulted in covalent dimerization of hSOD1\textsuperscript{WT}. Clearly, the Trp residue at position 32 plays a crucial role in the oxidation, aggregation, and covalent cross-linking of hSOD1\textsuperscript{WT} caused by HCO\textsubscript{3}\textsuperscript{−}-mediated peroxidase activity.

The Effect of Molecular Oxygen on the Fluorescence Spectra of Tryptophan-derived Oxidation Products—The UV-visible and fluorescence spectral changes in the oxidation products of tryptophan in a bovine SOD1/H\textsubscript{2}O\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{−} system are shown in Fig. 9. Bicarbonate accelerated the rate of oxidation of tryptophan in the presence of SOD1 and H\textsubscript{2}O\textsubscript{2} (Fig. 9, A (middle) and B (left)). Similar spectral changes due to oxidation of Trp-32 residue were observed in the hSOD1\textsuperscript{WT}/H\textsubscript{2}O\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{−} system (Fig. 9, A (right) and B (middle)). Fluorescence spectra (Fig. 9B, right) revealed at least three different products that are formed during HCO\textsubscript{3}\textsuperscript{−}/H\textsubscript{2}O\textsubscript{2}-dependent oxidation of Trp-32 in hSOD1\textsuperscript{WT}. The fluorescent intensities of two products (Fig. 10, A and B) were monitored under aerobic and anerobic conditions in the hSOD1\textsuperscript{WT}/H\textsubscript{2}O\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{−} system. Product formation was considerably enhanced in air. Bicarbonate/H\textsubscript{2}O\textsubscript{2}-dependent dimerization of hSOD1\textsuperscript{WT} was enhanced in air and diminished in N\textsubscript{2} (Fig. 10C). These results indicate that molecular oxygen enhances HCO\textsubscript{3}\textsuperscript{−}/H\textsubscript{2}O\textsubscript{2}-dependent aggregation and covalent cross-linking of hSOD1\textsuperscript{WT} via formation of the tryptophanyl carbon-centered and peroxyl radicals (see Scheme 1).

**DISCUSSION**

This study revealed that HCO\textsubscript{3}\textsuperscript{−} augments human SOD1\textsuperscript{WT} peroxidase activity through formation of CO\textsubscript{2}\textsuperscript{−}, a potent one-electron oxidant, at the active site of the enzyme. Indeed, secondary formation of CO\textsubscript{2}\textsuperscript{−} provides biological relevance to the peroxidase activity via the formation of a strong oxidant that can diffuse away from the active site, unlike the hydroxyl radical. Because of its selective reactivity, CO\textsubscript{2}\textsuperscript{−} diffuses out of the active site and oxidizes numerous substrates in the bulk solution. The rapid reaction between CO\textsubscript{2}\textsuperscript{−} and the tryptophan residue (Trp-32) located on the surface of the enzyme leads to a covalent aggregation that is nondisulfide in nature (i.e. dithiothreitol-resistant) and, therefore, more irreversible under biological conditions. The ESR spin trapping provided the spectroscopic proof for the formation of a tryptophan-derived radical. The site-directed mutation of Trp-32 by Phe-32 completely prevented HCO\textsubscript{3}\textsuperscript{−}/hSOD1\textsuperscript{WT} peroxidase-induced aggregation and covalent cross-linking, demonstrating that Trp-32 oxidation is responsible for the covalent aggregation of hSOD1\textsuperscript{WT}.

**In Vivo Peroxidase Activity of SOD1: The Role of CO\textsubscript{2}\textsuperscript{−} Radical**—The peroxidase activity of SOD1 has previously been in-

![Fig. 10. The effect of molecular oxygen on bicarbonate-dependent aggregation of human SOD and H\textsubscript{2}O\textsubscript{2}. A. hSOD1\textsuperscript{WT} (1 mg/ml) was incubated with H\textsubscript{2}O\textsubscript{2} (1 mM) with and without HCO\textsubscript{3}\textsuperscript{−} (25 mM) in phosphate buffer (100 mM, pH 7.4) containing DTPA (0.1 mM). Incubations were performed in air or under N\textsubscript{2}. The fluorescence intensity was measured using excitation and emission wavelengths (320 and 420 nm). B. Same as A except that the fluorescent intensity was measured after 30 min using excitation and emission wavelengths 365 and 460 nm, respectively. C, after incubating for 45 min, samples obtained from incubations performed under conditions shown in A were analyzed by SDS-PAGE.](image-url)
cells and tissues, HCO$_3^-$-induced oxidative and nitrative reactions are biologically relevant. Recently, evidence for CO$_3^-$-dependent enhanced peroxidase activity was shown in an in vivo yeast model expressing hSOD1 (24). The spin trap POBN was used to trap the hydroxyethyl radical derived from the reaction between CO$_3^-$ and ethanol generated in situ in yeast cultures (24). The first in vivo spin trapping evidence for increased generation of oxygen-centered radicals was provided in a mouse model (25). The spin trap azulenyl nitrone was administered to ALS SOD1 transgenic mice (SOD1G93A), and the corresponding oxidative metabolite azulenyl aldehyde was detected (25). The proposed mechanism involved trapping of a peroxyl radical, followed by a decomposition of the nitrone-peroxyl adduct (25, 26). Based on the present findings obtained with an analogous open-chain nitrone, POBN, it is conceivable that the azulenyl aldehyde is formed from oxidation and hydrolysis of azulenyl nitrone by CO$_3^-$ (6). Thus, it is also conceivable that the G93A mutant of hSOD1, which causes ALS in humans and motor neuron disease in transgenic mice, may be producing such oxidants in vivo.

Comparison between CO$_3^-$ and 'OH Reactivities—Using a steady-state ESR spin trapping measurements, it is difficult to differentiate between 'OH and CO$_3^-$ based on their reaction with conventional hydroxyl radical scavengers (e.g. azide, formate, ethanol) (Fig. 1). Both 'OH and CO$_3^-$ react with these scavengers rapidly to generate the corresponding scavenger-derived radicals, which are then trapped by the nitronate spin traps, yielding a characteristic ESR spectrum of the resulting spin adduct. Thus, in order to differentiate between CO$_3^-$ and 'OH radical formation, it is necessary to monitor the initial rates of spin adduct formation as a function of trap concentrations and calculate the rate constants for 'OH and CO$_3^-$ reaction with the relevant traps (27). However, Me$_2$SO appears to be an exception. As shown in Fig. 1, hydroxyl radicals but not CO$_3^-$ react with Me$_2$SO to form the methyl radicals that were trapped by DMPO. It is pertinent to point out that Sato et al. reported similar results using ethanol and Me$_2$SO as radical scavengers in bicarbonate buffers containing bovine SOD1, DMPO, and H$_2$O$_2$ (28). The investigators observed the formation of a DMPO-CH(CH$_3$)OH but not a DMPO-CH$_3$ adduct in bicarbonate buffers containing bovine SOD1, DMPO, and H$_2$O$_2$ (28). This was attributed to the lack of reaction between copper-bound hydroxyl radical and Me$_2$SO.

In the present work, we obtained independent evidence for CO$_3^-$-mediated oxidation reactions by UV-photolysis of the pentammine carbonato complex of Co(III) as shown below. Irradiation of this complex in the presence of DMPO and azide, formate, etc. yielded ESR spectra of adducts that were similar to those obtained from a SOD1/H$_2$O$_2$/HCO$_3^-$ system (6).

The spin trapping results also revealed that, in the presence of free tyrosine and tryptophan, the DMPO-OH signal intensity obtained in the SOD1/H$_2$O$_2$/HCO$_3^-$ system and from cobalt complex/UV light was markedly inhibited in the presence of tyrosine or tryptophan but not in systems containing glycine or alanine. These results are consistent with the observed rate
Bicarbonate Stimulates SOD1 Peroxidase Activity

Fig. 11. The effect of tryptophan and DBNBS on SOD1-induced oxygen release. A, bovine SOD1 (2 mg/ml) was incubated with H2O2 (0.5 mM) with or without HCO3 (25 mM) in phosphate buffer (100 mM, pH 7.4) containing DTPA (0.1 mM). Where indicated, incubations contained DBNBS (25 mM) or added tryptophan (5 mM). B, same as A, except that the incubation mixture contained hSOD1WT or hSOD1W3E. Oxygen uptake/release experiments were performed using a World Precision Oxygen electrode at room temperature. The electrode was calibrated using air-saturated water (240 ± 0.0 mOxygen was equated to 100%).

constants reported for CO3 and OH (Table II). Loss of tryptophan fluorescence in the hSOD1WT/H2O2/HCO3 system has been attributed to CO3-mediated oxidation of Trp-32 residue. Recently, Yamakura et al. (29) reported that peroxyxenitrite and bicarbonate treatment induced a loss in Trp-32 fluorescence in the hSOD1 WT enzyme is caused by the peroxyxenitrite-carbon dioxide adduct. However, it is well known that the peroxyxenitrite-carbon dioxide adduct or nitroso-peroxyxenitrite decomposes to CO3 and NO2 radicals at physiological pH (30, 31). Thus, the observed modification of Trp-32 in this study (29) could be explained on the basis of the reaction between CO3 and Trp-32 in hSOD1 WT.

Tryptophan Radical-mediated Covalent Aggregation of Human SOD1: Dependence on Molecular Oxygen.—Based on the previous work by Gunther et al. (20) and the present spin trapping results using bovine SOD1 and hSOD1W3E, we conclude that Trp-32 is the site of oxidation and that the radical adduct is formed from trapping of a carbon-centered radical at the C-3 of the indole ring. As reported previously (20) and reiterated in this study, the ESR simulation of the high resolution spectrum of hSOD1WT-DBNBS adduct (Table I) strongly supports the structural assignment. The primary carbon-centered radical associated with Trp-32 most likely reacts with molecular oxygen to form the peroxyl radical, as shown in Scheme 1. As reported by Gunther and co-workers (32), the oxygen evolution was detected in incubations containing bovine SOD1WT, HCO3, and H2O2 (Fig. 11A, trace 1). As indicated earlier, the bovine SOD1 lacks the tryptophan residue. In the presence of added tryptophan, the oxygen evolution was completely suppressed due to oxygen consumption by the tryptophan-derived radicals. In fact, there was a slight increase in oxygen consumption (Fig. 11A, trace 4). In the presence of DBNBS spin trap, tryptophan-induced oxygen consumption was inhibited (Fig. 11A, trace 2). Similar results were obtained with hSOD1WT (which contains a tryptophan residue) in the absence of added tryptophan (Fig. 11B, trace 3). In the presence of the DBNBS spin trap, oxygen evolution was observed from incubations containing hSOD1WT, HCO3, and H2O2 (Fig. 11B, trace 1). This is attributed to trapping of the Trp-32-derived carbon-centered radical inhibiting radical-mediated oxygen consumption. This result further supports the possibility that the DBNBS-hSOD1WT adduct is formed from trapping the Trp-32 radical by DBNBS and not from a non-radical-mediated "ene" reaction between DBNBS and the indole moiety in Trp-32 (33). Attempts to directly detect and characterize the peroxy radical at low temperatures, as had been demonstrated in the metmyoglobin/H2O2 system, were not possible in the hSOD1WT/H2O2/HCO3 system due to interference from copper ESR absorption. Increased oxygen evolution was observed from incubations containing hSOD1W3E (mutant lacking the tryptophan residue), HCO3, and H2O2 (Fig. 11B, trace 2).

Results from this study reveal that radical-mediated oxidation of Trp-32 in the presence of molecular oxygen is responsible for H2O2/HCO3-dependent covalent aggregation of hSOD1 WT. Anaerobic conditions prevented the covalent dimerization of hSOD1 WT induced by HCO3 and H2O2 (Fig. 10). The oxidative cleavage of the indole ring of tryptophan has been shown to yield multiple products, including hydroxyindole, N-formyl-kynurenine, and kynurenine (34–36). These products can cause multiple cross-linking reaction pathways, leading to protein aggregation. We have proposed a tentative reaction pathway involving CO3-dependent Trp-32 oxidation, leading to oligomerization of hSOD1 WT via oxidative derivatives of Trp-32 (Scheme 1). Fig. 9 (left) provides some evidence for formation of oxidative degradation products during HCO3/H2O2-dependent SOD1 peroxidase activity. Fluorescence emission spectrum excited at 320 nm yielded a product that had an emission maximum around 420 nm. This peak may be assigned to the N-formyl-kynurenine (excitation, 310–330 nm; emission, 410–430 nm). This spectrum also suggests that dimerization does not occur via ditryptophan formation, since ditryptophan exhibits a distinctly different fluorescence (excitation, 320 nm; emission, double peaks at 370–380 nm). Fig. 9B (right) also shows that excitation at 365 and 410 nm yielded characteristic emission at 450 and 520 nm, respectively. These spectra have previously been assigned to kynurenine-type (37, 38). Results from the preliminary matrix-assisted laser desorption ionization-time-of-flight experiments are consistent with the notion that Trp-32 is oxidized by hSOD1WT/HCO3/H2O2 to the kynurenine-type oxidative products (39) (not shown).

Implications of Covalent Aggregation of SOD1 in Familial ALS.—Results from this study may increase our understanding of the fundamental mechanism(s) by which some forms of SOD1 mutants induce ALS (40, 41). Although the mechanism of pathogenesis in ALS and the role of ALS SOD1 mutants remains controversial and poorly understood, several reports suggest that familial ALS, a dominantly inherited form of ALS, is linked to a toxic gain of function in mutant SOD1 (i.e., increased peroxidase activity, peroxyxenitrite-mediated nitration, or enhanced aggregation of SOD1) (42–44). It is now
becoming more evident that nitration and oxidation reactions mediated by peroxynitrite are modulated by CO2, a ubiquitous cellular component (see Ref. 44 and references therein). The peroxynitrite-CO2 intermediate decomposes into CO2 and NO2 radicals and causes oxidative modifications in amino acids (e.g., tryptophan and tyrosine). Thus, enhanced covalent aggregation of hSOD1 could be mediated by peroxynitrite/CO2 chemistry or by CO2-dependent peroxidase activity. ALS-associated SOD1 mutants were shown to exhibit increased peroxidase activity (42). It is reasonable to suggest that HCO3− could exacerbate the intrinsic difference between wild-type and familial ALS mutant SOD1 with respect to peroxidase activity. Although protein aggregation is frequently associated with several neurodegenerative diseases (41, 46), it is not known whether these aggregates were formed from noncovalent or covalent aggregation (47, 48). In the present study, we present evidence for covalent aggregation of ALS mutant as well as the expression and purification of the recombinant human enzyme.

Acknowledgment—We acknowledge the technical expertise of Dr. Yingxin Zhuang (Department of Anesthesiology, University of Alabama at Birmingham) with regard to preparation of the cDNA for W32F mutant mice (6). Published data also indicate that high molecular weight complexes of mutant SOD1 (apparent molecular mass of 100–150 kDa) are observed in the spinal cord extracts of SOD1(1993A) mice (6). It is not known whether these aggregates were formed from noncovalent or covalent aggregation (47, 48).

REFERENCES

1. Hodgson, E. K. & Fridovich, I. (1975) Biochemistry 14, 5294–5298
2. Hodgson, E. K. & Fridovich, I. (1975) Biochemistry 14, 5299–5303
3. Liochev, S. I. & Fridovich, I. (2002) J. Biol. Chem. 277, 34674–34678
4. Zhang, H., Joseph, J., Felix, C. & Kalyanaraman, B. (2000) J. Biol. Chem. 275, 14038–14045
5. Singh, R. J., Goss, S. P. A., Joseph, J. & Kalyanaraman, B. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 12912–12917
6. Zhang, H., Joseph, J., Gurney, M. D. & Kalyanaraman, B. (2002) J. Biol. Chem. 277, 1015–1020
7. Liochev, S. I. & Fridovich, I. (1999) Free Radic. Biol. Med. 27, 1444–1447
8. Kalyanaraman, B., Joseph, J. & Zhang, H. (2001) Adv. Exp. Med. Biol. 500, 175–182
9. Hinkle, H. U., Santanam, N., Dakalow, S., McCann, L., Nguyen, A. D., Parthasarathy, S., Harrison, D. G. & Fukui, T. (2002) Arterioscler. Thromb. Vase. Biol. 22, 1402–1408
10. Basolo, F. & Murmann, R. K. (1953) J. Am. Chem. Soc. 75, 171–172
11. Buettner, G. R. & Oberley, L. W. (1978) Biochem. Biophys. Res. Commun. 83, 69–74
12. Zhang, H., Joseph, J., Vasquez-Vivar, J., Kasrai, H., Nunnzumurhe, C., Martinack, P., Tordo, P. & Kalyanaraman, B. (2000) FEBS Lett. 473, 56–62
13. Crow, J. P., Sampson, J. B., Zhuang, Y., Thompson, J. A. & Beckman, J. S. (1997) J. Neurochem. 69, 1936–1944
14. Zhao, L. J., Zhang, Q. X. & Padmanabhna, R. (1993) Methods Enzymol. 217, 218–227
15. McQuillan, G., Goss, S. P. A., Joseph, J. & Kalyanaraman, B. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 12912–12917
16. Basolo, F. & Murmann, R. K. (1953) J. Biol. Chem. 204, 6049–6055
17. Kety, V. & Janzen, E. G. (1999) J. Am. Chem. Soc. 113, 9053–9056
18. Copes, V. W., Chen, S. N. & Hoffman, M. Z. (1973) J. Biol. Chem. 248, 7458–7461
19. Kalyanaraman, B., Joseph, J. & Zhang, H. (2001) Free Radic. Biol. Med. 32, 544–550
20. Basolo, F. & Murmann, R. K. (1953) J. Biol. Chem. 204, 6049–6055
21. Kim, K. S., Choi, S. Y., Kwon, H. Y., Won, M. H., Kang, T. C. & Kang, J. H. (2002) Free Radic. Biol. Med. 32, 544–550
22. Konya, S. N. & Hoffman, M. Z. (1973) J. Biol. Chem. 248, 7458–7461
23. Okado-Matsumoto, A., Myint, T., Fuji, J. & Taniguchi, N. (2000) Free Radic. Res. 33, 65–73
24. Roe, J. A., Wiedau-Panke, M., Yen, V. N., Goto, J. T., Gralla, E. B. & Valentine, J. S. (2002) Free Radic. Biol. Med. 32, 169–174
25. Liu, R., Althaus, J. S., Ellerbrock, B. R., Becker, D. A. & Gurney, M. E. (1998) Ann. Neurol. 44, 763–770
26. Becker, D. A. (1996) J. Am. Chem. Soc. 118, 905–906
27. Morehouse, K. M. & Mason, R. P. (1988) J. Biol. Chem. 263, 1204–1211
28. Sato, K., Akaike, T., Kohno, M., Ando, M. & Maeda, H. (1992) J. Biol. Chem. 267, 25571–25577
29. Yamakura, F., Matsumoto, T., Fujimura, T., Taka, H., Murayama, I., Imai, T. & Uchida, K. (2001) Biochim. Biophys. Acta 1548, 38–46
30. Timmins, G. S., Barlow, G. K., Silvester, J. A., Wei, X. & Whitwood, A. C. (1997) Redox Rep. 3, 125–133
31. Stochl, S. J., Habeeb, R. L. & Van Vranken, D. A. (1996) J. Am. Chem. Soc. 118, 1225–1226
32. Aquilina, J. A., Carver, J. A. & Truscott, R. J. (2000) Biochemistry 39, 16176–16184
33. Aquilina, J. A., Carver, J. A. & Truscott, R. J. (1999) Biochemistry 38, 11455–11464
34. Vazquez, S., Aquilina, J. A., Jamie, J. F., Skef, M. M. & Truscott, R. J. (2002) J. Biol. Chem. 277, 4867–4873
35. Anderson, L. B., Madera, M., Ouellette, A. J., Putnam-Evans, C., Higgins, L., Krick, T., MacCoss, M. J., Lim, H., Yates, J. R., III & Barry, B. A. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 14676–14681
36. Cleveland, D. W. & Rothstein, J. D. (2001) Nat. Rev. Neurosci. 2, 806–819
37. Julien, J. P. (2001) Cell 104, 581–591
38. Crow, J. P., Ye, Y. Z., Strong, M., Kirk, M., Barnes, S. & Beckman, J. S. (1997) J. Neurochem. 69, 1945–1953
39. Wiedau-Panke, M., Goto, J. T., Rabizadeh, S., Gralla, E. B., Roe, J. A., Lee, M. K., Valentine, J. S. & Breidenes, D. E. (1996) Science 271, 515–518
40. Johnston, J. A., Dalton, M. J., Gurney, M. E. & Kopito, R. R. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 12571–12576
41. Augusto, O., Benini, M. G., Amason, A. M., Linares, E., Santos, C. C. & De Menezes, L. S. (2002) Free Radic. Biol. Med. 32, 841–859
42. Basolo, F. & Murmann, R. K. (1953) J. Biol. Chem. 204, 6049–6055
43. Okado-Matsumoto, A. & Fridovich, I. (2000) Proc. Natl. Acad. Sci. U. S. A. 99, 9010–9014
44. Brum, L., Houseweart, M. K., Kato, S., Anderson, K. L., Anderson, S. D., Ohama, E., Reaume, A. G., Scott, R. W. & Cleveland, D. W. (1998) Science 281, 1851–1854