DMPO Spin Trapping of Superoxide Anion in Strong Alkaline DMSO Solution

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Superoxide anion ($O_2^{\bullet-}$), generated in a strong alkaline DMSO solution and detected by direct 77 K EPR, was trapped with 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) and the resultant nitroxide radical, DMPO/$O_2^{\bullet-}$, has been found to be sufficiently stable for the subsequent EPR detection.

Superoxide anion ($O_2^{\bullet-}$)[1] is generated in living bodies *via* electron transfer reactions and scavenged by superoxide dismutases[2] forming H_2O_2, which is further converted into much more toxic hydroxyl radicals (•OH), actually the most harmful species, in the presence of transient metal ions or complexes.[3] Since such series of reactions are thought to induce serious diseases[4] such as atherosclerosis, chronic inflammation, ishaemia/reperfusion injury, cancer, and so on as well as aging, great efforts have been made to develop effective medicines which scavenge $O_2^{\bullet-}$.

In assays of test samples, the xanthine-xanthine oxidase (XOD) system generating $O_2^{\bullet-}$ is successfully coupled with EPR detection of the nitroxide radical (DMPO/$O_2^{\bullet-}$) which is produced, in the presence of samples, by the reaction of $O_2^{\bullet-}$ with a diamagnetic spin trap, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO).[5] By calculating the spin number, the scavenging efficiency of the test compounds can be estimated. This method, however, requires great skill of the operator because XOD converts dissolved O_2 into $O_2^{\bullet-}$ immediately[2] and, therefore, the sample preparation should be performed not only precisely but also extremely promptly so that one can always start the EPR measurements at the accurate time immediately after the preparation. One way to avoid this and make the assay more accurate is to prepare a stable $O_2^{\bullet-}$ pool.

A stable solution of $O_2^{\bullet-}$ ever reported is alkaline dimethylsulfoxide (DMSO).[6] This has been, however, rarely applied as a $O_2^{\bullet-}$ pool because nitroxide radicals have been reported to readily denature in alkaline solutions.[7] The half life of the •OH adduct of DMPO, for instance, is 6.5 min at pH 9.5. For DMPO/$O_2^{\bullet-}$, however, no detailed study has been conducted. Evidently, therefore, it is worthwhile to examine if the DMPO spin trapping method is applicable to this system. In the present study, DMPO was added to the alkaline DMSO solution, and examined if DMPO/$O_2^{\bullet-}$ generated by the reaction between DMPO and $O_2^{\bullet-}$ is successfully detected by EPR.

Reportedly, 1 mL of (L = dm^3) NaOH stock solution (0.5 *M*, *M* = mol dm^{-3}) was added to 99 mL DMSO (spectroscopy grade, Merck, Darmstadt, Germany) and aerated under the atmospheric condition.[6] The $O_2^{\bullet-}$ generation was confirmed by the UV spectrum (λ_{max} = 254 nm and ε = 1000).[8] For EPR measurements at 77 K, the aliquots were placed in a cylindrical EPR tube, which was then dipped in liq. N_2 in a dewar. The measurements were carried out on a TE EPR spectrometer (JEOL, Tokyo) with 100 kHz field modulation under the following settings: microwave power, 5 mW; field, 325±50 mT (9.12869 GHz); modulation, 0.5 mT; time constant, 0.1 sec; sweep time, 2 min. The resulting EPR spectrum consisted only of a broad line with g(perpendicular) = 2.007 and g(parallel) = 2.103, indicative of $O_2^{\bullet-}$ formation.[9] The EPR intensity increased for 90 min due to stability of $O_2^{\bullet-}$ in alkaline DMSO, and then started to decrease because of the reaction of $O_2^{\bullet-}$ with concomitant components in the solution as well as of the consumption of O_2, as shown in Figure 1. The maximum spin concentration calculated at 90 min using tetramethylpiperidine-N-oxyl (Aldrich, Milwaukee) as a standard electron spin was 10 μM.

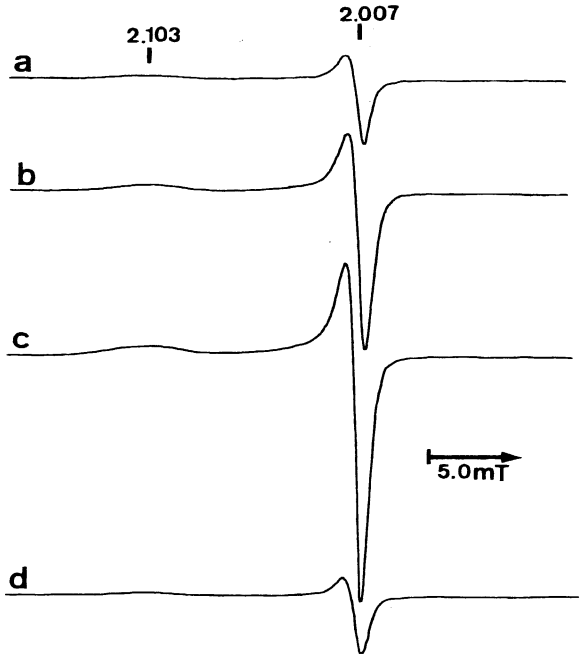

Figure 1. EPR spectra obtained at 77 K for a mixture of 1 mL NaOH (0.5 *M*) and 99 mL DMSO at (a) 1, (b) 30, (c) 90, (d) 120 min. EPR settings were: microwave power, 5 mW; field, 325±50 mT (9.12869 GHz); modulation, 0.5 mT; time constant, 0.1 sec; sweep time, 2 min Both the g values, g(perpendicular) = 2.007 and g(parallel) = 2.103, are indicated.

To the same solution, DMPO[10] (50 *mM*) (Labotec, Tokyo) was added, and subsequently EPR measurements were performed in an aqueous flat EPR cell at room temperature. The EPR settings were as follows: microwave power, 8 mW; field, 336±5 mT (9.42751 GHz); modulation, 0.079 mT; time constant, 0.1 sec; sweep time, 2 min. In this system, however,

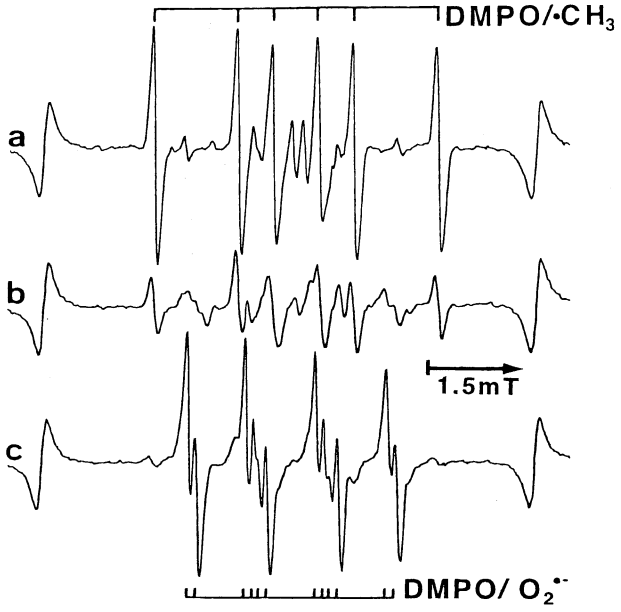

Figure 2. EPR spectra obtained at room temperature for a mixture of 1 mL NaOH (0.5 *M*) and 99 mL DMSO in the presence of 50 *mM* DMPO at (a) 1, (b) 45 and (c) 90 min. EPR settings were: microwave power, 8 mW; field, 336±5 mT (9.42751 GHz); modulation, 0.079 mT; time constant, 0.1 sec; sweep time, 2 min. EPR components for DMPO/$O_2^{\bullet-}$ and DMPO/$^{\bullet}CH_3$ spin adducts are indicated by the stick diagrams.

the EPR signals of DMPO/$O_2^{\bullet-}$ did not emerge immediately but at least 60 min after the sample preparation, as represented in Figure 2. The intensity was rather poor compared to the expected value based on the maximum spin concentration of $O_2^{\bullet-}$, although the EPR components were in good agreement of the values reported for DMPO/$O_2^{\bullet-}$: the hyperfine splitting constants obtained were a(N) = 1.27, a(βH) = 1.03, and a(γH) = 0.15 mT for the reported values of a(N) = 1.26, a(βH) = 1.04, and a(γH) = 0.13 mT.[11] It is indicated in Figure 2 that $^{\bullet}CH_3$ is generated from DMSO in the early stage of the $O_2^{\bullet-}$ formation and trapped by DMPO, and therefore possible that this species may attack the produced DMPO/$O_2^{\bullet-}$ to denature it because the concentration of $^{\bullet}CH_3$ is much larger than that of DMPO/$O_2^{\bullet-}$ whose generation is controlled by the slow reaction between DMPO and $O_2^{\bullet-}$ ($k_{DMPO+O_2^{\bullet-}}$ = 10 and 15.7 $M^{-1}s^{-1}$ in aqueous solutions at pH 7.8 and 8.0, respectively).[12] The responsibility of hydroxyl radical ($^{\bullet}OH$) can be ruled out since $^{\bullet}OH$ is readily scavenged by DMSO abundant in this system. Since the generation of $^{\bullet}CH_3$ is dependent on the OH^- concentration,[13] the influence of the NaOH concentration on the EPR signal was explored and 1 *mM* NaOH was found to lead to the immediate production of DMPO/$O_2^{\bullet-}$ signals whose EPR intensity was 4 fold larger than that obtained at 5 *mM* NaOH. Only in the stronger NaOH solution of 5 *mM*, the $^{\bullet}CH_3$ formation was confirmed by measuring the EPR signals obtained using 2,5-dibromonitrosobenzene sulfonic acid instead of DMPO: The hyperfine splitting constants were a(N) = 1.39 and $a(H^{CH_3})$ = 1.28, and a(γH) = 0.07 mT.[14] Also on addition of toluene as a scavenger, a DMPO system produced 3 x 2 x 3 line EPR signals characteristic of the methylene group generated by hydrogen abstraction from the methyl group of toluene and directly attached to the nitroxide center.

In an alkaline DMSO solution, it has been known that the water content is critical for the efficiency of $O_2^{\bullet-}$ generating reaction. It is, therefore, possible that sufficient hydration of $O_2^{\bullet-}$ as well as the spin trap leads to the improved spin trapping efficiency. To see this, we employed well-established $O_2^{\bullet-}$ generation system consisting of KO_2 and $_{18}C_6$-crown ether.[12] To a saturated KO_2/DMSO solution, added was 0.2 M $_{18}C_6$-crown ether/DMSO solution. When the system contained 50% H_2O, EPR signals of DMPO/$O_2^{\bullet-}$ was much higher while in the 77 K measurements, the $O_2^{\bullet-}$ EPR intensity was 10 fold less.

Evidently, as shown above, when alkaline DMSO is used as a $O_2^{\bullet-}$ pool and added to aqueous systems in the presence of test compounds and DMPO, EPR detection can serve as an efficient monitor of $O_2^{\bullet-}$ scavenging of such compounds.

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