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DIRECT DETECTION OF PEROXYL RADICALS FORMED IN THE REACTIONS OF METMYOGLOBIN AND METHAEMOGLOBIN WITH t-BUTYL HYDROPEROXIDE

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The reaction of metmyoglobin and methaemoglobin with t-butyl hydroperoxide has been investigated using stopped-flow e.s.r. spectroscopy. The major species observed immediately after mixing has been identified as the basis of its g value (2.014) and line width as being due to a peroxyl radical. The detection of this species under anaerobic conditions suggests that this species is the t-butyl peroxyl radical rather than a secondary species. The immediate observation of this species on mixing suggests that this species is arising via a reaction involving the intact haem moiety rather than via 'free iron' produced via oxidative damage. Detection of a second, oxygen-dependent, species, which is formed at a slower rate, in the metmyoglobin reaction suggests that the initial peroxyl radical subsequently reacts with the protein to give a protein-derived radical.

KEY WORDS: Free radicals, electron spin resonance, peroxyl, hydroperoxide, metmyoglobin, methaemoglobin.

INTRODUCTION

The metal-ion catalysed decomposition of either hydrogen peroxide or alkyl hydroperoxides to give free radicals has been suggested to be of considerable importance in a large number of pathological conditions.[1] The radical species produced and their rate of formation have been shown by a variety of methods to be dependent on a number of factors including the oxidation state of the metal ion, its chelating ligands, and, in the case of alkyl hydroperoxides, their structure.[2–6] Controversy exists as to the availability of low-molecular weight metal-ion chelates in biological systems[7] and attention has been focussed on the possible catalytic role of endogenous iron chelates such as iron-storage molecules and haem-proteins.[8–11] Both product studies[12,13] and e.s.r. spin trapping experiments[6,14–16] have shown that certain haem-proteins can catalyse the decomposition of alkyl hydroperoxides; the mechanism of production of the observed radicals has yet to be fully elucidated, though it is believed to occur via reactions at the *intact* haem group. In contrast, the radical-induced degradation of 'detector' molecules, such as deoxyribose, when certain haem-proteins are incubated with hydrogen peroxide has been suggested to occur via reactions of 'free' iron formed by release of the metal from the protein by oxidative damage.[8,10,11]

In view of the possible importance of haem-proteins as catalytic agents in, for example, both reperfusion injury[17] and erythrocyte disorders,[6,18,19] a direct method has

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been employed to determine whether the radical species observed in the reaction of alkyl hydroperoxides with haem-proteins arise via reactions at the *intact* haem moiety or via reactions of 'free' iron released from oxidatively damaged proteins. In this report it is shown that reaction of a model alkyl hydroperoxide with either metmyoglobin (MetMb) or methaemoglobin (MetHb) gives peroxyl radicals, which can be observed using an e.s.r. stopped-flow system, within seconds of mixing the two components, suggesting that these radicals arise via reactions at the intact haem.

MATERIALS AND METHODS

Chemicals

Horse heart myoglobin (MetMb) and bovine methaemoglobin (MetHb) were obtained from Sigma (Poole, Dorset, U.K.) and purified before use as described previously.[20] t-BuOOH was obtained from Aldrich (Gillingham, Dorset, U.K.) and used as supplied.

E.s.r. Spectroscopy

Reactions were carried out in 25 mM phosphate buffer (pH 7.4) at 22°C using a custom built stopped-flow system inserted into the cavity of a Bruker ESP300 e.s.r. spectrometer equipped with 100 KHz modulation. This system allowed rapid mixing of two reagent streams under either anoxic or normoxic conditions. g values were measured relative to a standard nitroxide radical (di-t-butyl nitroxide).

RESULTS

Methaemoglobin

Rapid mixing of air-saturated solutions of MetHb (500 μM) with t-BuOOH (10 mM) in the cavity of the e.s.r. spectrometer led to the immediate detection of a single broad peak with a g value of 2.014 and a peak to peak line width of 18 Gauss (Figure 1). The low field side of this signal is assymetrical suggesting the presence of a further radical species (arrowed in Figure 1), though it proved impossible to differentiate this signal from the main species. Omission of either of the two reagents resulted in the complete loss of both of these signals. Fast scanning of the magnetic field was necessary for the observation of these species as they both decayed rapidly. The time course of build-up and decay of the major species was monitored by setting the spectrometer to the magnetic field where the signal was of maximum intensity and recording the peak height with time after mixing (Figure 2). By use of this methodology it can be shown that the radical is formed extremely rapidly (within seconds) and that the half-life of this species is of the order of 8 seconds. The decay of this initial signal allowed the observation of a further species which appeared as a relatively weak absorption with a g value of 2.005 (Figure 1). This species though longer lived than the initial radical also decayed rapidly.

When similar experiments were carried out with nitrogen-saturated solutions no significant changes were observed. Pretreatment of MetHb with either 5 mM NaN_3 or 4 mM KCN led to the loss of all signals.

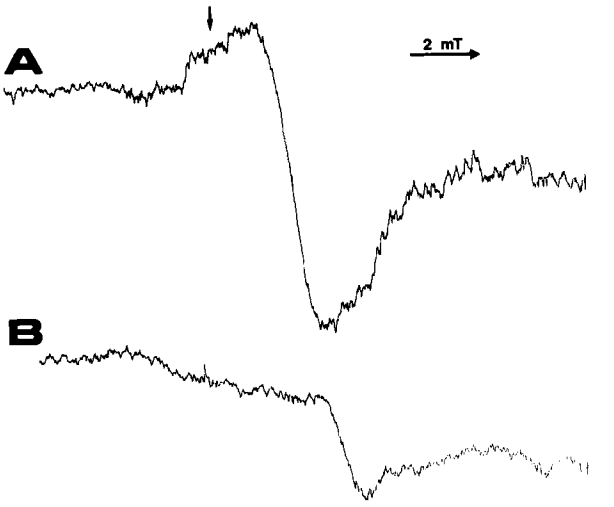

FIGURE 1 E.s.r. spectra observed immediately (A) and 5 minutes (B) after mixing of 500 μM MetHb with 10 mM t-BuOOH in normoxic 25 mM pH 7.4 phosphate buffer at 22°C. Main absorption in (A) assigned to t-BuOO$^{\cdot}$, g 2.014. Absorption in (B) unassigned, g 2.005. E.s.r. spectrometer conditions: gain 1×10^6, modulation amplitude 0.5 mT, time constant 320 ms, scan time 100 s, field 345.7 mT, scan range 20 mT, power 31 mW.

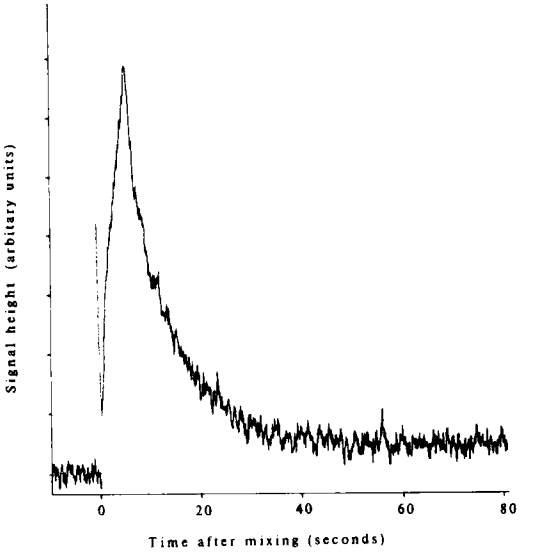

FIGURE 2 Build up and decay of signal assigned to t-BuOO$^{\cdot}$ radical. Reaction system as in Fig. 1. E.s.r. spectrometer conditions as Fig. 1 except modulation amplitude 1 mT, scan time 300 s, field 345.775 mT, scan range 0 mT.

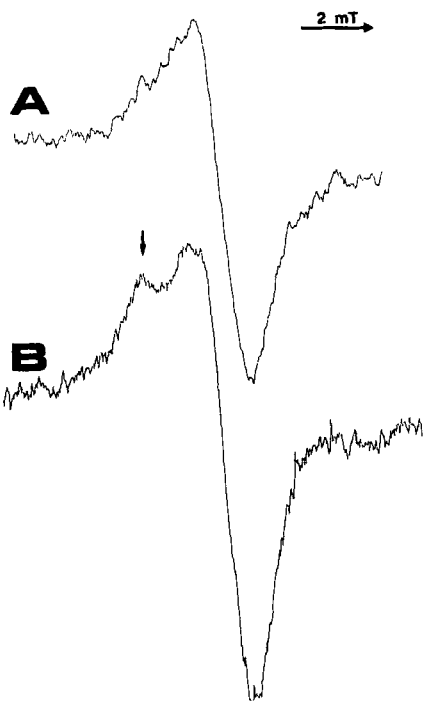

FIGURE 3 E.s.r. spectra observed immediately (A) and 1 minute after mixing of 1 mM MetMb with 10 mM t-BuOOH in normoxic 25 mM pH 7.4 phosphate buffer at 22°C. Main absorption in (A) assigned to t-BuOO$^{\cdot}$. Low field shoulder on main absorption in (B) tentatively assigned to a protein-derived peroxyl radical. E.s.r. spectrometer conditions as Fig. 1 except modulation amplitude 0.2 mT, scan time 50 s, and, in (B), gain 2×10^6.

Metmyoglobin

Reaction of 1 mM MetMb with 10 mM t-BuOOH under normoxic conditions led to the initial observation of a broad singlet signal, similar to that observed with MetHb, though with a slightly smaller peak to peak line width (15 Gauss). As with MetHb this signal decayed rapidly and a second radical signal appeared as a low field shoulder (arrowed peak, Figure 2). This second species, which has a g value of 2.026, decayed rapidly though its half life was longer than that of the initial species.

Omission of either of the two reagents led to the loss of all signals (suggesting, as with MetHb, that reactions of contaminating trace metals are not significant), as did pretreatment of MetMb with either 5 mM NaN_3 or 4 mM KCN. Repetition of these experiments (in the absence of azide or cyanide) under *anoxic* conditions resulted in similar behaviour with the exception that the low field shoulder on the main signal was present at considerably lower concentrations.

DISCUSSION

The major species observed immediately after mixing in both the MetMb and MetHb systems has a high g value (2.014) and no resolvable hyperfine couplings. This type

of signal is characteristic of peroxyl radicals and is identical to that observed on reaction of t-BuOOH with free haem (Hematin);[21] this absorption is therefore assigned to such a species. The detection of this species immediately after mixing in both the normoxic and anoxic experiments suggests that this transient species is the t-BuOO$^{\cdot}$ radical rather than a protein derived species. This radical would appear to be formed via a direct reaction between the intact haem moiety and the hydroperoxide as both CN^- and N_3^-, which are known to strongly chelate iron (III) haems,[2,16] result in the loss of all the signals. Further evidence for this reaction occuring at the intact haem rather than via released iron comes from the observation that this species is observed immediately on mixing (probably within the dead time of the system used) when the extent of haem degradation is extremely low or undetectable.[8] The isotropic nature of the initial signal shows that this species is capable of unconstrained motion, suggesting that this species is being observed outside the haem pocket. These conclusions are in agreement with data obtained from spin trapping experiments.[16]

The subsequent fate of this initial species appears to be dependent on the protein structure. With MetHb a second species is observed under both normoxic and anoxic conditions; the identity of this species is unknown. Similar species have been observed in previous experiments,[22,23] but little definitive evidence as to the nature of the radical(s) is available; the g value of this species and its lifetime suggest that one possible assignment is to a tyrosine phenoxyl radical with the hyperfine couplings not observed due to over modulation and power saturation of the signal. Tyrosine phenoxyl radicals observed in other proteins, such as ribonucleotide reductase,[24] have a g value of 2.0047 and, under conditions of low modulation and non-saturating power levels (< mW), a poorly resolved doublet structure. Further speculation as to the nature of this species is unwarranted. In the case of MetMb the second signal has a very high g value (2.026), is anisotropic in nature, is oxygen dependent, and has a lifetime of tens of seconds. This signal is tentatively assigned to a highly-constrained protein-derived peroxyl radical arising via reaction (1). Previous work[25] has shown that immobilised peroxyl radicals have highly anisotropic spectra with g values of the order of 2.03 (g_{zz}) and 2.006–9 (g_{xx} and g_{yy}). The latter features, if present in these spectra, would be hidden beheath the absorption from the primary radical. The weak signal from this species observed in experiments where nitrogen-saturated solutions were employed, may arise from the production of oxygen during the reaction due to the breakdown of tetroxides formed on peroxyl radical dimerisation[26] (reaction 2).

$$\text{t-BuOO}^{\cdot} + \text{protein} \longrightarrow \text{t-BuOOH} + \text{protein} \xrightarrow{{}^{\cdot}O_2} \text{protein–O–O}^{\cdot} \qquad (1)$$

$$2\text{t-BuOO}^{\cdot} \longrightarrow \text{t-Bu–O–O–O–O–t-Bu} \longrightarrow 2\text{t-BuO}^{\cdot} + O_2 \qquad (2)$$

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References

1. Yagi, K. *Chem. Phys. Lipids,* **45,** 337–351, (1987).
2. O'Brien, P.J. *Can. J. Biochem.,* **47,** 485–492, (1969).
3. Kochi, J.K. in: Free Radicals, edited by J.K. Kochi, vol. 1, pp. 591–683. New York: Wiley-Interscience, (1973).

4. Gardner, H.W. *J. Agr. Food Chem.*, **23,** 129–136, (1975).
5. Schaich, K.M. and Borg, D.C. in: Autoxidation in Food and Biological Systems, edited by M.G. Simic and M. Karel, pp. 45–70. New York and London: Plenum Press, (1980).
6. Thornalley, P.J., Trotta R.J. and Stern, A. *Biochim. Biophys. Acta,* **759,** 16–22, (1983).
7. Halliwell, B. and Gutteridge, J.M.C. *Biochem. J.,* **219,** 1–14, (1984).
8. Gutteridge, J.M.C. *FEBS Lett.*, **201,** 291–295, (1986).
9. Aruoma, O.I. and Halliwell, B. *Biochem. J.,* **241,** 273–278, (1987).
10. Puppo, A. and Halliwell, B. *Biochem. J.,* **249,** 185–190, (1988).
11. Puppo, A. and Halliwell, B. *Free Rad. Res. Comms.,* **4,** 415–422, (1988).
12. Weiss, R.H. and Estabrook, R.W. *Arch. Biochem. Biophys.,* **251,** 348–360, (1986).
13. Vaz, A.D.N. and Coon, M.J. *Proc. Natl. Acad. Sci. USA,* **84,** 1171–1176, (1987).
14. Griffin, B.W. and Ting, P.L. *FEBS Lett.*, **89,** 196–200, (1978).
15. Schreiber, J., Mason, R.P. and Eling, T.E... *Arch. Biochem. Biophys.*, **257,** 17–24, (1986).
16. Davies, M.J. *Biochim. Biophys. Acta,* **964,** 28–35, (1988).
17. Weiss, S.J. *Acta Physiol. Scand. Suppl.*, **548,** 9–37, (1986).
18. Rice-Evans, C. and Baysal, E. *Biochem. J.,* **244,** 191–196, (1987).
19. Rice-Evans, C. *Bioelectrochem. Bioenerg.*, **18,** 257–262, (1987).
20. Harada, K. and Yamazaki, I. *J. Biochem.,* **101,** 283–286, (1987).
21. Kalyanaraman, B., Mottley, C. and Mason, R.P. *J. Biol. Chem.*, **258,** 3855–3858, (1983).
22. Kelso King, N., Looney, F.D. and Winfield, M.E. *Biochim. Biophys. Acta,* **133,** 64–82. (1967).
23. Yonetani, T. and Schleyer, H. *J. Biol. Chem.*, **242,** 1974–1979, (1967).
24. Sjöberg, B.-M., Reichard, P., Gräslund, A. and Ehrenberg, A. *J. Biol. Chem.*, **252,** 534–541, (1977).
25. Howard, J.A. in: Magnetic Properties of Free Radicals, edited by H. Fischer and K.-H. Hellwege, Landolt-Bornstein New Series Group II, vol. 9, part C2, pp. 5–28. Berlin: Springer-Verlag, (1979).
26. Howard, J.A. in Free Radicals, edited by J.K. Kochi, vol. 2, pp. 3–62. New York: Wiley-Interscience, (1973).

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