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THE EFFECT OF MYOGLOBIN ON THE STABILITY OF THE HYDROXYL-RADICAL ADDUCTS OF 5,5 DIMETHYL-1-PYROLLINE-N-OXIDE (DMPO), 3,3,5,5 TETRAMETHYL-1-PYROLLINE-N-OXIDE (TMPO) AND 1-ALPHA-PHENYL-TERT-BUTYL NITRONE (PBN) IN THE PRESENCE OF HYDROGEN PEROXIDE

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The hydroxyl radical adducts of 5,5 dimethyl-1-pyrolline-N-oxide (DMPO) and 3,3,5,5 tetramethyl-1-pyrolline-N-oxide (TMPO) formed in the presence of hydrogen peroxide and Fe^{II} are normally quite stable, but in the presence of 5-20 micromolar myoglobin their ESR signals decay rapidly. This decay probably reflects further oxidation of the adduct to nonparamgnetic products.

The ESR signal of the hydroxyl radical adduct of 1-alpha-phenyl-tert-butyl nitrone (PBN) formed under similar conditions is subject to non-heme dependent attenuation, possibly via hydroxyl radical scavenging, but not to heme dependent decay. Hydrogen peroxide readily converts myoglobin to its ferryl (Fe^{IV}) derivative, and this centre may be responsible for the oxidation of the DMPO and TMPO adducts. The different behaviour of PBN may be due to differences in susceptibility to ferrylmyoglobin mediated oxidation, or to steric differences controlling access to the heme pocket of myoglobin, and is relevant to the choice of spin trap for biological experiments aimed at detecting hydroxyl radicals in the presence of myoglobin or other heme proteins.

KEY WORDS:

INTRODUCTION

Spin trapping, the detection by electron spin resonance (ESR) spectroscopy of the relatively stable paramagnetic adducts formed between hydroxyl radicals and certain organic molecules, is a well recognised technique for measuring the formation of hydroxyl radicals in biological systems [1, 2]. The precautions necessary in interpreting results of biological spin-trapping experiments have been widely discussed [3]. If the spin-trap or its adducts react with some component of the biological system to form nonparamagnetic products, then interpretation may become impossible. We have previously shown that when 5,5 dimethyl-1-pyrolline-N-oxide (DMPO) reacts with hydrogen peroxide and ferrous iron in aqueous solution the ESR signal attributable to the DMPO-OH adduct formed decays rapidly on the addition of small

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quantities of myoglobin or haemoglobin. On the basis of the behaviour of the ESR signal of the stable radical 2,2,6,6 tetramethyl-1-piperidinyl-N-oxyl when exposed to both myoglobin and hydrogen peroxide, we have suggested that DMPO-OH may be oxidised by ferrylmyoglobin to non-paramagnetic products [4]. In this paper we extend these observations to the effect of myoglobin and hydrogen peroxide on two other nitrone spin traps, 3,3,5,5 tetramethyl-1-pyrolline-N-oxide (TMPO) and phenyl-tert-butyl nitrone (PBN), with the aim of identifying the most appropriate spin trap for biological studies where interaction with myoglobin or haemoglobin can be expected.

MATERIALS AND METHODS

Metmyoglobin (type III, from horse heart) was purchased from Sigma and purified on Sephadex G50 before use. Oxymyoglobin was prepared by adding an excess of sodium dithionite (Sigma) in the presence of air, and purified on a Sephadex G50 column. Apomyoglobin was purchased from Sigma and used after overnight dialysis against Hanks solution, or prepared by acid-acetone precipitation of metmyoglobin, further washing with acid acetone, and G50 filtration of the redissolved apomyoglobin (5). Hydroxyl radicals were generated from hydrogen peroxide (30% solution, Sigma) in the presence of ferrous ammonium sulphate (ACS grade, Sigma). Hydrogen peroxide concentrations were measured by titration against potassium permanganate. Total myoglobin concentrations were determined by Drabkin's method.

5,5'dimethyl-1-pyrroline-N-Oxide (DMPO), 3,3,5,5 tetramethyl-1-pyrroline-N-oxide (TMPO) and 1-alpha-phenyl-tert-butyl nitrone (PBN) were purchased from Sigma, and purified before use (1). The stable radical 2,2,6,6 tetramethyl-1-piperidine-N-oxyl (TEMPO) was also purchased from Sigma. ESR spectra were recorded using a JEOL JES-REIX spectrometer with a flat quartz cell. Spin trap adducts were monitored from 1 to 60 min after adding hydrogen peroxide to the mixture containing spin trap, ferrous ion, and myoglobin or apomyoglobin. For the purposes of the present study, we defined the signal amplitude or intensity measured 1 minute after mixing as the peak amplitude, and the apparent half life of the signal as the time for it to decay to half the peak value. All water was purified by the Milli-Q system.

RESULTS

ESR spectra of the hydroxyl radical adducts of DMPO, TMPO and PBN formed in the presence of hydrogen peroxide and Fe^{II} are shown in figure 1. The ESR spectra and their parameters were identical with those described in the literature. Under the conditions used, the amplitude of the ESR signals were approximately linearly related to the concentration of ferrous ion and of hydrogen peroxide.

In the presence of low concentrations of myoglobin (5–20 micromolar) the ESR signal of the hydroxyl radical adducts of both DMPO and TMPO decayed rapidly (figure 2, table 1). This decay was not seen when apomyoglobin was used instead of myoglobin. Hydrogen peroxide together with oxymyoglobin also cause decay of the amplitude of the ESR signal from the stable radical 2,2,6,6 tetramethyl-1-piperidine-N-oxyl. The rate of decay was proportional to the hydrogen peroxide concentration (figure 3).

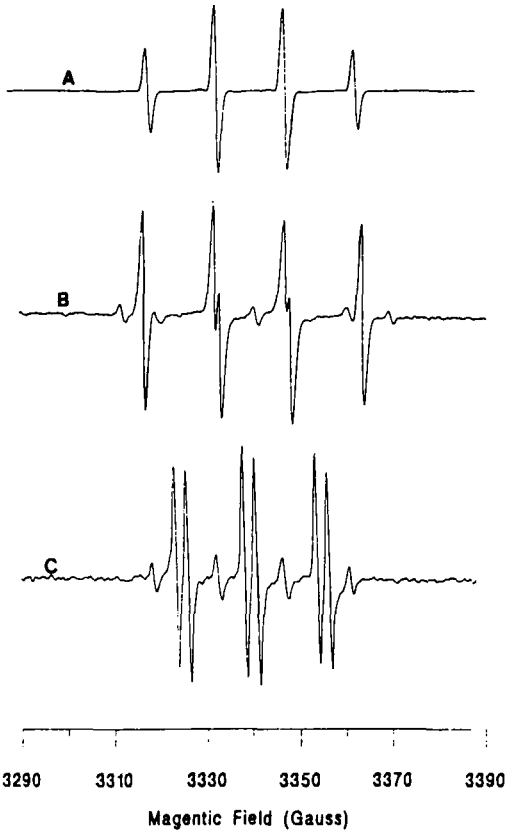

FIGURE 1 ESR spectra of the hydroxyl radical adducts of DMPO (a), TMPO (b) and PBN (c). Conditions were as follows: (a) DMPO 100 mM dm^{-3}, H_2O_2 1mM dm^{-3}, Fe^{++} 1 mM dm^{-3}, in phosphate buffered saline pH7.4. $A_N = A_H = 14.7G$. Gain 100, power 1 mW. (b) TMPO 100 mM dm^{-3}, H_2O_2 1 mM dm^{-3}, Fe^{++} 1 mM dm^{-3}. In phosphate buffered saline pH 7.4. $A_N = 15.3G$ $A_H = 16.8G$. Gain 500, power 1 mW. (c) PBN 300 mM dm^{-3}, H_2O_2 10 mM dm^{-3}, Fe^{++} 10 mM dm^{-3} in phosphate buffered saline pH 7.4. $A_N = 15.5G$, $A_H = 2.7G$ Gain 500, power 20 mW. All spectra were recorded at room temperature 1 minute after adding hydrogen peroxide. Centre Field 3340G, Scan range 100G, time constant 0.1 second, modulation 1G, frequency 9.42 GHz, scan time 1 minute.

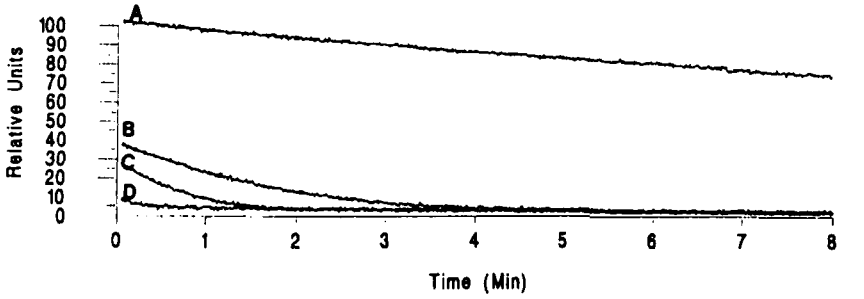

FIGURE 2 Time course of decay of amplitude of the ESR signal of the hydroxyl radical adduct of TMPO in the presence of oxymyoglobulin. A) TMPO 100 mM dm^{-3}, Fe^{++} 1 mMol dm^{-3}, H_2O_2 1 mMol dm^{-3}; B,C,D as for A plus 5, 10, 20 M^{-6} dm^{-3} oxymyoglobulin respectively. Centre field 3318G, other conditions as for figure 1b.

TABLE 1
Half-lives of hyrdoxyl radical adducts of DMPO, TMPO and PBN in the presence of myoglobin

Spin trap	H_2O_2	Myoglobin $M^{-6}dm^{-3}$	Half-life (min) t_1	t_2^*
DMPO	1 $M^{-3}dm^{-3}$	0	16	
	1	5	3.2	
	1	10	0.5	
TMPO	1 $M^{-3}dm^{-3}$	0	34	
	1	5	1.3	
	1	10	0.4	
PBN	10 $M^{-3}dm^{-3}$	0	1.2	90
	10	100	0.9	85
	10	200	0.8	83

*Decay of DMPO and TMPO signals is monophasic, of PBN signal biphasic.

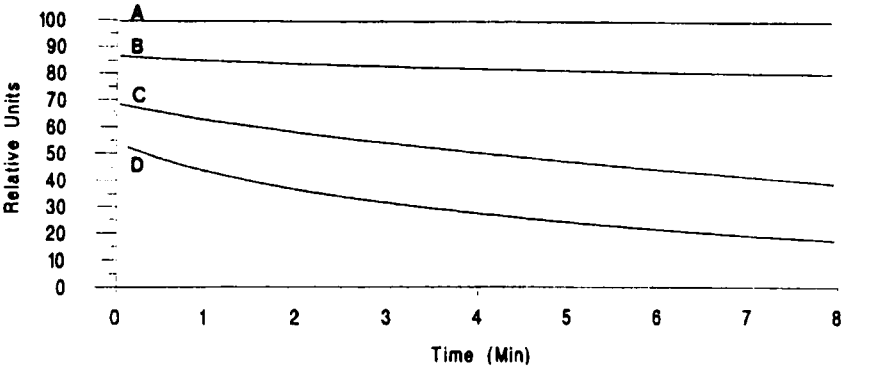

FIGURE 3 Effect of different concentrations of H_2O_2 on the stability of the ESR signal for 2,2,6,6 tetramethyl-piperidine-N-oxyl (TEMPO). A) TEMPO 10 $mM^{-3}dm^{-3}$ + oxymyoglobin 0.5 mM dm^{-3}, B-D as for A plus H_2O_2 1, 5, 10 mM dm^{-3} respectively.

In contrast, after an initial fall over a time course of about two minutes, the amplitude of the PBN-OH signal decayed only slightly in the presence of myoglobin concentrations as high as 200 micromolar (figure 4). There was a diminution in signal amplitude proportional to the concentration of myoglobin, but this was also seen with apomyoglobin. Similar results to those described with myoglobin were seen in corresponding experiments with haemoglobin.

DISCUSSION

We have shown that the hydroxyl radical adducts of the pyrroline nitrone spintraps DMPO and TMPO are unstable in the presence of myoglobin (or haemoglobin) and hydrogen peroxide, whereas the adduct of phenyl-tert-butyl nitrone is relatively stable. We have previously attributed the instability of DMPO-OH under these conditions to its oxidation to non-paramagnetic products by ferrylmyoglobin, formed by the reaction of oxy (or met) myoglobin with H_2O_2 [4]. The formation of ferrylmyoglobin under conditions of cardiac ischaemia and reperfusion has been

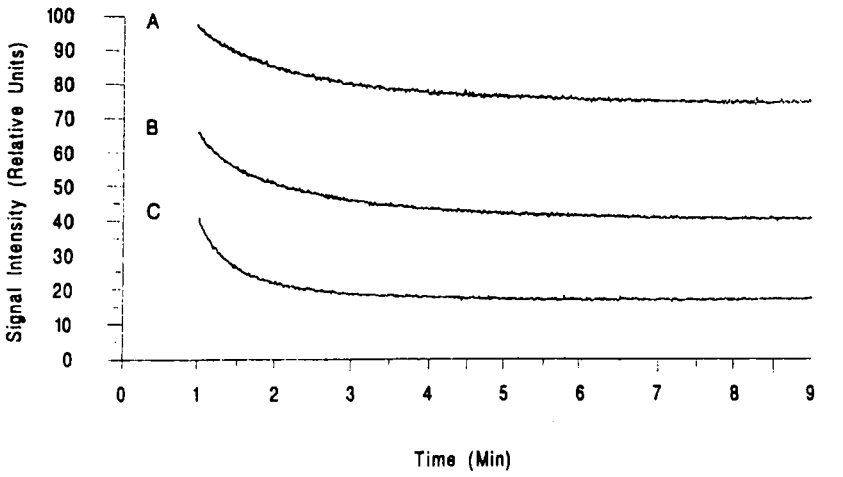

FIGURE 4 Effect of oxymyoglobin on the amplitude of the ESR signal from the hydroxyl radical adduct of PBN. A) PBN 300 mM dm^{-3} plus H_2O_2 10 mM dm^{-3}. B,C as for A but with the addition of 0.1, 0.2 mM dm^{-3} oxymyoglobin respectively. Centre field 3342G, conditions as for figure 1c.

shown by reflectance spectroscopy [6]. The ESR signal of the hydroxyl radical adduct of PSN formed under similar conditions is subject to non-heme dependent attenuation, possibly by hydroxyl radical scavenging and/or slow hydrogen peroxide scavenging, but not to heme dependent decay. The different behaviour of the spin traps may be due to differences in susceptibility to ferrylmyoglobin mediated oxidation, or to steric differences controlling access to the heme pocket of myoglobin, and is relevant to the choice of spin trap for biological experiments aimed at detecting hydroxyl radicals in the presence of myoglobin or other heme proteins. Clearly PBN is the spin trap of choice in systems containing myoglobin, despite its lack of sensitivity and the slow decay of the ·OH adduct.

Pyrroline nitrone spin traps have been used successfully to detect hydroxyl radical formation in biological ischaemia/reperfusion experiments, for example bv Pietri, Culcasi and Cozzone (7), but our results underline the difficulty of interpreting such results quantitatively. Reperfusion associated hydroxyl radical generation is likely to be accompanied or preceded by the production of superoxide anion and hydrogen peroxide. In myocytes, which have high myoglobin and superoxide dismutase but low catalase or free iron concentrations, conditions favour the formation of ferrylmyoglobin, and pyrroline nitrone spin trapping may considerably underestimate the formation of hydroxyl radicals: indeed, such adducts as are detected in perfusion experiments may well be formed extracellularly from hydrogen peroxide which has diffused out of myocytes. The use of PBN is less susceptible to such interference, although the cell permeability and diffusion characteristics of this agent are far from optimal.

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