DMPO-OH Radical Formation from 5,5-Dimethyl-1-pyrroline *N*-Oxide (DMPO) in Hot Water

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When an aqueous solution of 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) was heated at 70°C for 30 min, formation of DMPO-OH was observed by ESR. This DMPO-OH radical formation was suppressed under an argon atmosphere. When water was replaced with ultra-pure water for ICP-MS experiments, DMPO-OH radical formation was also diminished. Under an argon atmosphere in ultra-pure water, the intensity of the DMPO-OH signal decreased to about 1/20 of that observed under aerobic conditions with regular purified water. The addition of hydroxyl radical scavengers such as mannitol did not affect the formation of DMPO-OH, but the signal turned faint in the presence of EDTA. We suggest that DMPO reacted with dissolved oxygen to form DMPO-OH.

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Introduction

The spin-trapping method with 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) has been widely accepted as an assay method to measure hydroxyl radical formation, and to detect the hydroxyl radical scavenging activity of a compound. Although instability of DMPO is claimed because it turns yellow with time even at -20°C in a sealed tube under vacuum,¹ the basic chemistry of DMPO and DMPO-OH has not been well understood.

In a previous work,² we noticed very weak DMPO-OH signals in the baseline of a negative control spectrum which was obtained after standing DMPO solution at 37°C for 24 h. We suspected that in a DMPO aqueous solution at a higher concentration and at a higher temperature, an appropriate amount of DMPO-OH might generate. And we considered that this reaction should provide a practical preparation method to obtain DMPO-OH radical as a simple aqueous solution which contains no other materials except unreacted DMPO. Also this DMPO-OH aqueous solution may be utilized in the investigation of the chemical or physicochemical properties of DMPO-OH radicals.

When DMPO was dissolved in purified water and the solution was heated at 70°C for 30 min, a sufficient amount of DMPO-OH radical formation was observed by ESR (Fig. 1). Under an argon atmosphere, DMPO-OH formation was minimized to 1/4, and this slightly increased under a dioxygen atmosphere. This indicates that dioxygen participates in the reaction. Previously, Makino *et al.* observed DMPO-OH formation in an aqueous mixture of DMPO and 1 mM FeCl₃. Based on this observation

of the formation of iron chelate with DMPO at 77 K, and DMPO-OCH₃ formation in the presence of CH₃OH, researchers elucidated that nucleophilic attachment of water to DMPO should occur in the presence of Fe³⁺ ion.³ To study the effect of a small number of metal ions in purified water, we replaced the purified water with ultra-pure water for ICP-MS measurements; the latter should contain iron of no more than 1 ppb. In ultra-pure water, and under an argon atmosphere, DMPO-OH signal was still observed after heating, although its intensity decreased to about 1/20. In this communication, we describe DMPO-OH radical formation in hot water, in which dissolved dioxygen participates in the reaction and metal ions such as Fe³⁺ might catalyze DMPO-OH formation.

Experimental

Chemicals

5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was obtained from

Fig. 1 DMPO-OH radical formed in a heated aqueous solution of DMPO.

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Fig. 2 The ESR spectrum of DMPO-OH radical observed in an aqueous solution of DMPO after heating at 70°C for 30 min (a). Four strong signals in the mid-section are of the DMPO-OH radical (A_H 1.50 mT, A_N 1.50 mT), and two side signals are of Mn used as an external reference. The ESR spectrum of DMPO aqueous solution heated under an argon atmosphere (b); under aerobic conditions in ultra-pure water (c); under an argon atmosphere in ultra-pure water (d).

Labotec Co. (Tokyo, Japan), and used without further purification.

The water used in this study was purified by ion-exchange resin (18.2 M Ω). Ultra-pure water for ICP-MS was obtained by two successive distillations of purified water with an all-quartz apparatus, in which Fe³⁺ ion was not detected (at most 1 ppb). In experiments under an argon atmosphere, water was deaerated under reduced pressure using an aspirator, and then stored under argon by attaching an argon balloon.

ESR measurement

An electron spin resonance spectrometer, JES-RE1X (JEOL, Tokyo, Japan), and a JEOL flat quartz cell were used. The conditions were: field, 336 $\pm$ 5 mT width; power, 4 mW; field modulation, 0.200 mT; time constant, 0.1; and amplitude, 500. A manganese signal was used for the external standard. Pyrex glassware was washed with 1 M nitric acid by sonication, rinsed well with purified water, and air dried.

DMPO-OH radical formation in hot water

Neat DMPO was dissolved in purified water to make a 2.5% aqueous solution. In cases of pH controlled experiments, 2.5% DMPO solution in an acetate buffer or a phosphate buffer was used. A 2-mL volume of sample was heated in a test tube for 30 min in a water bath at 70°C. Under an argon atmosphere or a dioxygen atmosphere, 10 mL of 2.5% DMPO aqueous solution was introduced into a 25 mL round-bottomed flask, and a three-way stopcock with an argon (or dioxygen) balloon was attached. The solution was deaerated under reduced pressure (aspirator), and argon (or dioxygen) was introduced from the balloon. After heating at 70°C for 30 min, an aliquot was removed and was measured by ESR just 1 min after the tube was taken out of the water bath.

Results and Discussion

After heating the aqueous solution of DMPO at 70°C for 30

Fig. 3 Effect of pH on the relative intensity of DMPO-OH vs. Mn (external reference), which is the highest at around pH 8.5.

min, we observed typical ESR signals of DMPO-OH radical with an intensity ratio of 1:2:2:1 (A_H 1.50 mT, A_N 1.50 mT; Ref. 4, A_H 1.53 mT, A_N 1.53 mT) (Figs. 1 and 2).

It was reported that Fe³⁺ ions catalyze the addition of water to DMPO to produce DMPO-OH radical.³ In Fig. 2, however, its formation decreased under Ar atmosphere (Figs. 2(b) and (d)), also in ultra-pure water under aerobic conditions (Fig. 2(c)). In all spectra in Fig. 2, unknown weak signals can be seen. DMPO-OH radical formation by heating was not suppressed by the addition of a hydroxyl radical scavenger, mannitol (1 – 100 mM) or DMSO (1 – 100 mM). This indicates that DMPO-OH radical should not be formed *via* hydroxyl radical attachment to DMPO. However, DMPO-OH signal intensity was decreased to about 1/6 in the presence of 1 mM of EDTA. Thus, it was suspected that dissolved dioxygen, which is a biradical molecule, should be added directly to DMPO, where catalytic participation of metal ions might exist (Eq. (1)). Biradical might produce a DMPO-OOH radical, and this would develop into a DMPO-OH radical quickly at higher temperatures.

DMPO-OH signals were also observed in acetate buffer (pH 5.0 – 6.8), phosphate buffer (pH 7.0 – 10.0) or disodium phosphate solution of which the pH was adjusted with sodium hydroxide solution (pH 13.0) (Fig. 3). The half-lives of DMPO-OH⁵ or DMPO-OOH^{6,7} at basic pH are much shorter than those at neutral or acidic conditions, and the decrease of the intensity of DMPO-OH signal can be considered as the unstability of these radical species. The optimum pH was found to be around pH 8.5, thus, the catalytic action of H⁺ may not be plausible.

Even at low concentrations, transition metal ions such as Fe³⁺ may catalyze the first step (Eq. (1)), or catalyze the reaction of [DMPO-O₂] biradical with water to [DMPO-OOH] (Eq. (2)), although we could not detect [DMPO-OOH] signals nor OH radical formation by the addition of DMSO. DMPO-OOH radical is known to be labile so as to decompose to DMPO-OH with a half-time of 50 s at pH 7^{6,7} (Eq. (3)), and the decomposition mechanisms of DMPO-OOH adducts to DMPO-OH are discussed by Villamena *et al.*⁸

The formation of DMPO-OH with time was traced at 70°C (Fig. 4). It was found that the reaction reached almost steady state after 60 min. After dioxygen in the reaction mixture is consumed, the supply of dioxygen to the reaction solution

Fig. 4 The relative intensity of DMPO-OH vs. Mn (external reference) with time. The ESR signal of DMPO-OH radical in a 2.5% DMPO solution adjusted at pH 7.4 using a phosphate buffer, was recorded just 1 min after taking out the reaction tube which was heated at 70°C and then cooled at 0°C for 15 s in a water-bath.

Fig. 5 ESR signal of DMPO-OH radical observed in an aqueous solution of DMPO (dissolved in milliQ water) under aerobic conditions.

should be the rate-determining step.

DMPO-OH formation in an aqueous solution of DMPO was also observed at 37°C after 24 h, indicating that radical trapping experiments in an intact animal using DMPO over 20 h may provide misleading results (Fig. 5). However, in the standard experimental procedure, spin-trapping experiments should be carried out at room temperature or lower, and over a short time,

DMPO-OH formation could be neglected.

The mechanism of DMPO-OH radical formation remains uncertain; however, we report here the observed evidence that DMPO should react with dioxygen in water to generate DMPO-OH radical under aerobic conditions at 70°C.

Conclusions

In conclusion, DMPO-OH radical was detected after heating a DMPO aqueous solution at 70°C for 30 min. In the presence of dioxygen, DMPO-OH production was enhanced. The catalytic participation of metal ions was also suspected, because decreased formation of DMPO-OH was observed when regular purified water was replaced with ultra-pure water.

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