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Superoxide Dismutase versus Ferricytochrome C: Determining Rate Constants for the Spin Trapping of Superoxide by Cyclic Nitrones

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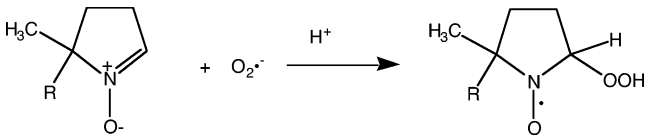
Given that spin trapping/electron paramagnetic resonance (EPR) spectroscopy has become the primary technique to identify important biologically generated free radicals, such as superoxide ($O_2^{\bullet-}$), in vitro and in vivo models, evaluation of the efficiency of specific spin traps to identify this free radical is paramount. Recently, a family of ester-containing nitrones has been prepared, which appears to have distinct advantages for spin trapping $O_2^{\bullet-}$ compared to the well-studied spin traps 5,5-dimethyl-1-pyrroline *N*-oxide **1** and 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide **2**. An important determinant in the selection of a spin trap is the rate constant (k_{app}) for its reaction with $O_2^{\bullet-}$, and several different methods have been employed in estimating this k_{app}. In this paper, the two most frequently used scavengers of $O_2^{\bullet-}$, ferricytochrome *c* and Cu/Zn-SOD, were evaluated as competitive inhibitors for spin trapping this free radical. Data presented herein demonstrate that SOD is the preferred compound when determining the k_{app} for the reaction of $O_2^{\bullet-}$ with spin traps. Using this model, the k_{app} for the reaction of nitrone **1**, 5-*tert*-butoxycarbonyl-5-methyl-1-pyrroline *N*-oxide **3**, and 5-methoxycarbonyl-5-methyl-1-pyrroline *N*-oxide **4** with $O_2^{\bullet-}$ was estimated to be 24.6 ± 3.1, 73.0 ± 12, and 89.4 ± 1.0 M^{-1} s^{-1} at pH 7.0, respectively. Several other comparative studies between known spin traps were also undertaken.

Introduction

Detection and characterization of biologically generated free radicals, such as superoxide ($O_2^{\bullet-}$) and nitric oxide ($NO^{\bullet}$), have been pivotal to our understanding of their cell signaling properties.[1] The identification of these free radicals has relied on a few well-tested methods, one of which is spin trapping and electron paramagnetic resonance (EPR) spectroscopy.[2] Currently, for instance, the measurement of nitric oxide synthase (NOS; EC 1.14.13.39) generated $O_2^{\bullet-}$ is best estimated using spin trapping/EPR spectroscopy.[3]

While nitrones are the primary spin traps for $O_2^{\bullet-}$, the efficiency of this reaction is, surprisingly, poor with rate constants typically ≤ 100 M^{-1} s^{-1}. In the case of 5,5-dimethyl-1-pyrroline *N*-oxide **1** (Scheme 1), for instance, the apparent rate constant (k_{app}) for the reaction of $O_2^{\bullet-}$ with this nitrone, affording aminoxyl **5**, ranges from 60 to 10 M^{-1} s^{-1} over the narrow pH range of 7.0−9.0.[4] This finding is further troublesome when one considers that the disproportionation rate constant for $O_2^{\bullet-}$ at pH 7.4 is $\sim 3 \times 10^5$ M^{-1} s^{-1},[5] and the reaction of $O_2^{\bullet-}$ with bovine superoxide dismutase (Cu/Zn-SOD) over this same pH range is even faster with a rate constant (k_{SOD}) ranging from 2.4×10^9 to 3.8×10^9 $M^{-1}s^{-1}$ as determined by pulse radiolysis.[6]

SCHEME 1. Schematic that Depicts the Reaction of Nitrones with $O_2^{\bullet-}$



R = CH_3, **1**
R = $P(O)(OC_2H_5)_2$, **2**
R = $CO_2C(CH_3)_3$, **3**
R = CO_2CH_3, **4**

R = CH_3, **5**
R = $P(O)(OC_2H_5)_2$, **6**
R = $CO_2C(CH_3)_3$, **7**
R = CO_2CH_3, **8**

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Attempts to enhance the efficiency of spin trapping have resulted in the synthesis of nitrones with markedly improved reactivity toward this free radical. For example, the k_{app} for spin trapping $O_2^{\bullet-}$ by 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide **2** (Scheme 1) is 60−90 M^{-1} s^{-1} at pH 7.0.[7] However, difficulties encountered isolating pure nitrone **2** have inspired the synthesis of a family of ester-containing nitrones,[8] which appear to have distinct advantages for spin trapping $O_2^{\bullet-}$ as compared to nitrone **2**. This conclusion is based, in part, on the ease of isolating pure nitrones, essential in spin trapping small concentrations of free radicals. Other important determinants in selection of a spin trap are the rate constant for its reaction with $O_2^{\bullet-}$ and the stability of the corresponding aminoxyls. In the case of 5-*tert*-butoxycarbonyl-5-methyl-1-pyrroline *N*-oxide **3** (Scheme 1), the calculated k_{app} for the reaction of $O_2^{\bullet-}$ with this nitrone widely differs, ranging from 0.24[9] to 7 M^{-1} s^{-1} [7c] and to 77 M^{-1} s^{-1}.[8d,10] Since these papers[7c,8d,9,10] report the use of competitive kinetics to estimate rate constants for spin trapping $O_2^{\bullet-}$ by nitrone **3**, the disparity in these numbers must undoubtedly arise from the choice of the competitive inhibitor, whether ferricytochrome *c*, Cu/Zn-SOD, or the self-dismutation of $O_2^{\bullet-}$. Therefore, we reevaluated the use of ferricytochrome *c* and Cu/Zn-SOD, as competitive inhibitors for determining the k_{app} for the reaction of $O_2^{\bullet-}$ with nitrones **1**, **3**, and **4**. Based on these data, we recommend experimental designs, along with the strengths and weaknesses of each model, which may be helpful for future investigations on this topic.

Results and Discussion

Pulse radiolytic production of $O_2^{\bullet-}$ is the best source of this free radical to accurately estimate rate constants for the spin trapping of $O_2^{\bullet-}$. As few scientists have the equipment available to undertake such experiments, we recommend that apparent rate constants be determined using a competitive inhibitor. Based on the original studies of Sawada and Yamazaki[11] and Asada et al.,[12] SOD and ferricytochrome *c* were selected as competitive inhibitors in estimating k_{app} for the reaction of nitrones with $O_2^{\bullet-}$. These inhibitors were chosen since the rate constants for the reaction of each with $O_2^{\bullet-}$ have been determined using pulse radiolysis.[6,13] The competitive kinetic model previously described[11,12] will not be further elaborated herein. Interested readers should consult an earlier paper on this subject.[8d]

In developing an appropriate kinetic scheme, one must be aware of the source of $O_2^{\bullet-}$. While KO_2 is commercially available, it is not without significant limitations. First, the purity of KO_2 is questionable; many sources of this compound contain peroxide, as a contaminant. Second, the preparation of a stock solution of KO_2 in aprotic solvents, such as DMF or DME, requires inclusion of crown ethers, although KO_2 is sparingly soluble in DMSO.[13d] Third, great care should be followed when adding an organic solution of KO_2 to water or a buffer, as a violent reaction may ensue, depending on the amount of KO_2 added to the reaction. Fourth, estimating the concentration of $O_2^{\bullet-}$ in aqueous solutions of KO_2 is not without considerable effort, requiring the inclusion of catalase to eliminate H_2O_2.[13d] If, however, one were to require a chemical source of $O_2^{\bullet-}$, $(CH_3)_4NO_2$ is a reasonable alternative, void of many of the above-mentioned problems with KO_2.[14]

Considering the enormous disparity in rate constants between disproportionation of $O_2^{\bullet-}$ and the reaction of this free radical with nitrones, a continued slow flux of $O_2^{\bullet-}$, typically in the range of 1 μM/min, is recommended. At physiological pH, this criterion can easily be achieved by using xanthine oxidase and a number of substrates such as xanthine, hypoxanthine, or acetaldehyde.[15] Recombinant NADPH-oxidase from neutrophils[16] is an alternative physiologic source of $O_2^{\bullet-}$, even though care is required to isolate the functional enzyme. When it is necessary to conduct spin trapping experiments at a pH beyond the physiologic range,[8d] we have found that NADPH/FMN[17] is a preferable source of $O_2^{\bullet-}$.

The initial concentration of the nitrone is set sufficiently high, e.g., 100 mM, so that during the lifetime of the kinetic experiment the reaction of $O_2^{\bullet-}$ with either itself[5] or the aminoxyl[18] can be excluded. When using enzymes to generate $O_2^{\bullet-}$, it is important to determine whether the spin trap inhibits the production of this free radical.[4] This is an especially critical series of experiments to undertake. For example, nitrone **3** has been

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TABLE 1. Apparent Rate Constant (k_{App}) for the Reaction of $O_2^{\bullet-}$ with Nitrone 3

competitive inhibitor	k_{app} (M^{-1} s^{-1})[a]	$k_{inhibitor}$ (M^{-1} s^{-1})
Cu/Zn-SOD	49.9 ± 8.2 (32.5 kDa)	2.6×10^{9} [6a]
	53.9 ± 7.2 (31.2 kDa)	
	59.5 ± 9.8 (32.5 kDa)	3.1×10^{9} [6b]
	64.2 ± 8.6 (31.2 kDa)	
	73.0 ± 12 (32.5 kDa)	3.8×10^{9} [6c]
	78.7 ± 11 (31.2 kDa)	
ferricytochrome c	57.8 ± 4.4	8.0×10^{5} [13c]

[a] Rate constants are the average of three independent experiments, expressed as the means and standard deviations.

TABLE 2. Apparent Rate Constant (k_{app}) for the Reaction of $O_2^{\bullet-}$ with Nitrone 1 and Nitrone 4 at pH 7.0

	k_{app} ($M^{-1}s^{-1}$)[a]		
spin trap	k_{SOD} = 2.6×10^{9} [6a]	k_{SOD} = 3.1×10^{9} [6b]	k_{SOD} = 3.8×10^{9} [6c]
nitrone **1**	16.8 ± 2.1	20.1 ± 2.5	24.6 ± 3.1
nitrone **4**	61.2 ± 0.70	72.9 ± 0.83	89.4 ± 1.0

[a] Rate constants are the average of three independent experiments, expressed as the means and standard deviations using the 32.5 kDa preparation of SOD.

found to inhibit the nNOS oxidation of L-arginine, and presumably production of $O_2^{\bullet-}$, at concentrations above 100 mM.[10] Experimentally, such studies can be undertaken by, in the case of xanthine oxidase, monitoring the production of uric acid from xanthine oxidation.[4] For kinetic experiments presented herein, concentrations of nitrone **1**, nitrone **3** and nitrone **4** were set at 100 mM, a concentration of which did not inhibit xanthine oxidase (data not shown). Of note, the flux of $O_2^{\bullet-}$ remained constant over the duration of all experiments, demonstrating that enough substrate (hypoxanthine) was available throughout the reaction and not a limiting factor.

In our initial series of experiments, ferricytochrome *c* was used as the competitive inhibitor. This decision was based on several factors. First, this protein is commercially available from a number of different venders at reasonable prices. Second, the rate constant (k_{cytoc}) for the reaction of ferricytochrome *c* by $O_2^{\bullet-}$ has been determined using pulse radiolytic experiments. However, k_{cytoc} is dependent on the ionic strength and pH of the buffer, ranging from 4.0×10^5 to 1.1×10^6 M^{-1} s^{-1}.[13,16] Third, the concentration of ferricytochrome *c* in these kinetic experiments can be set sufficiently high, without interfering with the enzymatic activity of xanthine oxidase, so that the catalytic concentration is not significantly diminished.

Despite the above, one should be attentive to the limitations of ferricytochrome *c*, in this experimental paradigm. For instance, ferrocytochrome *c* can be oxidized by H_2O_2, the dismutation product of $O_2^{\bullet-}$. While this one-electron oxidation can be, in some experimental models, a serious problem,[19] in these kinetic studies we have not observed a loss in linearity over the short time period of 2 min for the experiment. Typically, inclusion of catalase (50 U/mL) to scavenge H_2O_2 was found not to alter the calculated k_{app} for the reaction of nitrone **3** with $O_2^{\bullet-}$ (Table 1). In addition, ferricytochrome *c* may accept an electron from the enzyme that is the source of $O_2^{\bullet-}$. Such is the case with NOS,[3a,20] where the inability of SOD to inhibit the reduction of ferricytochrome *c* led to the conclusion that NOS only produced H_2O_2.[21] Nevertheless, with ferricytochrome *c*, we have determined that the k_{app} for the reaction of $O_2^{\bullet-}$ with nitrone **3** falls within a range of 29−79 M^{-1} s^{-1}, using the literature values for k_{cytoc} of 4.0×10^5 M^{-1} s^{-1} to 1.1×10^6 M^{-1} s^{-1}.[13,16] The value of ~58 M^{-1} s^{-1} for k_{app} obtained using $k_{cytoc} = 8.0 \times 10^{5}$ [13c] was closest to our experimental conditions (Table 1) and similar to other reports.[8d,10]

In our second series of experiments, Cu/Zn-SOD was used as the competitive inhibitor for the determination of k_{app}. This enzyme was chosen for these kinetic studies, as it is specific for this free radical; there are no other substrates for SOD.[22] The reaction is first order with regard to SOD and its substrate, and the rate constant is uniform and independent of pH over a broad range from 5.0 to 9.5. In addition, SOD reacts with $O_2^{\bullet-}$ at near diffusion-controlled rates. The rate constant is independent of the oxidation state of the metal ion, whether Cu^{2+} or Cu^{1+}. The enzyme is, for all practical purposes, nonsaturable with a K_m > 5 mM.[23] And finally, the catalytic removal of $O_2^{\bullet-}$ does not diminish the effective concentration of the enzyme.

There are, nevertheless, concerns associated with the use of SOD in this kinetic model. First, we found that purity of the commercial sources of SOD varied, requiring that each preparation be standardized to determine the exact enzymic activity. For illustrative purposes, we conducted experiments with two different commercial preparations of Cu/Zn-SOD from the same vendor. SOD activity, equivalent to 1 unit of enzyme activity as defined in the literature,[24] varied slightly between these preparations (see the Experimental Section). These SOD activity values were then used to determine the concentration range of SOD suitable for the inhibition experiments. Based on data from these studies, we determined that the k_{app} for nitrone **3** was ~70 M^{-1} s^{-1}, depending on the k_{SOD} chosen[6] (Table 1). Additional experiments were conducted to determine the k_{app} for nitrone **1** and nitrone **4** using SOD as a competitive inhibitor. Based on these findings, we determined that the k_{app} for nitrone **1** and nitrone **4** to be ~25 M^{-1} s^{-1} and ~90 M^{-1} s^{-1}, respectively, using 3.8×10^9 M^{-1} s^{-1}as k_{SOD} (Table 2), comparable to those found previously.[4,8d]

While the k_{app} for nitrone **3** using ferricytochrome *c*, as the competitive inhibitor, is comparable with k_{app} calculated using SOD (Table 1), we believe that in this kinetic model SOD is the more appropriate of the two. First, one should be mindful of the primary side reactions that occur when using ferricytochrome *c*−one-electron reduction of this probe. For instance, NOS will reduce ferricytochrome *c* as well as acetylated and succinylated ferricytochrome *c* at a rate that is faster than the reaction of $O_2^{\bullet-}$ with ferricytochrome *c* and its analogues.[20] Second,

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the rate constant for ferricytochrome *c* reaction with $O_2^{\bullet-}$ is dependent on the pH and the ionic strength of the buffer,[13,16] and thus, small changes in experimental design will significantly alter the calculated k_{app}. On the other hand, the rate constant for SOD with $O_2^{\bullet-}$ is uniform and independent of pH over a broad pH range from 5.0 to 9.5 under various experimental conditions, and this enzyme is specific to $O_2^{\bullet-}$.

A review of literature shows a broad range of determined k_{app} for several nitrones.[4,7c,8d,9,25] For instance, a recent publication[9] reported the k_{app} for nitrone **1**, nitrone **2**, and nitrone **3** to be 2.4, 0.53, and 0.24 M^{-1} s^{-1}, respectively, while a second report[25] determined the k_{app} of nitrone **1** and nitrone **2** to be 2.0 M^{-1} s^{-1} and 4.0 M^{-1} s^{-1}, respectively. On the basis of the rate constants presented[9] and time course studies described therein, it was predicted that nitrone **1** would be more efficient at spin trapping $O_2^{\bullet-}$ in short-term studies (up to 100 s). This finding is unexpected given the short lifetime of aminoxyl **5**[26] compared to aminoxyl **6** and aminoxyl **7**.[10] In contrast, data presented[25] predicts a different scenario with nitrone **2** being more efficient than nitrone **1**. The difference in experimental design between these studies[9,25] and data presented herein is the flux of $O_2^{\bullet-}$ used for the determination of k_{app} (22 μM/min,[9] and unknown,[25] compared to 1 μM/min, herein) and the competitive inhibitor (self-dismutation of $O_2^{\bullet-}$,[9,25] versus ferricytochrome *c* and SOD, herein). Unlike these studies,[9,25] our data suggest that nitrone **3** is a more efficient spin trap for $O_2^{\bullet-}$ than is nitrone **1** and is comparable to nitrone **2**.[7] Also, the calculated k_{app} for these nitrones (Tables 1 and 2) and those presented previously[7,8d] are higher than that reported in the above studies.[9,25]

Currently, we cannot explain the disparity between the data herein and those reported using the self-dismutation of $O_2^{\bullet-}$ as a competitive inhibitor model. However, the observed pH dependence of k_{app} of the reaction of nitrones with $O_2^{\bullet-}$ in a previous study[4] may provide insight into some of the discrepancies between current investigations. Recently, the k_{app} for the spin trapping of $O_2^{\bullet-}$ by nitrone **3** was estimated to be ~3 M^{-1} s^{-1} at pH 10 in which pulse radiolysis was used as a source of $O_2^{\bullet-}$.[27] Given that the k_{app} for spin trapping $O_2^{\bullet-}$ by nitrone **1** is pH dependent,[4] the pH dependence on the k_{app} for the spin trapping of nitrone **3** with this free radical was investigated using SOD as a competitive inhibitor. We determined that the pH dependence on k_{app} for nitrone **3** was comparable to the trend observed with nitrone **1**[4] (Table 3). The k_{app} for nitrone **3** increased by ~45% at pH 5 from that observed at pH 7 and decreased by about ~25% at pH 10 (Table 3). The k_{app} is noticeably dependent upon pH, increasing with decreasing pH. Of note, the k_{app} observed at pH 10 for nitrone **3** (Table 3) is slightly higher compared to that calculated with pulse radiolysis at pH 10, which predicts that our calculated k_{app} at pH 7 may also be slightly higher. However, these data do suggest that the k_{app} for nitrone **3** at pH 7 must be significantly greater than 3 M^{-1} s^{-1}, observed at pH 10 by pulse radiolysis, following this pH trend (herein and ref 4).

TABLE 3. Apparent Rate Constant (k_{App}) for the Reaction of $O_2^{\bullet-}$ with Nitrone 3 at Various pHs

pH	k_{app}[a] (M^{-1} s^{-1})
5.0[b]	80.1 ± 6.6 to 117.0 ± 9.8
7.0[c]	49.9 ± 8.2 to 73.0 ± 12
10.0[d]	12.4 ± 1.9 to 18 ± 2.8

[a] Rate constants are the average of three independent experiments, expressed as the means and standard deviations using the 32.5 kDa preparation of SOD and k_{SOD} of $(2.6-3.8) \times 10^9$.[6] [b] FMN, NADPH 120−160 μM, 50 mM phosphate buffer, 1 mM DTPA. [c] Hypoxanthine/xanthine oxidase (1 μM/min of $O_2^{\bullet-}$), 50 mM phosphate buffer, 1 mM DTPA. [d] FMN, NADPH 1 mM, 50 mM phosphate buffer, 1 mM DTPA.

TABLE 4. EPR Spectral Peak Heights (mm) of Aminoxyl 5 and Aminoxyl 7

aminoxyl[a]	1 μM/min of $O_2^{\bullet-}$	20 μM/min of $O_2^{\bullet-}$
aminoxyl (**5**)	51.7 ± 5.1	77.7 ± 12
aminoxyl (**7**)	68.7 ± 9.0	147 ± 13

[a] The low-field EPR spectral peak height of aminoxyl **5** and aminoxyl **7**, derived from the reaction of $O_2^{\bullet-}$ with nitrone **1** and nitrone **3**, respectively, at pH 7.4, are measured. Data are the average of three independent experiments, expressed as the means and standard deviations. EPR spectra were recorded continually with the highest point of the first spectral peak appearing at 70 s. For more details, see the Experimental Section.

To ascertain if the significant differences in flux rate of $O_2^{\bullet-}$ could have contributed to dissimilar values for k_{app}, the spin trapping efficiency of nitrone **1** and **3** was monitored at 20 and 1 μM/min (Table 4 and Figure 1). For comparison purposes, 50 mM of each nitrone was used, as this concentration falls within the range of kinetic experiments previously reported.[7,8d,9,10,25] In Figure 1, we present EPR spectra of aminoxyl **5** and aminoxyl **7** recorded with the highest point of the first peak at 110 s, a time point when maximal EPR spectral peak height for aminoxyl **5** has previously been noted.[9] Since EPR spectra of aminoxyl **5** and aminoxyl **7** are similar,[8d,28] we compared their low-field and high-field spectral peak heights. We found that at the 1 μM/min of $O_2^{\bullet-}$, the EPR spectral peak height of aminoxyl **5** (Figure 1B) remained constant, whereas that of aminoxyl **7** (Figure 1A) grew during the 5 min scan. However, at 20 μM/min flux of $O_2^{\bullet-}$, the EPR spectral peak height of aminoxyl **5** (Figure 1D) decreased during the EPR scan. And at the end of the spectrum, the ratio of the high-field to the low-field spectral peak height had declined by 50%, demonstrating significant decomposition of aminoxyl **5** as previously noted.[4] In contrast, at this same $O_2^{\bullet-}$ flux, the ratio of the high-field to the low-field EPR spectral peak height of aminoxyl **7** had essentially remained unchanged (Figure 1C). We then measured the low-field EPR spectral peak height for aminoxyl **5** and aminoxyl **7** at 70 s at a flux of 1 and 20 μM/min (Table 4). This time scan would closely match the early time point used for the determination of k_{app} in ref 9. At 1 μM/min of $O_2^{\bullet-}$, there is no significant difference in spectral peak height between aminoxyl **5** and aminoxyl **7**. On the

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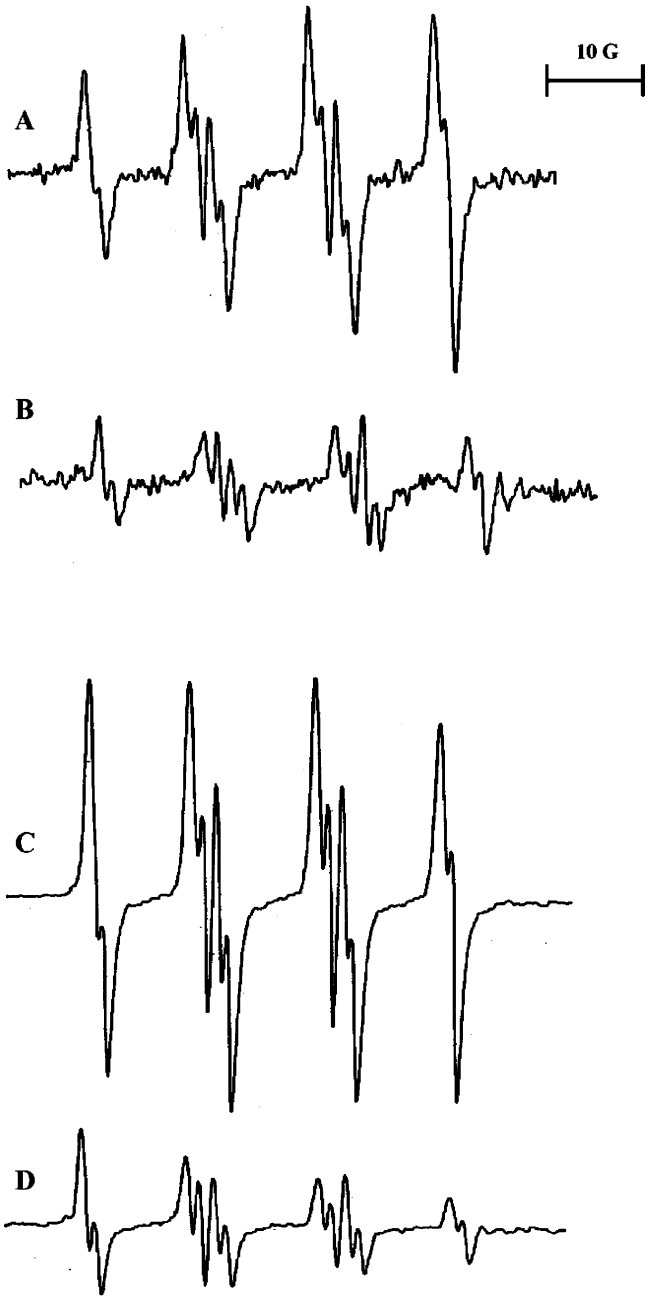

FIGURE 1. Typical EPR spectra for the reaction of nitrone **1** and nitrone **3** with $O_2^{\bullet-}$ using the hypoxanthine/xanthine oxidase reaction system. The reaction mixture contained nitrone (50 mM), hypoxanthine (400 μM), and sufficient xanthine oxidase to generate $O_2^{\bullet-}$ at specified concentrations in potassium phosphate buffer (50 mM, pH 7.0, containing 1 mM DTPA). EPR spectra were recorded at room temperature with the highest point of the first peak at 110 s, after the reaction was initiated by the addition of xanthine oxidase. (A) Nitrone **3**:, 1 μM/min of $O_2^{\bullet-}$, receiver gain was 10×10^4. (B) Nitrone **1**: 1 μM/min of $O_2^{\bullet-}$, receiver gain was 10×10^4. (C) Nitrone **3**: 20 μM/min of $O_2^{\bullet-}$, receiver gain was 4×10^4. (D) Nitrone **1**: 20 μM/min of $O_2^{\bullet-}$, receiver gain was 4×10^4. Scan speed for all experiments was 12.5 G/min.

other hand, at $O_2^{\bullet-}$ flux of 20 μM/min, the spectral peak height of aminoxyl **7** is nearly doubled that of aminoxyl **5**. In our hands, nitrone **3** appears to be the superior spin trap for $O_2^{\bullet-}$ as compared to nitrone **1** at early and longer time points based on the determination of k_{app} for each using SOD (Tables 1 and 2) and EPR spectral data presented in Figure 1 and Table 4.

In the Introduction of this paper, we mentioned that three different competitive kinetic models have been used to estimate rate constants for spin trapping $O_2^{\bullet-}$ by nitrones. Thus far, we have focused exclusively on experiments that compare ferricytochrome *c* with SOD. In fact, we believe that using the self-dismutation of $O_2^{\bullet-}$ as a competitive reaction has serious limitations. First, the rate of disappearance of $O_2^{\bullet-}$ is dependent on the concentration of $O_2^{\bullet-}$. Higher concentrations of $O_2^{\bullet-}$ promote a faster rate of disappearance, thereby providing less $O_2^{\bullet-}$ to react with the nitrone. Second, as shown in Figure 1D, high fluxes of $O_2^{\bullet-}$ increase the rate of aminoxyl decomposition, whether this is through direct reaction of the aminoxyl with either $O_2^{\bullet-}$ or H_2O_2.[4,18] These secondary reactions can result in an underestimation of k_{app}.

In contrast, in the experiments in which either ferricytochrome *c* or SOD is used as a competitive inhibitors, the flux of $O_2^{\bullet-}$ is kept low, while the concentration of the nitrone and inhibitor are high, eliminating the self-dismutation of $O_2^{\bullet-}$ reaction. Decomposition of aminoxyls in the absence (Figure 1) or the presence of competitive inhibitor (data not shown) was not observed under our experimental conditions.

Results presented[4,7,8d,9,10,25] report on the use of competitive kinetics to estimate k_{app} for spin trapping $O_2^{\bullet-}$. The disparity in k_{app} must arise from the choice of a competitive inhibitor, whether ferricytochrome *c*, Cu/Zn-SOD, or the self-dismutation of $O_2^{\bullet-}$ is used for the studies. Although we do agree with the conclusions made[25] that the uncertainty in k_{cyto} makes it a less favorable choice for the determination of k_{app}, the lowest k_{cyto} still gives a significantly higher k_{app} than using the self-dismutation of $O_2^{\bullet-}$. Therefore, we recommend that Cu/Zn-SOD be used as a competitive inhibitor in determining rate constants for the reaction of $O_2^{\bullet-}$ with nitrones.

Experimental Section

Ferricytochrome *c* (bovine heart), hypoxanthine, xanthine oxidase (EC 1.1.3.22), bovine Cu/Zn-SOD (EC 1.15.1.1), flavin adenine mononucleotide (FMN), nicotinamide adenine dinucleotide phosphate (NADPH), and 5,5-dimethyl-1-pyrroline *N*-oxide **1** (freshly distilled prior to use) were purchased from Sigma Chemical Co. (St. Louis, MO). 5-*tert*-Butoxycarbonyl-5-methyl-1-pyrroline *N*-oxide **3** and 5-methoxycarbonyl-5-methyl-1-pyrroline *N*-oxide **4** were prepared according to methods described in the literature.[8c,d] All other chemicals were used as purchased without further purification.

Generation of $O_2^{\bullet-}$. Superoxide was generated from the action of xanthine oxidase on hypoxanthine (400 μM, final concentration) in potassium phosphate buffer (50 mM, pH 7.0, containing 1 mM DTPA). Initial rates of $O_2^{\bullet-}$ generation were estimated spectrophotometrically by measuring the SOD (30 U/mL)-inhibitive reduction of ferricytochrome *c* (10 μM) at 550 nm using an extinction coefficient of 21 $mM^{-1}cm^{-1}$.[29] The flux of $O_2^{\bullet-}$ was set to 1 μM/min or 20 μM/min for each experiment by adjusting the amount of xanthine oxidase added to the reaction mixture. For experiments performed at pH 5 and 10, an NADPH/FMN $O_2^{\bullet-}$ generating system was employed.[8d,17]

Standardization of SOD. SOD was standardized according to the method described by McCord and Fridovich[24] using

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the hypoxanthine/xanthine oxidase system described above as a continued source of $O_2^{\bullet-}$. Under these experimental conditions, the amount of SOD required to inhibit the reduction of ferricytochrome *c* by 50% was defined as 1 unit of enzyme activity. Two different commercial sources of SOD were used. SOD activity, equivalent to 1 unit of enzyme activity, varied from ~0.2 U/mL (I_{50} = 0.05 μg/ml) for a 32.5 kDa preparation to ~0.8 U/mL (I_{50} = 0.16 μg/mL) for a 31.2 kDa preparation.

Rate Constant for Spin Trapping of Superoxide. The k_{app} for the spin trapping of $O_2^{\bullet-}$ by nitrone **1**, nitrone **3**, and nitrone **4**, generating the corresponding spin trapped adduct of $O_2^{\bullet-}$, aminoxyl **5**, aminoxyl **7**, and aminoxyl **8**, was estimated using hypoxanthine/xanthine oxidase as a source of $O_2^{\bullet-}$. The reaction mixture contained nitrone (100 mM), hypoxanthine (400 μM), and sufficient xanthine oxidase in potassium phosphate buffer (50 mM, pH 7.0, containing 1 mM DTPA) to generate $O_2^{\bullet-}$ at a rate of 1 μM/min, as was determined by the SOD-inhibitive reduction of ferricytochrome *c*. Five concentrations of the competitive inhibitor, ferricytochrome *c* (0−20 μM) or SOD (0−4.0 nM), were chosen for the determination of k_{app} in the presence of the nitrone (100 mM). Since the catalytic concentration of SOD is not diminished during the period the experiment was performed, steady-state conditions, e.g., reaction of $O_2^{\bullet-}$ with either SOD or the spin trap, were achieved at ~3 min, i.e., after 3 min. no increase in peak height of the respective aminoxyl was observed and the peak height remained the same until the decay of the corresponding aminoxyl. Therefore, EPR spectrum of the aminoxyls was recorded 2 min after the reaction was initiated to ensure reliable and reproducible data before steady state conditions were achieved. Of note, a decrease in peak height of any of the aminoxyls as a result of the decomposition of the corresponding aminoxyl was not observed during the time course of the experiments. We previously reported that peak heights and peak areas of the EPR spectrum give essentially the same data.[30] The k_{app} for the spin trapping of $O_2^{\bullet-}$ by nitrone **1**, nitrone **3**, and nitrone **4** are given in Tables 1−3 as the average of three independent experiments, expressed as the means and standard deviations with a confidence level of 95%.

Reaction mixtures were immediately transferred to an open EPR flat quartz cell and introduced into the cavity of the EPR spectrometer. EPR spectra were recorded at room temperature 2 min after the reaction was initiated by the addition of xanthine oxidase. Instrument settings were: microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 0.5 G; sweep time, 12.5 G/min; and response time, 0.5 s.

Spin Trapping $O_2^{\bullet-}$. For the EPR spectra depicted in Figure 1, the reaction mixture contained spin trap (50 mM), hypoxanthine (400 μM), and sufficient xanthine oxidase to generate $O_2^{\bullet-}$ at a rate of 1 or 20 μM/min in potassium phosphate buffer (50 mM, pH 7.0, containing 1 mM DTPA). This concentration of spin trap (50 mM) falls within the range of concentrations used in other studies for comparison between results presented herein and those works.[7,8d,9,10,25] The reaction mixture was immediately transferred to an EPR flat quartz cell opened and introduced into the cavity of the EPR spectrometer. Considering that the concentration of O_2 is 250 μM in the buffer[31] and two fluxes of $O_2^{\bullet-}$ used herein were either 1 or 20 μM/min, it would take a minimum of 12 min to consume all the O_2 present, were there no diffusion of O_2 into the open ended EPR flat cell. Since experiments depicted in Figure 1 occur over a 6 min time course, we do not believe that the concentration of O_2 was sufficiently low to decrease the rate of $O_2^{\bullet-}$ production when the experiment was terminated. EPR spectra were continuously recorded at room temperature. Those EPR spectra shown in Figure 1 were recorded with the highest point of the first peak at 110 s, after the reaction was initiated by the addition of xanthine oxidase. The receiver gain is given in the legend of Figure 1.

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