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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 ($O_2^{\bar{}}$/$^{\cdot}$OOH) with the superoxide spin adduct of 5,5-dimethylpyrroline-N-oxide, DMPO/$^{\cdot}$OOH. This rate constant is estimated to be 4.9 ($\pm$ 2.2) $\times$ 10^6 M^{-1} 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^{obs} = 30 M^{-1} s^{-1} at pH 7.4[1]) producing a spin adduct, DMPO/$^{\cdot}$OOH, that decays by a first-order process, and is relatively short-lived ($t_{1/2}$ = 50 s at pH 7.4, 25°C[2]). It has recently been shown that the reaction of $O_2^{\bar{}}$ with DMPO/$^{\cdot}$OH and DMPO/$^{\cdot}CH_3$ 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/$^{\cdot}$OOH. However, the short lifetime of DMPO/$^{\cdot}$OOH precludes a simple direct determination of the rate constant for the reaction:

$$O_2^{\bar{}} + \text{DMPO}/^{\cdot}\text{OOH} \rightarrow \text{products.}$$

However, by: 1) determining the rate of production of superoxide in a superoxide-generating system; 2) determining the steady-state concentration of DMPO/$^{\cdot}$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with charcoal[2] and its concentration determined using ε_{228} = 7.8 $\times$ 10^3 M^{-1} cm^{-1} (G.R. Buettner, unpublished).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.

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*In this paper, I use superoxide (or $O_2^{\bar{}}$) to represent the equilibrium mixture of $O_2^{\bar{}}$ and $^{\cdot}$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($\Delta\varepsilon_{550}$ = 2.1 × 10^4 M^{-1} cm^{-1}) was followed in a system containing 0.5 mM hypoxanthine, 0.1 mM cytochrome c, 50 μM DETAPAC, and xanthine oxidase (≈ 0.25-20 mU/ml) in 50 mM phospate buffer, pH 7.4.

The spin trapping incubations used to determine [DMPO/˙OOH]$_{ss}$ contained 0.10 M DMPO, 0.5 mM hypoxanthine, 50 μM DETAPAC, and varying amounts of X.O. such that the rate of $O_2^{\cdot -}$ production varied from 9–71 nMs^{-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 repetative scans of the high field doublet of

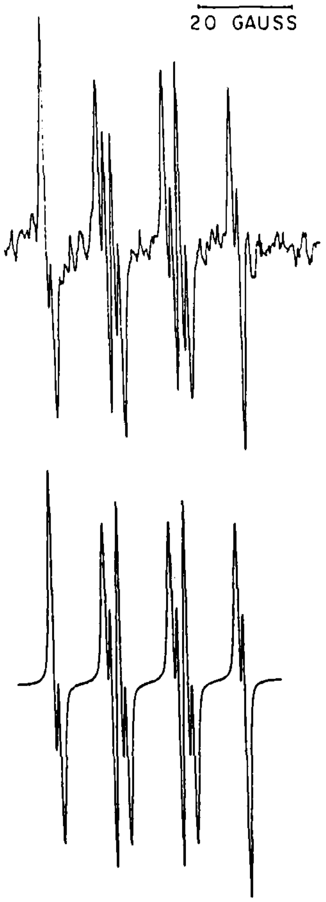

FIGURE 1 *Top*: The superoxide spin adduct spectrum of DMPO produced by a solution of 0.1 mM xanthine, 150 μM DETAPAC, 70 mM DMPO and ≈ 12 mU/ml of X.O. in 50 mM phosphate buffer, pH 7.8. Instrument settings were: power, 20 mW; scan rate 25 G min^{-1}; modulation amplitude, 0.3 G, time constant 0.5 s.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

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-carboxyproxyl 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 $\Delta Hpp = 1.275\,G$ and a 70% Gaussian-30% Lorentzian shape function. The high field doublet of DMPO/˙OOH was simulated using $a^H = 1.15\,G$, $\Delta Hpp = 1.11\,G$ for the low field component and $\Delta Hpp = 0.96\,G$ 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:

$$O_2 + \text{hypoxanthine} \xrightarrow{\text{X.O.}} O_2^{\bar{\cdot}} + \text{other products} \tag{1}$$

rate$_1$ measured with cytochrome c[5]

$$O_2^{\bar{\cdot}} + O_2^{\bar{\cdot}} + 2H^+ \longrightarrow O_2 + H_2O_2 \tag{2}$$

$k_2^{obs} = 2.4 \times 10^5\ M^{-1}\ s^{-1}$; pH 7.4[9]

$$O_2^{\bar{\cdot}} + \text{DMPO} + H^+ \longrightarrow \text{DMPO/˙OOH} \tag{3}$$

$k_3^{obs} = 30\ M^{-1}\ s^{-1}$; pH 7.4[1]

$$\text{DMPO/˙OOH} \longrightarrow \text{products} \tag{4}$$

$k_4^{obs} = 1.4 \times 10^{-2}\ s^{-1}$; pH 7.4[2]**

$$\text{DMPO/˙OOH} + O_2^{\bar{\cdot}} \longrightarrow \text{products} \tag{5}$$

$k_5^{obs} = ?$

**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]

TABLE 1

$rate_1/nMs^{-1}$	$[DMPO/{}^{\cdot}OOH]_{ss}/nM$	$k_5/10^6\ M^{-1}\ s^{-1}$
9.1	212	7.2
13.8	285	5.8
21.2	357	5.2
40	590	3.4
71	765	2.9
	$k_5 = 4.9(\pm 2.2) \times 10^6\ M^{-1}\ s^{-1}$	

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

Use of the steady state assumption that $d[O_2^{\overline{\cdot}}]/dt = 0$ and $d[DMPO/{}^{\cdot}OOH]/dt = 0$ 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 $\sim 5 \times 10^6\ M^{-1}\ s^{-1}$. This high rate constant implies that DMPO/·OOH can compete effectively for $O_2^{\overline{\cdot}}$ in the typical superoxide spin trapping experiment that uses DMPO(0.1 M). For example, the $[O_2^{\overline{\cdot}}]_{ss}$ in the experiment where $rate_1 = 21.2\ nM\ s^{-1}$ was calculated to be 4.4 nM. Thus, at pH 7.4 $rate_3 = 13.2\ nM\ s^{-1}$ while $rate_5 = 8.2\ nM\ s^{-1}$ and $rate_4 = 4.9\ nM\ s^{-1}$, i.e., the loss of DMPO/·OOH due to self-decay is only one half that due to the superoxide-induced decay. That $rate_5$ is greater than $rate_4$ accounts for the low levels of DMPO/·OOH seen in spin trapping experiments.

If in this kinetic model k_5 were zero, then

$$k_3[DMPO][O_2^{\overline{\cdot}}]_{ss} = k_4\,[DMPO/{}^{\cdot}OOH]_{ss}$$

After calculating $[O_2^{\overline{\cdot}}]_{ss}$ (7.1 nM) we have $[DMPO/{}^{\cdot}OOH]_{ss} = 1.5\ \mu M$, 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 $O_2^{\overline{\cdot}}$ 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 $[DMPO/{}^{\cdot}OOHJ]_{ss}$ would occur if the diamagnetic products of reaction (5) could be reoxidized to DMPO/·OOH by superoxide, i.e.,

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$$***[O_2^{\overline{\cdot}}]_{ss} = \frac{2k_3[DMPO] - ((2k_3[DMPO])^2 - 4(-2k_2)(rate_1 + k_4[DMPO/{}^{\cdot}OOH]))^{1/2}}{2(-2k_2)}$$

and

$$k_5 = \frac{k_4[DMPO/{}^{\cdot}OOH] - k_3[DMPO][O_2^{\overline{\cdot}}]}{-[DMPO/{}^{\cdot}OOH][O_2^{\overline{\cdot}}]}$$

$$\text{products}_5 \xrightarrow[k_6 \text{ is small}]{\text{slow}} \text{irreversible ESR-silent products} \quad (6)$$

$$\text{products}_5 + O_2^{\bar{\cdot}} + 2H^+ \xrightarrow[k_7 \text{ is large}]{\text{fast}} \text{DMPO/}^{\cdot}\text{OOH} + H_2O_2 \quad (7)$$

In essence, reaction (7) could occur if the initial ESR-silent products of reaction (5) could be efficiently reoxidized by $O_2^{\bar{\cdot}}$/'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 DMPO/'OH + $O_2^{\bar{\cdot}}$ with either ferricyanide or H_2O_2/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 $\approx 10^7\ M^{-1}s^{-1}$. Nonetheless, even with the kinetic model used to arrive at k_5^{obs}, the value of $5 \times 10^6\ M^{-1}s^{-1}$ 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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