

ON THE REACTION OF SUPEROXIDE WITH DMPO/•OOH

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A kinetic model has been used to estimate the rate constant for the reaction of superoxide (O_2^- /•OOH) with the superoxide spin adduct of 5,5-dimethylpyrroline-N-oxide, DMPO/•OOH. This rate constant is estimated to be $4.9 (\pm 2.2) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, pH 7.4 and 25°C.

KEY WORDS: Spin trapping, 5,5-dimethylpyrroline-N-oxide, superoxide, free radical.

ABBREVIATIONS: DETAPAC, diethylenetriaminepentaacetic acid; DMPO, 5,5-dimethylpyrroline-N-oxide; 3-CP, 3-carboxy-proxyl; OXANO, 2-ethyl-2,5,5-trimethyl-3-oxazolidinoxyl; OXANOH, 2-ethyl-1-hydroxy-2,5,5-trimethyl-3-oxazolidine; X.O., xanthine oxidase.

INTRODUCTION

Superoxide* reacts slowly with DMPO at neutral pH ($k^{\text{obs}} = 30 \text{ M}^{-1} \text{ s}^{-1}$ at pH 7.4¹) producing a spin adduct, DMPO/•OOH, that decays by a first-order process, and is relatively short-lived ($t_{1/2} = 50 \text{ s}$ at pH 7.4, 25°C²). It has recently been shown that the reaction of O_2^- with DMPO/•OH and DMPO/•CH₃ may be a significant process and should be considered when interpreting spin trapping data.^{3,4} In general, it was found that 5-membered ring nitroxides react with superoxide to produce diamagnetic products. Thus, it is reasonable to suspect that superoxide will react with DMPO/•OOH. However, the short lifetime of DMPO/•OOH precludes a simple direct determination of the rate constant for the reaction:



However, by: 1) determining the rate of production of superoxide in a superoxide-generating system; 2) determining the steady-state concentration of DMPO/•OOH; and 3) using an appropriate kinetic model, I have estimated the rate constant for this reaction.

MATERIALS AND METHODS

Xanthine oxidase, hypoxanthine, cytochrome c, 3-carboxy-proxyl, and DMPO were from Sigma. DMPO was purified with charcoal² and its concentration determined using $\epsilon_{228} = 7.8 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ (G.R. Buettner, unpublished). Adventitious metals were removed from the buffer with chelating resin (sodium form, dry mesh 50-100, from Sigma, St. Louis, MO). In the demetalled buffer, the loss of ascorbate was 0.3% or less in the standard 15 minute test,⁵ indicating effective removal of catalytic metals.

*In this paper, I use superoxide (or O_2^-) to represent the equilibrium mixture of O_2^- and •OOH.

The rate of production of superoxide in a xanthine oxidase system was determined as outlined by Fridovich.⁶ Briefly, the change in absorbance of cytochrome c ($\Delta\epsilon_{550} = 2.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) was followed in a system containing 0.5 mM hypoxanthine, 0.1 mM cytochrome c, 50 μM DETAPAC, and xanthine oxidase ($\approx 0.25\text{--}20 \text{ mU/ml}$) in 50 mM phosphate buffer, pH 7.4.

The spin trapping incubations used to determine $[\text{DMPO}/\text{OOH}]_{\text{ss}}$ contained 0.10 M DMPO, 0.5 mM hypoxanthine, 50 μM DETAPAC, and varying amounts of X.O. such that the rate of O_2^- production varied from 9–71 nM s^{-1} . These X.O. concentrations produced a constant rate of superoxide production in the time range of 3–8 minutes after the introduction of X.O. and an apparent steady-state concentration of DMPO/OOH as determined by repetitive scans of the high field doublet of

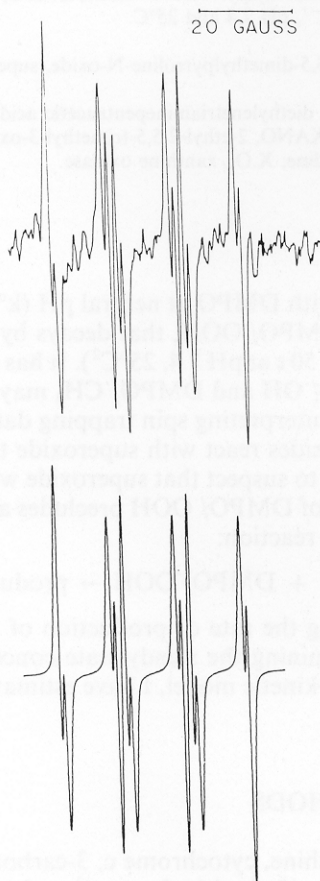


FIGURE 1 *Top*: The superoxide spin adduct spectrum of DMPO produced by a solution of 0.1 mM xanthine, 150 μM DETAPAC, 70 mM DMPO and $\approx 12 \text{ mU/ml}$ of X.O. in 50 mM phosphate buffer, pH 7.8. Instrument settings were: power, 20 mW; scan rate 25 G min^{-1} ; modulation amplitude, 0.3 G, time constant 0.5 s. *Bottom*: Simulation of DMPO/OOH spectrum assuming two species are present at equal population. The parameters used were: $a_{\text{N}}^1 = 14.25 \text{ G}$, $a_{\text{H}}^1 = 12.45 \text{ G}$ and $\Delta H_{\text{pp}}^1 = 0.96 \text{ G}$, $a_{\text{N}}^2 = 14.25 \text{ G}$, $a_{\text{H}}^2 = 10.10 \text{ G}$, and $\Delta H_{\text{pp}}^2 = 1.11 \text{ G}$. A 50% Lorentzian-50% Gaussian shape function was used.

TABLE 1

rate ₁ /nMs ⁻¹	[DMPO/·OOH] _{ss} /nM	k ₅ /10 ⁶ M ⁻¹ s ⁻¹
9.1	212	7.2
13.8	285	5.8
21.2	357	5.2
40	590	3.4
71	765	2.9

$$k_5 = 4.9(\pm 2.2) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$$

The data of columns 1 and 2 represent the median of at least three determinations.

Use of the steady state assumption that $d[\text{O}_2^-]/dt = 0$ and $d[\text{DMPO}/\cdot\text{OOH}]/dt = 0$ allows an exact solution for the unknown rate constant, k_5 ***.

As seen in Table 1, the second-order rate constant determined with this kinetic model is $\sim 5 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$. This high rate constant implies that DMPO/·OOH can compete effectively for O_2^- in the typical superoxide spin trapping experiment that uses DMPO(0.1 M). For example, the $[\text{O}_2^-]_{ss}$ in the experiment where rate₁ = 21.2 nM s⁻¹ was calculated to be 4.4 nM. Thus, at pH 7.4 rate₃ = 13.2 nM s⁻¹ while rate₅ = 8.2 nM s⁻¹ and rate₄ = 4.9 nM s⁻¹, i.e., the loss of DMPO/·OOH due to self-decay is only one half that due to the superoxide-induced decay. That rate₅ is greater than rate₄ accounts for the low levels of DMPO/·OOH seen in spin trapping experiments.

If in this kinetic model k_5 were zero, then

$$k_3[\text{DMPO}][\text{O}_2^-]_{ss} = k_4[\text{DMPO}/\cdot\text{OOH}]_{ss}$$

After calculating $[\text{O}_2^-]_{ss}$ (7.1 nM) we have $[\text{DMPO}/\cdot\text{OOH}]_{ss} = 1.5 \mu\text{M}$, but I only observed 0.36 μM DMPO/·OOH. Therefore, this value of k_5 accounts for the less than predicted concentration of DMPO/·OOH seen in spin trapping experiments.

These results point to the difficulty in attempting to do quantitative work by observing DMPO/·OOH. In addition, it may be an experimental advantage in some experiments to arrange a low rate of superoxide generation so that reaction (5) can be minimized. Then, use of a slower scan rate with longer time constant or signal averaging can be employed because oxygen depletion will be delayed significantly.

A close examination of the results presented in Table 1 reveals that as the rate of superoxide generation increases, k_5^{obs} decreases. This trend suggests that the kinetic model may not be complete. The lower values of k_5^{obs} at higher O_2^- fluxes, i.e., high X.O. concentrations, suggest that a relative increase in DMPO/·OOH concentration occurs. This would not be the case if X.O. were reducing DMPO/·OOH. (Samuni *et al.*⁴ found no evidence that X.O. directly destroys DMPO/·OH.) A relative increase in $[\text{DMPO}/\cdot\text{OOH}]_{ss}$ would occur if the diamagnetic products of reaction (5) could be reoxidized to DMPO/·OOH by superoxide, i.e.,

$$***[\text{O}_2^-]_{ss} = \frac{2k_3[\text{DMPO}] - ((2k_3[\text{DMPO}])^2 - 4(-2k_2)(\text{rate}_1 + k_4[\text{DMPO}/\cdot\text{OOH}]))^{1/2}}{2(-2k_2)}$$

and

$$k_5 = \frac{k_4[\text{DMPO}/\cdot\text{OOH}] - k_3[\text{DMPO}][\text{O}_2^-]}{-[\text{DMPO}/\cdot\text{OOH}][\text{O}_2^-]}$$

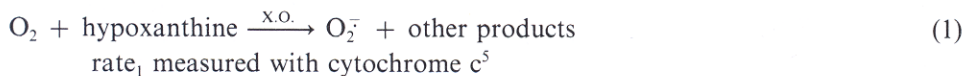
DMPO/·OOH. (The high field doublet was chosen because it is least affected by the DMPO/·OH signal that is also present.)

The determination of the concentration was accomplished by using 3-carboxy-proxyl as a standard. Because there are many factors that affect ESR signal area measurements,⁷ the 3-CP standard and DMPO/·OOH experimental spectra were obtained using identical instrument settings, except for receiver gain, and identical physical arrangement of the samples in the cavity: Linearity of the receiver gain was verified. Double integration of the spectra was accomplished with the aid of the free radical simulation program of Oehler and Janzen.⁸ The relative area for each species was found by simulation of the midfield line of 3-CP and the high field doublet of DMPO/·OOH. The 3-CP lineshape was simulated using $\Delta H_{pp} = 1.275$ G and a 70% Gaussian-30% Lorentzian shape function. The high field doublet of DMPO/·OOH was simulated using $a^H = 1.15$ G, $\Delta H_{pp} = 1.11$ G for the low field component and $\Delta H_{pp} = 0.96$ G for the high field component. For each component a 50% Gaussian-50% Lorentzian shape function was used.

The DMPO/·OOH ESR spectrum has an asymmetry that traditional simulation efforts do not reproduce. However, the asymmetrical DMPO/·OOH spectrum can be reproduced when two species of equal population, but different line widths, are used. An excellent fit is obtained if for species 1, $a_N^1 = 14.25$ G, $a_H^1 = 12.45$ G and $\Delta H_{pp}^1 = 0.96$ G for species 2, $a_N^2 = 14.25$ G, $a_H^2 = 10.10$ G and $\Delta H_{pp}^2 = 1.11$ G. See figure 1. These parameters showed that for DMPO/·OOH and 3-CP lines of *equal* height, the relative area for the two species is: (area DMPO/·OOH)/(area 3-CP) = 2.9. This information allows the calculation of the DMPO/·OOH concentration from ESR signal height measurements using 3-CP as a standard. ESR spectra were recorded using a Varian E-4 system.

RESULTS AND DISCUSSION

To estimate the rate of the reaction of superoxide with DMPO the following system of kinetic equations was used:



**It has been reported the DMPO/·OOH decomposes with a half-life of 8 minutes.¹⁰ However, this claim could not be substantiated using the riboflavin-DETAPAC system;² rather, at pH 7.4 I found a first-order half-life of 50 s, as previously reported.²



In essence, reaction (7) could occur if the initial ESR-silent products of reaction (5) could be efficiently reoxidized by $\text{O}_2^- / \text{'OOH}$. This possibility has precedent. It has recently been demonstrated that the nitroxide/hydroxylamine couple of OXANO/OXANOH undergoes a reaction sequence parallel to reactions (5) and (7) above.¹¹ However, Samuni *et al.*⁴ were not able to reoxidize the ESR-silent product of $\text{DMPO/'OH} + \text{O}_2^-$ with either ferricyanide or $\text{H}_2\text{O}_2/\text{Cu(II)}$. If reactions (6) and (7) were to be included in the reaction scheme, then gathering the experimental data for an exact solution becomes a problem. If reactions (6) and (7) are operative, then the value of k_5 at pH 7.4 is probably $\approx 10^7 \text{ M}^{-1} \text{ s}^{-1}$. Nonetheless, even with the kinetic model used to arrive at k_5^{obs} , the value of $5 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ is a very useful number that can be used as a guideline for researchers to help interpret spin trapping data dealing with DMPO/'OOH .

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