Free Rad. Res. Comms., Vol. 9, No. 3-6, pp. 233-240
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DMPO SPIN TRAPPING IN THE PRESENCE OF Fe ION

KEISUKE MAKINO,† TAKUYA HAGIWARA, HIROMASA IMAISHI, MASATOSHI NISHI,‡ SATOSHI FUJII,§ HIROAKI OHYA§ and AKIRA MURAKAMI

Department of Polymer Science and Engineering, Faculty of Textile Science, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606, Japan
‡Department of Pharmaceutical Science, Setsunan University, Nagaotouge-cho, Hirakata, Osaka 573-01, Japan
§Department of Chemistry, Faculty of Science, Kyoto University, Kitashirakawa, Sakyo-ku, Kyoto 606, Japan

Aminoxyl radical formation from DMPO in the presence of Fe ion was studied to clarify the ambiguous ESR signals obtained by spin trapping with DMPO. It was found that when DMPO was used in a Fenton system, a Fe-DMPO complex was formed immediately. This complex was subsequently attacked by oxidative species originating from H_2O_2 and thus oxidative degradation of DMPO was induced in the Fenton system. On the other hand, in the case of M_4PO, the degradation was found to be very slow, indicating that the 3 position of DMPO was favorably attacked by the oxidative species. Some of the degradation products are probably aminoxyl radicals. This series of the degradation products are probably aminoxyl radicals. This series of reactions may compete with spin trapping and make it difficult to analyze ESR spectra obtained in the presence of Fe ion.

KEY WORDS: Spin trapping, 5,5-dimethyl-1-pyrroline-N-oxide, Fenton system, Fe complex of DMPO, artifact ESR signals, ESR.

INTRODUCTION

From a diagnostic and therapeutic point of view, interest exists concerning free radical formation in living bodies.[1,2] Spontaneous generation of these reactive species is now considered a cause of various diseases such as cancer, heart attack, and diabetes. Therefore, it is essential to detect and identify free radicals produced in biological systems. Unfortunately, however, such labile species have so short lifetimes of gratitude of less than μsec that conventional ESR technique can not be applied at biological temperature. Therefore, the spin trapping method is employed in experiments with these labile species.[3-9]. In the method, diamagnetic compounds, namely spin traps, are used to convert short-lived labile free radicals into relatively long-lived aminoxyl radicals, referred to spin adducts, which can be measured by conventional electron spin resonance (ESR) spectrometry.[10] Characteristic ESR spectra of spin adducts enable us to identify the initial free radicals. A typical spin trap used in biological systems is 5,5-dimethyl-1-pyrroline-N-oxide (DMPO).[3,5-9,11] This spin trap is characteristic of forming relatively stable oxygen-centered aminoxyl radicals.[12-15] Therefore, this spin trap has been exclusively utilized for the detection of oxygen-

† To whom correspondence should be addressed.

centered labile species, such as superoxide anion radical and hydroxyl radical, which are closely related to the lipid peroxidation in living bodies, a process considered essential for initiation of various diseases.

Although combining spin trapping with DMPO and ESR spectrometry is a useful tool to study free radical formation in biological systems, its basic chemistry is not clear, which has prevented unambiguous conclusions concerning the free radical initiation of diseases. For instance, an impurity found in DMPO may disturb quantitative measurement of the produced spin adducts.[16] As we have previously reported,[16] an unknown doublet of triplet ($a(N) = 1.57\,mT$ and $a(\beta H) = 2.28\,mT$) is frequently observed in biological systems and is due to the hydroxylamine impurity having an epoxy ring. This compound competes with lipids for O_2 to change the yield of oxygen radical formation.[16] Also, the presence of metal ions in these samples may change the structure of ESR signals.

In the present study, therefore, we focused on the effect of Fe ions on the production of these ESR artifact signals.

MATERIALS AND METHODS

DMPO with high purity was obtained from Mitsui-Toatsu Co. (Tokyo, Japan) and used without further purification. 2,2,5,5-Tetramethyl-1-pyrroline-N-oxide (M_4PO) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals of reagent grade were purchased from Nacalai Tesque (Kyoto, Japan).

ESR measurements were performed at room temperature and 77 K using a JEOL Model PE-3X (X-band, 100 kHz field modulation, JEOL, Tokyo, Japan). The markers used for the measurements of the hyperfine splitting constants and g-values were Mn^{2+} and Cr^{3+}. In the room temperature experiments, an aqueous flat quartz cell (1.6 mm in thickness, 10.0 mm in width, and 180.0 mm in length) was used while a quartz round cell (5.0 mm i.d. and 262.5 mm long) was utilized for experiments performed at 77 K.

High-performance liquid chromatography (HPLC) experiments utilized a CCPM (Tosoh, Tokyo, Japan) equipped with a computer controlled gradient programmer and a UV-8000 detector (Tosoh, tuned to 260 nm). A TSKgel ODS-120T (4 mm × 15 cm, Tosoh) column was used. The eluent was acetonitrile/water; the gradient was maintained at 4% for 10 min and then increased from 4 to 20% for 30 min at a flow rate of 0.5 mL/min.

RESULTS AND DISCUSSION

In order to explore the effect of Fe ions, coexisting in the sample solutions, on the ESR spectrum obtained by spin trapping with DMPO, we took Fenton system where following reactions are involved.[15,17–19]

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + \cdot OH + OH^- \quad (1)$$

$$\cdot OH + H_2O_2 \longrightarrow \cdot OOH + H_2O \quad (2)$$

$$\cdot OOH + Fe^{3+} \longrightarrow Fe^{2+} + O_2 + H^+ \quad (3)$$

In this system, ferrous ion (Fe^{2+}) is oxidized to be converted into ferric ion (Fe^{3+}).

We have already determined that the Mitsui Toatsu DMPO used in this study does not contain a hydroxylamine.[21] Therefore, the spin trap was utilized without further purification. Theoretically, DMPO should trap ·OH in aqueous solutions containing $FeSO_4$ and H_2O_2. The formation of the hydroxyl radical adduct of DMPO (DMPO–OH) will result in the reduction of reactions (2 and 3) because of the scavenging of hydroxyl radicals. Consequently only ESR signals of DMPO–OH should appear in the spectrum. ESR signals of aminoxyl radicals other than DMPO–OH and larger than that of DMPO–OH (indicated by the stick diagrams in the figure and referred to DMPO–X and –Y) were observed in systems containing DMPO (100 mM), $FeSO_4$ (5 mM), and H_2O_2 (300 mM) (Figure 1). These unidentified ESR signals increased in intensity as a function of time; DMPO–X intensity increased for 45 min, before declining while DMPO–Y intensity steadily increased (Figure 2). On the other hand, DMPO–OH was not detectable after 45 min (Figure 2). This result suggests that free radicals other than ·OH were produced in the Fenton system and trapped with DMPO or that DMPO was degraded in the Fenton system generating ESR detectable aminoxyl radicals. The intensities of these three ESR signals were dependent on the amount of Fe^{2+} (Figure 3). As Fe^{2+} increased, the intensity of DMPO–OH decreased and those of DMPO–X and DMPO–Y increased, implying the involvement of Fe ions in this unusual behavior of spin trapping with DMPO.

In order to determine which species were produced in the Fenton systems containing DMPO, ESR measurements were performed at 77 K. Figure 4a shows an ESR spectrum obtained from the Fenton system, comprising 100 mM $FeSO_4$, 300 mM H_2O_2 and 100 mM DMPO. There is a signal of g = 4.2, typical of non-heme Fe^{3+} complex. This observation indicates the formation of Fe^{3+}-DMPO complex in the solution. In order to confirm this observation, DMPO (10 mM) was added to aqueous $FeNH_4(SO_4)_2$. We obtained an ESR signal with g = 4.2 and having intensity similar to that of Figure 4b was obtained from a solution containing 10 mM Fe^{3+}. From the

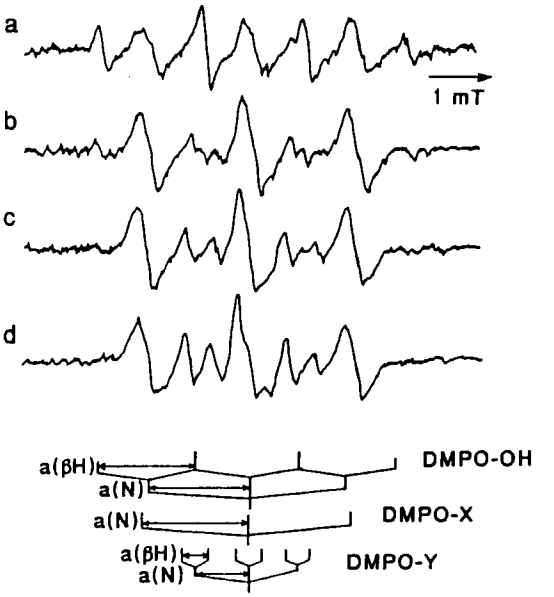

FIGURE 1 ESR spectra obtained from the Fenton system in the presence of DMPO. Incubation time: a, 1 min; b, 20 min; c, 40 min; d, 60 min. System: DMPO (100 mM); $FeSO_4$ (5 mM); H_2O_2 (300 mM).

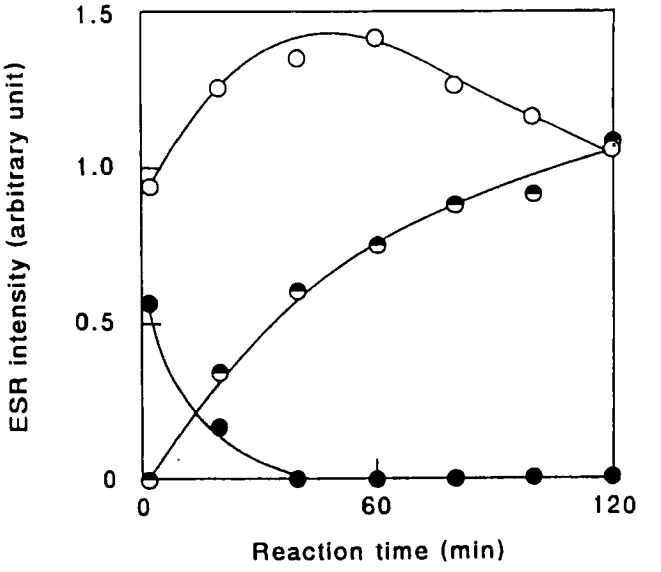

FIGURE 2 Change in the ESR intensities versus time of DMPO–OH, –X, and –Y produced in a Fenton system containing DMPO. System: DMPO (100 mM); $FeSO_4$ (5 mM); H_2O_2 (300 mM). Symbols: (●) DMPO–OH; (○) DMPO–X; (◓) DMPO–Y.

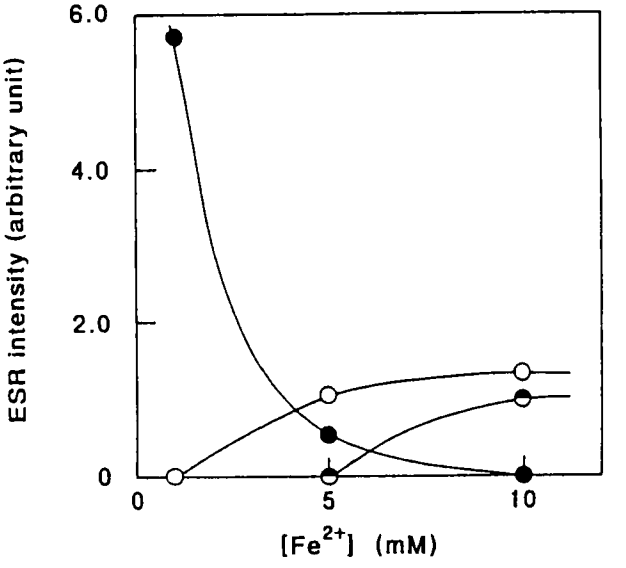

FIGURE 3 Effect of the amount of Fe^{2+} on the ESR intensities of DMPO–OH, –X, and –Y formed in the Fenton system containing DMPO. System: DMPO (100 mM); H_2O_2 (300 mM). Symbols: (●) DMPO–OH; (○) DMPO–X; (◓) DMPO–Y.

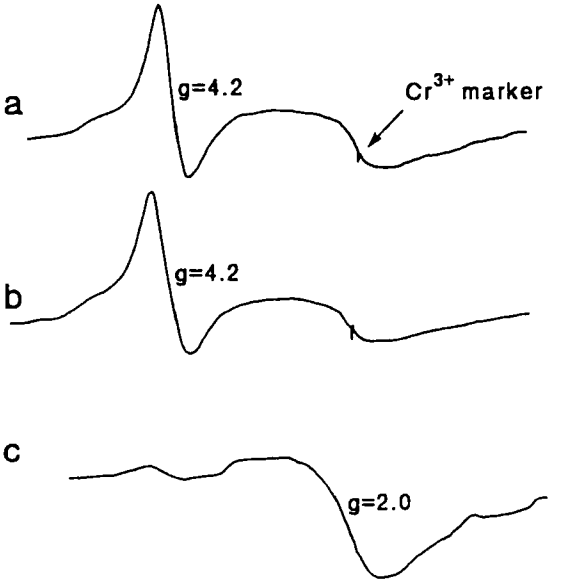

FIGURE 4 ESR spectra obtained at 77 K from: a, Fenton system containing DMPO. System: DMPO, 100 mM; $FeSO_4$, 100 mM; H_2O_2, 300 mM. b, an aqueous DMPO solution containing Fe^{3+} ion. System: DMPO, 10 mM; $FeNH_4(SO_4)_2$, 10 mM. c, an aqueous $FeNH_4(SO_4)_2$ solution (10 mM).

above results, it is implied that ferric ion generated from ferrous ion by the reaction with H_2O_2 forms non-heme complex with DMPO in the Fenton system and it is suggested that DMPO-X and/or -Y may be produced through this complex.

In order to explore what compounds are produced from Fe^{3+}-DMPO complex in the presence of H_2O_2, reverse-phase HPLC was applied. To an aqueous DMPO solution (100 mM), $FeNH_4(SO_4)_2$ (100 mM) was added followed by H_2O_2 (100 mM). In Figure 5a, a chromatogram was obtained from an aqueous solution containing only DMPO, where the large Peak 8 is DMPO and the small Peak 9 is a DMPO impurity. Sixty minutes after Fe^{3+} addition, for Peak 8 a decrease in height occurred. Peak 11 having an absorption maximum at 520 nm (see Table 1) was observed (Figure 5b). This peak appears to be due to a Fe-DMPO complex: Fe^{2+} is already produced from Fe^{3+} within the complex by the intramolecular charge transfer (The details will be published elsewhere[21]). In addition, other peaks (Peaks 2, 3, and 6) were observed (Figure 5b). After H_2O_2 addition to this system containing this complex, peaks (6, 8, 11) due to DMPO and the complex disappeared, and a number of peaks due to the products, generated by the reaction between the complex and H_2O_2 appeared (Figure 5c). An absorption spectrum of each chromatographic peak was observed by the photo-diode array detector and the absorption maximum was measured. The values obtained are listed in Table 1. Some compounds have λmax of around 230 nm which is characteristic of DMPO derivatives or aminoxyl radicals, suggesting that DMPO-X and -Y are probably artifacts due to the reaction products of Fe-DMPO complex and H_2O_2.

The site of DMPO complexing with Fe ion which is attacked by H_2O_2 was explored. Usually the allyl position is preferably attacked by oxidative species. In the Fenton system, degradation of M_4PO[20] was compared with that of DMPO. 50 mM $FeNH_4$-

TABLE I
Absorption maxima of the chromatographic peaks appearing in Figure 5

Peak No.	λ max (nm)
1	205
2	214
3	220
4	205
5	190
6	232
7	190
8 (DMPO)	227
9	230
10	241
11 (Complex)	197, 520
12	203
13	195
14	200
15	200
16	210
17	200

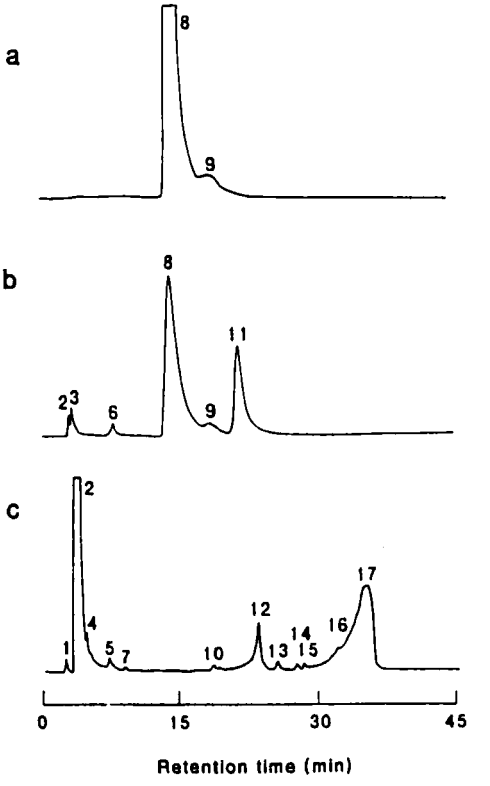

FIGURE 5 HPLC chromatograms obtained from: a, an aqueous DMPO solution (50 mM). b, an aqueous DMPO solution containing Fe^{3+} ion. System: DMPO, 50 mM; $FeNH_4(SO_4)_2$, 50 mM. c, an aqueous DMPO solution containing Fe^{3+} ion in the presence of H_2O_2. System: DMPO (50 mM); $FeNH_4(SO_4)_2$ (50 mM); H_2O_2, 300 mM. Reaction time: 2 hr. Chromatographic conditions: column, TSKgel ODS-120 T (4 mm × 15 cm, Tosoh); eluent, (a) 4% CH_3CN and (b) 24% CH_3CN; gradient, holded in (a) for 10 min and linear from (a) to (b) for 30 min; flow rate, 0.5 mL/min; detection, 210 nm.

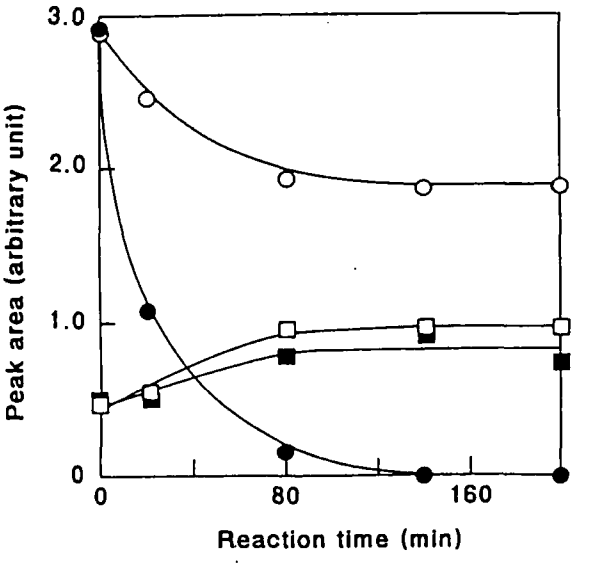

FIGURE 6 Change in the chromatographic peak areas of DMPO, M_4PO, and their complexes formed by the reaction with Fe^{3+} in the presence of H_2O_2, as a function of reaction time. System: DMPO or M_4PO (50 mM); $FeNH_4(SO_4)_2$ (50 mM); H_2O_2, 20 mM. Symbols: (●), DMPO; (○), M_4PO; (■), Fe-DMPO complex; (□), Fe-M_4PO complex.

$(SO_4)_2$ was added to an aqueous solution of either DMPO or M_4PO and followed by H_2O_2 addition. Progressive changes of the chromatographic peaks due to DMPO, M_4PO, Fe-DMPO complex, and Fe-M_4PO complex were measured (Figure 6). M_4PO was subjected to much slower degradation compared to DMPO while the increase rates of Fe complexes were identical. This result indicates that the formation of the complexes is at steady-state and that after the formation of Fe complex, attack of H_2O_2 is made at the 3 position of the pyrroline spin traps. This oxidative reaction may result in the formation of some aminoxyl radicals.

In summary, when DMPO is used in the Fenton system, DMPO forms Fe-DMPO complex in the system and by the attack of oxidative species at the 3 position of complexing DMPO, degradation of DMPO is induced in the complex. Some of the degradation product may be aminoxyl radicals. Since this reaction cycle occurs quite effectively, analysis of the results obtained by spin trapping with DMPO in the presence of Fe ions must be carried out very carefully.

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Accepted by Prof. E.G. Janzen