Free Rad. Res. Comms., Vol. 10, No. 6, pp. 361–370
Reprints available directly from the publisher
Photocopying permitted by license only

DETECTION OF MYOGLOBIN-DERIVED RADICALS ON REACTION OF METMYOGLOBIN WITH HYDROGEN PEROXIDE AND OTHER PEROXIDIC COMPOUNDS

MICHAEL J. DAVIES

Department of Chemistry, University of York, Heslington, York, YO1 5DD, U.K.

(Received March 20, 1990; accepted May 3, 1990)

The reaction of metmyoglobin with equimolar concentrations of hydrogen peroxide has been studied using both electron spin resonance (e.s.r.) and optical spectroscopy. Using the former technique a strong anisotropic e.s.r. signal is observed, in the presence of the spin trap DMPO, which decays relatively rapidly. This previously unobserved signal, which is also observed on reaction of metmyoglobin with a number of other powerful oxidants (peracetic acid, 3-chloroperoxybenzoic acid, monoperoxyphthalic acid, iodosyl benzene, ^{t}BuOOH and cumene hydroperoxide) is assigned to a slowly-tumbling, metmyoglobin-derived, spin adduct. The parameters of this signal (a_N 1.45, a_H 0.83 mT) are consistent with the trapped radical having a heteroatom centre; this is believed to be oxygen. The concentration of this species is not affected by compounds such as 2-deoxyribose, mannitol and phenylalanine which are all efficient hydroxyl radical scavengers, demonstrating that the formation of this radical is not due to reaction of "free" HO· generated by breakdown of H_2O_2 by released iron ions. The concentration of this species is however decreased by desferal, ascorbate, Trolox C, salicylate and, to a lesser extent, linoleic acid; with the first three of these compounds further substrate-derived radicals are also observed. Examination of similar reaction systems (though in the absence of DMPO) by optical spectroscopy shows that the myoglobin (IV) species is formed and that this species behaves in a somewhat different manner with these added compounds. These results suggest that the radical trapped in the e.s.r. experiments is a myoglobin-derived species, probably a tyrosine peroxyl radical, arising from oxidative damage to the globin moiety.

The diminution of both the e.s.r. signal of the spin adduct and the optical absorption of the myoglobin (IV) species in the presence of linoleic acid suggests that these myoglobin-derived species can initiate oxidative damage but that this process can be ameliorated by the presence of a number of water-soluble compounds such as ascorbate, Trolox C, desferal and salicylate.

KEY WORDS: Electron spin resonance, radicals, hydrogen peroxide, hydroperoxides, myoglobin, spin trapping.

INTRODUCTION

Considerable evidence has been obtained to suggest that the damage observed in cells and organs after increased generation of $O_2^{-\cdot}$ (and hence H_2O_2) is due to the production of HO· from H_2O_2 in a transition-metal catalysed reaction.[1] Similar metal-ion catalysed processes are believed to be responsible for the production of ROO·, RO· and R· radicals from a variety of alkyl hydroperoxides and related compounds.[2] The transition-metal catalysts responsible for this radical production have not been definitively identified, though attention has focussed on the possible role of endogenous

Correspondence: to the author at the above address. Tel: (0904) 432535.

iron chelates such as iron-storage molecules and haem-proteins.[3-9] Studies on the reactions of hydrogen peroxide with haemoglobin and myoglobin have concluded that neither of these haem-proteins directly give rise to HO· "in free solution"; instead they react to give short-lived, high oxidation state (ferryl) species which have one oxidising equivalent in the haem group and one in the surrounding protein.[4,8,9] Subsequent reactions result in damage to the haem moiety and release of iron ions which can react with excess H_2O_2 to give "free" HO·.[4,9,10] Similar processes, and high oxidation state intermediates, have been observed with some alkyl hydroperoxides,[4] though there is evidence that "free" peroxyl radicals can be produced by reactions at the *intact* haem centre.[7,11] These radicals are believed to arise from reaction of the ferryl haem species (which have some similar characteristics to Compounds I and II of peroxidases[12]) with excess peroxide (e.g. reaction 1); a similar process may occur with H_2O_2 (reaction 2) though the rate of reaction and/or rapid dismutation of the $O_2^{\cdot-}$ generated, may make this process difficult to detect. Similar reactions have been directly observed in model systems.[13,14]

$$\text{Por-Fe(IV)-OH} + {}^{t}\text{BuOOH} \rightarrow \text{Por-Fe(III)} + H_2O + {}^{t}\text{BuOO}\cdot \quad (1)$$

$$\text{Por-Fe(IV)-OH} + H_2O_2 \rightarrow \text{Por-Fe(III)} + H_2O + H^+ + O_2^{\cdot-} \quad (2)$$

In view of the possible role of myoglobin as a catalyst of radical generation in cardiac reperfusion injury where peroxides are known to be generated[15] it is important to know what intermediates are formed at low oxidant:haem ratios (i.e. when the peroxide is not in great excess), whether the ferryl haem species and/or the globin radical are capable of initiating damage and how the formation of such species gives rise to iron release, and whether processes such as reactions (1) and (2) occur. A study has therefore been carried out, using both e.s.r. and optical spectroscopy, to determine what intermediates are formed on reaction of metmyoglobin (MetMb) with low concentrations of H_2O_2 and other peroxidic compounds.

EXPERIMENTAL

E.s.r. spectra of samples contained in a standard aqueous cell were recorded at room temperature (21°C) 70 s after mixing, with a Bruker ESP 300 spectrometer equipped with 100 kHz modulation and a Bruker ERO35M gaussmeter for field calibration. Hyperfine coupling constants were measured directly from the field scan. g values were calculated by comparison with spectra from a standard solution of di-t-butyl nitroxide. Where necessary, spectra were scanned repeatedly at fixed time intervals to obtain data on the stability of the radical adducts. Signal intensities were determined by measurement of peak-to-peak line heights for the relevant adducts on spectra recorded with the use of identical spectrometer settings, and are the means of at least 3 measurements. U.v./visible spectra were recorded at 21°C on either a Pye Unicam 554 spectrophotometer or a Shimadzu UV260 spectrometer using 1 mm path-length cells.

All the chemicals used were commercial samples of the highest available purity and used as supplied with the excepton of DMPO, which was purified before use as described previously,[16] iodosylbenzene, which was prepared by a standard procedure[17] and peracetic acid, which was treated with catalase to remove H_2O_2.[18] All solutions were prepared in air-saturated, deionised water, unless stated otherwise.

RESULTS

E.s.r. Spectroscopy

Reaction of MetMb (300 μM) with H_2O_2 (300 μM) in the presence of 33 mM-DMPO in air-saturated, 33 mM-phosphate buffer, pH 7.4, resulted in the observation, immediately after mixing, of an intense e.s.r. signal (Figure 1). The concentration of this species (as measured by peak-to-peak line heights) decreased rapidly with time, being reduced to about 10% of its initial intensity within 10 min. (Figure 1). The observed signal, which consists of a triplet of doublets with hyperfine coupling constants a_N 1.45, a_H 0.83 mT, is highly anisotropic with considerable broadening of the high field lines; this is consistent with the trapping, by DMPO, of a very large radical, resulting in the formation of a slowly-tumbling nitroxide radical adduct. Omission of any of the components of the reaction mixture resulted in the loss of this signal.

Increasing the concentration of hydrogen peroxide used had a dramatic effect on the observed species; when 1.5 mM-H_2O_2 was employed (i.e. an oxidant:haem ratio of 5:1) the intensity of the signal decreased by approximately 50%. Further decreases in signal intensity were observed as the oxidant:haem ratio was increased in stages to 50:1. At ratios greater than 50:1 (i.e. > 15 mM-H_2O_2) the signal was very weak and difficult to observe. No additional species were observed.

Addition of various organic compounds to a standard 300 μM-MetMb, 300 μM-H_2O_2 system in the presence of 33 mM-DMPO produced marked changes in the observed spectra in some cases. Inclusion of 2-deoxyribose (6.6 mM), phenylalanine (10 mM) mannitol (20 mM) had no significant effect on the intensity of the signal from the slowly-tumbling adduct, and no other additional signals were observed. In contrast, inclusion of salicylate (5 mM) or linoleic acid (150 μM) reduced the intensity of the signal from the slowly-tumbling adduct to about 15% and 90% of control values respectively; no other additional species were observed. With Trolox C (3 mM) the signal from the slowly-tumbling adduct was not observed, though weak signals (assigned on the basis of the hyperfine coupling constants and comparison with previously reported values[19]) from the Trolox C phenoxyl radical were detected (Figure 1). Similar behaviour (i.e. partial or complete loss of the DMPO adduct, and the observation of additional radical species) occurred with ascorbic acid and desferal: with 100 μM ascorbic acid the intensity of the DMPO adduct was reduced to 45% of control values and no signals from the ascorbyl radical were observed (though low concentrations of this species would not be observed as the lines from this species occur at the same field position as one of the lines from the DMPO adduct), at higher concentrations (1 mM) the DMPO adduct was not observed and strong signals from the ascorbyl radical were. With desferal (75 μM) weak signals from both the DMPO adduct and the desferal nitroxide radical (with hyperfine coupling constants as previously reported[20]) were detected; at higher concentrations of desferal (150 and 300 μM) only strong signals from the desferal nitroxide radical were detected (Figure 1). Ferrioxamine at identical concentrations did not give these effects.

Substitution of a number of other powerful oxidants in place of H_2O_2 in the absence of these additional scavengers and chelators, also gave rise to this DMPO adduct signal. Thus inclusion of peracetic acid (300 μM), 3-chloroperoxybenzoic acid (300 μM), magnesium monoperoxyphthalic acid (300 μM), iodosyl benzene (as a saturated aqueous solution), ^{t}BuOOH (300 μM), or cumene hydroperoxide ($PhC(CH_3)_2OOH$, 300 μM) all gave this species, with the intensity of the signal being dependent on the oxidant used in the order peracetic acid > iodosyl benzene >

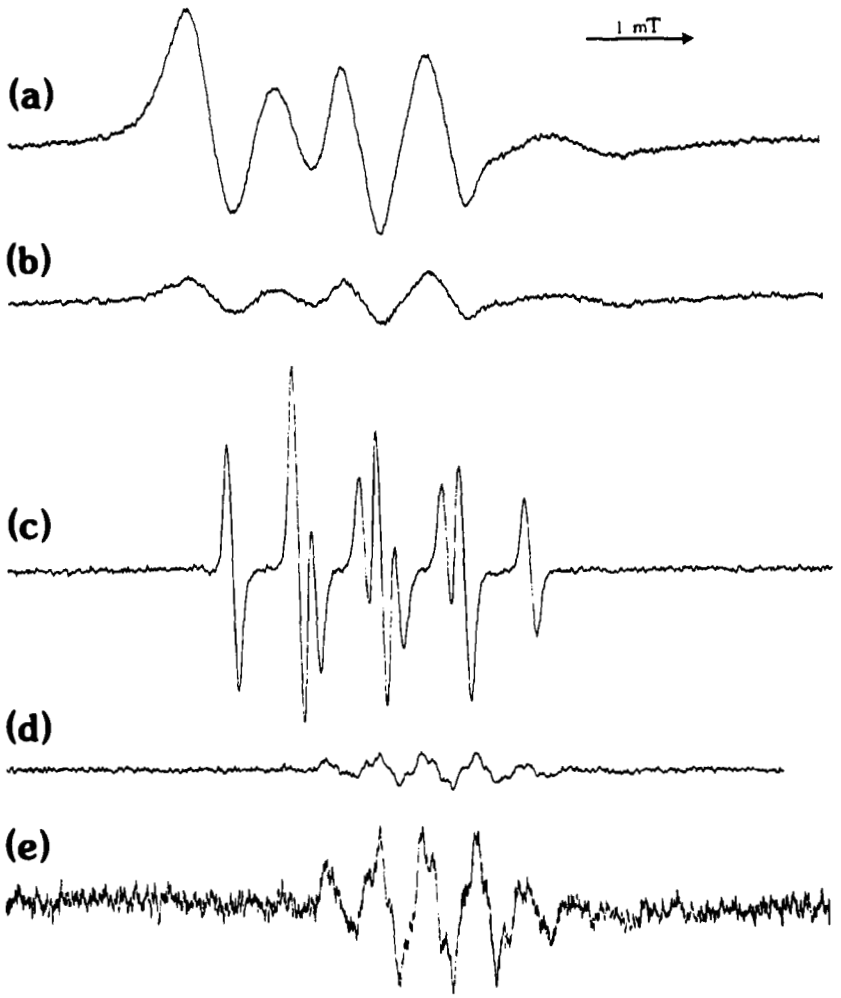

FIGURE 1 E.s.r. spectra observed on reaction of metmyoglobin with H_2O_2 in the presence of DMPO. Metmyoglobin (300 μM) was incubated with H_2O_2 (300 μM) in presence of 33 mM-DMPO in 33 mM-phosphate buffer, pH 7.4. Spectra were recorded 70 s after mixing with the exception of (b) which was recorded 7 mins. after mixing. (*a*) and (*b*) complete system. (*c*) As (*a*) except that 300 μM-desferal was added, signal assigned to the desferal nitroxide radical. (*d*) As (*a*) except that 3 mM-Trolox C was added. (*e*) As (*d*) except gain was $\times 4$. Signals in (*d*) and (*e*) assigned to the Trolox C phenoxyl radical.

magnesium monoperoxyphthalic acid > 3-chloroperoxybenzoic acid > ^{t}BuOOH > cumene hydroperoxide. No additional signals were observed with any of these oxidants at these concentrations; at higher concentrations of the last two oxidants additional signals assigned to the corresponding peroxyl radical adduct to the spin trap were observed as described previously.[7]

In order to characterize this novel spin adduct further, additional studies on similar reaction mixtures were carried out using optical spectroscopy.

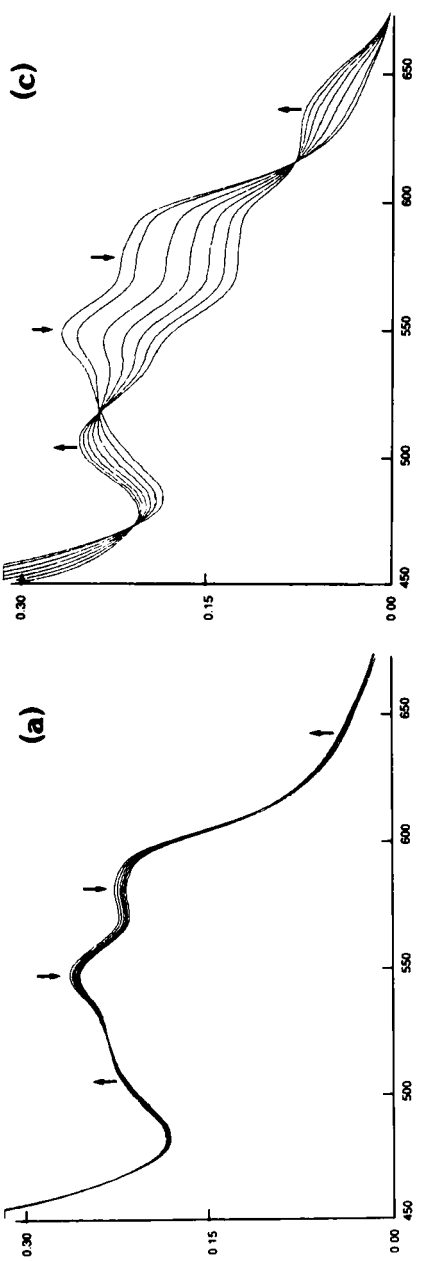

FIGURE 2 Visible spectra observed on reaction of metmyoglobin with H_2O_2 in absence and presence of added substrates. Metmyoglobin (300 μM) was incubated with H_2O_2 (300 μM) in 33 mM-phosphate buffer, pH 7.4. Spectra (absorbance versus wavelength) were recorded 70 secs. after mixing and then at 3 minute intervals. (*a*) control system. (*b*) As control except that 33 mM-DMPO was added. (*c*) As control except that 5 mM-salicylate was added. (*d*) As control except that 300 μM-desferal was added.

Optical Spectroscopy

Reaction of MetMb (300 μM final concentration) with H_2O_2 (300 μM, final concentration) in 33 mM-phosphate buffer, pH 7.4, led to the observation, by visible spectroscopy, of the myoglobin (IV) spectrum as described previously.[21] The formation of this species was essentially complete within the first 5 min. and was stable for a considerable period of time. Analysis of the spectra using the Whitburn equations[22] showed that the major species present was myoglobin (IV). No significant loss of haem could be detected during the first 20 minutes, though the ratio of myoglobin (IV) to MetMb decreased slowly (Figure 2).

Qualitatively similar results were obtained on replacement of H_2O_2 with other powerful oxidants (peracetic acid, 3-chloroperoxybenzoic acid, magnesium monoperoxyphthalic acid, iodosyl benzene, t-butylhydroperoxide, cumene hydroperoxide) when the MetMb:oxidant ratio was 1:1. In each of these cases the formation of the myoglobin (IV) species was observed though in some cases, particularly with the more sterically bulky oxidants (3-chloroperoxybenzoic acid and magnesium monoperoxyphthalic acid), the rate and extent of formation of the myoglobin (IV) species was considerably reduced; in such cases MetMb accounted for the rest of the species present at short times after mixing. At longer times ($>$ 15 minutes) significant haem loss was observed.

Addition of various organic compounds to a standard MetMb/H_2O_2 reaction system (both 300 μM final concentration) in 33 mM-phosphate bufer, pH 7.4, led to significant changes in the observed visible spectra in some cases. 2-Deoxyribose (6.6 mM), phenylalanine (10 mM) and mannitol (20 mM, all final concentrations), which are all efficient HO$\cdot$ scavengers, had no significant effect on either the extent of myoglobin (IV) formation or its lifetime; this is in agreement with previous observations.[9] Inclusion of desferal (300 μM), salicylate (5 mM), Trolox C (3 mM) or the spin trap DMPO (33 mM, all final concentrations) all led to an increased rate of decay of the myoglobin (IV) species back to MetMb (Fig. 2), with the efficiency of these compounds in inducing this change being desferal $>$ Trolox C $>$ salicylate $>$ DMPO. Similar observations with desferal and ascorbate have been previously described.[23] No additional species which absorb in the region of the spectrum investigated (700–450 nm) were detected, which is in contrast to previously reported experiments with salicylate where much larger ratios of oxidant:haem were employed,[9] and no significant loss of haem was observed. None of the compounds studied had any effect on the absorption spectrum of MetMb in the absence of hydrogen peroxide.

DISCUSSION

Reaction of metmyoglobin with equimolar concentrations of H_2O_2 gives the myoglobin (IV) species which can be detected by its characteristic optical spectrum as described previously.[21] This intermediate is stable for a considerable period of time in the absence of other reagents. No reaction was observed between this species and 2-deoxyribose, phenylalanine and mannitol which are all potent hydroxyl radical scavengers. This confirms previous reports which have demonstrated that degradation of these substrates only occurs when iron is released from the tetrapyrrolic ligand and becomes available for reaction with excess H_2O_2 by the Haber-Weiss or Fenton reactions.[4,9,10] Other reagents however, do appear to react with the myoglobin (IV)

species; the presence of Trolox C, salicylate, desferal and, to a much lesser extent, DMPO, all lead to a significant reduction in the lifetime of the high oxidation-state species and the reappearance of MetMb. This type of process has been previously observed with desferal and ascorbate (though in the latter case myoglobin (II) is observed instead of MetMb[23]).

E.s.r. studies on identical reaction systems (though in the presence of DMPO) gave a strong, anisotropic, signal consisting of a triplet of doublets. Such a pattern of lines is consistent with the observed signal being due to the addition of a large radical to the spin trap DMPO to give a slowly-tumbling, nitroxide radical; the identification of this species as an adduct is confirmed by the loss of this signal on omission of DMPO from the reaction mixture. The hyperfine coupling constants of this species are difficult to determine accurately due to the anisotropic nature of the signal, but are approximately a_N 1.45, a_H 0.83 mT from measurements on the low field lines. These values, particularly the low a_H value, are what would be expected from the spin trapping, by DMPO, of a heteroatom-centred radical (c.f. previously reported values of a_N 1.448, a_H 1.088, a_H 0.130 mT for the trapping of $^tBuOO\cdot$ radicals and a_N 1.43, a_H 1.17, a_H 0.125 mT for the trapping of $O_2^- \cdot$ radicals[7,24]).

Mason and co-workers have recently reported the spin trapping, by DMPO and PBN, of a haemoglobin-derived species, formed on reaction of haemoglobin with both a variety of hydrazine drugs and certain hydroperoxides.[25] This species, which has been assigned to a haemoglobin-derived thiyl-radical adduct to DMPO as a result of experiments carried out with reagents which react with free sulphydryl groups, is somewhat similar to the species observed in these experiments though the hyperfine coupling constants reported in these previous studies are somewhat larger. Despite the similarities between these two systems, the signal observed in these experiments cannot be due to the trapping of a myoglobin thiyl radical as horse heart myoglobin contains no cysteine residues and hence no free -SH groups.[26] Trapping of a nitrogen-centered radical (e.g. from oxidation of N–H groups) is thought to be unlikely as such species would be expected to give an adduct with a further triplet splitting due to the nitrogen nucleus which has a nuclear spin of 1.[27] Exclusion of these two possibilities leaves oxygen as the prime candidate for the heteroatom centre; this oxygen-centred species, if it is such, could be a substrate-derived species, a radical from the haem centre or a radical arising from the globin.

The first of these possibilities is excluded by the observation of the same anisotropic signal (albeit at different intensities) with a number of other peroxide compounds as well as H_2O_2. This suggests that the observed species is a MetMb-derived species and not, for example, an adduct arising from the peroxide component which is immobilised in some way by the protein. The fact that this signal is observed with all the compounds which have been shown by visible spectroscopy, to generate the myoglobin (IV) species suggests that the trapped radical is arising via a common mechanism which is somehow linked to the production of this high oxidation state iron-oxo species. The lack of peroxide-derived radicals in the e.s.r experiments suggests that the initial reaction between the MetMb and these peroxidic compounds is a two-electron process (which would be in agreement with the known chemistry of compounds such as peracetic acid and iodosyl benzene) rather than a one-electron process (as the latter would give rise to other radical species), with both the oxidising equivalents being present, at least initially, in the vicinity of, or at, the haem centre. Thus the observed adduct is thought to be due to the trapping of a radical arising from either of these two oxidising equivalents.

The results obtained in the presence of the added substrates demonstrate that the radical adduct observed in the e.s.r. experiments is not due to the trapping of the iron (IV)-oxo species itself as the effects of these compounds on the e.s.r. and optical spectra are markedly different in some cases. Thus the inclusion of desferal, Trolox C, or salicylate has a much more marked effect on the intensity of the e.s.r. spin adduct signal than on the optical absorpton of the myoglobin (IV) species suggesting that the species which reacts with the spin trap to give the observed adduct is much more readily scavenged than the iron (IV)-oxo centre itself; this may be due more to the accessibility of these reactive species to the added substrates than their absolute reactivities. The observation that 2-deoxyribose, mannitol and phenylalanine, which are all efficient HO· scavengers, have no effect on either the optical or e.s.r. signals suggests that the species observed in the e.s.r. experiments is *not* arising from the reaction of radicals (such as HO·) which might be generated in free solution by, for example, reaction of H_2O_2 with iron released from the haem ligand.

Trapping of a protein-derived radical, though possible, would necessitate a multi-step process. Oxidation of the globin would be expected to occur at an electron-rich site with the formation of a relatively stable, delocalised radical (c.f. radiation studies which have shown that tryptophan, tyrosine, histidine, phenylalanine, methionine and cysteine residues are most susceptible to oxidation and that such structures act as radical "sinks"[28]). There is evidence that this is indeed the case with myoglobin, where signals assigned to a tyrosyl radical on the globin surface have been detected;[29,30] this species has been suggested to arise as a result of intramolecular electron-transfer from the tyrosine residue to either an oxidised phenylalanine or histidine residue in the vicinity of the haem, or a porphyrin radical-cation species. Similar tyrosine-derived radicals have been observed in ribonucleotide reductase,[31] photosystem II,[32] and prostaglandin H synthase.[33] It is unlikely that the e.s.r. spin adduct observed in this case is due to the trapping of such a tyrosine phenoxyl radical as such delocalised species do not react rapidly with DMPO.

Recent studies have however suggested[34,35] that this tyrosine radical, once formed, can react with molecular oxygen to give a tyrosine peroxyl radical (which is believed to be responsible for some of the epoxidation of alkenes catalysed by haemoglobin and myoglobin in the presence of peroxides[34]), and it is postulated that it is this species which is being trapped by DMPO; the lifetime of the adduct, its hyperfine coupling constants and the effect of scavengers on the concentration of this species are all consistent with this assignment.

These myoglobin-derived species appear on the above evidence, to be, as expected, moderately powerful oxidants which are capable of oxidising ascorbic acid, desferal, salicylate, Trolox C and to a lesser extent linoleic acid. The mechanism of these processes (hydrogen-abstraction or electron-transfer) remain to be determined, but it would appear on this evidence that these species are capable of depleting natural and synthetic antioxidants, oxidising the iron-chelator desferal, and initiating lipid peroxidation through reaction with polyunsaturated fatty acids. These observations provide further evidence for a possible role of myoglobin in the initiation and propogation of damage in post-ischaemic reperfusion injury in the heart.[8,23,36–38] The reaction of both the myoglobin (IV) species and the tyrosine peroxyl radical with linoleic acid (observed by optical spectroscopy and the loss of the DMPO adduct by e.s.r. spectroscopy respectively) presumably results in the production of fatty acid radicals and the initiation of lipid peroxidation; this may be of considerable significance as it has been shown that the presence of membranes inhibits the H_2O_2 stimulated release of iron

from MetMb,[23] suggesting that although iron release from myoglobin can be a significant factor in the generation of radical damage *in vitro*, it may be much less significant *in vivo*. The above processes provide a mechanism by which damage can be initiated without the involvement of "free" iron ions. The rapid scavenging of the myoglobin-derived species by water-soluble antioxidants (such as ascorbate, salicylate and Trolox C) and the iron-chelator desferal may play a significant role in reducing lipid peroxidation both directly, by competition with fatty acids for the myoglobin-derived species, and indirectly by preventing haem degradation and subsequent iron release.

Acknowledgements

The author is grateful to Prof. B.C. Gilbert for helpful comments and discussions, to Dr. D. Leonard for the samples of iodosyl benzene, and to the Nuffield Foundation for financial support.

References

1. B. Halliwell and J.M.C. Gutteridge (1985). Free Radicals in Biology and Medicine, Clarendon Press, Oxford.
2. P.J. O'Brien (1969). Intracellular mechanisms for the decomposition of a lipid peroxide. I. Decomposition of a lipid peroxide by metal ions, heme compounds and nucleophiles, *Canadian Journal of Biochemistry*, **47**, 485–492.
3. P. Biemond, J.G. van Eijk, A.J.G. Swaak and J.F. Koster (1984). Iron mobilization from ferritin by superoxide derived from stimulated polymorphonuclear leukocytes. Possible mechanism in inflammation diseases. *Journal of Clinical Investigation*, **73**, 1576–1579.
4. J.M.C. Gutteridge (1986). Iron promoters of the Fenton reaction and lipid peroxidation can be released from haemoglobin by peroxides, *FEBS Letters*, **210**, 291–295.
5. M. O'Connell, B. Halliwell, C.P. Moorhouse, O.I. Aruoma, H. Baum and T.J. Peters (1986). Formation of hydroxyl radicals in the presence of ferritin and haemosiderin, *Biochemical Journal*, **234**, 727–731.
6. O.I. Aruoma and B. Halliwell (1987). Superoxide-dependent and ascorbate-dependent formation of hydroxyl radicals from hydrogen peroxide in the presence of iron, *Biochemical Journal*, **241**, 273–278.
7. M.J. Davies (1988). Detection of peroxyl and alkoxyl radicals produced by reaction of hydroperoxides with heme-proteins by electron spin resonance spectroscopy, *Biochimica et Biophysica Acta*, **964**, 28–35.
8. S. Harel and J. Kanner (1988). The generation of ferryl or hydroxyl radicals during interaction of haemproteins with hydrogen peroxide, *Free Radical Research Communications*, **5**, 21–33.
9. A. Puppo and B. Halliwell (1988). Formation of hydroxyl radical in biological systems. Does myoglobin stimulate hydroxyl radical formation from hydrogen peroxide?, *Free Radical Research Communications*, **4**, 415–422.
10. S. Harel, M.A. Salan and J. Kanner (1988). Iron release from metmyoglobin, methaemoglobin and cytochrome c by a system generating hydrogen peroxide, *Free Radical Research Communications*, **5**, 11–19.
11. M.J. Davies (1989). Direct direction of peroxyl radicals formed in the reactions of metmyoglobin and methaemoglobin with t-butyl hydroperoxide, *Free Radical Research Communications*, **7**, 27–32.
12. M. Chance, L. Powers, C. Kumar and B. Chance (1986). X-ray absorption studies of myoglobin peroxide reveal functional differences between globins and heme enzymes, *Biochemistry*, **25**, 1259–1265.
13. T. Yonetani and H. Schleyer (1967). Studies on Cytochrome c Peroxidase. IX. The reaction of ferrimyoglobin with hydroperoxides and a comparison of peroxide-induced compounds of ferrimyoglobin and cytochrome c peroxidase, *Journal of Biological Chemistry*, **242**, 1974–1979.
14. P.N. Balasubramanian, J.R. Lindsay Smith, M.J. Davies, T.W. Kaaret and T.C. Bruice (1989). Dynamics of reaction of (meso-tetrakis(2,6-dimethyl-3-sulfonatophenyl)porphinato)-iron (III) hydrate with tert-butyl hydroperoxide in aqueous solution. 2. Establishment of a mechanism that involves homolytic O–O bond breaking and one-electron oxidation of the iron (III) porphyrin, *Journal of the American Chemical Society*, **111**, 1477–1483.

15. M. Schlafer, C.L. Myers and S. Adkins (1987). Mitochondrial hydrogen peroxide generation and activities of glutathione peroxidase and superoxide dismutase following global ischaemia, *Journal of Molecular and Cellular Cardiology*, **19**, 1195–1206.
16. G.R. Buettner and L.W. Oberley (1978). Considerations in the spin trapping of superoxide and hydroxyl radical in aqueous systems using 5,5-dimethylpyrroline-1-oxide, *Biochemical and Biophysical Research Communications*, **83**, 69–74.
17. H. Saltzman and J.G. Sharefkin (1963). Iodosobenzene, *Organic Syntheses*, **43**, 60–61.
18. P. Jones and D.N. Middlemiss (1972). Formation of Compound I by the reaction of Catalase with Peroxacetic acid, *Biochemical Journal*, **130**, 411–415.
19. M.J. Davies, L.G. Forni and R.L. Willson (1988). Vitamin E analogue Trolox C. E.s.r. and pulse radiolysis studies of free-radical reactions, *Biochemical Journal*, **255**, 513–522.
20. M.J. Davies, R. Donker, C.A. Dunster, C.A. Gee, S. Jonas and R.L. Willson (1987). Desferrioxamine (Desferal) and superoxide free radicals. Formation of an enzyme-damaging nitroxide, *Biochemical Journal*, **246**, 725–729.
21. D. Keilin and E.F. Hartree (1950). Reaction of methaemoglobin with hydrogen peroxide, *Nature*, **166**, 513–514.
22. 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.
23. C. Rice-Evans, G. Okunade and R. Khan (1989). The suppression of iron release from activated myoglobin by physiological electron donors and desferrioxamine, *Free Radical Research Communications*, **7**, 45–54.
24. J.R. Harbour and J.R. Bolton (1975). Superoxide formation in spinach chloroplasts: electron spin resonance detection by spin trapping, *Biochemical and Biophysical Research Communications*, **64**, 803–807.
25. K.R. Maples, S.J. Jordan and R.P. Mason (1988). In vivo rat haemoglobin thiyl free radical formation following phenylhydrazine administration, *Molecular Pharmacology*, **33**, 344–350.
26. M. Dautrevaux, Y. Boulanger, K. Han and G. Bisterte (1969). Structure covalente de la myoglobine de cheval, *European Journal of Biochemistry*, **11**, 267–277.
27. G.R. Buettner (1987). Spin trapping: e.s.r. parameters of spin adducts, *Free Radicals in Biology and Medicine*, **3**, 259–303.
28. G.E. Adams, R.H. Bisby, R.B. Cundall, J.L. Redpath and R.L. Willson (1972). Selective free radical reactions with proteins and enzymes: the inactivation of ribonuclease. *Radiation Research*, **49**, 290–299.
29. 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.
30. H. Miki, K. Harada, I. Yamazaki, M. Tamura and H. Watanabe (1989). Electron spin resonance spectrum of Tyr-151 free radical formed in reactions of sperm whale metmyoglobin with ethyl hydroperoxide and potassium irridate, *Archives of Biochemistry and Biophysics*, **275**, 354–362.
31. A. Ehrenberg and P. Reichard (1972). Electron Spin Resonance of the iron-containing Protein B2 from ribonucleotide reductase, *Journal of Biological Chemistry*, **247**, 3485–3488.
32. B.A. Barry and G.T. Babcock (1987). Tyrosine radicals are involved in the photosynthetic oxygen-evolving system, *Proceedings of the National Academy of Science USA*, **84**, 7099–7103.
33. R.J. Kulmacz, A.-L. Tsai and G. Palmer (1987). Heme Spin states and peroxide-induced radical species in Prostaglandin H Synthase, *Journal of Biological Chemistry*, **262**, 10524–10531.
34. P.R. Ortiz de Montellano and C.E. Catalano (1985). Epoxidation of styrene by haemoglobin and myoglobin. Transfer of oxidising equivalents to the protein surface, *Journal of Biological Chemistry*, **260**, 9265–9271.
35. P.R. Ortiz de Montellano (1987). Control of the catalytic activity of prosthetic heme by the structure of hemoproteins, *Accounts of Chemical Research*, **20**, 289–294.
36. T. Shiga and K. Imaizumi (1975). Electron spin resonance study on peroxidase- and oxidase-reactions of horse radish peroxidase and methemoglobin, *Archives of Biochemistry and Biophysics*, **167**, 469–479.
37. J. Kanner and S. Harel (1985). Lipid peroxidation and oxidation of several compounds by H_2O_2 activated metmyoglobin, *Lipids*, **20**, 625–628.
38. M.B. Grisham (1985). Myoglobin-catalysed hydrogen peroxide dependent arachidonic acid peroxidation, *Journal of Free Radicals in Biology and Medicine*, **1**, 227–232.

Accepted by Prof. J.V. Bannister