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ESR-spin trapping in the presence of cyclodextrins. Detection of PBN-superoxide spin adduct

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Abstract—In phosphate buffer solution the half-life of the PBN-superoxide spin adduct is very short. However, as a result of the formation of inclusion complexes, its ESR signal was easily detected for up to 25 min when the trapping of superoxide anion with PBN was carried out in the presence of different cyclodextrins. Furthermore, the formation of these inclusion complexes results in a significant protection of the PBN-superoxide adduct against L-ascorbate monoanion reduction.

The ESR-spin trapping technique is a unique method to detect and identify free radicals and it is widely used to trap oxygen-centred radicals in biological milieu.[1,2] 5,5-Dimethyl-1-pyrroline *N*-oxide **1** (DMPO) and α-phenyl-*tert*-butyl nitrone **2** (PBN) (Fig. 1) are popular spin traps, which have been extensively used and the ESR features of their resulting spin adducts are well known.[3] PBN is commonly used to detect carbon-centred radicals resulting from radical reactions involving superoxide and hydroxyl radicals.[4,5] Pure crystalline PBN is readily available from many sources, its lipophilicity is significant ($K_p = 10$)[6] and it exhibits interesting protective properties against oxidative stress injuries in animal models.[7,8] However, PBN is a poor direct spin-trapping agent for hydroxyl and superoxide radicals, which are among the most important radicals involved in oxidative stress. At pH 7.4 the half-life of PBN-OH is 38 s[9,10] while it is even less for the superoxide spin adduct PBN-OOH.[11] We have recently shown that when the trapping of superoxide with either DMPO or 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO) was carried out in the presence of β-cyclodextrin, inclusion complexes were formed between the resulting nitroxide spin adducts, DMPO-OOH and DEPMPO-OOH, respectively, and the cyclodextrin.[12] The inclusion of these spin adducts into the cavity of the cyclodextrin resulted in a dramatic increase of their half-life and a change of their routes of decay. Kotake et al. have reported that stable linear nitroxides bearing alkyl and phenyl groups can form multimodal inclusion complexes with cyclodextrins.[13] Thus, it was tempting to perform the trapping of superoxide with PBN in the presence of cyclodextrins, to see if in these circumstances the PBN-superoxide could be easily detected. Hereafter we describe the results we obtained when the trapping was carried out in the presence of either methyl- or hydroxypropyl-β-cyclodextrin (Me-β-CD and HP-β-CD, respectively).

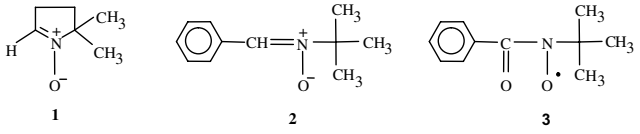

Figure 1. Chemical structures.

As previously reported,[11] in oxygenated-phosphate buffer at pH 7.4, the ESR signal ($a^N = 1.48$ mT and $a^{H\beta} = 0.27$ mT) (Fig. 2a) of an authentic sample of PBN-superoxide generated from the hypoxanthin (HX)/xanthin oxidase (XOD) system was very transient, and its kinetic of decay could not be measured. In the presence of Me-β-CD (50 mM) in the incubation mixture, a much more intense triplet of doublets accompanied with a less intense 1.1.1 triplet was obtained, and was observable for up to 25 min (Fig. 2b–f). This signal was inhibited in the presence of superoxide dismutase

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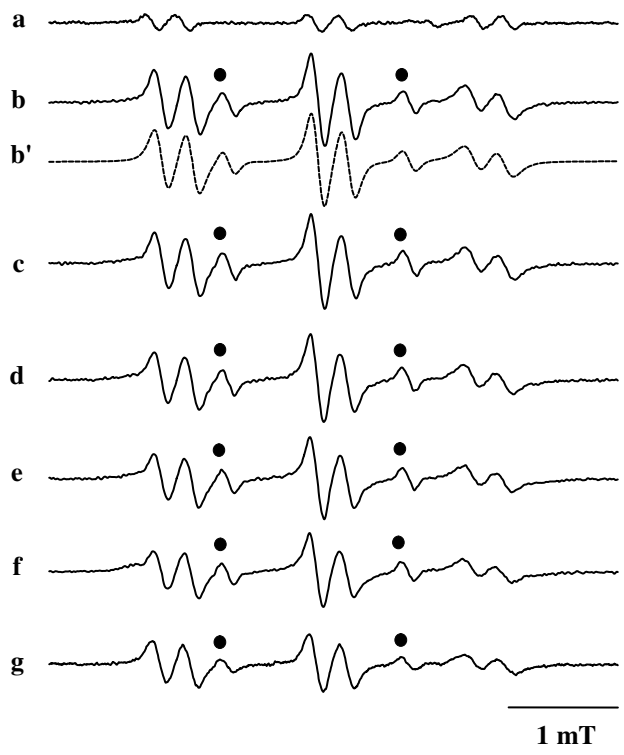

Figure 2. ESR signal of PBN-superoxide spin adduct in the presence of Me-β-CD. (a) Signal obtained after incubating for 5 min PBN (50 mM), DTPA (0.5 mM), HX (0.2 mM) and XOD (0.05 unit mL^{-1}) in oxygenated-phosphate buffer (0.1 M, pH 7.4) (four scans); (b) as (a) but in the presence of Me-β-CD (50 mM). (b′) Computer simulation of the spectrum (b); (c) as (b) but after 10 min of incubation; (d) as (b) but after 15 min; (e) as (b) but after 20 min; (f) as (b) but after 25 min; (g) PBN-superoxide spin adduct obtained after adding a 10% DMSO solution of KO_2 (0.1 M) in the presence of 1 equiv of 18-crown-6. Spectrometer settings: microwave power, 20 mW; modulation amplitude, 0.1 mT; time constant, 0.128 s; gain, 1×10^5; scan range, 6 mT and scan time, 82 s.

(SOD) (1250 units mL^{-1}), furthermore, the same signal was obtained when the HX/XOD system was replaced by an aliquot of a DMSO solution of KO_2 (0.1 M) containing 1 equiv of crown ether (18C6) (Fig. 2g). These observations indicate that the main signal observed in the presence of Me-β-CD, corresponds to the superimposition of both the free and the complexed PBN-superoxide spin adducts signals. In the presence of Me-β-CD, a much weaker intensity of the high-field doublet ($M_{IN} = -1$) was observed, which is in agreement with a slowing down of the tumbling motion expected for the PBN-OOH/Me-β-CD complex.

When the same experiment was carried out in the presence of increasing Me-β-CD concentrations from 3 to 150 mM, the intensity of the ESR signal increased, however, above 30 mM no significant changes of the coupling constant were observed. Thus, the spectrum shown in Figure 2b was calculated assuming a negligible contribution of the free PBN-OOH spin adduct. A very good agreement between the calculated (Fig. 2b′) and the experimental (Fig. 2b) spectra was obtained with the following parameters (complexed PBN-OOH, 91%, $a^N = 1.42$ mT and $a^{H\beta} = 0.28$ mT; three-line signal (•), 9%, $a^N = 0.83$ mT).[14] The nitrogen coupling is smaller for the complexed superoxide adduct ($\Delta a^N = 0.06$ mT), indicating that the nitroxyl group is partially inserted in the hydrophobic cavity of the Me-β-CD.

Owing to the large linewidth observed for the main triplet of doublets, we are aware that the assumption used for its calculation is questionable. The signal could correspond to the superimposition of the spectra of at least three species exhibiting very close coupling constants: the free PBN-OOH (very minor component at high concentration of cyclodextrin) and the 1:1 and 1:2 PBN-OOH/Me-β-CD complexes. Other experiments are in progress to yield a more detailed analysis of the signal.

The small nitrogen coupling value ($a^N = 0.83$ mT) measured for the three-line signal (•) indicates that it corresponds to an acyl alkylnitroxide, probably the benzoyl *tert*-butyl nitroxide **3** (Fig. 1), which has already been observed during the decomposition of PBN-peroxyl spin adducts in organic solvent.[15] However, because of its fast decomposition, the formation of **3** has never been observed in aqueous media. In the presence of Me-β-CD, the easy detection of **3** probably results from its inclusion into the cavity of the cyclodextrin. When the superoxide was generated from the HX/XOD system in the presence of PBN (25 mM) and Me-β-CD (50 mM), the signal of **3** was the only remaining signal after 40–45 min of incubation.

The signal of the complexed PBN-superoxide decayed rapidly when a large quantity of SOD (2500 units mL^{-1}) was used to quench the superoxide flux. At pH 7.4 and at room temperature, its half-life was estimated to be 180 s. The complexed PBN-superoxide adduct was protected towards L-ascorbate (AH^-) reduction. Addition of AH^- (0.10 mM) to the incubation mixture containing PBN (25 mM), HX (0.2 mM), DTPA (0.5 mM), Me-β-CD (50 mM) and XOD (0.05 unit mL^{-1}) slowly reduced the spin adduct signal, nevertheless it could be detected up to 6.5 min. The protection of the PBN-OOH adduct towards AH^- reduction increased with increasing concentrations of Me-β-CD. When Me-β-CD was used at 150 mM under the same experimental conditions described above, the PBN-OOH signal was still observed 9 min after the addition of AH^-. On the other hand, the addition of AH^- instantaneously removed the signal of **3**, thus indicating that its nitroxyl moiety is not included in the Me-β-CD cavity.

The use of other cyclodextrins such as α-CD and γ-CD did not yield the same results as those obtained with Me-β-CD. In the presence of α-CD, the PBN-superoxide adduct remained unobservable while a slight improvement was observed with γ-CD. The same experiments were also carried out in the presence of hydroxypropyl-β-cyclodextrin (HP-β-CD). As in the case of Me-β-CD, the presence of HP-β-CD in the incubation mixture stabilizes the PBN-superoxide spin adduct and an intense ESR signal was recorded. However, its intensity was three times lower than in the presence of Me-β-CD.

Among the different cyclodextrins that we have tested, Me-β-CD showed the best improvement in the results of experiments concerning the in vitro trapping of superoxide with PBN. The formation of inclusion complexes of PBN-OOH with Me-β-CD resulted in a significant stabilization of the nitroxide spin adduct and its partial protection towards L-ascorbate monoanion reduction. Besides its interest for in vitro spin trapping, the use of Me-β-CD could represent an interesting tool to detect superoxide formation with PBN during ex vivo experiments in biological fluids.

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