Characterization of Sulfur-centered Radical Intermediates Formed during the Oxidation of Thiols and Sulfite by Peroxynitrite

ESR-SPIN TRAPPING AND OXYGEN UPTAKE STUDIES*

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Using a novel phosphorylated spin trap, 5-diehtoxyphosphoryl-5-methyl-1-pyrroline N-oxide (DEPPO), an analog of the commonly used trap 5,5′-dimethyl-1-pyrroline N-oxide (DMPO), we have investigated the reactions of sulfur-centered radicals produced from the oxidation of thiols and sulfite by peroxynitrite. The predominant species trapped in all cases are the corresponding sulfur-centered radicals, i.e. glutathionyl radical (GS· from glutathione (GSH), N-acetyl-5-formimimidazolyl thiyl radical (S-NAP) from N-acetyl-5-formimimidazole (NAP) and sulfite anion radical (SO3·) from sulfite. These radicals can also be produced by hydrogen peroxide and the oxygen forming either peroxyl or superoxide anion radicals. GS·, S-NAP, and SO3· derived radicals react with ammonium formate to form the carbon dioxide anion radical (CO2·). Further support of spin adduct assignments and radical reactions are obtained from photolysis of S-nitrosothioglutathione and S-nitroso-N-acetyl-5-formimimidazolyl radicals. We conclude that the direct reaction of peroxynitrite with thiols and sulfite forms thiol and sulfite anions, respectively, by a hydroxyl radical-independent mechanism. Pathological implications of thiol radical formation and subsequent oxysubstituted chain reactions are discussed. Oxygen activation by thiol radicals formed during peroxynitrite-mediated oxidation of glutathione may limit the effectiveness of GSH against peroxynitrite-mediated toxicity in cellular systems.

The reaction between nitric oxide (NO) and superoxide anion (O2·−) generates peroxynitrite (1) at a near diffusion controlled rate (Equation 1) (1, 2).

\[
\text{NO} + \text{O}_2^{-} \rightarrow k_1 \rightarrow 4.3 \times 10^{7} \text{M}^{-1} \text{s}^{-1} \quad \text{ONOO}^{-} \quad \text{(Eq. 1)}
\]

This reaction has been implicated in many pathological conditions including reperfusion injury (3), atherosclerosis (4), and neurodegenerative diseases (5). There are many potential targets for peroxynitrite in biological systems including thiols, lipids, and DNA (6–8). Although free radical intermediates have been proposed in peroxynitrite-mediated oxidation reactions, they have been directly detected in only a few cases (9–13). Because of its role in pathophysiology, further understanding of the free radical reactions of peroxynitrite with potential biological targets is essential.

The chemistry of decomposition of peroxynitrite is complex (14). ONOO− is stable in alkaline solutions. However, upon protonation (pKa = 6.9), ONOOH decays rapidly (t½ ~ 1 s at pH 7.4) (15). The decay of ONOOH has been shown to have two free radical character as evidenced by malondialdehyde formation from deoxyribose by a process inhibitable by hydroxyl radical scavengers and aromatic hydroxylation of sodium benzoate (15). This activity has been shown to have many similarities to the oxidant produced in the Fenton reaction and is often referred to as a “hydroxyl radical”–like species. Köppenol et al. (14) have shown that homolytic decomposition of ONOOH is unlikely and that the oxidant is probably an active conformer produced during internal rearrangement to nitric acid. The oxidative effects of peroxynitrite are not confined to this species, however, as ONOO− and ONOOH can act as both one-electron and two-electron oxidants (6, 10, 16). For example, ONOO− will oxidize glutathione (GSH) to glutathione disulfide (GSSG) (6).

In this study we have attempted to characterize the reactions of sulfur-centered radicals formed from the one-electron oxidation of thiols by peroxynitrite using the spin-trapping technique. Previous electron spin resonance (ESR) studies of the decomposition of peroxynitrite at physiological pH have used 5,5′-dimethyl-1-pyrroline N-oxide (DMPO) as the spin trap and the results are controversial. Shi et al. (13) reported the formation of 5,5′-dimethyl-1-pyridin-2-one-1-oxyl, a further oxidation product of DMPO, at high concentration of peroxynitrite and concluded that free hydroxyl radical is not released. However, Augusto et al. (9) reported the formation of DMPO·OH from DMPO using low peroxynitrite concentrations and a strong and persistent DMPO·OH signal at higher concentrations only in the presence of GSH. Recently, Pou et al. (11) reported that the yield of formation of HO· from peroxynitrite decomposition was about 1 to 4% based on the methylene sulfonic acid assay. A more recent study from Lemercier et al. (12) claimed that DMPO·OH adduct detected during the reaction between DMPO and ONOO− is formed from a molecule-induced homolysis mechanism and not from trapping of HO·.

The use of DMPO does not allow easy discrimination be-
between DMPO/\(\cdot\)OH and DMPO/\(\cdot\)SG, the radical adduct formed from trapping of the glutathionyl radical (GS'). To obviate this problem, we have used a novel spin trap, 5-diethoxyphosphoryl-5-methyl-1-pyrroline N-oxide (DEPMPO) (17–19, 45), to examine the oxidative reactions of peroxynitrite. The phosphorus nucleus (\(I = 1/2\)) in DEPMPO spin adducts has a large hyperfine splitting (\(a^H\) between 45 and 53 G) that is sensitive to the nature of the radical (\(R^*\)) trapped and to the nitroxide spin adduct conformation (Scheme 1) (18). For the DEPMPO/\(R^*\) spectrum, the extra hyperfine coupling to phosphorus (\(I = 1/2\)) results, in most cases, in a spectrum that resembles a juxtaposition of two DMPO/\(R^*\) spin adduct spectra.

In this paper, using ESR, gas chromatography, and oxygen uptake studies, we have characterized the reactions of sulfur-centered radicals formed from the reaction of peroxynitrite with sodium bisulfite and several thiols including glutathione. Biological implications of thiyl radical production from peroxynitrite in cellular systems are discussed.

### EXPERIMENTAL PROCEDURES

**Chemicals—** GSH, GS\(G\)SH, Chelex 100, N-acetyl-\(\beta\)-penicillamine (NAP), ferrous sulfate, EDTA, diethylenetriaminepentaacetic acid (DTPA), and manganese dioxide were obtained from Sigma. Sodium nitrite, hydrogen peroxide, and sodium hydroxide were obtained from Fischer Scientific (Pittsburgh, PA). S-Nitrosothioglutathione (GSNO) and S-nitroso-N-acetyl-\(\beta\)-penicillamine (SNAP) were synthesized according to the published procedure (20). 2,2,5,5-Tetramethyl-3-pyrroline-1-oxyl-3-carboxamide was obtained from Molecular Probes Inc. (Eugene, OR). The manganese macrocyclic superoxide dismutase mimics, SC-52608 (21), was obtained from the Monsanto Co. (St. Louis, MO).

**Peroxynitrite Synthesis—** Peroxynitrite was synthesized by mixing ice-cold solutions of 1 M NaNO\(2\) and 1 M H\(_2\)O\(_2\) in 0.3 M H\(_2\)SO\(_4\) and rapidly quenched by 1.4 M NaOH (15). Excess hydrogen peroxide was removed by adding a small amount (\(-1\) mg) of MnO\(_2\). After filtering, the solution was kept frozen at \(-20^\circ\)C. The yellow top layer contained 170–250 mM peroxynitrite as determined by the absorbance at 302 nm (\(\epsilon = 1670\) M \(^{-1}\) cm\(^{-1}\)). Peroxynitrite solutions were prepared by dilution to the desired concentration in 0.1 M NaOH.

ESR Measurements—ESR spectra were recorded at room temperature on a Varian E109 spectrometer at 9.5 GHz employing 100 kHz field modulation. Reaction mixtures were prepared in Chelex-treated 0.2 M phosphate buffer, pH 7.4. After the addition of peroxynitrite to the solution mixture, the solution was incubated for 30 s, transferred into a 100-\(\mu\)l capillary (Corning, NY) and placed in a 4-mm quartz tube inside the ESR cavity. The final pH was 7.4–7.5. The phosphate buffer used for the spin-trapping experiments was saturated with argon. Authentic spin adducts were generated by (i) photolysis of nitrosothiols, the quartz tube containing the capillary was irradiated with visible light (\(\lambda = 350–350\) nm) inside the ESR cavity; and (ii) a Fenton reaction system consisting of H\(_2\)O\(_2\) (1 mM), Fe\(^{2+}\) (0.05 mM), and EDTA (1 mM). Authentic DEPMPO/\(\cdot\)OH was generated by a nucleophilic addition of H\(_2\)O\(_2\) to DEPMPO in pyridine. Data were collected using the VIKING software, developed by the Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, NC. Simulations were used to deconvolute the complex ESR spectra from DEPMPO. As with all fitting procedures, we recognize that the computer simulations may not represent a unique solution to the data. ESR spectra were approximately quantified by double integration and comparison to a nitroxide standard (2,2,5,5-tetramethyl-3-pyrroline-1-oxyl-3-carboxamide). The relative contribution of the different radical adducts to the total integrated area was derived from the simulated spectrum.

Gas Chromatography—CO\(_2\) analysis was performed using a Varian 3700 Gas Chromatograph equipped with a 6 \(\times\) 1/8 inch Porapak Q column and thermal conductivity detector operating at 30 °C with a flow rate of 5 ml/min. Samples were prepared in a sealed vial and peroxynitrite was injected through the seal immediately prior to measurement. Headspace gas (100 \(\mu\)l) was injected into the gas chromatograph with an airtight Hamilton syringe. The CO\(_2\) retention time was 2.1–2.3 min.

Oxygen Uptake—Oxygen uptake experiments were performed using a YSI Oxygen electrode (Yellow Springs Instruments, Columbus, OH) at 37 °C. The electrode was calibrated using air-saturated water (240 \(\mu\)M oxygen) and sodium dithionite-treated water (0 \(\mu\)M oxygen). All experiments were performed using phosphate buffer (200 mM, pH 7.4) with a final volume of 5 ml. Data are given as mean \(\pm\) S.D. (\(n = 3\)).

### RESULTS

**Spin-trapping of HO' and GS' by DEPMPO—** Fig. 1, a and b, shows the ESR spectra of authentic DEPMPO/\(\cdot\)OH (\(a^H = 14.0\) G, \(a^H = 13.2\) G, \(a^H = 0.27\) G (\(\cdot\)H), and \(a^H = 47.3\) G) and DEPMPO/\(\cdot\)SG (\(a^H = 14.1\) G, \(a^H = 14.9\) G, and \(a^H = 45.8\) G) adducts generated by a Fenton system and by photolysis of GSNO, respectively. There is a clear difference between these two spin adduct spectra that is mainly due to the variations in phosphorus hyperfine splittings. A simulated spectrum of a mixture of DEPMPO/\(\cdot\)OH (20%) and DEPMPO/\(\cdot\)SG (80%) is shown in Fig. 1c. The majority of the spectral lines overlap and cannot be used as diagnostic indicators of either radical. However, the central portion of the spectrum clearly shows the contributions of DEPMPO/\(\cdot\)OH and DEPMPO/\(\cdot\)SG to the signal.

**FIG. 1.** ESR spectra of the authentic hydroxyl and glutathionyl radical adducts of DEPMPO. a, spectrum obtained upon incubating DEPMPO (20 mM), H\(_2\)O\(_2\) (0.2 mM), EDTA (0.2 mM), and FeSO\(_4\) (0.1 mM); b, spectrum obtained upon photolysis of GSNO (1 mM) in the presence of DEPMPO (20 mM) and DTPA (0.1 mM); c, computer simulation of a mixture of DEPMPO/\(\cdot\)SG (80%) and DEPMPO/\(\cdot\)OH (20%) adducts. The spectra in a and b were obtained at room temperature in phosphate buffer (0.2 M, pH 7.4).
Spin-trapping of Radicals Formed from the Reaction between Peroxynitrite, Glutathione, and DEPMPO—Incubation of DEPMPO with peroxynitrite in phosphate buffer (200 mM, pH 7.4) did not result in any ESR detectable spin adduct (Fig. 2a). This is in contrast to previous studies with DMPO wherein the investigators reported formation of DMPO/ •OH adducts (9, 11, 12). The addition of HO• scavengers (ethanol, 1.8 M; Me2SO, 1.4 M; ammonium formate, 1 M; mannitol, 1 M, or t-butanol, 1.1 M) also gave no ESR detectable adduct. In the presence of GSH (1 mM), the spectrum shown in Fig. 2b was observed. This signal is complex and consists of several spin adducts. The predominant species is DEPMPO/GSH which corresponds to 55 to 60% of the signal. This represents a concentration of 12 to 14 μM DEPMPO/GSH indicating that the efficiency of the one-electron oxidation of GSH to GS• by peroxynitrite is about 1–2%. The high performance liquid chromatography data indicate that DEPMPO does not affect the yield of GSSG during ONOO•-dependent oxidation suggesting that GS• is a minor product from the reaction between GSH and ONOO•. The origin of the remaining signal in Fig. 2b is uncertain, but the whole spectrum can be simulated (Fig. 2b, dotted line) by assuming the presence of two radical adducts with hyperfine splitting constants characteristic of carbon-centered adducts (17) (aH = 14.5 G, aH = 21.5 G, and aH = 45.9 G; aH = 14.8 G, aH = 20.7 G, and aH = 47.8 G) and a species (from 10 to 19%) with parameters similar to DEPMPO/OH (aH = 14.0 G, aH = 13.4 G, aH = 0.6 G (3H), and aH = 47.5 G). Both DEPMPO/GSH and DEPMPO/OH adducts decayed with time leaving only the signal assigned to the carbon-centered radical adducts (Fig. 2c). This spectrum can be simulated (Fig. 2c, dotted line) using the same ESR parameters for the carbon-centered radicals shown in Fig. 2b. We also observed a similar behavior after the decay of the DEPMPO/ •G adduct obtained during photolysis of GSNO (data not shown). Augusto et al. (9) also reported the detection of an unidentified carbon-centered radical during oxidation of GSH by peroxynitrite in the presence of DMPO. The source of these carbon-centered radicals is unknown but Grierson et al. (22) and Zhao et al. (23) have reported formation of a carbon-centered radical from an intramolecular rearrangement of the glutathionyl radical.

In order to examine whether the DEPMPO/OH spin adduct in Fig. 2b is formed from trapping of HO•, the effects of HO• scavengers were investigated. Fig. 3 shows that a large excess of t-butanol (1.1 M)Fig. 3b), mannitol (1 M)Fig. 3c), and also ethanol (1.8 M) and Me2SO (1.4 M) (data not shown) had only minor effects on the DEPMPO/OH component of the ESR spectrum in Fig. 3a. The effects of ethanol and Me2SO were identical irrespective of DEPMPO concentration (20 to 100 mM). This suggests that the signal with DEPMPO/OH parameters (Figs. 2b and 3a) does not arise from the trapping of free hydroxyl radical.

An alternate route by which DEPMPO/OH can be formed is via decomposition of DEPMPO/OOH to DEPMPO/OH. However, unlike DMPO/OOH, the DEPMPO/OOH adduct does not decay spontaneously to DEPMPO/OH (18, 24). It is conceivable that this conversion is facilitated by high concentrations of GSH. This reaction is similar to the GSH-dependent reduction of hydroperoxide to alcohol (Equation 2). In support of this, GSH was shown to facilitate the conversion of chemically or enzymatically synthesized DEPMPO/OOH to DEPMPO/OH (data not shown).

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3 H. Karoui, N. Hogg, and B. Kalyanaraman, unpublished data.
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Fig. 4. Effect of superoxide dismutase mimic on the radical adduct formation during the reaction between GSH, ONOO\(^-\), and DEPMPO. a, spectrum obtained upon incubating DEPMPO (20 mM), GSH (1 mM), peroxynitrite (0.75 mM), and DTPA (0.1 mM) in phosphate buffer (0.2 M, pH 7.4) at room temperature; b, as a in the presence of superoxide dismutase mimic (100 \(\mu\)M). The dotted lines in a and b show a computer simulation of the respective spectra. Instrument settings: microwave power, 5 mW; modulation amplitude, 1 G; time constant, 0.128 s; gain, 8 \(\times 10^4\); scan range, 200 G; and scan time, 120 s. Contributions of the various radical adducts to the simulated spectra are described in the text.

Fig. 5. Effect of ammonium formate on formation of spin adducts during oxidation of GSH by ONOO\(^-\). a, spectrum obtained upon incubating DEPMPO (20 mM), GSH (1 mM), HCO\(_2\)NH\(_4\) (400 mM), ONOO\(^-\) (0.8 mM), and DTPA (0.1 mM) in phosphate buffer (0.2 M, pH 7.4); b, as a but in the presence of HCO\(_2\)NH\(_4\) (400 mM), c, same as a but containing HCO\(_2\)NH\(_4\) (1 mM). The dotted line in c shows a computer simulation of the spectrum; and d, the authentic DEPMPO/CO\(_2\) spin adduct prepared by oxidation of formate anion in a Fenton system. Spectrometer settings: microwave power, 5 mW; modulation amplitude, 1 G; time constant, 0.128 s; gain, 8 \(\times 10^4\) (2.5 \(\times 10^4\) for d); scan range, 200 G; and scan time, 120 s. Open and closed circles indicate the low-field lines of DEPMPO/SG and DEPMPO/CO\(_2\), respectively.

To further verify that DEPMPO/OH is actually formed from DEPMPO/OOH, we investigated the effect of superoxide dismutase mimic in peroxynitrite/GSH system.\(^4\) The spectrum obtained in the absence of superoxide dismutase mimic was simulated assuming the presence of DEPMPO/SG (44%), DEPMPO/OH (19%), and two carbon-centered adducts (37%) (Fig. 4a). No ESR spectrum was obtained in the absence of peroxynitrite. The spectrum obtained in the presence of superoxide dismutase mimic (1 mM) shows no evidence for the presence of DEPMPO/OH adduct (Fig. 4b) as determined by simulation using DEPMPO/SG (88%) and one carbon-centered adduct (12%) (Fig. 4b, dotted line). The spectral intensity of DEPMPO/SG was enhanced in the presence of superoxide dismutase mimic (Fig. 4b), perhaps indicating a superoxide-mediated destruction of the DEPMPO/SG adduct. This is consistent with a previous report where it was shown that addition of superoxide dismutase enhanced the signal intensity of DMPO/SG adduct formed during horseradish peroxidase/H\(_2\)O\(_2\)-catalyzed oxidation of GSH (25).

Effect of Ammonium Formate (HCO\(_2\)NH\(_4\)) on Spin Adduct Formation during the Reaction between Peroxynitrite, GSH, and DEPMPO—As mentioned above, the reaction between peroxynitrite (0.8 mM) and HCO\(_2\)NH\(_4\) (1 mM) in the presence of DEPMPO (20, 50, and 100 mM) generated no ESR signal. However, in the presence of GSH (1 mM), DEPMPO/CO\(_2\) was observed (Fig. 5, a–c). Lower concentrations of HCO\(_2\)NH\(_4\) (100 mM) gave an ESR spectrum that consisted of a mixture of DEPMPO/SG (marked \(\bigcirc\)) and DEPMPO/CO\(_2\) (marked \(\bullet\)) adducts (Fig. 5a). At higher concentrations of HCO\(_2\)NH\(_4\) (0.4 and 1 mM), the DEPMPO/CO\(_2\) adduct dominated the spectrum (Fig. 5, b and c, respectively). The dotted line signal represents the computer simulation of the spectrum 5c which is a mixture of DEPMPO/CO\(_2\) (80%) and DEPMPO/SG (20%). The authentic DEPMPO/CO\(_2\) spin adduct (\(a^H = 14.5\) G, \(a^N = 17.3\) G, and \(a^o = 51.6\) G) was generated by the Fenton reaction in the presence of DEPMPO (20 mM) and HCO\(_2\)NH\(_4\) (1 mM) (Fig. 5d). The generation of GS\(^-\) from photolysis of GSNO (1 mM) in the presence of DEPMPO (20 mM) and HCO\(_2\)NH\(_4\) (400 mM) led to a time-dependent formation of DEPMPO/CO\(_2\) (Fig. 5, a–e). These results indicate that GS\(^-\) generated from reaction of peroxynitrite with GSH reacts with HCO\(_2\)NH\(_4\) to form the CO\(_2\) radical (Equations 3 and 4).

\[
\text{GSH + ONOO}^- \xrightarrow{k_3 = 10^3 - 10^4 M^{-1}s^{-1}} \text{GS}^- + \text{NO}_2^- + \text{H}_2\text{O}
\]  

(Eq. 3)

\[
\text{GS}^- + \text{HCOO}^- \xrightarrow{k_4 = 10^4 M^{-1}s^{-1}} \text{GSH} + \text{CO}_2
\]  

(Eq. 4)

\[
\text{CO}_2 + \text{O}_2 \xrightarrow{k_5 = 10^6 M^{-1}s^{-1}} \text{CO}_2 + \text{O}_2^-
\]  

(Eq. 5)

Glutathionyl Radical-mediated CO\(_2\) Formation—In the absence of DEPMPO, CO\(_2\) will react with O\(_2\) to give CO\(_2\) and the superoxide anion, O\(_2^-\) (Equation 5) (26). No detectable CO\(_2\) was formed from the reaction between peroxynitrite and HCO\(_2\)NH\(_4\). However, in the presence of GSH, CO\(_2\) formation was observed (Fig. 7). DEPMPO (30 mM) completely inhibited CO\(_2\) formation in this reaction system (fig. 7), suggesting that GS\(^-\) is respon-

\(^4\) Unlike superoxide dismutase, the superoxide dismutase mimic does not react directly with peroxynitrite (personal communication with Dr. Randy Weiss of the Monsanto Co.).
sible for CO₂ formation. GSNO photolysis in the presence of HCO₂NH₄ also resulted in the evolution of CO₂ which was inhibited by DEPMPO (data not shown).

Spin-trapping of Radicals Formed during the Reaction between N-Acetyl-DL-penicillamine and Peroxynitrite—The DEPMPO/•SNAP adduct (DEPMPO/S-NAP) exhibits a distinctly different ESR spectrum than other DEPMPO thiyl adducts. Authentic DEPMPO/S-NAP adduct was obtained by photolysis of SNAP (1 mM) in the presence of DEPMPO (50 mM) and DTPA (0.1 mM) in Chelex-treated phosphate buffer (Fig. 8a). The spectrum shown in Fig. 8a was simulated by assuming the presence of two diastereoisomeric DEPMPO/S-NAP adducts with slightly different hyperfine splitting constants (aN = 14.3 G, aH = 16.5 G, and aP = 44.9 G (32%); aN = 14.4 G, aH = 15.2 G, and aP = 46.2 G (38%)) and a carbon-centered radical adduct (aN = 14.8 G, aH = 20.8 G, and aP = 48.0 G (30%)) (Fig. 8a, dotted line).

The incubation of NAP (10 mM) with DEPMPO (50 mM) and peroxynitrite (0.8 mM) in phosphate buffer led to the formation of a spectrum (Fig. 8b) which was essentially identical to the spectrum obtained by photolysis of SNAP (1 mM) in the presence of DEPMPO (50 mM) and DTPA (0.1 mM) in Chelex-treated phosphate buffer (Fig. 8a). No evidence for DEPMPO/OH adduct was observed and the addition of HO scavengers such as ethanol (1.8 mM) or Me₂SO (1.4 mM) had no effect on the signal. DEPMPO/OH was also not observed when N-acetyl-ο-l-cysteine was used (data not shown). Addition of peroxynitrite to a mixture containing DEPMPO (50 mM), NAP (10 mM), and ammonium formate produced an ESR spectrum consisting of both DEPMPO/S-NAP and DEPMPO/•CO₂ adducts. The spectrum was simulated by assuming the presence of two diastereoisomers of DEPMPO/S-NAP (33%) and DEPMPO/•CO₂ (67%) (Fig. 8c, dotted line). These results indicate that S-NAP also reacts with ammonium formate to form CO₂.

5 The abbreviations SNAP and S-NAP indicate S-nitroso-N-acetyl-ο-l-penicillamine and the N-acetyl-ο-l-penicillamine thiyl radical, respectively. Note that the “S” in SNAP refers to the S-nitroso functional group which is lost upon conversion to S-NAP. In S-NAP the “S” indicates the presence of a thiyl radical.
Thiyl Radical-mediated Oxygen Uptake—Decomposition of peroxynitrite results in the evolution of $O_2$ (7). The concentration of oxygen formed was an approximately linear function of peroxynitrite concentration up to 1 mM (10% yield) and was unaffected by DEPMPO (20 mM). However, in the presence of GSH (1 mM), rapid consumption of $O_2$ was observed (Fig. 9). The addition of DEPMPO (20 mM) inhibited $O_2$ consumption by 62 ± 2% (Fig. 9, inset), indicating the possible involvement of thiyl radicals.

The $O_2$ uptake during the reaction between peroxynitrite and NAP in phosphate buffer (200 mM, pH 7.4) at 37°C is shown in Fig. 10. The $O_2$ uptake increased as a function of NAP concentration (Fig. 10, inset). Under similar experimental conditions, the $O_2$ uptake with NAP was much higher than with GSH, suggesting differences in the reaction mechanism of the corresponding thiyl radicals.

Spin-trapping of Sulfite Radical Anion Formed during the Reaction between Sodium Bisulfite and Peroxynitrite—The reaction between peroxynitrite and NaHSO$_3$ in the presence of DEPMPO resulted in the formation of DEPMPO/SO$_3^-$ adduct (a$^0$ = 13.3 G, a$^m$ = 14.9 G, and a$^d$ = 48.9 G) (Fig. 11a). For comparison, the DEPMPO/NO$_3$ adduct formed from HO$^•$ and NaHSO$_3$ is shown in Fig. 11c. Hydroxyl radical scavengers (ethanol, 1.8 M, and Me$_2$SO 1.4 M) had only a slight effect (5 to 10%) on the ESR signal intensity shown in Fig. 11a indicating that SO$_3^-$ probably arises from a direct reaction between HSO$_3^-$ and peroxynitrite. The signal intensity of DEPMPO/NO$_3$ adduct was diminished in oxygen saturated buffer. This supports trapping of free SO$_3^-$ by DEPMPO to form the DEPMPO/NO$_3$ adduct, as SO$_3^-$ radical reacts rapidly with oxygen (27).

Sulfite Radical Anion-mediated Oxygen Uptake—Addition of peroxynitrite to various concentrations of NaHSO$_3$ (from 0.1 to 1 mM) caused a concentration-dependent increase in $O_2$ consumption (Fig. 12). During the reaction between 200 µM NaHSO$_3$ and 300 µM peroxynitrite, DEPMPO (20 mM) inhibited oxygen consumption by 69 ± 6% (Fig. 12, inset). The inhibition in $O_2$ uptake observed in the presence of DEPMPO is probably due to effective trapping of SO$_3^-$ by DEPMPO.

Figure 9. Oxygen consumption during the reaction between GSH and peroxynitrite: effect of spin trap. Peroxynitrite was added into a chamber of an oxygen electrode containing GSH (1 mM) and DTPA (0.1 mM) in phosphate buffer (0.2 M, pH 7.4) and the total $O_2$ consumed was recorded. Inset, $O_2$ uptake traces obtained by the addition of GSH (1 mM) alone (+), with peroxynitrite (0.3 mM) in the absence of DEPMPO (●) and in the presence of DEPMPO (20 mM) (○). The arrow (↓) indicates the point of addition of peroxynitrite.

Figure 10. Oxygen consumption during the reaction between NAP and peroxynitrite: effect of spin trap. Peroxynitrite (280 µM) was added into a chamber of an oxygen electrode containing varying concentrations of NAP and DTPA (0.1 mM) in phosphate buffer (0.2 M, pH 7.4) and the total $O_2$ consumed was recorded. Inset, $O_2$ uptake traces obtained by the addition of NAP (1 mM) alone (+), with peroxynitrite (280 µM) in the absence of DEPMPO (●) and in the presence of DEPMPO (30 mM) (○). The arrow (↓) indicates the point of addition of peroxynitrite.

Figure 11. Addition of peroxynitrite to sodium bisulfite in the presence of DEPMPO. a, spectrum obtained upon the incubation of DEPMPO (20 mM), NaH$_2$SO$_3$ (1 mM), peroxynitrite (0.36 mM), and DTPA (0.1 mM) in phosphate buffer (200 mM, pH 7.4); b, as in 100% $O_2$-saturated buffer; c, the ESR spectrum of an authentic DEPMPO/NO$_3$; obtained in a Fenton reaction (H$_2$O$_2$ (0.1 mM), NaH$_2$SO$_3$ (30 mM), EDTA (0.1 mM), and FeSO$_4$ (0.05 mM)). The dotted line in c shows a computer simulation of the spectrum; and d, as in the absence of peroxynitrite. Spectrometer settings: microwave power, 5 mW; modulation amplitude, 1 G; time constant, 0.128 s; gain, 8 × 10$^6$; scan range, 200 G; and scan time, 120 s.
under these conditions. This is trapped by DMPO and O₂ could also oxidize radical. DEPMPO to DMPO radical cation followed by hydrolysis, as shown below (12):

\[ \text{HCO}_2^+ + \text{SO}_3^2^- \rightarrow \text{HCO}_2 + \text{SO}_3 \] (Equation 6)

It is likely that SO₃²⁻ formed from SO₂ and O₂ could also oxidize formate anion to the CO₂ radical.

**DISCUSSION**

Peroxynitrite-mediated Oxidation of DEPMPO and DMPO—Our data show that incubating DEPMPO with peroxynitrite does not form the DEPMPO/OH adduct (Fig. 2a). This is in contrast to previous findings using DMPO (9, 11, 12). Several investigators have detected DMPO/OH adduct during the reaction between DMPO and peroxynitrite (9, 11, 12). Proposed mechanisms of formation of DMPO/OH include oxidation of DMPO to DMPO radical cation followed by hydrolysis, as shown below (12):

\[ \text{H}^+ + \text{DMPO} + \text{ONOO}^- \rightarrow \text{DMPO}^+: + \text{NO}_2 + \text{HO}^- \] (Eq. 7)

\[ \text{DMPO}^+: + \text{H}_2\text{O} \rightarrow \text{DMPO}/\text{OH} + \text{H}^+ \] (Eq. 8)

The inactivity of peroxynitrite to oxidize DEPMPO is of interest. The oxidation potentials of DMPO and DEPMPO were measured to be about 1.87 V (versus NHE) and 2.24 V (versus NHE), respectively. It is likely that the 400 mV difference in oxidation potential could account for the lack of oxidation of DEPMPO by peroxynitrite. The present data show that the DEPMPO/OH adduct that is formed from the reaction between peroxynitrite and DEPMPO is likely to be derived from GSH-dependent decomposition of DEPMPO/OOH.

Peroxynitrite-dependent Sulfur Radical Formation—Thyli radical formation in peroxynitrite-mediated oxidation of thiols has been previously detected using DMPO (9, 12, 13). This observation is now confirmed using DEPMPO, as thyli radical adducts of GSH, cysteine, and NAP were observed. The formation of thyli adducts was independent of the hydroxyl radical-formation or represents an independent pathway of oxidation by one of the conformers of ONOOH.

As discussed earlier, the DEPMPO-dependent inhibition of O₂ uptake observed during DMPO-mediated oxidation of GSH and NAP is due to

\[ \text{RS}^- + \text{O}_2 \rightarrow \text{RSO}_2^- \] (Eq. 9)

\[ \text{RS}^- + \text{DEPMPO} \rightarrow \text{DEPMPO}/\text{SR} \] (Eq. 10)

trapping of thyli radicals (Equations 9 and 10). Most thyli radicals, such as GS, react with O₂ at nearly diffusion-controlled rate (≈10⁹-10¹⁰ M⁻¹ s⁻¹) (22, 23). Since peroxynitrite...
decomposition was unaffected by DEPMPO, the nearly complete inhibition \( \text{O}_2^- \) uptake observed at higher concentrations of DEPMPO (100 mM) could be explained if \( k_{10} \ll k_{10} \) [DEPMPO], where \( k_{10} = 10^{9} \text{ M}^{-1} \text{s}^{-1} \), \( \text{O}_2^- = 2.4 \times 10^{-4} \text{ M} \), and [DEPMPO] = 1 \times 10^{-1} \text{ M} \). Hence, \( k_{10} \) can be estimated to be greater than \( 10^{6} - 10^{7} \text{ M}^{-1} \text{s}^{-1} \) (25, 29).

Peroxynitrite oxidized sulfite by an one-electron mechanism to generate the radical. This again appears to be a direct reaction as hydroxyl radical scavengers did not affect the ESR spectral intensity of DEPMPO/\( SO_2^- \) spectrum and the signal was diminished in the presence of 100% oxygen.

Formation of DEPMPO/Carbon-centered Adducts during Peroxynitrite-mediated Oxidation of GSH and NAP—Our results indicate that both DEPMPO/SR and DEPMPO/R (DEPMPO-carbon centered adduct) were detected during oxidation of thiols by peroxynitrite (Figs. 2b and 7a). Evidence for formation of \( \alpha \)-amino acid carbon-centered radicals from glutathionyl and other sulfur-centered radicals had been obtained using low-temperature (28) and fast-flow ESR spectroscopy (22). This mechanism involves an intramolecular abstraction of an \( \alpha \)-hydrogen atom of the glutamyl residue by the thyl radical center. A diagrammatic representation of this process is shown in Scheme 2.

It is possible that the thyl radical, once formed, undergoes a conformational change which brings the thyl radical center closer to the \( \alpha \) -hydrogen atom. The equilibrium between GS' and 'GSH is dependent on the pH and is shifted to the right side at higher pH (22, 23). However, a considerable fraction of GS' presumably exists as the \( \alpha \)-amino acid radical at physiological pH. A similar reaction mechanism can also be proposed for formation of the NAP-derived carbon-centered radical from 'S-NAP. The \( \alpha \)-amino acid carbon-centered radical has an asymmetric carbon center and the resulting DEPMPO-carbon-centered adduct will exist as diastereoisomers. Further ESR structural characterization of these DEPMPO-carbon-centered adducts must await synthesis of carbon-13 labeled GSNO and SNAP.

Peroxynitrite-dependent Oxidation of Formate—No spin adducts were observed from the reaction between peroxynitrite and formate in the absence of GSH. However, in the presence of GSH, the formation of \( \text{CO}_2^- \) was observed. Augusto et al. (9) reported similar results using DMPO and indicated that formate was acting as a typical hydroxyl radical scavenger. The observation that GS', generated from the photolysis of GSNO, is also able to abstract a hydrogen atom from formate to form \( \text{CO}_2^- \) indicates another mechanism whereby formate can affect spin adduct formation (30). Our results indicate that \( \text{CO}_2^- \) formation from formate cannot be used as a diagnostic indicator of hydroxyl radical in the presence of GSH.

Mechanism of Oxygen Activation by Sulfur-centered Radicals—The oxygen consumption observed during the oxidation of GSH by peroxynitrite can be explained by Equations 11-15. GS' reacts with \( \text{O}_2^- \) at a diffusion-controlled rate to form GSOO' which then abstracts a hydrogen from GSH to produce GS'. The reaction between GS' and GS will readily form GSSG' which can reduce \( \text{O}_2^- \) to \( \text{O}_2 \) (31). Equations 11, 12, and 15 constitute a chain reaction by which glutathione can be oxidized (Ref. 32 and references therein).

\[
\begin{align*}
\text{GS'} + \text{O}_2^- & \overset{k_{11}}{\longrightarrow} \text{GSOO}' \quad \text{(Eq. 11)} \\
\text{GSOO}' + \text{GSH} & \longrightarrow \text{GSOOH} + \text{GS}' \quad \text{(Eq. 12)} \\
\text{GS'} + \text{GS} & \overset{k_{13}}{\longrightarrow} \text{GSSG}' \quad \text{(Eq. 13)} \\
\text{GSSG}' + \text{O}_2^- & \overset{k_{14}}{\longrightarrow} \text{GSSG} + \text{O}_2 \quad \text{(Eq. 14)} \\
\text{O}_2^- + \text{GSH} + \text{H}^+ & \overset{k_{15}}{\longrightarrow} \text{\text{GSO}_2^- + \text{SO}_4^-} \quad \text{(Eq. 15)}
\end{align*}
\]

NAP elicited a greater consumption of oxygen than GSH for the same concentration of thiol and peroxynitrite. It is likely that the additional steric hindrance at the sulfur atom of NAP hinders dimerization of the corresponding thyl radical (i.e. 'S-NAP). Whereas rapid dimerization of GS' will reduce the probability of a reaction between GS' and oxygen.

Oxygen consumption during the reaction between sulfite and peroxynitrite can be explained by the following reactions (27). \( \text{SO}_2^- \) formed from the reaction between peroxynitrite and \( \text{Na}_2\text{SO}_3 \) can react with oxygen to generate a number of higher oxidation species including '\text{OOSO}_2^- \) and '\text{SO}_4^- \). During this process, chain propagation reactions can occur as shown in Equations 18-20.

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*H. Karoui, N. Hogg, C. J. Fréjaville, P. Tordo, and B. Kalyanaraman, unpublished data.*
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**Scheme 3. Hypothetical scheme showing the sustained production of peroxynitrite in the neurotoxicity associated with nitric oxide.**

Both NO and O$_2^-$ can be generated enzymatically during inflammation. The reaction between ONOO$^-$ and GSH can amplify the oxidative damage via formation of O$_2^*$ and may limit the antioxidant potential of GSH.

$$\text{HSO}_3^- + \text{ONOO}^- \rightarrow \text{SO}_3^2^- + \text{NO}_2^- + \text{HO}^- \quad \text{(Eq. 16)}$$

$$\text{SO}_3^2^- + \text{O}_2^- \quad k_{17} = 1.5 \times 10^7 \text{M}^{-1} \text{s}^{-1} \quad \text{O}_2^* \quad \text{(Eq. 17)}$$

$$\text{O}_2^* + \text{HSO}_3^- \quad k_{18} = 3 \times 10^7 \text{M}^{-1} \text{s}^{-1} \quad \text{O}_2 \text{SO}_2^- + \text{SO}_3^2^- \quad \text{(Eq. 18)}$$

$$\text{O}_2^* \quad \text{SO}_3^2^- + \text{SO}_3^2^- \rightarrow \text{SO}_4^2^- + \text{O}_2^- \quad \text{(Eq. 19)}$$

$$\text{SO}_4^2^- + \text{SO}_3^2^- \rightarrow \text{SO}_4^2^- + \text{SO}_3^2^- \quad \text{(Eq. 20)}$$

Superoxide is also formed as a minor product of reaction between SO$_3^2^-$ and O$_2^-$ (Equation 21):

$$\text{SO}_3^2^- + \text{O}_2^- + \text{SO}_3^2^- \quad \text{(Eq. 21)}$$

Biological Implications of Peroxynitrite-Mediated Oxidation of Thiols—It has been suggested that nitric oxide-induced cytotoxicity is amplified by thiol depletion (33). Nitric oxide-mediated neurotoxicity has been thought to be due to peroxynitrite formation (34). Although the critical biological target of toxicity for peroxynitrite is not known, it has been suggested that thiols and protein sulfhydryls are major targets for peroxynitrite in neurons (35). It was recently shown that intact neurons are more susceptible than astrocytes to peroxynitrite formation (34). Although the critical biological target of oxidative damage via formation of O$_2^*$ and may limit the antioxidant potential of GSH.

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Characterization of Sulfur-centered Radical Intermediates Formed during the Oxidation of Thiols and Sulfite by Peroxynitrite: ESR-SPIN TRAPPING AND OXYGEN UPTAKE STUDIES

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