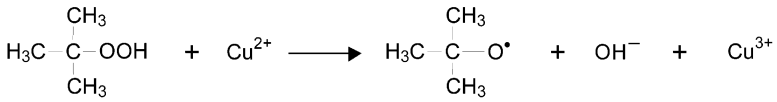
Article

EPR Spin-Trapping Evidence for the Direct, One-Electron Reduction of *tert*-Butylhydroperoxide to the *tert*-Butoxyl Radical by Copper(II): Paradigm for a Previously Overlooked Reaction in the Initiation of Lipid Peroxidation

Clare M. Jones, and Mark J. Burkitt

J. Am. Chem. Soc., **2003**, 125 (23), 6946-6954• DOI: 10.1021/ja034416z • Publication Date (Web): 10 May 2003



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 1 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

J|A|C|S
ARTICLES
Published on Web 05/10/2003

EPR Spin-Trapping Evidence for the Direct, One-Electron Reduction of *tert*-Butylhydroperoxide to the *tert*-Butoxyl Radical by Copper(II): Paradigm for a Previously Overlooked Reaction in the Initiation of Lipid Peroxidation

Clare M. Jones and Mark J. Burkitt*

Contribution from the Gray Cancer Institute, P.O. Box 100, Mount Vernon Hospital, Northwood, Middlesex, HA6 2JR, United Kingdom

Received January 30, 2003; E-mail: burkitt@gci.ac.uk

Abstract: Lipid peroxidation is often initiated using Cu^{II} ions. It is widely assumed that Cu^{II} oxidizes preformed lipid hydroperoxides to peroxyl radicals, which propagate oxidation of the parent fatty acid via hydrogen atom abstraction. However, the oxidation of alkyl hydroperoxides by Cu^{II} is thermodynamically unfavorable. An alternative means by which Cu^{II} ions could initiate lipid peroxidation is by their one-electron *reduction* of lipid hydroperoxides to alkoxyl radicals, which would be accompanied by the generation of Cu^{III}. We have investigated by EPR spectroscopy, in conjunction with the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide, the reactions of various Cu^{II} chelates with *tert*-butylhydroperoxide. Spectra contained signals from the *tert*-butoxyl, methyl, and methoxyl radical adducts. In many previous studies, the signal from the methoxyl adduct has been assigned incorrectly to the *tert*-butylperoxyl adduct, which is now known to be unstable, releasing the *tert*-butoxyl radical upon decomposition. This either is trapped by 5,5-dimethyl-1-pyrroline *N*-oxide or undergoes β-scission to the methyl radical, which either is trapped or reacts with molecular oxygen to give, ultimately, the methoxyl radical adduct. By using metal chelates that are known to be specific in either their oxidation or reduction of *tert*-butylhydroperoxide (the Cu^{II} complex of bathocuproine disulfonic acid and the Fe^{II} complex of diethylenetriaminepentaacetic acid, respectively) for comparison, we have been able to deduce, from the relative concentrations of the three radical adducts, that the Cu^{II} complexes tested each reduce *tert*-butylhydroperoxide directly to the *tert*-butoxyl radical. These findings suggest that a previously overlooked reaction, namely the direct reduction of preformed lipid hydroperoxides to alkoxyl radicals by Cu^{II}, may be responsible for the initiation of lipid peroxidation by Cu^{II} ions.

Introduction

Copper(II) ions are widely used to initiate lipid peroxidation in model systems, particularly in the study of low density lipoprotein (LDL) oxidation.[1−5] In the presence of a suitable reducing agent for Cu^{II}, such as ascorbic acid or α-tocopherol, peroxidation is believed to involve the reductive decomposition of preformed lipid hydroperoxides to alkoxyl radicals by the reduced metal ion (eq 1, in which ROOH is a lipid hydroperoxide and $RO^{\bullet}$ an alkoxyl radical). Peroxidation of the parent polyunsaturated fatty acid (RH) is then initiated by hydrogen atom abstraction, the process being propagated through oxygenation (eqs 2−4). Alkoxyl radicals can also undergo β-scission to carbon-centered radicals and allyloxyl radicals cyclization to oxiranylcarbinyl radicals,[6,7] both of which are also expected to initiate RH oxidation.

$$Cu^{+} + ROOH \rightarrow Cu^{2+} + RO^{\bullet} + OH^{-} \quad (1)$$

$$RO^{\bullet} + RH \rightarrow ROH + R^{\bullet} \quad (2)$$

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet} \quad (3)$$

$$ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet} \quad (4)$$

Various mechanisms have been proposed to account for the ability of Cu^{II} to induce lipid peroxidation in the absence of preformed lipid hydroperoxides. For example, it has been proposed that peroxidation can be initiated by the α-tocopheroxyl radical, which is generated in the reaction between Cu^{II} and α-tocopherol.[8−10] Alternatively, a role has been suggested for the highly reactive hydroxyl radical ($^{\bullet}OH$), which is generated through the sequential reduction of molecular oxygen by Cu^{I}, generated in the reaction between Cu^{II} and α-tocopherol.[5,11] Copper(II) ions can also induce lipid peroxi-

(1) Esterbauer, H.; Gebicki, J.; Puhl, H.; Jürgens, G. *Free Radical Biol. Med.* **1992**, *13*, 341−390.
(2) Maiorino, M.; Zamburlini, A.; Roveri, A.; Ursini, F. *Free Radical Biol. Med.* **1995**, *18*, 67−74.
(3) Lynch, S. M.; Frei, B. *J. Biol. Chem.* **1995**, *270*, 5158−5163.
(4) Abuja, P. M.; Albertini, R.; Esterbauer, H. *Chem. Res. Toxicol.* **1997**, *10*, 644−651.
(5) Burkitt, M. J. *Arch. Biochem. Biophys.* **2001**, *394*, 117−135.
(6) Porter, N. A. *Methods Enzymol.* **1984**, *105*, 273−282.
(7) Grossi, L.; Strazzari, S.; Gilbert, B. C.; Whitwood, A. C. *J. Org. Chem.* **1998**, *63*, 8366−8372.
(8) Bowry, V. W.; Stocker, R. *J. Am. Chem. Soc.* **1993**, *115*, 6029−6044.
(9) Iwatsuki, M.; Niki, E.; Stone, D.; Darley-Usmar, V. M. *FEBS Lett.* **1995**, *360*, 271−276.
(10) Neužil, J.; Thomas, S. R.; Stocker, R. *Free Radical Biol. Med.* **1997**, *22*, 57−71.
(11) Burkitt, M. J.; Milne, L. *FEBS Lett.* **1996**, *379*, 51−54.

10.1021/ja034416z CCC: $25.00

dation in the absence of an additional reducing agent. It has often been assumed that this involves the reduction of Cu^{II} by a preformed lipid hydroperoxide (eq 5), resulting in the generation of a peroxyl radical ($ROO^{\bullet}$), which may then initiate further cycles of peroxidation (eq 4).[2,4,12,13]

$$Cu^{2+} + ROOH \rightarrow Cu^{+} + ROO^{\bullet} + H^{+} \qquad (5)$$

However, this reaction is thermodynamically unfavorable, at least for Cu^{II} complexes of biological relevance, which are generally only mild oxidants (the $E^{\circ\prime}$ for the Cu^{II}/Cu^{I} couple is typically 0.15 V at pH 7).[1,5,12,14] Although the Cu^{II} complex of bathocuproine disulfonate (BCDS) has been demonstrated to undergo reduction by a fatty acid hydroperoxide,[15] this is of limited physiological relevance because copper chelation by BCDS results in an unusually high reduction potential for the Cu^{II}/Cu^{I} couple (0.615 V in the pH range 4−7).[5,16]

An alternative explanation for the ability of Cu^{II} to induce lipid peroxidation in the presence of a lipid hydroperoxide, but not a reducing agent, involves the oxidation of Cu^{II} by the hydroperoxide, forming Cu^{III} and an alkoxyl radical (eq 6).

$$Cu^{2+} + ROOH \rightarrow Cu^{3+} + RO^{\bullet} + OH^{-} \qquad (6)$$

Several copper(III) complexes have been prepared, and their redox properties studied using cyclic voltammetry.[17−19] In the LDL particle, copper binding is believed to occur primarily at the apoprotein B 100 moiety.[20] Therefore, it may be particularly relevant that the trivalent oxidation state of the metal in copper−peptide complexes is known to be stabilized by deprotonation, resulting in a lowering of the reduction potential for the Cu^{III}/Cu^{II} couple (values for the representative complexes of Gly-Gly-His, Gly-Gly-His-Gly, and Asp-Ala-His-Lys are 0.98, 1.02, and 0.96 V, respectively).[17] Since the reduction potential for $ROOH,H^{+}/RO^{\bullet},H_2O$ couples is around 2.0 V at pH 7.0,[21] lipid hydroperoxides should be able to oxidize Cu^{II}−peptide complexes, with the formation of Cu^{III} and the corresponding alkoxyl radical (eq 6). Thermodynamically, this reaction would appear to be more favorable than that involving the reduction of Cu^{II} by ROOH (eq 5).

In the present study, we have investigated using the electron paramagnetic resonance (EPR) spin-trapping technique the reactions of various of Cu^{II} complexes (including those of simple peptides) with a model alkyl hydroperoxide, *tert*-butylhydroperoxide (^{t}BuOOH). Our findings provide evidence that typical Cu^{II} complexes reduce alkyl hydroperoxides to alkoxyl radicals rather than generate peroxyl radicals via oxidation, suggesting that the species responsible for the initiation of lipid peroxidation in the absence of a reductant (e.g., in liposomes or LDL deficient in α-tocopherol) is the alkoxyl radical. Since it has been estimated that the reduction potential of the $L^{\bullet},H^{+}/LH$ couple (where L is a bisallylic methylene group in a polyunsaturated fatty acid) is 0.6 V at pH 7.0,[21] Cu^{III}−peptide complexes should be capable of hydrogen atom abstraction from membrane fatty acids. Thus, both the lipid alkoxyl radical and Cu^{III} generated according to eq 6 must be viewed as potential initiators of lipid peroxidation.

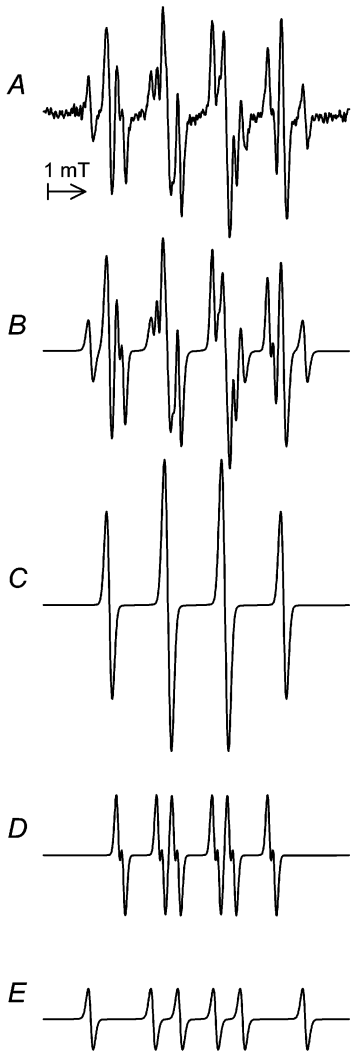


Figure 1. EPR spectrum obtained following the reaction of 1 mM $CuCl_2$ with 20 mM ^{t}BuOOH in 0.1 M Tris buffer (pH 7.4) containing 50 mM DMPO. (A) Experimental spectrum; (B) computer simulation of spectrum A with the following components: (C) DMPO/$^{\bullet}$O^{t}Bu [a(N) = 1.49 mT, a(β-H) = 1.57 mT] (47.0% relative area); (D) DMPO/$^{\bullet}$OMe [a(N) = 1.45 mT, a(β-H) = 1.05 mT, a(γ-H) = 0.13 mT] (38.0%); and (E) DMPO/$^{\bullet}$Me [a(N) = 1.63 mT, a(β-H) = 2.34 mT] (15.0%).

Results and Discussion

The EPR spectrum obtained following the addition of Cu^{II} to ^{t}BuOOH in the presence of the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) consisted of signals from at least three trapped radicals, which were identified on the basis of their hyperfine coupling constants (Figure 1). The detection of the *tert*-butoxyl (DMPO/$^{\bullet}$O^{t}Bu) and methyl (DMPO/$^{\bullet}$Me) radical adducts suggests that the peroxide has undergone a single-

(12) Yoshida, Y.; Furuta, S.; Niki, E. *Biochim. Biophys. Acta* **1993**, *1210*, 81−88.
(13) Yoshida, Y.; Tsuchiya, J.; Niki, E. *Biochim. Biophys. Acta* **1994**, *1200*, 85−92.
(14) Cadenas, E.; Sies, H. *Free Rad. Res.* **1998**, *28*, 601−609.
(15) Patel, R. P.; Svistunenko, D.; Wilson, M. T.; Darley-Usmar, V. M. *Biochem. J.* **1997**, *322*, 425−433.
(16) Sayre, L. M. *Science* **1996**, *274*, 1933−1934.
(17) Bossu, F. P.; Chellappa, K. L.; Margerum, D. W. *J. Am. Chem. Soc.* **1977**, *99*, 2195−2203.
(18) Lappin, A. G.; Youngblood, M. P.; Margerum, D. W. *Inorg. Chem.* **1980**, *19*, 407−413.
(19) Lamour, E.; Routier, S.; Bernier, J.-L.; Catteau, J.-P.; Bailly, C.; Vezin, H. *J. Am. Chem. Soc.* **1999**, *121*, 1862−1869.
(20) Chen, K.; Frei, B. *Redox Rep.* **1997**, *3*, 175−181.
(21) Koppenol, W. H. *FEBS Lett.* **1990**, *264*, 165−167.

electron reduction, followed by β-scission (eqs 7 and 8, where $k_8 = 1.5 \times 10^6\ s^{-1}$).[22]

$$(CH_3)_3COOH + e^- \rightarrow (CH_3)_3CO^\bullet + OH^- \quad (7)$$

$$(CH_3)_3CO^\bullet \rightarrow {}^\bullet CH_3 + (CH_3)_2C{=}O \quad (8)$$

In principle, the electron needed could be provided by either Cu^{II} or Cu^{I} (eqs 9 and 10).

$$Cu^{2+} + (CH_3)_3COOH \rightarrow Cu^{3+} + (CH_3)_3CO^\bullet + OH^- \quad (9)$$

$$Cu^{+} + (CH_3)_3COOH \rightarrow Cu^{2+} + (CH_3)_3CO^\bullet + OH^- \quad (10)$$

Reduction of the peroxide by Cu^{I} would require the initial reduction of the metal ion, being accompanied by generation of the *tert*-butylperoxyl radical (eq 11).

$$Cu^{2+} + (CH_3)_3COOH \rightarrow Cu^{+} + (CH_3)_3COO^\bullet + H^+ \quad (11)$$

Although the spectrum shown in Figure 1A contains a 12-line signal (component D) with hyperfine coupling constants that correspond to those assigned previously to the *tert*-butylperoxyl adduct (DMPO/•OO^{t}Bu),[22−24] recent studies by Dikalov and Mason have indicated that this spectral assignment is incorrect.[25] They proposed that the DMPO/•OO^{t}Bu adduct is extremely unstable, undergoing rapid decomposition via a series of reactions that lead to the formation of the methoxyl adduct (DMPO/•OMe), the species to which they have assigned the 12-line signal. Our own recent studies, in which we directly observed the rapid decay ($t_{1/2} < 0.1$ s) of the DMPO/•OO^{t}Bu adduct under fast-flow conditions, support this proposal.[26]

To check the stability of the spin adducts, spectra were also recorded after prolonged reaction periods of up 30 min (data not shown). Although a slight increase in the intensities of the DMPO/•O^{t}Bu and DMPO/•OMe adducts relative to DMPO/•Me was noted, none of the signals decayed over this period. Indeed, an overall increase in signal intensity was noted, which we attribute to redox cycling of the metal ion by excess peroxide.

The initial step in the decomposition of DMPO/•OO^{t}Bu involves the release of free *tert*-butoxyl radicals, which undergo β-scission followed by oxygen addition (eqs 8 and 12). The resultant methylperoxyl radicals are then converted to methoxyl radicals by at least two possible mechanisms.[25] According to the first mechanism, two •OOMe radicals combine to give a tetraoxide (eq 13), which decomposes with the release of oxygen and either two methoxyl radicals (eq 14a) or methanol and formaldehyde (eq 14b, the Russell mechanism). Alternatively, the methoxyl radical can be generated by decomposition of the unstable DMPO/•OOMe adduct, resulting from the initial trapping of the methylperoxyl radical.[25] In either case, reaction with DMPO yields the DMPO/•OMe adduct (eq 15).

$$(CH_3)_3CO^\bullet \rightarrow {}^\bullet CH_3 + (CH_3)_2C{=}O \quad (8)$$

$$O_2 + {}^\bullet CH_3 \rightarrow {}^\bullet OOCH_3 \quad (12)$$

$$2\ {}^\bullet OOCH_3 \rightarrow CH_3OOOOCH_3 \quad (13)$$

$$CH_3OOOOCH_3 \rightarrow 2\ {}^\bullet OCH_3 + O_2 \quad (14a)$$

$$CH_3OOOOCH_3 \rightarrow CH_2O + CH_3OH + O_2 \quad (14b)$$

$$DMPO + {}^\bullet OCH_3 \rightarrow DMPO/{}^\bullet OCH_3 \quad (15)$$

Therefore, although the detection of DMPO/•O^{t}Bu, DMPO/•Me, and DMPO/•OMe is consistent with the initial oxidation of ^{t}BuOOH by Cu^{II} (eq 11), the same radical adducts could also arise following the initial *reduction* of ^{t}BuOOH by Cu^{II} (eq 9). A similar spectrum was observed when the reaction was performed in the presence of the peptide Gly-Gly-Gly, but only very weak signals were obtained when using Gly-Gly-His (vide infra). Spectra recorded over a 100-mT field confirmed the chelation of Cu^{II} by both peptides (not shown). In the absence of the peptides, it is assumed that the metal is chelated by the Tris buffer.

In principle, it should also be possible to obtain further insight into the above reactions by using DMPO over a range of concentrations. At high concentrations, the spin trap should intercept the initially formed radicals before they can undergo secondary reactions, whereas, at low concentrations of spin trap, the initially formed radicals will undergo conversion to secondary species before being trapped. At a DMPO concentration of 400 mM, the spectra in all cases were dominated by the signal from the DMPO/•O^{t}Bu adduct (Figures 2A, 3A, and 4A). In addition, the spectrum obtained in the presence of Gly-Gly-Gly contained weak signals from an additional species, which is most likely an adduct of a radical derived from the peptide (Figure 3A).[27] In the absence of the peptides, lowering the spin-trap concentration resulted in a marked increase in the relative intensity of the DMPO/•OMe adduct, which became the dominant species at 20 mM DMPO. A modest increase in the intensity of the DMPO/•Me signal was also evident (Figure 2B−D). Broadly similar (though less marked) responses were seen in the presence of Gly-Gly-Gly (Figure 3B and C). The signals obtained with this peptide and 20 mM DMPO were too weak to be discerned (not shown). With Gly-Gly-His, discernible signals were obtained only at high DMPO concentrations (Figure 4A and B).

The above findings indicate that the •O^{t}Bu radical is formed very early during the interaction of all three Cu^{II} complexes with ^{t}BuOOH. At low concentrations of DMPO, however, it is apparent that •O^{t}Bu scavenging by the spin trap is less efficient, allowing the radical to decompose via secondary reactions that lead to the generation of •Me and •OMe. Although it is clear that •O^{t}Bu is the first species to form a *stable* adduct with DMPO, the mechanism by which the radical is generated remains unresolved: either it is generated via the direct, one-electron reduction of ^{t}BuOOH by Cu^{II} (eq 9) or it is released upon decomposition of the unstable DMPO/•OO^{t}Bu adduct. In the latter case, the peroxide would have to undergo an initial one-electron oxidation by Cu^{II} (eq 11).

(22) Bors, W.; Michel, C.; Stettmaier, K. *J. Chem. Soc., Perkin Trans. 2* **1992**, 1513−1517.
(23) Thornalley, P. J.; Trotta, R. J.; Stern, A. *Biochim. Biophys. Acta* **1983**, *759*, 16−22.
(24) Timmins, G. S.; Davies, M. J. *Carcinogenesis* **1993**, *14*, 1615−1620.
(25) Dikalov, S. I.; Mason, R. P. *Free Radical Biol. Med.* **1999**, *27*, 864−872.
(26) Jones, C. M.; Burkitt, M. J. *J. Chem. Soc., Perkin Trans. 2* **2002**, 2044−2051.
(27) Ettinger, K. V.; Forrester, A. R.; Hunter, C. H. *Can. J. Chem.* **1982**, *60*, 1549−1559.

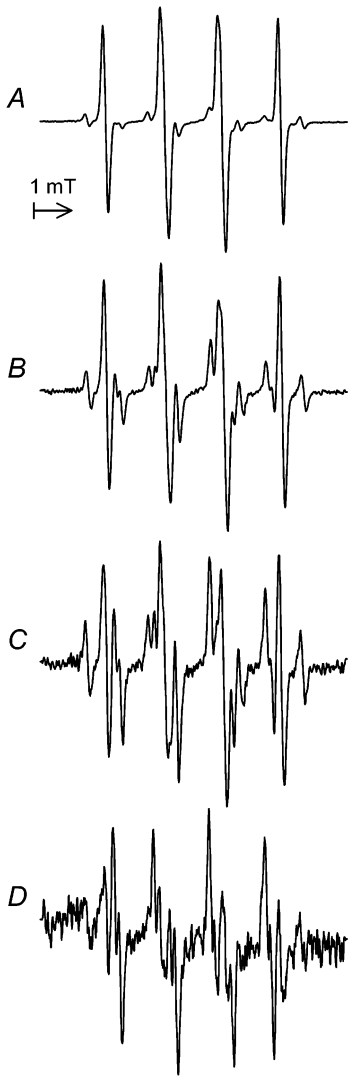


Figure 2. Spectra obtained following the reaction of 1 mM $CuCl_2$ with 20 mM ^{t}BuOOH in 0.1 M Tris buffer (pH 7.4) containing DMPO at the following concentrations: (A) 400 mM, (B) 100 mM, (C) 50 mM, and (D) 20 mM. The spectra B−D are shown expanded in the *y*-axis 6-, 20-, and 50-fold, respectively, relative to spectrum A.

To explore the possibility that these reactions may be distinguished, complementary experiments were performed using metal complexes having well-defined redox properties: the Cu^{II} complex of bathocuproine disulfonate, $Cu^{II}(BCDS)_2$, and the Fe^{II} complex of diethylenediaminepentaacetate, Fe^{II}-DTPA. The 2,9-dimethyl substituents on the phenanthroline moiety of BCDS sterically prevent distortion of the $Cu^{II}(BCDS)_2$ complex to the energetically favored tetragonal/planar coordination usually seen in d^9 complexes. Instead, a Cu^{I}-preferring tetrahedral geometry is forced upon the complex, which accounts for the ease with which $Cu^{II}(BCDS)_2$ is reduced to the stable $Cu^{I}(BCDS)_2$ complex.[16] Indeed, the facile reduction of $Cu^{II}(BCDS)_2$ by an alkylhydroperoxide (13-hydroperoxyoctadecadienoic acid) has already been demonstrated, by both electronic absorption and EPR spectroscopies.[15] Therefore, the $Cu^{II}(BCDS)_2$ complex was selected for use as a positive control for the investigation of the one-electron oxidation of ^{t}BuOOH by Cu^{II} (eq 11).

The spectra obtained following the addition of Cu^{II} to ^{t}BuOOH in the presence of BCDS always contained a strong

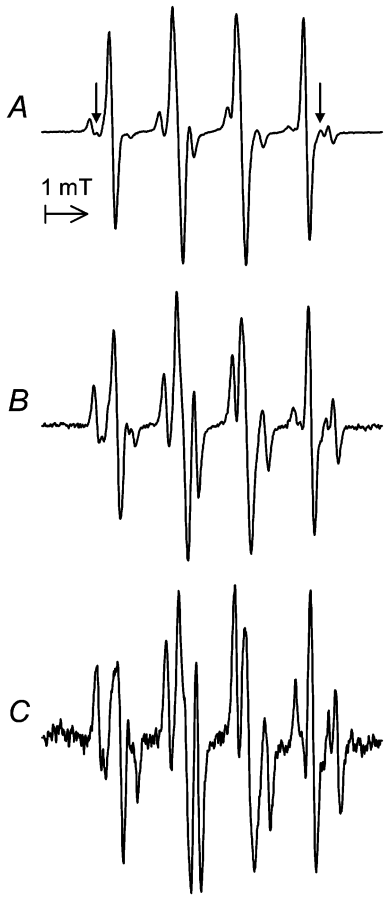


Figure 3. Spectra obtained following the reaction of 1 mM $CuCl_2$ with 20 mM ^{t}BuOOH in 0.1 M Tris buffer (pH 7.4) containing 4 mM Gly-Gly-Gly and DMPO at the following concentrations: (A) 400 mM, (B) 100 mM, and (C) 50 mM. The spectra B and C are shown expanded in the *y*-axis 5- and 25-fold, respectively, relative to spectrum A. The arrows in spectrum A indicate the outermost lines of an adduct believed to result from the trapping of a radical derived from the peptide.

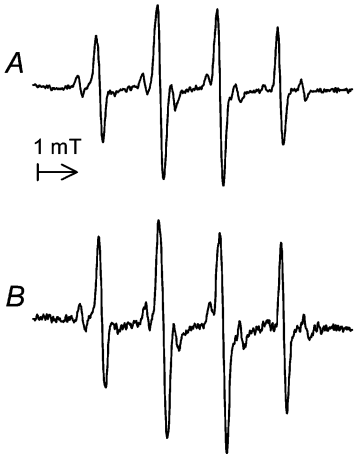


Figure 4. Spectra obtained following the reaction of 1 mM $CuCl_2$ with 20 mM ^{t}BuOOH in 0.1 M Tris buffer (pH 7.4) containing 4 mM Gly-Gly-His and either 400 or 100 mM DMPO (A and B, respectively). Spectrum B is shown expanded in the *y*-axis 2-fold relative to spectrum A.

12-line signal from the DMPO/$^\bullet$OMe adduct, even at very high DMPO concentrations (Figure 5). The DMPO/$^\bullet$O^{t}Bu adduct was also present, but DMPO/$^\bullet$Me was never detected in this reaction system. Lowering the concentration of DMPO resulted in a lowering of the overall signal intensity, which was associated

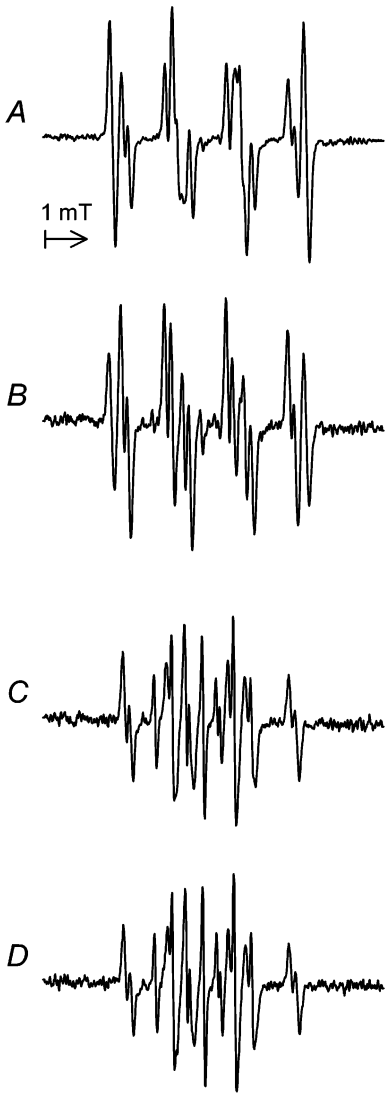


Figure 5. Spectra obtained following the reaction of 1 mM $CuCl_2$ with 20 mM ^{t}BuOOH in 0.1 M Tris buffer (pH 7.4) containing 4 mM BCDS and DMPO at the following concentrations: (A) 800 mM, (B) 400 mM, (C) 100 mM, and (D) 50 mM. Spectra B−D are shown expanded in the *y*-axis 2-fold relative to spectrum A. Spectra C and D contain a prominent signal from the DMPO oxidation product DMPOX [a(N) = 0.72 mT, $a(\beta$-H)(2) = 0.41 mT].

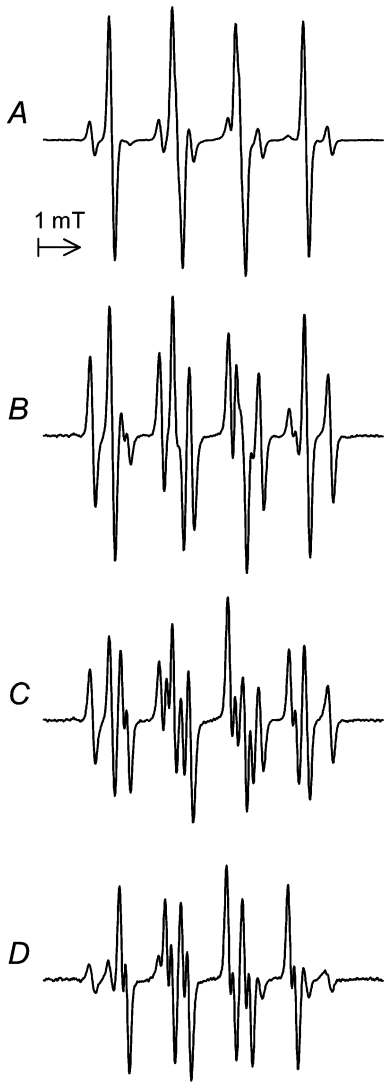


Figure 6. Spectra obtained following the reaction of 0.4 mM $Fe(NH_4)_2(SO_4)_2$ with 0.4 mM ^{t}BuOOH in 0.1 M Tris buffer (pH 7.4) containing 0.8 mM DTPA and DMPO at the following concentrations: (A) 400 mM, (B) 100 mM, (C) 50 mM, and (D) 20 mM. Spectra B−D are shown expanded in the *y*-axis 4-, 4-, and 5-fold, respectively, relative to spectrum A.

with the loss of the DMPO/$^{\bullet}$O^{t}Bu signal (but not DMPO/$^{\bullet}$OMe) and the appearance of the DMPO oxidation product 5,5-dimethyl-1-pyrrolidone-2-oxyl (DMPOX)[26,28] (Figure 5B−D).

The ferrous complex of DTPA was used as a positive control for the one-electron reduction of ^{t}BuOOH by a metal complex. Due to the low reduction potential of the Fe^{III}DTPA/ Fe^{II}DTPA couple (0.03 V at pH 7),[29] Fe^{II}DTPA undergoes facile one-electron oxidation by ^{t}BuOOH, giving the $^{\bullet}$O^{t}Bu radical (eq 16).[30]

$$Fe^{2+} + (CH_3)_3COOH \rightarrow Fe^{3+} + (CH_3)_3CO^{\bullet} + OH^- \quad (16)$$

The addition of Fe^{II} to ^{t}BuOOH in the presence of DTPA and 400 mM DMPO resulted in the observation of a spectrum dominated by the DMPO/$^{\bullet}$O^{t}Bu adduct. In contrast to the corresponding experiment using $Cu^{II}(BCDS)_2$, a prominent signal from the DMPO/$^{\bullet}$Me adduct was also present and only a very weak signal from DMPO/$^{\bullet}$OMe was apparent (Figure 6A). Upon lowering the concentration of the spin trap, we observed a marked increase in the relative intensity of the signal from DMPO/$^{\bullet}$OMe (Figure 6B−D). Although Fe^{III}DTPA is relatively difficult to reduce, care was taken to avoid the possibility of any reaction between the Fe^{III}DTPA produced (eq 16) and ^{t}BuOOH. Thus, equimolar concentrations of Fe^{II} and ^{t}BuOOH were used in the above reactions to ensure removal of all the peroxide in the initial reaction with Fe^{II}DTPA. Indeed, when Fe^{III}DTPA (at the concentration of Fe^{II}DTPA used above) was added directly to ^{t}BuOOH in the presence of 100 mM DMPO, no EPR signals were detectable (data not shown). Although a very weak signal from the hydroxyl radical adduct of DMPO was detected when the DMPO concentration was increased to 400 mM (not shown), this was < 3% the intensity of the DMPO/$^{\bullet}$O^{t}Bu adduct detected in the corresponding reaction with Fe^{II}DTPA (Figure 6A) and probably results from the nucleo-

(28) Hill, H. A. O.; Thornalley, P. J. *Inorg. Chim. Acta* **1982**, *67*, L35−L36.
(29) Koppenol, W. H.; Butler, J. *Adv. Free Radical Biol. Med.* **1985**, *1*, 91−131.
(30) Karlsson, J.; Emgård, M.; Brundin, P.; Burkitt, M. J. *J. Neurochem.* **2000**, *75*, 141−150.

philic addition of water to the spin trap.[31] Therefore, it was concluded that the radical adducts responsible for the spectra shown in Figure 6 all result from the trapping of radicals generated in a reaction series initiated by the one-electron reduction of ^{t}BuOOH by Fe^{II}DTPA (eq 16).

A striking difference between the spectra obtained from the $Cu^{II}(BCDS)_2$/^{t}BuOOH and Fe^{II}DTPA/^{t}BuOOH reactions is the prominence of the DMPO/$^{\bullet}$OMe signal and, in particular, the absence of the DMPO/$^{\bullet}$Me signal in the spectra from the $Cu^{II}(BCDS)_2$/^{t}BuOOH reaction, regardless of DMPO concentration (Figures 5 and 6). A fraction of the *tert*-butylperoxyl radicals generated upon the oxidation of ^{t}BuOOH by $Cu^{II}(BCDS)_2$ (eq 11) will be trapped by DMPO, forming the unstable DMPO/$^{\bullet}$OO^{t}Bu adduct, which will release the *tert*-butoxyl radical upon rapid decay. We have determined the second-order rate constant for the trapping of the *tert*-butylperoxyl radical by DMPO to be $\approx 30\ M^{-1}\ s^{-1}$.[26] It is, therefore, expected that a significant proportion of these radicals will evade spin trapping and undergo bimolecular decay (eq 17), for which a rate constant ($2k_{17}$) of $2 \times 10^4\ M^{-1}\ s^{-1}$ has been reported.[32]

$$2\ (CH_3)_3COO^{\bullet} \rightarrow \text{products} \qquad (17)$$

$$2\ (CH_3)_3COO^{\bullet} \rightarrow (CH_3)_3COOC(CH_3)_3 + O_2 \qquad (17a)$$

$$2\ (CH_3)_3COO^{\bullet} \rightarrow 2\ (CH_3)_3CO^{\bullet} + O_2 \qquad (17b)$$

The *tert*-butoxyl radicals generated through this reaction (eq 17b), and also those released during decomposition of the DMPO/$^{\bullet}$OO^{t}Bu adduct, will either be trapped by DMPO, forming DMPO/$^{\bullet}$O^{t}Bu, or undergo β-scission to the methyl radical (eq 8), depending on the concentration of DMPO.[26] It is relevant, therefore, that a prominent signal from DMPO/$^{\bullet}$O^{t}Bu was detected following the oxidation of ^{t}BuOOH by $Cu^{II}(BCDS)_2$ in the presence of 800 and 400 mM DMPO but not at the lower concentrations of the spin trap (Figure 5). The methyl radicals generated by the β-scission of the untrapped *tert*-butoxyl radicals could either be trapped, forming DMPO/$^{\bullet}$Me, or combine with molecular oxygen to give methylperoxyl radicals ($^{\bullet}$OOMe) (eq 12).

$$O_2 + {}^{\bullet}CH_3 \rightarrow {}^{\bullet}OOCH_3 \qquad (12)$$

The fact that no methyl radicals were trapped following the oxidation of ^{t}BuOOH by $Cu^{II}(BCDS)_2$, irrespective of the DMPO concentration (Figure 5), suggests that the oxygen addition reaction is faster. This is supported by the observation of prominent signals from the DMPO/$^{\bullet}$OMe adduct. The finding that appreciable amounts of the methyl radical are trapped when its precursor, the *tert*-butoxyl radical, is generated directly via the one-electron reduction of ^{t}BuOOH by Fe^{II}DTPA (Figure 6) suggests that the oxygen released during the bimolecular decay of peroxyl radicals[33] (eqs 17a and 17b) is responsible for the increased rate of methyl radical oxygenation following the oxidation of ^{t}BuOOH by $Cu^{II}(BCDS)_2$.

To investigate further the origin of the oxygen incorporated into the DMPO/$^{\bullet}$OMe adduct in both the $Cu^{II}(BCDS)_2$/^{t}BuOOH and Fe^{II}DTPA/^{t}BuOOH reaction systems, additional experiments were performed using deoxygenated solutions. The removal of dissolved oxygen from the $Cu^{II}(BCDS)_2$/^{t}BuOOH reaction in the presence of 400 mM DMPO had essentially no effect on the resultant spectrum, other than to sharpen the spectral features due to the prevention of paramagnetic line broadening by O_2 (Figure 7). The same effect was seen using 20, 50, 100, and 800 mM DMPO (data not shown).

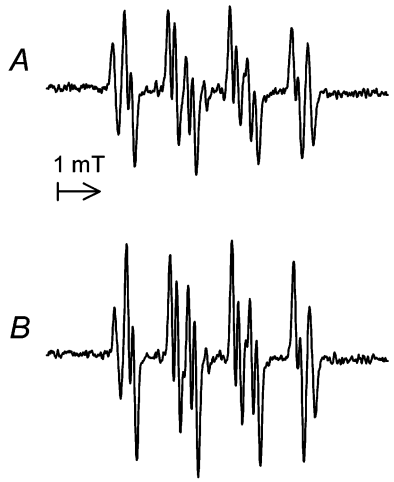


Figure 7. Effect of deoxygenation on the signals obtained following the reaction of 1 mM $CuCl_2$ with 20 mM ^{t}BuOOH in 0.1 M Tris buffer (pH 7.4) containing 4 mM BCDS and 400 mM DMPO. (A) Without deoxygenation, where simulation showed that the DMPO/$^{\bullet}$O^{t}Bu and DMPO/$^{\bullet}$OMe adducts contributed 30% and 70%, respectively, to the total spectral area; (B) with deoxygenation, where the DMPO/$^{\bullet}$O^{t}Bu and DMPO/$^{\bullet}$OMe adducts contributed 25% and 75%, respectively, to the spectrum (for hyperfine coupling constants, see Figure 1). Both spectra are shown at the same scale.

In contrast, the omission of oxygen from the Fe^{II}DTPA/^{t}BuOOH reaction (20 mM DMPO) resulted in the almost complete removal of the DMPO/$^{\bullet}$OMe signal and a very marked enhancement of the DMPO/$^{\bullet}$Me signal (Figure 8). The spectrum shown in Figure 8B also contains a weak signal from a fourth species, of which the outermost lines are marked by arrows. Although these additional lines could result from carbon-13 coupling in the methyl adduct, the natural abundance of this isotope (1.1%)[34] is too low to account for the high ratio of, what would be, DMPO/$^{\bullet}{}^{13}CH_3$ relative to DMPO/$^{\bullet}CH_3$ (0.022, estimated by simulation). Alternatively, the outermost spectral lines could be due to the trapping of a nitrogen-centered DTPA radical, formed by oxidation at a tertiary nitrogen, though such a species would be expected to undergo a rapid 1,2-shift, thereby losing the nitrogen-15 coupling that would be needed to account for the wide EPR spectrum of its DMPO adduct. Whatever the identity of this minor species ($\sim$2% relative area of the total spectrum), the fact remains that deoxygenation prevents formation of the DMPO/$^{\bullet}$OMe adduct and causes a marked enhancement in the intensity of the DMPO/$^{\bullet}$Me signal in this system. Similar effects were observed at higher concentrations of DMPO (not shown). These observations indicate that the DMPO/$^{\bullet}$OMe adduct is generated via different routes in the two reaction systems. In the Fe^{II}DTPA/^{t}BuOOH system, it is obvious that this involves the addition of dissolved molecular oxygen to the methyl radical, generated through β-scission of the $^{\bullet}$O^{t}Bu radical. In contrast, the finding that the DMPO/$^{\bullet}$OMe and DMPO/$^{\bullet}$Me adducts are insensitive to the presence of dissolved oxygen in

(31) Burkitt, M. J. *Free Radical Res. Commun.* **1993**, *18*, 43−57.
(32) Bennett, J. E. *J. Chem. Soc., Faraday Trans.* **1990**, *86*, 3247−3252.
(33) Thomas, J. R. *J. Am. Chem. Soc.* **1965**, *87*, 3935−3940.
(34) Norman, R. O. C.; Gilbert, B. C. *Adv. Phys. Org. Chem.* **1967**, *5*, 53−119.

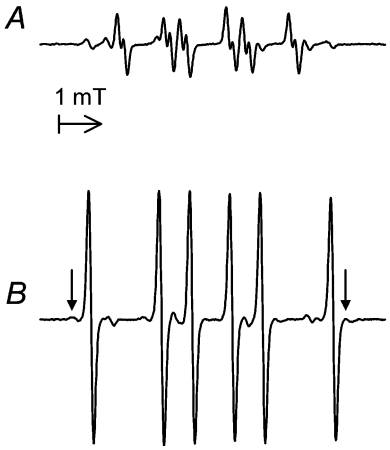


Figure 8. Effect of deoxygenation on the signals obtained following the reaction of 0.4 mM $Fe(NH_4)_2(SO_4)_2$ with 0.4 mM ^{t}BuOOH in 0.1 M Tris buffer (pH 7.4) containing 0.8 mM DTPA and 20 mM DMPO. (A) Without deoxygenation, where simulation showed that the DMPO/$^{\bullet}$O^{t}Bu, DMPO/$^{\bullet}$Me, and DMPO/$^{\bullet}$OMe adducts contributed 13%, 11%, and 76%, respectively, to the total spectral area; (B) with deoxygenation, where the DMPO/$^{\bullet}$O^{t}Bu, DMPO/$^{\bullet}$Me, and DMPO/$^{\bullet}$OMe adducts contributed 7%, 90%, and 1%, respectively, to the spectrum (for hyperfine coupling constants, see Figure 1). There is an additional species present in spectrum B (2% area), of which the outer lines are indicated by arrows. This species is tentatively assigned to the carbon-13 containing DMPO/$^{\bullet}$Me adduct [$a(^{13}C) \approx 0.7$ mT] and/or a DTPA-derived radical adduct (see text for details). Both spectra are shown at the same scale.

the $Cu^{II}(BCDS)_2$/^{t}BuOOH system indicates that the oxygen required for the generation of $^{\bullet}$OMe from $^{\bullet}$Me is generated internally, from the peroxide, which we propose originates from the bimolecular decay of free $^{\bullet}$OO^{t}Bu radicals (eqs 17a and 17b).

On the above basis, we propose that the absence of a signal from the DMPO/$^{\bullet}$Me adduct during the metal-catalyzed decomposition of ^{t}BuOOH is diagnostic of the initial one-electron oxidation of the peroxide to the *tert*-butylperoxyl radical. Similarly, the detection of the DMPO/$^{\bullet}$Me adduct is diagnostic of the initial one-electron reduction of the peroxide. Applying these criteria to the analysis of the spectra obtained following the reactions of the peptide (and Tris) complexes of Cu^{II} with ^{t}BuOOH, we find it immediately apparent that the peroxide has undergone reduction to the *tert*-butoxyl radical (eq 9) in all cases: had peroxyl radicals been generated through oxidation of the peroxide, then the oxygen released during their bimolecular decay would have added rapidly to any methyl radicals, resulting in the failure to detect DMPO/$^{\bullet}$Me, which was clearly not the case (Figures 2−4). To confirm this proposal, the effects of deoxygenation were examined. As shown in Figure 9, deoxygenation had the effect of suppressing the signal from DMPO/$^{\bullet}$OMe while enhancing that from DMPO/$^{\bullet}$Me, in the reaction of Tris-complexed Cu^{II} with ^{t}BuOOH (50 mM DMPO), as observed in the corresponding reaction using Fe^{II}DTPA, thereby confirming the proposal that ^{t}BuOOH is reduced directly to $^{\bullet}$O^{t}Bu by Cu^{II}. Similar effects were observed using DMPO at other concentrations (data not shown). The finding that the DMPO/$^{\bullet}$OMe signal was not completely removed by deoxygenation (Figure 9B) suggests that some peroxyl generation can still occur in this system, presumably via oxidation of the excess ^{t}BuOOH by *tert*-butoxyl radicals (eqs 9 and 18). In contrast, the metal and peroxide were present in equimolar amounts in the Fe^{II}DTPA system, thereby accounting for the complete suppression of DMPO/$^{\bullet}$OMe generation upon deoxygenation.

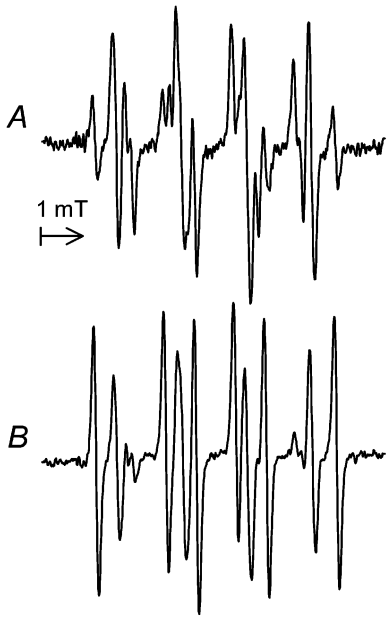


Figure 9. Effect of deoxygenation on the signals obtained following the reaction of 1 mM $CuCl_2$ with 20 mM ^{t}BuOOH in 0.1 M Tris buffer (pH 7.4) containing 50 mM DMPO. (A) Without deoxygenation, where simulation showed that the DMPO/$^{\bullet}$O^{t}Bu, DMPO/$^{\bullet}$Me, and DMPO/$^{\bullet}$OMe adducts contributed 47%, 15%, and 38%, respectively, to the total spectral area; (B) with deoxygenation, where the DMPO/$^{\bullet}$O^{t}Bu, DMPO/$^{\bullet}$Me, and DMPO/$^{\bullet}$OMe adducts contributed 36%, 51%, and 13%, respectively, to the spectrum (for hyperfine coupling constants, see Figure 1). Both spectra are shown at the same scale.

$$Cu^{2+} + (CH_3)_3COOH \rightarrow Cu^{3+} + (CH_3)_3CO^{\bullet} + OH^- \quad (9)$$

$$(CH_3)_3CO^{\bullet} + (CH_3)_3COOH \rightarrow (CH_3)_3COO^{\bullet} + (CH_3)_3COH \quad (18)$$

A similar experiment showed that this also occurs when the metal is complexed to Gly-Gly-Gly. In this reaction system, however, the yield of DMPO/$^{\bullet}$OMe was always relatively low, but it was clear that deoxygenation prevents removal of the methyl radical (data not shown). Similar effects were observed when using the Cu^{II} complex of Gly-Gly-His, though here the signal-to-noise ratio was much poorer (not shown).

Conclusions

Using metal chelates that undergo well-defined reactions with alkyl hydroperoxides, we have shown, despite the inherent difficulties associated with the detection of peroxyl radicals by the technique, that EPR spin trapping can be used to distinguish between the one-electron reduction and oxidation of a model alkylhydroperoxide by Cu^{II} and Fe^{II} chelates. Specifically, we have shown that the detection of the DMPO methyl radical adduct is diagnostic of the one-electron reduction of *tert*-butylhydroperoxide to the *tert*-butoxyl radical. In contrast, methyl radicals originating from the initial oxidation of ^{t}BuOOH are not trapped but undergo rapid oxygenation ($k_{12} = 4.7 \times 10^9$ M^{-1} s^{-1})[22] by oxygen evolved during the bimolecular decay of the initially formed *tert*-butylperoxyl radical.[33]

Using these criteria, we have demonstrated that Cu^{II} ions, including those complexed to simple peptides, reduce ^{t}BuOOH to the *tert*-butoxyl radical (eq 9). These conclusions are supported by the findings from our recent studies on the

oxidation of ^{t}BuOOH by the cerium(IV) ion $[Ce(SO_4)_3]^{2-}$, which proceeds with a second-order rate constant of 1.3×10^4 M^{-1} s^{-1}.[26] Since the cerium(III) generated in this reaction undergoes no further redox reaction, the Ce^{IV}/^{t}BuOOH couple provides a clean method for the efficient generation of the *tert*-butylperoxyl radical, which we observed both directly and by spin trapping under continuous-flow conditions. Significantly, the DMPO methyl radical adduct was never detected in this reaction system, despite the detection of DMPO/$^{\bullet}$O^{t}Bu and DMPO/$^{\bullet}$OMe. Since the initially formed *tert*-butylperoxyl radicals were shown to decay exclusively through bimolecular decay (eq 17), this supports the conclusion that rapid oxygenation prevents the trapping of methyl radicals by DMPO. It is noteworthy that the detection of DMPOX, a three-electron oxidation product of DMPO, also appears to be diagnostic of peroxyl radical trapping. In both the $Cu^{II}(BCDS)_2$/^{t}BuOOH reaction reported here and the Ce^{IV}/^{t}BuOOH system reported earlier,[26] the generation and trapping of the *tert*-butylperoxyl radical was accompanied by the generation of DMPOX, which we have proposed is a breakdown product of the DMPO/$^{\bullet}$OO^{t}Bu adduct (as well as an oxidation product of the DMPO hydroxyl radical adduct).[26]

Previously, it had been widely assumed that Cu^{II} ions oxidize alkyl hydroperoxides to peroxyl radicals (eq 5), albeit slowly, particularly when used to initiate lipid peroxidation in liposomes and LDL particles.[2,4,12,13]

$$Cu^{2+} + ROOH \rightarrow Cu^{+} + ROO^{\bullet} + H^{+} \quad (5)$$

The thermodynamically more favorable reduction of alkyl hydroperoxides by Cu^{II} ions, demonstrated here, has been entirely overlooked in such studies, probably because trivalent copper is generally considered to be an uncommon oxidation state (eq 6). However, chelation by certain peptides has been shown to lower the reduction potential of the Cu^{III}/Cu^{II} couple sufficiently to allow access to the oxidation state using relatively mild oxidants.[17,18]

$$Cu^{2+} + ROOH \rightarrow Cu^{3+} + RO^{\bullet} + OH^{-} \quad (6)$$

By the same thermodynamic criteria, it cannot be assumed that the Cu^{III} generated in this reaction undergoes reduction back to Cu^{II} by the remaining peroxide. In the Cu^{II}/*tert*-butylhydroperoxide system, we suggest that Cu^{III} could be removed through reaction with the highly reducing α-hydroxymethyl radical,[35] formed from the methoxyl radical by a formal 1,2-H-shift (eqs 18 and 19).[36,37]

$$^{\bullet}OCH_3 \rightarrow {}^{\bullet}CH_2OH \quad (18)$$

$$Cu^{3+} + {}^{\bullet}CH_2OH \rightarrow Cu^{2+} + CH_2O + H^{+} \quad (19)$$

Alternatively, an α-hydroxy alkyl radical, which would rapidly reduce Cu^{III}, may be generated from the Tris buffer upon oxidation by the *tert*-butoxyl radical (eqs 20 and 21).

$$(CH_3)_3CO^{\bullet} + (CH_2OH)_3CNH_2 \rightarrow (CH_3)_3COH + {}^{\bullet}CH(OH)(CH_2OH)_2CNH_2 \quad (20)$$

$$Cu^{3+} + {}^{\bullet}CH(OH)(CH_2OH)_2CNH_2 \rightarrow Cu^{2+} + CHO(CH_2OH)_2CNH_2 + H^{+} \quad (21)$$

In contrast, because the reduction potential for electron abstraction from the bisallylic methylene groups of polyunsaturated fatty acids is particularly low (0.6 V at pH 7.0),[21] it is expected that, in biological membranes, Cu^{III} will directly attack the parent fatty acid (eq 22). Therefore, both the alkoxyl radical and Cu^{III} generated according to eq 6 must be considered capable of initiating membrane lipid peroxidation.

$$Cu^{3+} + RH \rightarrow Cu^{2+} + R^{\bullet} + H^{+} \quad (22)$$

In the presence of a suitable reducing agent, such as ascorbate or α-tocopherol, the reduction of Cu^{II} to Cu^{I} (which reacts rapidly with ROOH, eq 1) will occur in competition with its oxidation to Cu^{III} by ROOH.[5] However, in LDL particles, for example, reductants confined to the aqueous phase (e.g., ascorbate) may have limited access to the bound metal ion, and the α-tocopherol present in LDL (only ~6 molecules per particle[1]) is known to be rapidly depleted by copper.[9,13] We suggest, therefore, that, in addition to its clear relevance to the widespread use of Cu^{II} ions in the induction of lipid peroxidation in liposomal and micellar systems,[2,12,15,38,39] the biological relevance of ROOH reduction by Cu^{II} warrants exploration through further studies into the kinetic and compartmentalization aspects of the above reactions.

Experimental Section

Reagents. All reagents, which were of the highest grade available, were obtained from Sigma−Aldrich (Poole, Dorset, UK). *tert*-Butylhydroperoxide was purchased as a 70% aqueous solution. The stock solution of tris(hydroxymethyl)aminomethane (Tris) buffer (0.2 M, pH 7.4) and Millipore-filtered water used in all reactions were treated with chelating resin (batch method[40]) before use to remove contaminating metal ions. DMPO was purified by vacuum distillation (Kugelrohr) and stored at −80 °C.

Reaction Conditions. The basic reaction mixture, prepared in Eppendorf microcentrifuge tubes, contained 0.1 M tris(hydroxymethyl)-aminomethane, DMPO at the concentrations shown in the figure legends, 20 mM *tert*-butylhydroperoxide, and 1 mM $CuCl_2 \cdot 2H_2O$ (added last) in a final volume of 0.5 mL, pH 7.4. Where indicated, glycyl-glycyl-glycine (Gly-Gly-Gly), glycyl-glycyl-histidine (Gly-Gly-His), or bathocuproine disulfonic acid (BCDS) was included at a final concentration of 4 mM. Similar reactions were also performed using the iron(II) complex of diethylenetriaminepentaacetic acid (DTPA). This involved the final addition of an aliquot of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ from a stock solution prepared using nitrogen-purged water, to give the final reagent concentrations indicated in the legend to Figure 6. Reactions involving deoxygenated solutions were prepared directly in the EPR flat cell: before the final addition of the metal, reaction mixtures were purged with a gentle stream of N_2 gas for 15 min via a needle placed through a polypropylene bung replacing the bottom stopper of the flat cell.

EPR Spectroscopy. After thorough mixing, reactions were transferred to a quartz flat cell and spectra (X-band) recorded within 6 min

(35) Asmus, K.-D.; Möckel, H.; Henglein, A. *J. Phys. Chem.* **1973**, *77*, 1218−1221.
(36) Gilbert, B. C.; Holmes, R. G. G.; Laue, H. A. H.; Norman, R. O. C. *J. Chem. Soc., Perkin Trans. 2* **1976**, 1047−1052.
(37) Elford, P. E.; Roberts, B. P. *J. Chem. Soc., Perkin Trans. 2* **1996**, 2247−2256.
(38) Murase, H.; Moon, J.-H.; Yamauchi, R.; Kato, K.; Kunieda, T.; Yoshikawa, T.; Terao, J. *Free Radical Biol. Med.* **1998**, *24*, 217−225.
(39) Zago, M. P.; Oteiza, P. I. *Free Radical Biol. Med.* **2001**, *31*, 266−274.
(40) Buettner, G. R. *J. Biochem. Biophys. Methods* **1988**, *16*, 27−40.

using a Bruker EMX spectrometer (Bruker UK Ltd, Coventry, UK) equipped with a cylindrical cavity (HS model) and operating with the following instrument settings: modulation frequency, 100 kHz; sweep width, 8 mT; microwave power, 20 mW; modulation amplitude, 0.05 mT; sweep time, 42 s; time constant, 10.24 ms; and a suitable receiver gain. Four spectra were accumulated and added. To improve spectral resolution, the broad background signals from Cu^{II} were removed by spectral subtraction using appropriate spectra acquired in the absence of *tert*-butylhydroperoxide and DMPO. After obtaining the 8-mT spectra, wide field spectra (100-mT sweep width, 0.5-mT modulation amplitude) were routinely performed to confirm the chelation of Cu^{II} by Gly-Gly-Gly and Gly-Gly-His. The spectrometer field calibration was checked using the signal from a dilute solution of Fremy's salt [(a(N) = 1.3091 mT] and was accurate to within ±0.005 mT. Hyperfine coupling constants were determined from spectral simulations performed using software available through the Internet (http://epr.niehs.nih.gov/) and described elsewhere.[41]

Acknowledgment. The authors thank Prof. P. Wardman for helpful comments on the manuscript and Cancer Research UK for financial support (Grant No. C134/A2001).

JA034416Z

(41) Duling, D. *J. Magn. Reson., Ser. B* **1994**, *104*, 105−110.