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THE EFFECTS OF MYOGLOBIN AND APOMYOGLOBIN ON THE FORMATION AND STABILITY OF THE HYDROXYL RADICAL ADDUCT OF 5,5′-DIMETHYL-1-PYRROLINE-N-OXIDE

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When aqueous solutions of the spin trap 5,5′-dimethyl-1-pyrroline-N-oxide (DMPO) are treated with hydrogen peroxide in the presence of either Fe^{II} or light, the hydroxyl radical adduct DMPO-OH is formed, with a characteristic 4 line ESR spectrum. When oxy- or metmyoglobin is added to such a system the initial yield and the halife of DMPO-OH are reduced, and at high myoglobin concentrations (about 0.1 mmol dm^{-3}) DMPO-OH becomes undetectable. Using the stable nitroxide 2,2,6,6-tetramethyl-1-piperidinyloxy-N-oxyl (TMPO) for comparison it was found that neither hydrogen peroxide nor myoglobin alone caused a loss of signal, but together a marked loss of signal was induced. From the evidence of these and other experiments it was concluded that the DMPO-OH adduct reacts with hydrogen peroxide and myoglobin to give non-paramagnetic products, and hence that the use of the DMPO spin trap to detect hydroxyl or other active radicals in systems containing physiological concentrations of myoglobin may give misleading results.

KEY WORDS: Myoglobin, spin-trapping, myoglobin oxidase activity, DMPO.

INTRODUCTION

The formation of a relatively stable radical adduct with a "spin trap" such as 5,5′-dimethyl-1-pyrroline-N-oxide (DMPO) and its quantitation by electron spin resonance (ESR) spectroscopy is a widely used technique for estimating short lived free radicals such as .OH in biological systems.[1,2] This approach may be invalid if there are redox reactions between the DMPO-OH adduct and other components of the system. Myoglobin is a ubiquitous protein in cardiac and skeletal muscle, occuurring physiologically at concentrations of up to 0.3 mmol dm^{-3}. It is known that myoglobin reacts, via its heme group, with hydrogen peroxide,[3] and it forms at least one radical adduct with DMPO through a globin-centred radical.[4] In the present study, we show that the use of DMPO to estimate the formation of hydroxyl (.OH) radicals is not valid in systems containing physiological concentrations of myoglobin or, by inference, haemoglobin. When oxymyoglobin or metmyoglobin are added to solutions containing DMPO-OH adducts, the ESR signal is reduced, and its rate of decay greatly accelerated.

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.[6] Hydroxyl radicals were generated from hydrogen peroxide (30% solution, Sigma) in the presence of ferrous ammonium sulphate (ACS grade, Sigma), or by photolysis of hydrogen peroxide. Hydrogen peroxide concentrations were measured by titration against potassium permanganate.

Concentrations of oxymyoglobin, metmyoglobin and ferrylmyoglobin were assessed spectrophotometrically.[3] Total myoglobin concentrations were determined by Drabkin's method. 5,5'-dimethyl-1-pyrroline-N-oxide (DMPO) (Sigma) was purified before use.[1] In all experiments the initial concentration of DMPO was 100 mmol dm^{-3} in phosphate-buffered saline. ESR spectra were recorded using a JEOL JES-RE1X spectrometer with a flat quartz cell. DMPO adducts were monitored from 1 to 60 min after adding hydrogen peroxide to the mixture containing DMPO, 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; no DMPO adducts were detected on mixing hydrogen peroxide and DMPO (without added iron). DMPO-OH was also produced from DMPO by photolysis of hydrogen peroxide in the absence of ferrous ion. TMPO (2,2,6,6-tetramethyl-1-piperidinyloxy-N-oxyl) was purchased from Sigma and used at a concentration of 0.1 mmol dm^{-3}. The ESR spectrum of TMPO was monitored in a similar way to that of DMPO-OH.

RESULTS

Peak amplitude of the central ($M_I(HN)=0$) features for DMPO-OH were linearly related to initial hydrogen peroxide concentration (Figure 1). Oxymyoglobin both diminshed the peak signal intensity and shortened the apparent half life, in a concentration dependent manner (Figure 2, Table 1). The effect of metmyoglobin was similar to oxymyoglobin. Apomyoglobin reduced peak DMPO-OH concentration, but did not affect the rate of signal decay (Figure 3).

The effect of apomyoglobin depended on the initial hydrogen peroxide concentration. At low initial hydrogen peroxide concentrations (1 mmol dm^{-3}), 5×10^{-3} mmol dm^{-3} apomyoglobin produced about 50% of the inhibition of the DMPO-OH signal produced by the same concentration of oxymyoglobin. Increasing apomyoglobin concentration however led to a plateau, with some DMPO-OH remaining detectable at high apomyoglobin concentration. Bovine serum albumin behaved in a similar way to apomyoglobin. With high concentrations of hydrogen peroxide, the *relative* inhibition of the DMPO-OH signal by apomyoglobin or albumin compared with oxymyoglobin was much less.

Increasing hydrogen peroxide concentrations from 1 to 10 mmol dm^{-3} in the

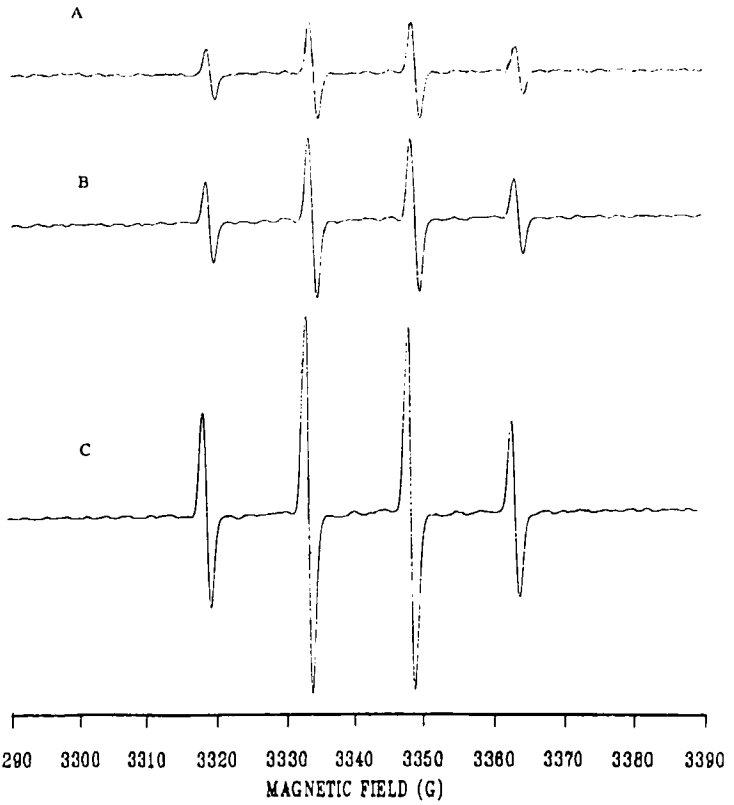

Figure 1 Typical ESR signals from DMPO-OH generated in the presence of (a) 0.5 mmol dm^{-3}, (b) 1 mmol dm^{-3}, (c) 2 mmol dm^{-3} hydrogen peroxide. Signal amplitudes (arbitrary units) a) 25, b) 48, c) 99. Initial concentrations DMPO 100 mmol dm^{-3} Fe^{2+} 1 mmol dm^{-3}. ESR spectra were recorded at 20°C, starting 60 s after mixing. The amplitudes of the signals were linearly related to initial hydrogen peroxide concentration.

Table 1 Effects of oxymyoglobin and apomyoglobin on the ESR signal of DMPO-OH (100 mmol dm^{-3} DMPO)

	Concentration ($\times 10^{-3}$ mmol dm^{-3})	1 mmol dm^{-3} HOOH Peak[a]	1 mmol dm^{-3} HOOH Halflife[b]	5 mmol dm^{-3} HOOH Peak	5 mmol dm^{-3} HOOH Halflife
Control	0	20.4	16.2	100.0	12.1
OxyMb	5	11.6	3.0	58.0	3.5
	10	6.1	0.5	29.6	0.2
	20	1.4	0.2	8.2	0.2
	50	0	0	0	0
ApoMb	5	11.4	13.7	93.8	11.6
	10	9.2	12.2	90.8	11.4
	20	8.6	11.0	80.6	11.3
	50	7.6	7.1	68.4	11.2
	100	6.9	5.0	56.1	10.5

All results average of 4 experiments.
[a]Peak amplitude measured 1 min after mixing, expressed as % of signal amplitude for 5 mmol dm^{-3} HOOH control.
[b]Apparent half life defined as time for signal amplitude to decline to half its amplitude at 1 min.

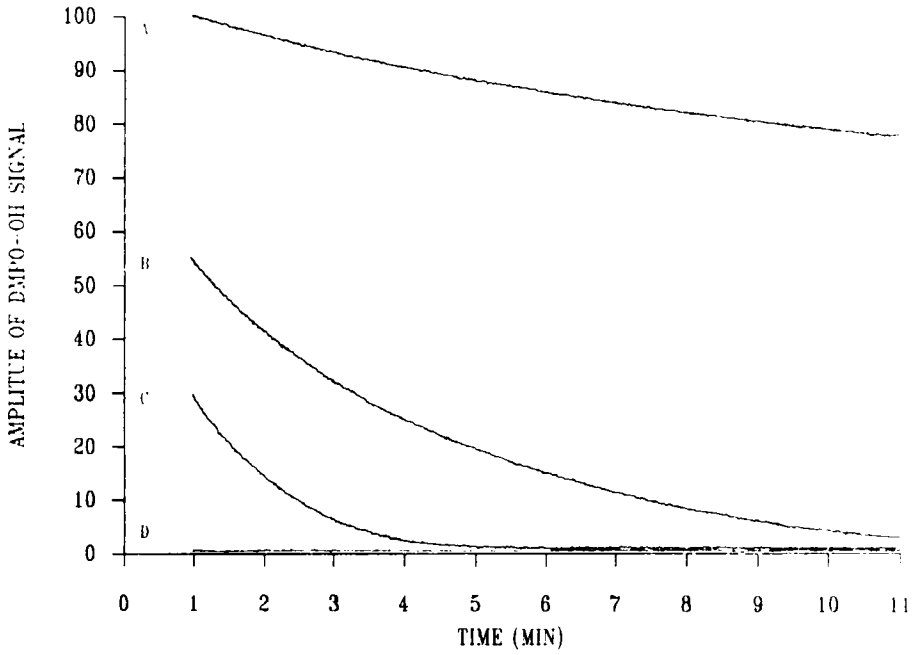

Figure 2 Time dependence of the change in amplitude of the DMPO-OH ESR signal in the presence of different concentrations of oxymyoglobin. A) control, B) 5×10^{-3} mmol dm^{-3}, C) 10×10^{-3} mmol dm^{-3}, D) 20×10^{-3} mmol dm^{-3}. Initial hydrogen peroxide concentration 5 mmol dm^{-3}, Fe^{++} 5 mmol dm^{-3}, DMPO 100 mmol dm^{-3}. Other conditions were as for Figure 1. Results are the average of four experiments.

presence of sufficient oxymyoglobin completely to suppress the DMPO-OH signal at the original hydrogen peroxide concentration led to reappearance of the DMPO-OH signal. Decreasing ferrous iron concentration from 1 mmol dm^{-3} to 0.1 mmol dm^{-3} diminished the size of the DMPO-OH signal and also diminished the quantity of myoglobin required to suppress it from 0.1 mmol dm^{-3} to 5×10^{-3} mmol dm^{-3}. Oxy- or metmyoglobin also suppressed the DMPO-OH signal produced by photolysing hydrogen peroxide in the presence of DMPO but the absence of ferrous ion.

High concentrations (5×10^{-1} mmol dm^{-3}) of oxy- or metmyoglobin, but not apomyoglobin, gave a DMPO adduct with a characteristic "slow tumbling" ESR spectrum in the presence of hydrogen peroxide but the absence of ferrous iron (Figure 5). The signal was reduced if ferrous iron was added, but no DMPO-OH signal was detected.

During the course of the reaction with hydrogen peroxide in the presence of Fe^{II} and DMPO, oxymyoglobin was converted initially to ferrylmyoglobin, and subsequently to metmyoglobin over a time course of several minutes (Figure 6). There was no loss of myoglobin under these conditions.

Experiments with Myoglobin and the Stable Nitroxyl Compound TMPO

The ESR signal of TMPO was unaffected by either 1 mmol dm^{-3} hydrogen peroxide

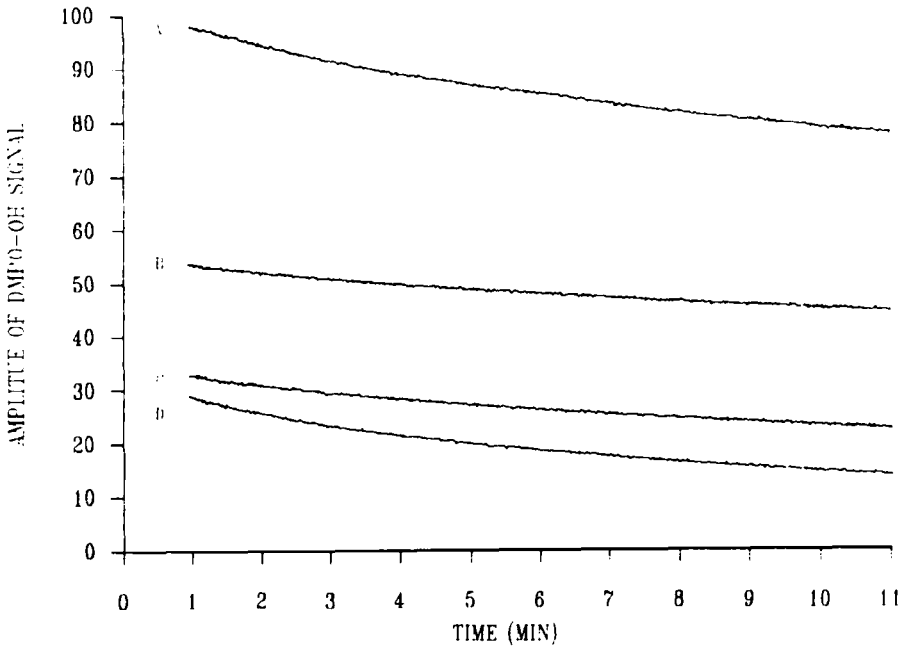

Figure 3 DMPO-OH signal strength against time in the presence of different concentrations of apomyoglobin: A) control, B) 10×10^{-3} mmol dm^{-3}, C) 20×10^{-3} mmol dm^{-3}, D) 100×10^{-3} mmol dm^{-3}. Other conditions as for Figure 2.

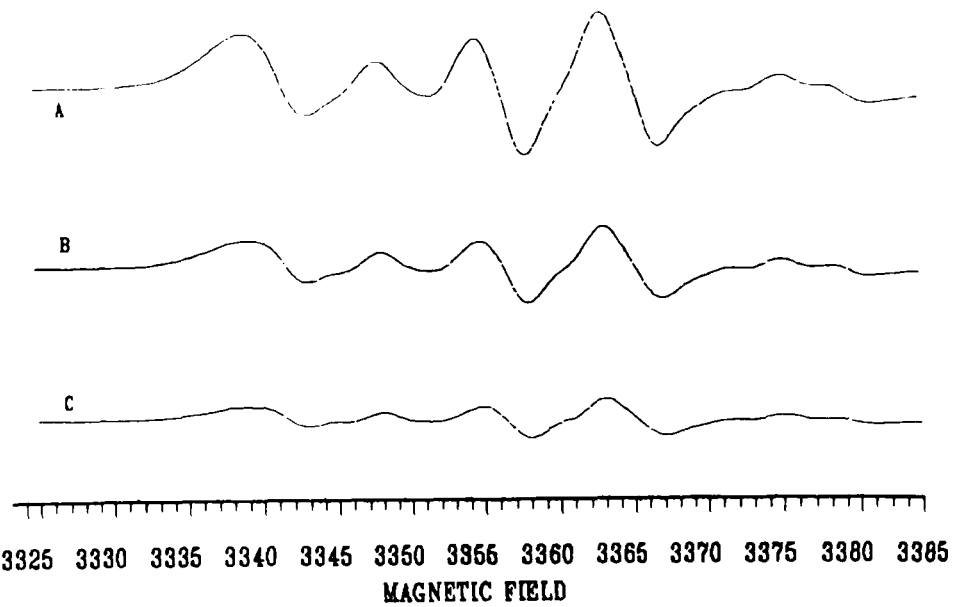

Figure 4 ESR spectra of DMPO-myoglobin adduct. A) DMPO (100 mmol dm^{-3}) + 200×10^{-3} mmol dm^{-3} oxymyoglobin + 5 mmol dm^{-3} hydrogen peroxide, B) as for (A) + 1 mmol dm^{-3} Fe^{2+}, C) as for (A) + 2 mmol dm^{-3} Fe^{2+}

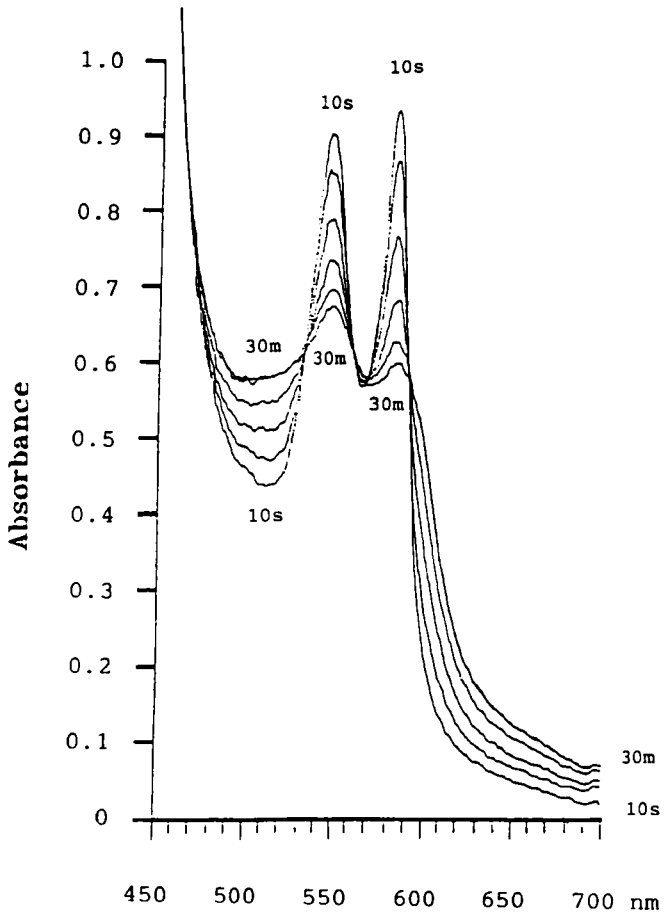

Figure 5 Absorption spectra of oxymyoglobin after incubation with 5 mmol dm^{-3} hydrogen peroxide and 5 mmol dm^{-3} ferrous iron. Spectra recorded at 10 seconds and 2, 5, 10, 20 and 30 minutes, showing progressive conversion of oxymyoglobin to ferryl and then metmyoglobin.

Table 2 Effect of oxymyoglobin and hydrogen peroxide on ESR signal of TMPO (TMPO 0.1 mmol dm^{-3})

	Half life (min)
100 μM Mb	Stable
10 mM HOOH	Stable
100 μM Mb + 1 mM HOOH	32
100 μM Mb + 5 mM HOOH	6.22
100 μM Mb + 10 mM HOOH	3.77
100 μM Mb + 5 mM HOOH + catalase	12.2

All results average of 4 experiments.

or 10^{-1} mmol dm^{-3} oxymyoglobin alone, but the combination of both hydrogen peroxide and oxymyoglobin resulted in signal loss. Ferrylmyoglobin, prepared by adding hydrogen peroxide to metmyoglobin followed by catalase to remove free hydrogen peroxide, also diminished the TMPO signal (Table 2).

DISCUSSION

There are three clear ways in which myoglobin could reduce the concentration of the DMPO-OH adduct—by scavenging hydroxyl radicals, by catalysing the breakdown of hydrogen peroxide, or by reacting directly with DMPO-OH. Nonspecific hydroxyl radical scavenging probably explains the effect of apomyoglobin, which behaves in a similar fashion to bovine serum albumin.

The reaction between myoglobin and hydrogen peroxide has been described by several authors.[3,4,5,7,8] It leads to the initial formation of ferrylmyoglobin, which may subsequently react with oxymyoglobin to give metmyoglobin, or break down in other possible ways, including sacrifical autooxidation with the eventual loss of iron. Davies[4,5] described the formation of an adduct between DMPO and myoglobin in the presence of relatively high concentrations of hydrogen peroxide which was attributed to the formation of an amino-acid based globin radical following the breakdown of ferrylmyoglobin. Prasad and colleagues[8] have confirmed that myoglobin will react with hydrogen peroxide with the eventual formation of metmyoglobin and the non-stochiometric release of iron. In their experiments, iron release from myoglobin was much less than from haemoglobin under similar conditions.

There are two objections to hydrogen peroxide scavenging as the principal mechanism for the effect of myoglobin on DMPO-OH concentration observed in the present study. First, the reaction between hydrogen peroxide and ferrous iron is known to be fast, and is certainly much faster than the reaction with myoglobin. Second, we know from the photolysis experiment that Fe^{II} is not necessary for the effect of myoglobin on DMPO-OH. However, decreasing Fe^{II} concentration in the absence of light both diminishes DMPO-OH concentration and enhances the effect of myoglobin. This strongly suggests that myoglobin is reacting with the product, DMPO-OH, rather than producing its effect by reducing the concentration of the initial reactants.

The most likely mechanism is a myoglobin catalysed oxidation of DMPO-OH by hydrogen peroxide to yield nonparamagntic products. The stable nitroxyl compound TMPO is a potential model for this: we found that the signal for TMPO was stable in the presence of either hydrogen peroxide or oxymyoglobin alone, but decayed when both were added. The decay of the TMPO signal was much slower than that of DMPO-OH: this partly reflects the greater stability of TMPO, and partly perhaps steric hindrance by the 2,6 methyl groups of TMPO in interacting with the heme pocket of myoglobin.

"Oxidase" reactions of myoglobin have previously been described by Grisham[9] and by Ozawa and Korzewka.[10] Presumably they involve formation of ferryl myoglobin as an intermediate, followed by reaction with the substrate and regeneration of metmyoglobin.[11,12]

The observation of diminished DMPO-OH formation in the presence of myoglobin is a potential complicating factor in the interpretation of experiments which use

spin-trapping to detect free radical formation in biological systems. Myoglobin could react with both intra- and extracellular hydrogen peroxide, since hydrogen peroxide readily crosses cell membranes. The effect on species such as DMPO-OH however would be confined to the intracellular compartment.[13,14] Evidence for the formation of ferrylmyoglobin in biological models of oxidative stress in heart muscle has been provided by Arduini,[15] this would suggest that hydrogen peroxide concentrations high enough to affect DMPO-OH in the presence of myoglobin may be generated *in vivo*.

In conclusion, we have observed and explained an interaction between myoglobin and a common "spin trap" radical adduct which should lead to caution in interpreting the results of spin trapping experiments in systems containing heme proteins such as myoglobin and, by extension, haemoglobin.

Acknowledgements

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References

1. G.R. Burttner and W. Oberley (1978) Considerations in the spin trapping of superoxide and hydroxyl radical in solutions using 5,5'-dimethyl-1-pyrroline-N-oxide. *Biochemistry Biophysics Research Communications*, **83**, 69–74.
2. S. Pou, M.S. Cohen, B.E. Britigan *et al.* (1989) Spin trapping and human neutrophils. *Journal of Biological Chemistry*, **264**, 12299–12302.
3. K.D. Whitburn (1987) The interaction of oxymyoglobin with hydrogen peroxide: the formation of ferrylmyoglobin at moderate excesses of hydrogen peroxide. *Archives of Biochemistry and Biophysics*, **253**, 419–430.
4. M.J. Davies (1990) Detection of myoglobin derived radicals on reaction of metmyoglobin with hydrogen peroxide and other peroxidic compounds. *Free Radical Research Communications*, **10**, 361–379.
5. M.J. Davies (1991) Identification of a globin free radical in equine myoglobin treated with peroxides. *Biochimia Biophysica Acta*, **1077**, 86–90.
6. F. Ascoli, M.R. Rossi Fanelli and E. Antonini (1981) Preparation and properties of apohaemoglobin and reconstituted hemoglobins. *Methods in Enzymology*, **76**, 72–88.
7. A. Puppo and B. halliwell (1988) Formation of hydroxyl radicals in biological systems. Does myoglobin stimulate hydroxyl radical formation from hydrogen peroxide? *Free Radical Research Communications*, **4**.
8. M.R. Prasad, R.M. Engelman, R.M. Jones and D.K. Das (1989) Effects of oxyradicals on oxymyoglobin. *Biochemical Journal*, **263**, 731–736.
9. M.B. Grisham (1985) Myoglobin catalysed hydrogen peroxide dependent arachidonic acid peroxidation. *Free Radicals in Biology and Medicine*, **1**, 227–232.
10. Y. Osawa and K. Korzewka (1991) Oxidative modification by low levels of HOOH can transform myoglobin to an oxidase. *Proccedings of the National Academy of Sciences of the USA*, **88**, 7081–7085.
11. D. Galaris, E. Cadenas and P. Hochstein (1989) Redox cycling of myoglobin and ascorbate: a potential protective mechanism against oxidative reperfusion injury in muscle. *Archives of Biochemistry and Biophysics*, **273**, 497–504.
12. C. Rice Evans, G. Okunade and R. Khan (1989) The suppression of iron release from activated myoglobin by physiological electron donors and by desferrioxamine. *Free Radical Research Communications*, **7**, 45–54.
13. S. Pou, D.J. Hassett, B.E. Britigan, M.S. Cohen and G.M. Rosen (1989) Problems associated with spin-trapping oxygen centered free radicals in biological systems. *Analytical Biochemistry*, **177**, 1–6.
14. A. Samuni, A.J. Carmichael, A. Russo, J.B. Mitchell and P. Reisz (1986) On the spin trapping and ESR detection of oxygen derived free radicals generated inside cells. *Proccedings of the National Academy of Science, USA*, **83**, 7593–7597.
15. A. Arduini, L. Eddy and P. Hochstein (1990) Detection of ferrylmyoglobin in the isolated ischaemic rat heart. *Free Radicals in Biology and Medicine*, **9**, 511–513.

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