This student paper was written as an assignment in the graduate course

Free Radicals in Biology and Medicine

(77:222, Spring 2003)

offered by the

Free Radical and Radiation Biology Program
B-180 Med Labs
The University of Iowa
Iowa City, IA 52242-1181
Spring 2003 Term

Instructors:
GARRY R. BUETTNER, Ph.D.
LARRY W. OBERLEY, Ph.D.

with guest lectures from:
Drs. Freya Q . Schafer, Douglas R. Spitz, and Frederick E. Domann

The Fine Print:

Because this is a paper written by a beginning student as an assignment, there are no guarantees that everything is absolutely correct and accurate.

In view of the possibility of human error or changes in our knowledge due to continued research, neither the author nor The University of Iowa nor any other party who has been involved in the preparation or publication of this work warrants that the information contained herein is in every respect accurate or complete, and they are not responsible for any errors or omissions or for the results obtained from the use of such information. Readers are encouraged to confirm the information contained herein with other sources.

SUPEROXIDE IN APROTIC SOLVENTS

By
Kimberly Krager

B180 ML
Department of Free Radical and Radiation Biology
The University of Iowa
Iowa City, IA 52242-1181

Abbreviations:
AE: Acetonitrile
DEPMPO: 5-(dimethoxyphosphorly)-5'-methyl-1-pyrroline *N*-oxide
DMF: Dimethyl formamide
DMPO: 5,5-dimethyl-pyrroline *N*-oxide
DMSO: Dimethyl Sulfoxide
EMPO: 5-ethoxycarbonyl-5methyl-pyrroline *N*-oxide
MTS: (5-(3-carboxymethyoxphenyl)-2-(4,5,-dimethyl-thiazolyl)-3-(4-sulphophenyl)tetrazolium, inner salt)
$O_2^{\bullet -}$ Superoxide
SOD: Superoxide Dismutase
XTT: (2,3-bis(2-methoxy-4nitro-5-sulphophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide, sodium salt)

Contents

Abstract

Superoxide ($O_2^{\bullet -}$) is the one electron reduction of ground state O_2. Superoxide is important in many biological systems, including lipid peroxidation, cytotoxicity certain drug toxicities and tissue inflammation [1]. The reduction of dioxygen leads to hydrogen peroxide or water unfortunately the reaction is very fast and the intermediate superoxide is hard to detect. The characterization of superoxide in aprotic solvents such as dimethyl sulfoxide however can stabilize superoxide for several hours. Detection is then performed using several methods.

Introduction

Superoxide is a radical that carries a negative charge due to the gain of an electron by ground state oxygen in the following reaction (reaction one):

$$O_2 + e- \longrightarrow O_2^{\bullet -} \qquad (1)$$

Vitamin C was found to inactive viruses *in vitro*, oxygen was, somehow, involved in the inactivation [2]. It was suggested that the inactivation occurred as a result of the formation of superoxide radical, or the hydroxyl radical [2]. The existence of superoxide was the subject of much debate, it was predicted by the theory of quantum mechanics [2]. Superoxide has shown to be involved in many biological mechanisms, such as phagocytosis, lipid peroxidation, and tissue inflammation.

Physical and Chemical Characteristics

The reduction of ground state oxygen to superoxide requires a powerful reducing system with an effective reduction potential of +0.33V [3]. Superoxide can act in both an oxidizing and reducing reaction by accepting or donating electrons respectively, depending on its environment. Superoxide is found to be the precursor for many other species such as peroxynitrite and singlet oxygen, and other radicals like hydroxyl radical (reaction 2).

The reaction that produced hydroxyl radical:

$$O_2^{\bullet -} + Fe^{3+} \longrightarrow {}^3O_2 + Fe^{2+}$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^{\bullet} + HO^- \qquad (2)$$

Reaction 3 describes the superoxide reaction that produces the peroxynitrite molecule:

$$O_2^{\bullet-} + NO^{\bullet} \longrightarrow OONO^- \qquad (3)$$

Superoxide is however a far less reactive radical compared to other radicals such as the hydroxyl radical [2].

In aqueous solution the disappearance of superoxide is very rapid, and this is due to the dismutation reaction, which is mediated by a group of enzymes know as superoxide dismutases (SOD). The dismutation reaction occurs because of the protonation of superoxide and to form $HO_2^{\bullet}$, which will continue to form H_2O_2 and O_2 equation 4.

$$O_2^{\bullet-} + HO_2^{\bullet} + H^+ \longrightarrow O_2 + H_2O_2 \qquad (4)$$

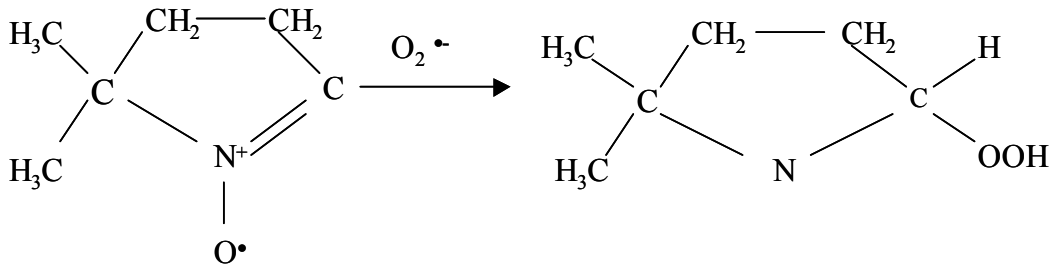
The second order rate constant for this reaction is 9.7×10^7 M^{-1} s^{-1} and has pK_a 4.8 at pH 7.4 [4]. Superoxide is absorbed at the ultraviolet range of 245nm. Superoxide displays different charactertics in aprotic solvents such as dimethyl sulfoxide and dimethylformamide compared to protic solutions. The redox potential for the $O_2/ O_2^{\bullet-}$ by water was found to be –0.16 V compared to -0.60 V when in dimethylformamide [5]. The redox potential of reaction 1 was found to be -.33V in aqueous conditions but the redox potential was –0.60V when in an aprotic solvent [6]. The UV absorption spectrum for DMSO and DMF are 250 nm and 257 nm compared to 245 nm in aqueous conditions, as seen in Table 1.

Detection of Superoxide

Several methods are utilized to detect and quantify superoxide. Some of the current assays rely on measurement of cytochrome c reduction, chemiluminescence from lucigemin and related dyes [7]. Superoxide is able to reduce tetrazolium dyes MTS and XTT to formazans. The rate constants for the MTS and XTT reactions with superoxide were estimated at 1.3×10^5 M^{-1} s^{-1} and 8.6×10^4 M^{-1} s^{-1} respectively [8]. Electron spin resonance (ESR) spin trapping is a

technique that is also used. For the Figures in this paper, ESR was performed with 5,5-dimethyl-pyrroline *N*-oxide (DMPO). ESR is able to detect superoxide but only in extremely low temperatures or by a rapid freezing technique [3]. The rate constant for the superoxide in DMPO is approximately 10 M^{-1} s^{-1}. DMPO traps the superoxide anion in the system (reaction 5), however the DMPO-OOH adduct is subject to spontaneous decay [3].

DMPO DMPO-OOH



(5)

The problem has recently been overcome with the generation of a phosphorylated analog, 5-(dimethoxyphosphorly)-5'-methyl-1-pyrroline *N*-oxide (DEPMPO). DEPMPO adducts is stabilized with the substitution of the 5-methyl group and analog 5-ethoxycarbonyl-5methyl-pyrroline *N*-oxide (EMPO). EMPO superoxide adducts were up to eight times more stable than with DMPO and exhibited the same ESR spectrum [6].

Generation of Superoxide in Aprotic Solvents

As indicated earlier in this paper, superoxide rapidly disappears in aqueous solution and is therefore hard to detect. Dimethyl sulfoxide (DMSO) and other aprotic solvents are able to stabilize superoxide under certain conditions from anywhere of couple hours to several days. Table one represents UV absorption spectral parameters for several aprotic solvents [9].

The effective way of generating superoxide is by the addition of sodium hydroxide to DMSO [10]. An ESR spectrum was generated by rapidly freezing the sample at a specific time.

DMPO was mixed with DMSO before the generation of superoxide was started [10]. Figure 1 represents an ESR spectrum at 77^{o} K for alkaline DMSO when it is frozen. The g-factor (the splitting factor of the free electron) for the spectrum is characteristic of the superoxide radical ($g_{\perp}$ = 2.005 and g_{ll} = 2.098) [10]. The concentration of superoxide is calculated to be 4.1 x 10^{-4} M. Figure 2 depicts the increase in the stability of superoxide when devoid of water that was from the atmospheric humidity. Figure 2a is the generation of superoxide in open vessel and the initial amount was followed by a rapid decay within 7 minutes [10]. The concentration of superoxide that was detected was less than 1% of the maximal concentration. Figure 2b represents that slow decrease of superoxide which is stable for 30 minutes on the graph, it was stated in the article that it was stable for more than 24 hours [10].

Superoxide Ion in solutions

Table I. UV Spectral parameters of "free" and bound $O_2^{\bullet-}$

	Solvent	λ_{max} (nm)	ε $M^{-1}cm^{-1}$
$O_2^{\bullet-}$	crystal	250	
$O_2^{\bullet-}$	H_2O	245	2350 ±120
$O_2^{\bullet-}$	DMF	<257.5	2500 ±150
$O_2^{\bullet-}$	AN	249	1760 ±230
$O_2^{\bullet-}$	DMSO	250	2686 ±29
$Co(s\text{-}R_2en)_2(Hal)O_2^{\bullet}$ *		212 ±4	12000
$[(NH_3)_5Co(O_2^{\bullet})Co(NH_3)_5]^{5+}$	H_2O	~225	~2100
$[(NC)_5Co(O_2^{\bullet})Co(NN)_5]^{5-}$	H_2O	~225	~2100

* s-R_2en—N,N-dialkylethlenediamine

Table 1. Representation of spectral parameters of free and bound superoxide [9].

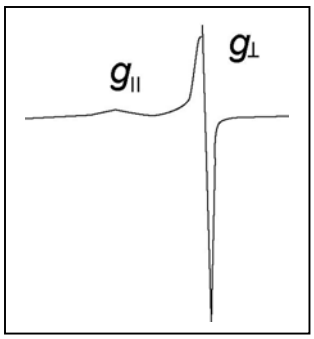


Figure 1 x-band ESR spectrum of superoxide in alkaline DMSO at 77^{o} K. Modulation amplitude, 0.4 mT; microwave power, 20 mW, time constant, 0.1 s. The concentration of superoxide was found to be 4.1 x 10^{-4} M. The g-factor of the spectrum ($g_{\perp}$ = 2.005 and g_{II} = 2.098) is characteristic of the superoxide radical [10].

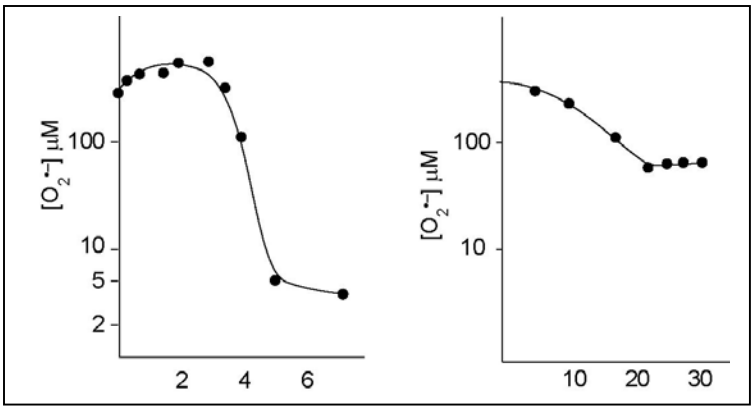


Figure 3a represents the open vessel generation of superoxide when mixed with DMSO.

Figure 3b represents the tightly stoppered vessel generation of superoxide when mixed with DMSO [10].

The concentration and stability is reliant on the concentration of oxygen in DMSO. The value of solubility of superoxide was calculated to be 2.2 mM atm^{-1} in DMSO. Oxygen occurs only in the reduced form after 3 minutes of mixing with DMSO and NaOH. The method of storage also was a factor and that water from the atmosphere may play a part in the rapid decay of superoxide [10].

Again the reduction of O_2 is the first step in the generation of superoxide. The consumption of O_2 through the addition of NaOH with DMSO is illustrated the next figure, figure 4. The consumption in figure 4 appears to be specific for DMSO since there is no oxygen consumption for the DMF and AE [11].

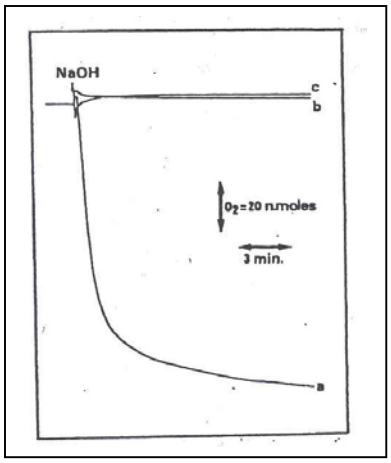


Figure 4. Oxygen consumption associated with the addition of NaOH to DMSO a) DMSO b) dimethyl formamide (DMF) c) and acetonitrile (AE). The final concentration of NaOH was 5 mM. The O_2 is equal to 20 nm for 3 min [11].

The last Figure, 5 shows the stabilization of superoxide in the presence of DMSO. The superoxide that is generated by NaOH in the presence of DMSO appears to be stable for up three days, and the concentration of the superoxide is inversely related to the H_2O concentration in the DMSO [11].

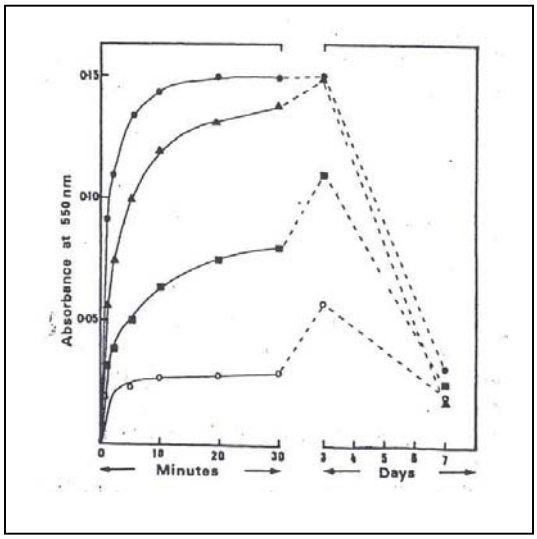


Figure 5. The effect of H_2O concentration of the kinetics of formation and stability of superoxide formed in DMSO. NaOH was added to DMSO to give final concentration of 5 mM NaOH. Final concentration of H_2O was 0.55 M (•), 1.1 M (Δ), 2.2 M (□), 4.4 M (o) [11].

Conclusion

Superoxide is reactive with many molecules, acting as an oxidant or reductant depending on the environment. The existence of superoxide was first only predicted, but with the development of more sensitive detection methods, the ability to detect superoxide became a reality. The methods of detection such a ESR helped to measure superoxide that was generated in many reactions. The generation of superoxide in aprotic solvents, such as DMSO is extremely useful because it stabilized the molecule for several days unlike seconds that was shown in aqueous solutions. Superoxide is found to be stable in aprotic solvents because of the weak solvation in that condition [5]. In water it is undergoes rapid hydrolysis and disproportionation to peroxide dianion. The disproportionation reaction to peroxide dianion is highly unfavorable in aprotic solvents rendering superoxide stable [5].

References

1. Stuehr D., Marletta M. (1985) Superoxide-promoted oxidation reactions of aniline and *N*-methylaniline in dimethyl sulfoxide. *J Org Chem*. **50**: 694-696.
2. Pauling L. (1979) The discovery of the superoxide radical. *TIBS*, 270-271.
3. Halliwell, B., Gutteridge J. (1999) *Free Radicals in Biology and Medicine*. Oxford New York: Oxford University Press.
4. Bieliski B., Cabelli D., Arudi, R.L. (1985) Reactivity of HO_2/O_2 radicals in aqueous solution. *J Phys Chem Ref Data*. **14:**1041-1100.
5. Sawyer T., Valentine J. (1981) How super is superoxide? *Acc. Chem. Res*., **14**:393-400.
6. Rodgers M., Powers E. (1981) *Oxygen and Oxy-Radicals in Chemistry and Biology*. New York, NY: Academic Press
7. Zhang H., Joseph J., Vasquez-Vivar V., Karoui H., Nsanzumuhire C., Martasek P., Tordo P., Kalyanaraman B. (2000) Detection of superoxide anion using an isotopically labeled nitrone spin trap: potential biological applications. *FEBS Letters***. 473**:58-62.
8. Sutherland M., Learmonth B. (1997) The tetrazolium dyes MTS and XTT provide new quantitative assays for superoxide and superoxide dismutase. *Free Rad Res*. **27**:283-289.
9. Afanasev B. Kuprianova N., Polozova N. (1983) Kinetics and mechanism of the reactions of the superoxide ion in solutions. I. Absorption spectra and interaction with solvents and cations. *Int J Chem Kinet*. **15**:1045-1056.
10. Haseloff R. (1989) Superoxide generation in alkaline dimethyl sulphoxide. *Analytica Chimica Acta*. **218**:179-184.
11. Hyland K. and Auclair C. (1981) The formation of superoxide radical anions by a reaction between O_2, OH^- and dimethyl sulfoxide. *Bioch Biophys Res Commun*. **102**:531-537.