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A HYDROGEN-DONATING MONOHYDROXAMATE SCAVENGES FERRYL MYOGLOBIN RADICALS

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The addition of 25μM hydrogen peroxide to 20μM metmyoglobin produces ferryl ($Fe^{IV}=O$) myoglobin. Optical spectroscopy shows that the ferryl species reaches a maximum concentration (60-70% of total haem) after 10 minutes and decays slowly (hours). Low temperature EPR spectroscopy of the high spin metmyoglobin ($g = 6$) signal is consistent with these findings. At this low peroxide concentration there is no evidence for iron release from the haem. At least two free radicals are detectable by EPR immediately after H_2O_2 addition, but decay completely after ten minutes. However, a longer-lived radical is observed at lower concentrations that is still present after 90 minutes. The monohydroxamate N-methylbutyrohydroxamic acid (NMBH) increases the rate of decay of the fenyl species. In the presence of NMBH, none of the protein-bound free radicals are detectable; instead nitroxide radicals produced by oxidation of the hydroxamate group are observed. Similar results are observed with the trihydroxamate, desferrioxamine. "Ferryl myoglobin" is still able to initiate lipid peroxidation even after the short-lived protein free radicals are no longer detectable (E.S.R. Newman, C.A. Rice-Evans and M.J. Davies (1991) *Biochemical and Biophysical Research Communications* **179**, 1414-1419). It is suggested that the longer-lived protein radicals described here may be partly responsible for this effect. The mechanism of inhibition of initiation of lipid peroxidation by hydroxamate drugs, such as NMBH, may therefore be due to reduction of the protein-derived radicals, rather than reduction of ferryl haem.

KEY WORDS: Ferryl, myoglobin, radical, hydroxamate, EPR, peroxide.
Abbreviations NMBH, N-methylbutyrohydroxamic acid; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide. DFO, desferrioxamine.

INTRODUCTION

The peroxidase action of haem proteins has been recognised for many years.[1] The interaction of metmyoglobin with peroxides involves activation to the ferryl species which consists of an iron (IV)-oxo complex and a protein radical on the surface of the protein.[2] It is not clear which of these species is responsible for oxidative damage to biological substrates. It has been proposed that the radical on the surface

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of the haem protein is located on tyrosine-103, in equine myoglobin.[3,4] The protein radical has been characterised by EPR spectroscopy, using both stopped-flow and spin trapping with DMPO. It forms rapidly (in 10 seconds) and decays rapidly, the DMPO-adduct being reduced to approximately 10% of its initial intensity after 10 minutes. It is suggested that the first detectable product is a tyrosine phenoxyl radical and that this can react with molecular oxygen to produce a peroxyl radical,[4] although the latter reaction is disputed.[5] These myoglobin-derived species can interact with a variety of substrates - low density lipoproteins,[6] erythrocyte membranes,[7] microsomes and model lipid membranes.[8] The precise identificafion of the initiating species is not yet established; our recent studies have suggested that it is the tyrosine-derived radicals on the surface of the haem protein which initiate oxidative damage to erythrocyte membranes.[7] In this work we have combined optical spectroscopy and low temperature EPR to assess the efficacy of the monohydroxamate drug, N-methylbutyrohydroxamic acid, NMBH.[9] Low temperature EPR confirms the presence of short-lived radicals associated with ferryl myoglobin formation, but also detects longer-lived radicals stable for over an hour subsequent to hydrogen peroxide addition. NMBH is shown to be an effective hydrogen-donating antioxidant, reducing both the ferryl haem and all protein radicals, thereby forming an NMBH radical. The latter decays rapidly.

MATERIALS AND METHODS

The myoglobin (ferric form, horse heart, type III) was purified by oxidation with potassium ferricyanide and subsequent separation on a Sephadex G-15 column. Visible spectroscopy was performed on a Beckman DU-65 spectrophotometer fitted with Quant 1 software and linked to an IBM computer. The formation and reduction of ferryl myoglobin species were assessed as described previously[10] by determining the concentration of the different myoglobin species using the Whitburn algorithms based on the extinction coefficients for ferryl, met- and oxymyoglobin.[11] The total haem concentration was assessed by visible spectroscopy. Lipid peroxidation studies of low-density lipoprotein were carried out as described previously.[12]

Continuous-wave EPR measurements were recorded on a Bruker ESP300 spectrometer fitted with a TE103 rectangular cavity, a Hewlett Packard microwave requency counter 5350B and an Oxford instruments liquid helium flow cryostat ESR90. Spectra were baseline corrected by subtraction of a cavity spectrum or water/buffer under identical conditions. The peroxide solution was added to metmyoglobin in a small tube at room temperature and aliquots removed at the required times for freezing in EPR tubes. The samples contained in the EPR tubes were rapidly frozen in liquid methanol, pre-cooled in a bath of liquid nitrogen. The tubes were stored in a standard liquid nitrogen storage dewar and although the spectra were recorded within 24 hours, all the EPR signals were found to be stable over a significantly longer period (days-weeks). Quantification of the concentration of free radical formed was determined by comparing the double integral of the derivative spectrum to that of a Cu(II) standard, both recorded under non-saturating conditions.

All reactions were carried out in phosphate-buffered saline (10mM phosphate, pH 7.4). Drugs were added to the metmyoglobin solution immediately prior to peroxide addition. Desferrioxamine mesylate was from CIBA-Geigy and N-methylbutyrohydroxamic acid was synthesised by standard laboratory methods.[13]

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RESULTS

On addition of hydrogen peroxide (1.25 molar excess) to metmyoglobin spectral changes characteristic of the formation of a significant proportion of ferryl myoglobin are observed.[10] In the presence of NMBH or desferrioxamine the ferryl myoglobin is reduced back to metmyoglobin.[14] We have previously shown using room temperature EPR that in the presence of hydroxamates the myoglobin protein radical is reduced and a nitroxide radical becomes detectable.[4] However, in order to detect the signals we used high (95 μM) protein concentrations. Using frozen samples at liquid helium temperatures greater sensitivity is achieved, both intrinsically and because signal averaging is possible (as the signals are stable at low temperature); low temperature experiments were therefore carried out using identical (20 μM) protein concentrations to those we have previously employed for optical spectroscopy.[14]

Figure 1 shows the $g = 2$ region of the EPR spectrum five seconds after peroxide treatment. In the absence of NMBH (Figure 1a) two distinct peaks are seen at $g = 2.035$ and $g = 2.004$. Similar results have been reported by other workers[2,15-18] and attributed to two distinct species, with the signal at $g = 2.004$ being the major component. This is consistent with our previous work suggesting that there is more than one radical formed at room temperature.[4] In agreement with previous studies[2,16,17] we were never able to detect a stoichiometric formation of the radical(s), relative to the initial level of metmyoglobin; the maximum total free radical signal being 8% in experiments where the maximum amount of ferryl formation was 60%.

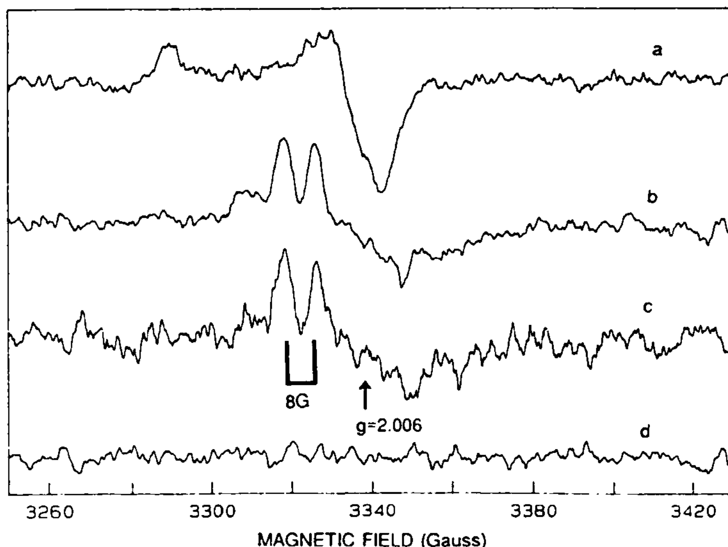

FIGURE 1 Identification of free radicals formed from the interaction of NMBH with ferryl myoglobin (a) 20 μM metmyoglobin + 25 μM H_2O_2, frozen after 10s; (b) 20 μM metmyoglobin + 25 μM H_2O_2 + 10 μM NMBH, frozen after 10s; (c) ferricyanide (crystals) + 800 μM NMBH, frozen after 60s; (d) spectrum *b* minus spectrum *c*. EPR conditions: temperature 100 K, microwave power 20 mW, microwave frequency 9.38 GHz, modulation frequency 100 KHZ, modulation amplitude 2 Gauss, Time constant 0.33 s, Sweep Time 5 Gauss/s, Signal is average of 20 scans. Relative gains of spectra displayed (a) × 1, (b) × 4 (c) × 2.

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In the presence of NMBH (Figure 1b) there is a much less intense free radical signal detected; it also has different characteristics. The radical is only observed when metmyoglobin, hydrogen peroxide and NMBH are all present. We attribute this signal to the anisotropically broadened (i.e. broadened due to slow molecular motion) NMBH nitroxide radical; both the g-value (2.006) and the one resolved hyperfine splitting observable (8 Gauss) are consistent with this assignment. This 8 Gauss coupling is consistent with a nitrogen coupling observed at room temperature for the NMBH nitroxide radical (a_N = 7.8 Gauss). Furthermore the low temperature spectrum obtained in the presence of metmyoglobin, H_2O_2 and NMBH is identical to that seen following addition of excess ferricyanide to NMBH (subtraction of the two spectra yield a flat baseline - Figure 1d). Therefore the radical seen in Figure 1b must be the product of a one electron oxidation of NMBH. This confirms that NMBH decreases the yield of ferryl myoglobin by acting as an electron donor.

It is also possible to use low temperature EPR to monitor both the haem oxidation state and the free radical signals simultaneously (Figure 2). The only oxidation state of myoglobin readily detectable by EPR spectroscopy is Fe^{III} (metmyoglobin). Therefore the technique can be used to directly detect the changes in concentration of metmyoglobin, without interference from other oxidation states. Figure 2a shows that following peroxide addition this high spin ferric signal (g = 6.0) decreases in intensity, reaching a minimum at around 20 minutes. It then gradually increases, but after 90 minutes it is still significantly smaller than its starting value. These results are consistent with the time course of metmyoglobin loss calculated from the optical spectra using the deconvolution algorithm of Whitburn[11] and thus provide independent evidence for the validity of this method. The kinetics of the decay and re-formation of the $g = 6$ signal are consistent with those observed optically (results not shown). The $g = 2$ region shows similar free radical signals to those seen in Figure 1a. However, under the conditions used to record these spectra the signal shapes are slightly different due to overmodulation and power saturation. There is also an effect due to the overlap of these free radical signals with the low field signal from the haem iron (g = 1.99). This latter effect can be compensated for by subtracting a proportion of the spectrum of metmyoglobin prior to peroxide addition (where no organic radicals are observed). Figure 2b shows these corrected spectra at low field. The initial free radicals are not present if the samples are frozen 10 minutes subsequent to peroxide treatment. However, there is a small free radical signal that is longer-lived, and is still detectable 90 minutes after peroxide addition.

Peroxide addition to metmyoglobin in the presence of NMBH (Figure 3a) show a smaller decrease in the high spin metmyoglobin signal, and a more rapid return to its initial intensity; this is again consistent with data from optical spectroscopy.[14] In the presence of NMBH, the NMBH radical is seen instead of the myoglobin protein radicals (the lack of hyperfine structure when compared to Figure lb is due to overmodulation and microwave power saturation of the radical signal under conditions optimised for detection of the haem iron). This radical is not detectable if the samples are frozen 20 minutes subsequent to peroxide addition. Furthermore, in the presence of NMBH there is *no* evidence of a longer-lived protein-derived radical.

The conditions used to generate ferryl myoglobin in Figures 1-3 (20 μM metmyoglobin +25 μM hydrogen peroxide) induce significant lipid peroxidation in

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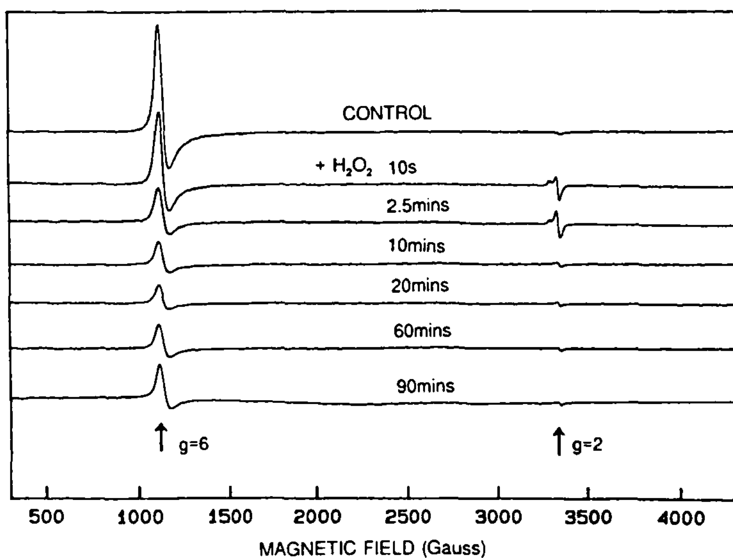

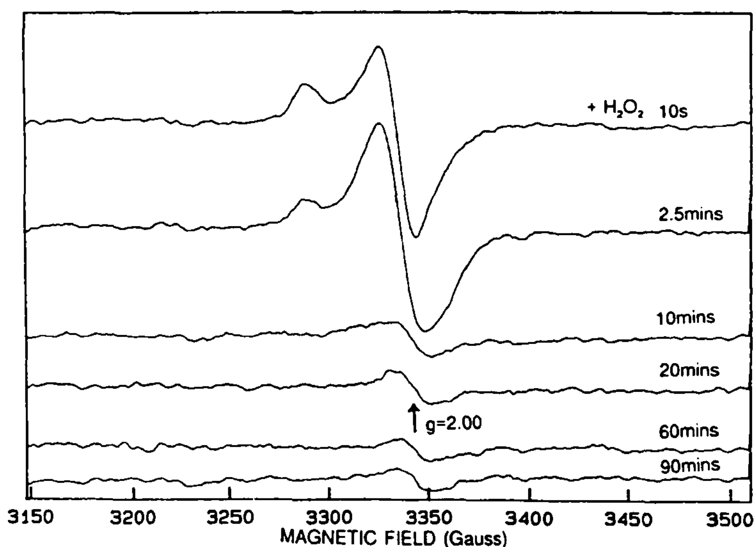

FIGURE 2 Effect of hydrogen peroxide addition on metmyoglobin EPR signals. (a) $[H_2O_2] = 25\ \mu M$, [metmyoglobin] = 20 μM, samples frozen at indicated times after peroxide addition. EPR conditions: temperature 30 K, microwave power 20 mW, microwave frequency 9.34 GHz, modulation frequency 100 KHz, modulation amplitude 9.7 Gauss, Time constant 0.33 s, Sweep Time 24 Gauss/s, Signal is average of 2 scans. (b) Expansion of $g = 2$ region from (a). Spectra were individually corrected for interference from $g = 1.99$ metmyoglobin signal by subtracting differing proportions of the control metmyoglobin spectrum ($t = 0$, minus H_2O_2) until the $g = 6$ signal was minimal.

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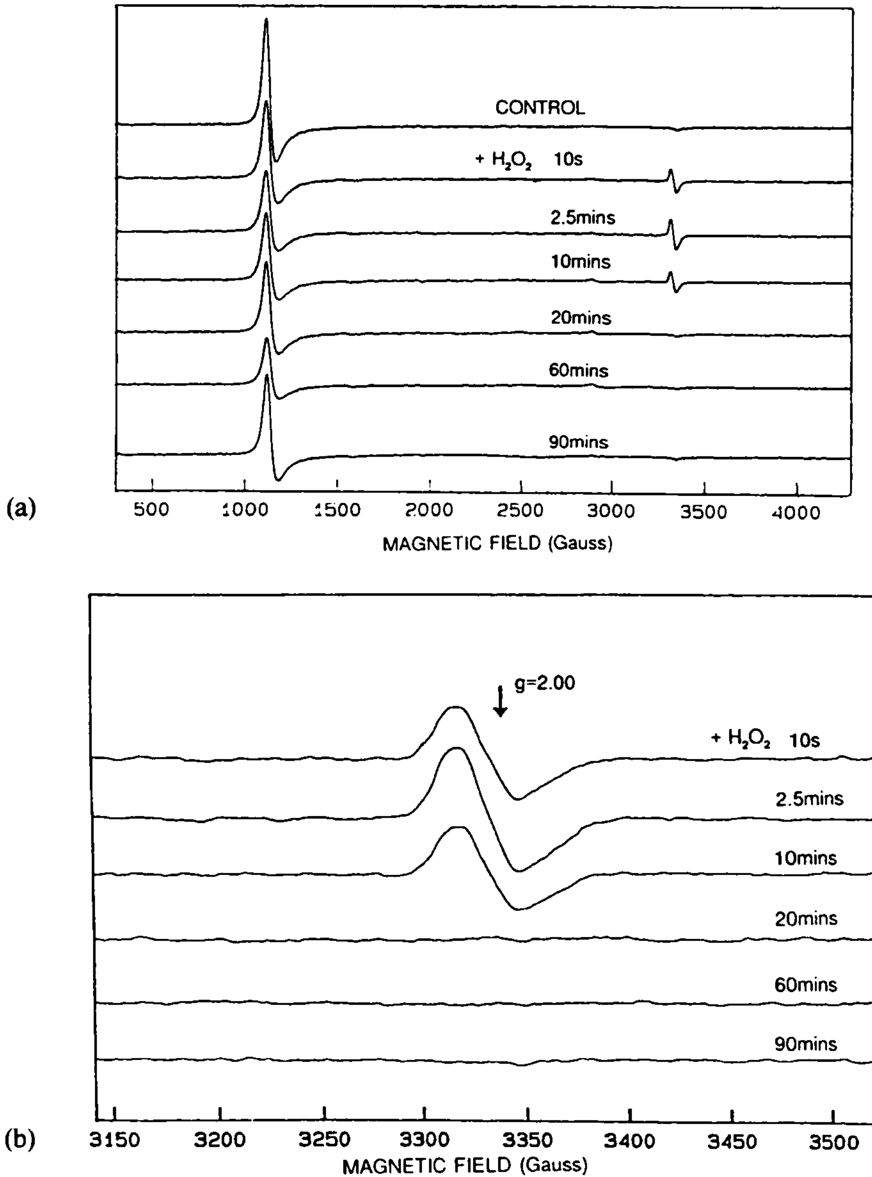

FIGURE 3 Effect of hydrogen peroxide addition on metmyoglobin EPR signals in the presence of NMBH. Conditions as for Figure 2 except that 10 μM NMBH was present.

0.25mg/ml low-density lipoprotein (measured 90 minutes after the simultaneous mixing of metmyoglobin, H_2O_2 and LDL). The presence of 10 μM or 100 μM NMBH at $t = 0$ inhibits this lipid peroxidation by 86% and 97% respectively. The reduction of protein radicals and/or ferryl myoglobin by NMBH is likely to be significant

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factor in this inhibition, although NMBH can also act as a chain-breaking antioxidant.[14]

We have also observed the desferrioxamine radical using low temperature EPR (Figure 4). Again immediately after peroxide addition the myoglobin protein radicals (a) are replaced by a nitroxide radical (b). This was confirmed by comparing this spectrum with that obtained via chemical oxidation of desferrioxamine with ferricyanide (c). In the absence of peroxide no EPR signals are detectable in the $g = 2$ region (d).

At high peroxide: haem ratios it is known that there is damage to the prosthetic group and release of iron.[19] However, at the low ratios used in this study no evidence for this process was observed, as evidenced by a lack of high spin non-haem iron EPR signals at $g = 4.3$. Separate experiments showed that under the conditions used the $g = 4.3$ EPR signal of ferrioxamine was detectable at concentrations at least as low as 1 μM. This is consistent with our previous optical studies[20] showing that there is no iron release at these low peroxide: haem ratios (1.25:1). Therefore the effect of both desferrioxamine[10] and NMBH (this paper) in decreasing ferryl myoglobin-induced lipid peroxidation is via reduction of the ferryl iron and/or the protein-free radicals, rather than via iron chelation.

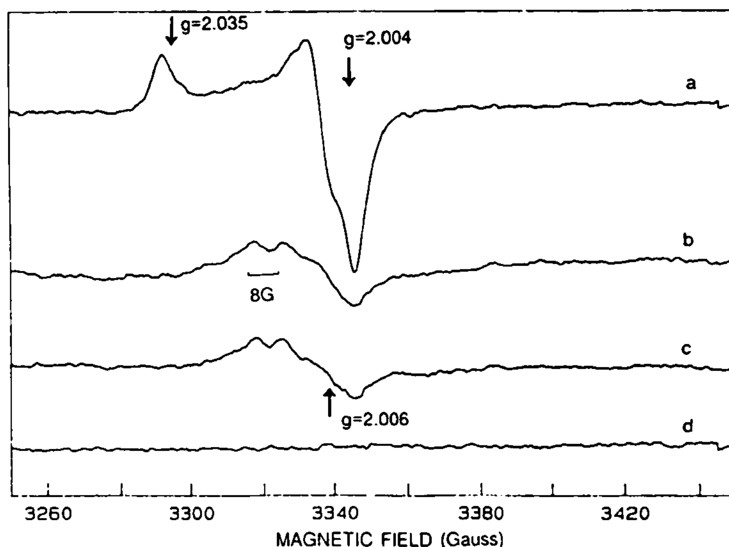

FIGURE 4 Effect of desferrioxamine on the free radical signals observed following H_2O_2 addition to metmyoglobin. (a) metmyoglobin + H_2O_2; (b) metmyoglobin + H_2O_2 + desferrioxamine; (c) desferrioxamine + excess (solid) ferricyanide; (d) metmyoglobin alone. Concentrations used: H_2O_2 = 119 μM, metmyoglobin = 95 μM, desferrioxamine = 100 μM. Samples frozen 5 seconds after peroxide addition. EPR conditions: As per Figure 1 except microwave frequency = 9.375 GHz. Spectra (b), (c) and (d) are displayed at 4× gain of spectra (a).

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DISCUSSION

These results show that monohydroxamates like NMBH can reduce both ferryl myoglobin and its associated protein radicals, and that this is likely to be the primary mechanism for their ability to inhibit lipid peroxidation *in vitro* and *in vivo*. The products of these reductions are the respective hydroxamate radicals. The ability of desferrioxamine and NMBH to inhibit ferryl myoglobin-catalysed lipid peroxidation suggests that both NMBH and DFO radicals are themselves unable to induce damage to lipids, as predicted from their delocalised structure.

The nature of the structure of the radical(s) on myoglobin is more controversial. The major radical observed at room temperature and low temperature EPR have the same g value (varying from 2.003–2.004, depending on the author) and are detectable at similar times following peroxide addition. The fine structure observed at room temperature appears indicative of a tyrosine-based radical. However, the lack of resolvable fine structure at low temperature is in contrast to the well-characterised tyrosine radicals of ribonucleotide reductase and photosystem II.[21] As suggested by Petersen *et al.*[18] the low temperature signal observable at $g = 2.03$ may be due to peroxyl radicals. The overlap of part of this signal with the tyrosine radical signal at $g = 2.003$ might explain the difficulty in observing resolvable fine structure at low temperature.

We have identified the presence of a longer-lived radical that is still detectable 90 minutes after peroxide addition. A similar signal has been detected previously by low temperature EPR.[16,18] We have not as yet been able to detect this signal at room temperature, presumably due to its low concentration. The amount of this long-lived radical was somewhat variable from experiment to experiment. This is most likely due to a variable rate of reduction as electrons enter the system - similar electron leaks are seen with other proteins in the absence of strong external oxidants.[22]

There are two possible catalysts for the damage initiated by the addition of ferryl myoglobin to lipids either ferryl haem or protein/porphyrin radicals: We have shown that ferryl myoglobin is able to catalyse similar levels of lipid peroxidation, whether membranes were added simultaneously with H_2O_2 or twenty minutes subsequent.[7] At the latter time the major free radical is not present, measured both by room temperature and low temperature EPR. We have previously stated that this is evidence for initiation of lipid peroxidation by ferryl species. The discovery of a longer-lived free radical, still detectable ninety minutes after peroxide addition, weakens this conclusion. The effects of desferrioxamine and NMBH in reducing lipid peroxidation may therefore be due primarily to reduction of long-lived radicals on the surface of the protein, rather than the ferryl iron.

Our previous studies have shown that ferryl myoglobin species can oxidise N-acetyl cysteine and N-mercapto propionyl glycine to thiyl radicals, which can be detected as the DMPO-thiyl adduct.[10] The similar reactions observed here with hydroxamates shows that there is now a wide variety of compounds available that can interact with ferryl myoglobin and its associated protein radicals. Their efficacy *in vivo* in inhibiting reperfusion damage is currently under investigation.

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References

1. J. Everse, K.E. Everse and M.B. Grisham (1991) *Peroxidases in Chemistry and Biology*. CRC Press, Boca Raton, USA.
2. N.K. King and M.E. Winfield (1963) The Mechanism of Metmyoglobin Oxidation. *Journal of Biological Chemistry*, **238**, 1520–1528.
3. D. Tew and P. Ortiz de Montellano (1988) The Myoglobin Protein Radical. Coupling of Tyr-103 to Tyr-151 in the H_2O_2-mediated cross-linking of sperm whale myoglobin. *Journal of Biological Chemistry*, **263**, 17880–17886.
4. M.J. Davies (1991) Identification of a globin free radical in equine myoglobin treated with peroxides. *Biochimica et Biophysica Acta*, **1077**, 86–90.
5. D.J. Kelman and R.P. Mason (1992) The myoglobin-derived radical formed on reaction of metmyoglobin with hydrogen peroxide is not a tyrosine peroxyl radical. *Free Radical Research Communications*, **16**, 27–33.
6. G. Dee, C. Rice-Evans, S. Obeyesekera, S. Mariji and M. Jacobs (1991) The modulation of ferryl myoglobin formation and its oxidative effect on low density lipoproteins by nitric oxide. *FEBS Letters*, **294**, 38–42.
7. E.S.R. Newman, C.A. Rice-Evans and M.J. Davies (1991) Identification of initiating agents in myoglobin-induced lipid peroxidation. *Biochemical and Biophysical Research Communications*, **179**, 1414–1419.
8. J. Kanner and S. Harel (1987) Desferrioxamine as an electron donor. Inhibition of membranal lipid peroxidation initiated by H_2O_2-activated metmyoglobin and other peroxidizing systems. *Free Radical Research Communications*, **3**, 309–317.
9. C.A. Rice-Evans and M.J. Davies (1991) *UK patent No. 90248204*.
10. J.J.O. Turner, C.A. Rice-Evans, M.J. Davies and E.S.R. Newman (1991) The formation of free radicals by cardiac myocytes under oxidative stress and the effects of electron-donating drugs. *Biochemical Journal*, **277**, 833–837.
11. K.D. Whitburn, J.J. Shieh, R.M. Sellers, M.Z. Hoffman and I.A. Taub (1982) Redox-transformations in ferrimyoglobin induced by radiation-generated free radicals in aqueous solution. *Journal of Biological Chemistry*, **257**, 1860–1869.
12. C. Rice-Evans, E. Green, G. Paganga, C. Cooper and J. Wrigglesworth (1993) Oxidised low density lipoproteins induce iron release from activated myoglobin. *FEBS Letters*, **326**, 177–182.
13. H. Ulrich and A.A.R. Sayigh (1963) Hydroxyamino-derivatives from formaldehyde-their reaction with acyl halides. *Journal of the Chemical Society*, 1098–1101.
14. E.S.R. Green, H. Evans, P. Rice-Evans, M.J. Davies, N. Salah and C. Rice-Evans (1993) The efficacy of monohydroxamates as free radical scavenging agents compared with di- and trihydroxamates. *Biochemical Pharmacology*, **45**, 357–366.
15. J.F. Gibson, D.J.E. Ingram and P. Nicholls (1958) Free Radical produced in the Reaction of Metmyoglobin with Hydrogen Peroxide. *Nature*, **181**, 1398–1399.
16. N.K. King, F.D. Looney and M.E. Winfield (1967) Amino acid free radicals in oxidised metmyoglobin. *Biochimica et Biophysica Acta*, **133**, 65–82.
17. T. Yonetani and H. Schleyer (1967) Studies on Cytochrome *c* Peroxidase. *Journal of Biological Chemistry*, **242**, 1974–1979.
18. R.L. Petersen, M.C.R. Symons and F.A. Taiwo (1989) Application of Radiation and Electron Spin Resonance Spectroscopy to the Study of Ferryl Myoglobin. *Journal of the Chemical Society, Faraday Transactions 1*, **85**, 2435–2443.
19. C.E. Catalano, Y.S. Choe and P.R. Ortiz de Montellano (1989) Reactions of the Protein Radical in Peroxide-treated Myoglobin. *Journal of Biological Chemistry*, **264**, 10534–10541.
20. C.A. 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.
21. B.A. Barry, M.K. El-Deeb, P.O. Sandusky and G.T. Babcock (1990) Tyrosine Radicals in Photosystem II and Related Model Compounds. *Journal of Biological Chemistry*, **265**, 20139–20143.
22. A.J. Moody, C.E. Cooper and P.R. Rich (1991) Characterisation of "fast" and "slow" forms of bovine heart cytochrome *c* oxidase. *Biochimica et Biophysica Acta*, **1059**, 189–207.

Accepted by Professor J.M.C. Gutteridge

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