Formation of peroxide- and globin-derived radicals from the reaction of methaemoglobin and metmyoglobin with t-butyl hydroperoxide: an ESR spin-trapping investigation

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The reaction of human methaemoglobin and horse metmyoglobin with t-butyl hydroperoxide (t-BuOOH) was investigated with the ESR spin-trapping technique. With the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) the formation of peroxy, alkoxy and methyl radicals derived from t-BuOOH could be detected. The relative contributions of these radicals were determined at various DMPO concentrations by computer simulation. From these data it could be concluded that the alkoxy radical was the initial radical produced, which indicates that the hydroperoxide is cleaved homolytically. Further investigations, with the nitroso spin trap 2-methyl-2-nitrosopropane (MNP), showed the formation of globin-centred radicals. Non-specific proteolysis of the MNP adducts revealed isotropic three-line

spectra, which means that the radical adducts were centred on a tertiary carbon with no bonds to a hydrogen or nitrogen. Comparison with MNP adducts of several amino acids indicated that in methaemoglobin the radical adduct was most probably located on a valine residue. With metmyoglobin the same adduct was obtained, whereas an additional adduct could be assigned to a tyrosyl radical. These protein radicals most probably resulted from hydrogen abstraction by the metal-oxo species, formed by heterolytic cleavage of the hydroperoxide. These results therefore show that homolytic cleavage of the hydroperoxide leads to the formation of peroxide-derived radicals, whereas concurrent heterolytic cleavage results in protein-derived radicals.

INTRODUCTION

Considerable attention has been focused on the formation, stabilities and reactions of protein radicals in biological systems and the pathological consequences of such processes. Recent interest in protein radicals also stems from their proposed roles as intermediates in the catalytic mechanisms of several enzymes, in particular in the reaction of haem proteins with peroxides. The reaction between haem proteins and H_2O_2 is thought to proceed via two-electron oxidation, resulting in the formation of ferryl haem ($Fe^{IV}=O$), which is one oxidizing equivalent above the initial level, and a protein radical [1–6]. The mechanism by which the protein radical is generated, its location in the protein and the mechanisms by which it is dissipated are poorly understood. The nature of the metmyoglobin and methaemoglobin radical site was originally proposed to be a tyrosine radical [6–9] but this assignment has been contested [10,11]. In studies by Ortiz de Montellano and colleagues, a direct ESR signal was observed when a mutant protein, which lacked all tyrosine residues, was exposed to H_2O_2 [12,13]. This signal was identical with that found when the native protein was exposed to H_2O_2 . However, observations such as the dityrosine cross-linking of sperm whale metmyoglobin by hydrogen peroxide and the covalent binding of tyrosine to haem indicate that oxidation of metmyoglobin by hydrogen peroxide forms one or more tyrosyl radicals [14,15]. Recently this was confirmed by Barr et al. [16], who showed that a tyrosyl radical could be trapped when horse metmyoglobin was exposed to hydrogen peroxide in the presence of the spin trap 2-methyl-2-nitrosopropane (MNP).

Less work has been done to determine what species are generated on the reaction of haem proteins with organic hydroperoxides. A complex ESR spectrum was observed in the reaction

of sperm whale metmyoglobin and ethyl hydroperoxide, and was found to be a mixture of a tyrosyl radical and an unidentified free radical [17,18]. The latter radical was also observed with horse heart metmyoglobin, and it was suggested that the tyrosyl radical was formed on Tyr-151, which is present only in sperm whale metmyoglobin. A study on the reaction of methaemoglobin and a number of organic hydroperoxides also described the formation of tyrosyl radicals, but the signals were weak and poorly resolved [9]. The half-life of this radical was estimated as approx. 50 s. It was suggested that the mechanism 'of their formation was similar to the mechanism with H_2O_2 '. However, recent ESR spin-trapping studies with cytochrome *c* and haematin showed that the reaction of haem proteins and organic hydroperoxides most probably proceeds through a one-electron reduction of the hydroperoxide to form an alkoxy radical as the first intermediate [19,20]. Thus there seems to be a difference between the reaction of haem proteins with H_2O_2 and the reaction with organic hydroperoxides. The reaction of haem proteins with organic peroxides was therefore studied further. Metmyoglobin and methaemoglobin were exposed to t-butyl hydroperoxide (t-BuOOH), and the formation of both peroxide- and protein-derived radicals was investigated with the spin traps 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) and MNP.

EXPERIMENTAL

Pronase was obtained from Boehringer. Human methaemoglobin, horse metmyoglobin, t-BuOOH, MNP and DMPO were purchased from Sigma. To improve its purity, DMPO (1:1 dilution in water) was passed through a column containing

activated charcoal [21]. The DMPO concentration was measured at 228 nm, assuming a molar absorption coefficient of $7800 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [22]. All other reagents were of analytical grade and used without further purification. All solutions were prepared in water that had been passed through a Millipore Milli-Q ultrapurification system, and were made up freshly before use. MNP (2 mg/ml) was dissolved in 50 mM sodium phosphate buffer, pH 7.4, by stirring overnight in the dark. All experiments were performed at room temperature in 50 mM sodium phosphate buffer, pH 7.4. Anaerobic conditions were obtained by bubbling with nitrogen.

ESR spectra were obtained with a JEOL RE2X spectrometer operating at 9.36 GHz with a 100 kHz modulation frequency, equipped with a TM110 cavity. The samples were transferred to the quartz flat cell by means of a rapid sampling device [23]. Hyperfine splittings were obtained with a program developed by David Duling (Laboratory of Molecular Biophysics, NIEHS, Research Triangle Park, NC, U.S.A.) [24]. The parameters used for simulating the experimental ESR spectra are described in the figure legends. The data for Figure 2 were obtained by simulating the low-field lines of each species. The component spectra were then double-integrated to give relative radical adduct concentrations. When ESR spectra were recorded at liquid nitrogen temperature, the spectrometer operating at 8.86 GHz was equipped with a TM011 cavity and a fingertip liquid-nitrogen Dewar flask.

When samples were subjected to dialysis, several ml of sample were placed in dialysis tubing (pore size 2.4 nm) and dialysed overnight at 4 °C against 50 mM sodium phosphate buffer, pH 7.4. After dialysis a small aliquot was removed and spectra were scanned. The remaining sample was treated with pronase (final concentration 2 mg/ml) for various periods at room temperature or 37 °C and subsequently scanned. Control experiments were performed in parallel with complete incubations.

RESULTS

Spin trapping of t-BuOOH-derived radicals with DMPO

The results here are described for methaemoglobin, but comparable results were obtained with metmyoglobin. Incubation of methaemoglobin with t-BuOOH in the presence of DMPO gave a spectrum consisting of several DMPO radical adducts (Figure 1A). Deconvolution of the composite spectrum was performed by simulating each species separately by using the relative intensities and hyperfine values obtained from the composite computer simulation (Figures 1B to 1F). From their respective hyperfine coupling constants, the adducts could be assigned as alkoxyl [$a^N = 15.0 \text{ G}$ and $a^H\beta = 16.5 \text{ G}$; 1 gauss = 10^{-4} tesla (T)], peroxy ($a^N = 14.4 \text{ G}$, $a^H\beta = 10.35 \text{ G}$ and $a^H\gamma = 1.4 \text{ G}$), methyl ($a^N = 16.5 \text{ G}$ and $a^H\beta = 23.6 \text{ G}$) and 5,5-dimethylpyrrolidone-2-(oxy)-(1) (DMPOX) [$a^N = 7.3 \text{ G}$ and $a^H\beta(2H) = 4.1 \text{ G}$]. These hyperfine splittings are in agreement with previous observations [19,20,25]. DMPOX is an oxidation product of DMPO that is thought to arise from the decomposition of DMPO/peroxy radical adducts and/or from the reaction of haem iron with DMPO [26]. Control experiments showed that the signal was completely dependent on the presence of methaemoglobin and t-BuOOH (Figures 1G and 1H). In addition, neither EDTA nor diethylenetriaminepenta-acetate had any effect on the concentration of radical adducts, which showed that the reactions were not mediated by free iron (results not shown).

The relative contributions of the peroxy, alkoxyl and methyl radical adducts to the composite ESR spectrum were influenced by the concentration of DMPO. This is illustrated in Figure 2.

Figure 1 Computer simulation and deconvolution of the ESR spectrum obtained from a reaction mixture containing methaemoglobin and t-BuOOH

Samples contained $100 \mu\text{M}$ methaemoglobin, 38 mM t-BuOOH and 58 mM DMPO. (A) Experimental spectrum; (B) composite computer simulation of (A); (C–F) individual simulations of each species in the composite spectrum. Hyperfine coupling constants are given in the text. (C) Simulated spectrum for DMPO/OR: linewidth 0.58 G; line shape 100% Gaussian; mole ratio 0.34. (D) Simulated spectrum for DMPO/OOR: linewidth 0.5 G; line shape 17% Lorentzian, 83% Gaussian; mole ratio 0.43. (E) Simulated spectrum for DMPO/ CH_3 : linewidth 0.77 G; line shape 80% Lorentzian, 20% Gaussian; mole ratio 0.17. (F) Simulated spectrum for DMPOX: linewidth 0.3 G; line shape 61% Lorentzian, 39% Gaussian; mole ratio 0.05. (G) As (A) but without methaemoglobin. (H) As (A) but without t-BuOOH. The spectrometer conditions were as follows: modulation amplitude, 0.5 G; microwave power, 20 mW; time constant, 0.3 s; scan time, 6 min; scan range, 80 G. [Note: 1 gauss = 10^{-4} tesla (T).]

Figure 2 Effect of DMPO on the relative contributions of the radical adducts in a methaemoglobin/t-BuOOH system

Reaction mixtures contained $100 \mu\text{M}$ methaemoglobin, 38 mM t-BuOOH and various DMPO concentrations. Symbols: $\circ$, DMPO/OR; $\triangle$, DMPO/OOR; $\square$, DMPO/ CH_3 ; $\bullet$, DMPOX.

The peroxy radical adduct comprised approx. 51% of the composite spectrum at a DMPO concentration of 13 mM, whereas this decreased to 16% at a DMPO concentration of 260 mM. At the same time, the methyl radical adduct decreased from 27% to 0%. The relative contribution of the alkoxyl radical adduct, in contrast, increased from approx. 17% to 65% of the composite spectrum. Thus as the DMPO concentration was increased, the relative contribution of the peroxy and methyl radical adducts decreased, whereas the contribution of the alkoxyl radical adduct increased (Figure 2).

Figure 3 ESR spectra obtained after addition of t-BuOOH to methaemoglobin in the presence of MNP

(A) ESR spectrum after incubation of 100 μ M methaemoglobin with 5 mM t-BuOOH in the presence of 23 mM MNP for 10 min. (B) ESR spectrum of an aliquot of the solution used to obtain spectrum (A) after dialysis overnight. (C) As (B) but without methaemoglobin. (D) As (B) but without t-BuOOH. (E) As (B) but without MNP. (F) An aliquot of the solution used to obtain spectrum (B) was treated with pronase (2 mg/ml) for 105 min. Instrument settings for scans (A–E) were as follows: modulation amplitude, 2.5 G; microwave power, 20 mW; time constant, 1 s; scan time, 10 min; scan range, 100 G. For scan (F) settings were: modulation amplitude, 1 G; time constant, 0.3 s.

Figure 4 ESR spectra obtained after addition of t-BuOOH to metmyoglobin in the presence of MNP

(A) Metmyoglobin (400 μ M) was incubated with 5 mM t-BuOOH in the presence of 23 mM MNP for 10 min and dialysed overnight. (B) An aliquot of the solution used to obtain spectrum (A) was treated with pronase (2 mg/ml) for 10 min. (C) An aliquot of the solution used to obtain spectrum (A) was treated with pronase (2 mg/ml) for 85 min. (D) As (B) but metmyoglobin was incubated with 400 μ M H_2O_2 . Spectrometer settings for (A) were as in Figure 3(A) and for (B–D) as in Figure 3(F).

Spin trapping of globin-derived radicals with MNP

Incubation of methaemoglobin with t-BuOOH in the presence of MNP resulted in the detection of an ESR spectrum characteristic of a highly immobilized nitroxide with $a_N = 32.7$ G (Figure 3A). Superimposed on the signal were the signals formed as a result of the spin trapping of t-BuOOH-derived radicals by MNP. Maximal signal intensity was reached after 10 min of incubation. Only moderate decay of the immobilized signal occurred on dialysis (Figure 3B). The formation of the immobilized nitroxide was completely dependent on the presence of methaemoglobin, t-BuOOH and MNP (Figures 3C to 3E). Treatment of the product with pronase resulted in the detection of a three-line spectrum with hyperfine coupling constant $a^N = 16.8$ G (Figure 3F). No

Figure 5 Computer simulation of the spectrum obtained after short pronase treatment of metmyoglobin exposed to t-BuOOH in the presence of MNP

(A) Metmyoglobin (400 μ M) was incubated with 5 mM t-BuOOH in the presence of 23 mM MNP for 10 min, dialysed overnight and treated with pronase (2 mg/ml) for 10 min. (B) Composite simulation of (A). (C) Simulated spectrum for the triplet with $a^N = 15.4$ G; linewidth 1.5 G; line shape 79% Lorentzian, 21% Gaussian; mole ratio 0.4. (D) Simulated spectrum for the triplet with $a^N = 16.8$ G; linewidth 0.9 G; line shape 100% Lorentzian; mole ratio 0.6.

signal could be detected in control samples, without either t-BuOOH or MNP, that were treated with pronase after dialysis (results not shown).

In contrast with H_2O_2 , with t-BuOOH peroxide-derived radicals are formed, which could oxidize amino acids in methaemoglobin and in this way give an MNP-radical adduct. Therefore the experiment with MNP (Figures 3A, 3B and 3F) was also performed in the presence of 200 mM DMPO, which traps t-BuOOH-derived radicals and thus prevents a reaction with the protein. In the presence of DMPO exactly the same results were obtained as without DMPO. It was also investigated whether iron, which can be released from the haem group on degradation, was involved in the formation of the globin-derived radicals. However, the same results were obtained when 100 μ M diethylenetriaminepenta-acetate was added to the incubation mixtures (results not shown).

When metmyoglobin was incubated with t-BuOOH in the presence of MNP, a highly immobilized nitroxide with $a_N = 28.8$ G was obtained, which was stable enough to be dialysed (Figure 4A). After treatment with pronase for 10 min the spectrum shown in Figure 4(B) was obtained, which was changed by further treatment with pronase into the spectrum shown in Figure 4(C). Simulation was difficult as the high-field lines were broadened as result of the slower molecular motion, which is consistent with the high molecular mass of the radical adduct from pronase-treated metmyoglobin. The simulation for the spectrum obtained after treatment with pronase for 10 min is shown in Figure 5(B), with the separate triplets in Figures 5(C) and 5(D). The spectrum consisted of two triplets: one with splitting constant $a^N = 16.8$ G, and another with $a^N = 15.4$ G. The latter was dominant after short incubation with pronase, whereas the former appeared more clearly after longer treatment with pronase (Figures 4B and 4C). For comparison the spectrum obtained by pronase treatment of metmyoglobin, exposed to hydrogen peroxide in the presence of MNP, is shown, which consists of one triplet with $a^N = 15.4$ G (Figure 4D). This result is in accordance with previous observations [16,27].

Figure 6 ESR spectra obtained after exposure of amino acids to hydroxyl radicals in the presence of MNP: comparison with methaemoglobin treated with t-BuOOH

(A) Isoleucine (20 mM) was incubated with 2 mM Fe^{2+} and 2.5 mM H_2O_2 in the presence of 23 mM MNP. (B) As (A) but with 20 mM leucine. (C) As (A) but with 20 mM valine. (D) Methaemoglobin (500 μM) was incubated with 10 mM t-BuOOH in the presence of 23 mM MNP, dialysed overnight, diluted 1:1 and subsequently treated with pronase (2 mg/ml) for 75 min at 37 °C. Spectrometer settings for scans (A–C) were: modulation amplitude, 0.5 G; microwave power, 20 mW; time constant, 0.1 s; scan time, 10 min; scan range, 100 G. For scan (D) these settings were: modulation amplitude, 1 G; time constant, 0.3 s.

Oxidation of amino acids

Structural information about trapped radicals can be obtained by using a nitroso spin trap such as MNP. Nitroso compounds have a distinct advantage over nitrones such as DMPO because the reactive free radical attaches directly to the nitroxide nitrogen atom, which will give rise to additional hyperfine splitting constants. The lack of additional hyperfine structure in the spectra obtained after pronase treatment of the haem proteins therefore indicates that the tertiary carbon atom in the adduct has no bonds to atoms with nuclear spin such as nitrogen or hydrogen. In the case of a one-electron oxidation reaction the most likely positions are C-3 of the indole ring of tryptophan and C-4 of the phenol ring of tyrosine. When tryptophan was incubated with methaemoglobin and t-BuOOH in the presence of MNP, a three-line spectrum was detected with hyperfine splitting constant $a^{\text{N}} = 15.8$ G (result not shown). With tyrosine, however, no radical was detected. However, when tyrosine was incubated with horseradish peroxidase and H_2O_2 in the presence of MNP a triplet was detected with $a^{\text{N}} = 15.4$ G (results not shown), as was also observed by Barr et al. [16].

A radical on a tertiary carbon atom can also be formed through abstraction of a hydrogen. Possible candidates are the amino acids valine, isoleucine, leucine, threonine and proline. Data in the literature showed that MNP adducts of tertiary carbon-centred radicals with a hyperfine splitting between 16.3 and 16.7 G can be obtained on exposure of valine, leucine and isoleucine to hydroxyl radicals, generated by photolysis of H_2O_2 [28]. Incubation of these amino acids with methaemoglobin and t-BuOOH in the presence of MNP did not give the formation of radical adducts.

In subsequent experiments isoleucine, leucine and valine were exposed to hydroxyl radicals, generated by Fe^{2+} and H_2O_2 , in the presence of MNP. The spectra shown in Figures 6(A) to 6(C) were compared with a spectrum that was obtained at a higher methaemoglobin concentration, which showed signals in addition to the main triplet of 16.8 G (Figure 6D). The hyperfine splitting

Table 1 Hyperfine coupling constants of radical adducts generated from the reaction of amino acids with hydroxyl radicals in the presence of MNP: comparison with methemoglobin treated with t-BuOOH

Reaction conditions were as described in the legend to Figure 6. Hyperfine coupling constants are given in gauss. [Note: 1 gauss = 10^{-4} tesla (T).]

Source	Trapped species	a^{N}	$a^{\text{N}}\beta$	$a^{\text{H}}\beta 1$	$a^{\text{H}}\beta 2$	Percentage of total
Valine*	$\cdot\text{CH}_2-$	16.75		24.9	0.65	12
	$\cdot\text{CH}_2-$	16.5		22.0	0.70	12
	$> \cdot\text{C}-$	16.5				29
	$\cdot\text{CN}-$	16.75	2.15			40
MNP		17.2				6
Leucine†	$\cdot\text{CH}_2-$	16.8		23.75	0.65	13
	$\cdot\text{CH}_2-$	16.6		27.4	0.60	5
	$> \cdot\text{C}-$	16.85				60
	$\cdot\text{CH}-$	16.45		1.9		19
MNP		17.2				3
Isoleucine‡	$\cdot\text{CH}_2-$	16.95		22.75	0.60	5
	$\cdot\text{CH}_2-$	16.9		21.45	0.60	6
	$\cdot\text{CH}-$	16.5		1.85		74
	$\cdot\text{C} <$	16.6				9
MNP		17.2				6
Methaemoglobin	$> \cdot\text{C}-$	16.8				37
	$\cdot\text{CN}-$	16.55	2.4			63

* Structure of valine: $(\text{CH}_3)_2\text{CHCH}(\text{NH}_3^+)\text{CO}_2^-$.
† Structure of leucine: $(\text{CH}_3)_2\text{CHCH}_2\text{CH}(\text{NH}_3^+)\text{CO}_2^-$.
‡ Structure of isoleucine: $\text{CH}_3\text{CH}_2\text{CH}(\text{CH}_3)\text{CH}(\text{NH}_3^+)\text{CO}_2^-$.

Figure 7 Computer simulation of the ESR spectrum obtained after exposure of valine to hydroxyl radicals

(A) Composite computer simulation of Figure 5(C). The hyperfine coupling constants of each species are listed in Table 1. (B) Simulated spectrum of $\text{MNP}/\cdot\text{CH}_2\text{CH}(\text{CH}_3)\text{CH}(\text{NH}_3^+)\text{CO}_2^-$: line width 0.2 G; line shape 81% Lorentzian, 19% Gaussian. (C) Simulated spectrum of $\text{MNP}/\cdot\text{CH}_2\text{CH}(\text{CH}_3)\text{CH}(\text{NH}_3^+)\text{CO}_2^-$: line width 0.25 G; line shape 65% Lorentzian, 35% Gaussian. (D) Simulated spectrum of $\text{MNP}/(\text{CH}_3)_2\text{CHC}(\text{NH}_3^+)\text{CO}_2^-$: line width 0.5 G; line shape 96% Lorentzian, 4% Gaussian. (E) Simulated spectrum of $\text{MNP}/(\text{CH}_3)_2\text{CH} \cdot\text{C}(\text{NH}_3^+)\text{CO}_2^-$: line width 0.64 G; line shape 100% Gaussian.

constants are listed in Table 1 and as illustration the simulation of the spectrum obtained with valine is given in Figure 7. All the spectra consisted of five species: four derived from the amino acid and a small contaminant signal from MNP, namely the di-t-butyl nitroxide radical ($a^{\text{N}} = 17.2$ G). Two radical adducts came from hydrogen abstraction from the methyl groups of each amino acid (Figures 7B and 7C). The non-equivalence in the

Figure 8 ESR spectrum of methaemoglobin at 77 K before and after addition of t-BuOOH

(A) Solution of 500 μ M methaemoglobin frozen in liquid nitrogen. (B) Methaemoglobin (500 μ M) was incubated with 10 mM t-BuOOH for 20 min and the sample was frozen in liquid nitrogen. Spectrometer settings were: modulation amplitude, 10 G; microwave power, 1 mW; time constant, 0.1 s; scan rate 625 G/min.

hyperfine splitting constants of the β -protons can be explained by the presence of two asymmetrical conformers produced by internal steric hindrance ([28], and references therein). The third species that was observed came from a tertiary carbon (Figure 7D). For leucine and isoleucine the final species came from a doublet, generated by hydrogen abstraction from a CH_2 group. For valine, in contrast, the last species was simulated by using a hyperfine splitting $a^N = 16.5$ G and an additional coupling $a^N = 2.1$ G, coming from the nitrogen of the amino group (Figure 7E). This species can also be interpreted as a doublet ($a^N = 16.7$ G and $a^H\beta = 4.3$ G), assuming that a centre line from a nitrogen triplet is not hidden under the strong triplet spectrum. However, the splitting for the β -proton is too high in comparison with the splittings obtained for leucine and isoleucine (Table 1).

The similarity between the methaemoglobin spectrum and the spectrum of valine was apparent (Figures 6C and 6D). With methaemoglobin, the hyperfine splittings of the second species could be simulated by using $a^N = 16.5$ G (linewidth 0.9 G) and $a^N\beta = 2.4$ G (linewidth 1.2 G). There were still some additional lines present in the methaemoglobin signal, but these could not be identified further.

ESR spectra at 77 K

Figure 8 shows the ESR spectrum of methaemoglobin in phosphate buffer frozen to 77 K. The major signal at $g = 6$ is typical of high-spin iron(III) in a haem environment [29]. Incubation of methaemoglobin with t-BuOOH resulted in a decrease in this signal (Figure 8).

DISCUSSION

Two mechanisms exist for the reduction of organic hydroperoxides by haem proteins. In heterolytic cleavage the hydroperoxide undergoes two-electron reduction to its corresponding alcohol and haem iron is oxidized to a compound I-type species:

This species could then abstract hydrogen, producing a peroxy

radical and the ferryl form of the haem protein as shown in reaction (2). Self-reaction of tertiary peroxy radicals will result in the formation of alkoxy radicals, as in reaction (3) [30,31]:

Homolytic cleavage of the hydroperoxide, in contrast, will yield the alkoxy radical as the initial radical:

Subsequent reaction of methyl radicals, formed through β -scission of the alkoxy radical [reaction (5)], with oxygen will give methylperoxy radicals, as in reaction (6):

Evidence has been obtained that heterolytic and homolytic cleavage can occur at the same time [32]. With the help of 4-hydroperoxy-4-methyl-2,6-di-*t*-butylcyclohexa-2,5-dien-1-one, a peroxide that is converted heterolytically to an alcohol and homolytically to a number of rearranged products, it was determined that the homolytic/heterolytic product ratio for several haem proteins was 0.5. This means that peroxide-derived radicals can be formed concurrently via reactions (2) and (4).

To determine what mechanism was responsible for the formation of peroxy and alkoxy radicals in the reaction of methaemoglobin or metmyoglobin with t-BuOOH, the following strategy was used: the reactions of the initial radical that occur after its formation can be suppressed by increasing the concentration of the spin trap, in this case DMPO. Thus as more spin trap is added, more of the initial radical is trapped, which prevents the initial radical from undergoing reactions that lead to secondary radicals. At low DMPO concentrations alkoxy, peroxy and methyl radical adducts were detected. At higher DMPO concentrations, however, the alkoxy radical dominated, whereas the peroxy and the methyl radical adducts decreased (Figure 2). These results strongly suggest that the alkoxy radical is the initial radical, which means that the hydroperoxide is cleaved homolytically [reaction (4)]. This is in accordance with the results obtained with haematin and cytochrome *c* [19,20]. These results also indicate that peroxy radicals are not formed via reaction (2). If such a reaction took place the peroxy radical concentration would not have decreased, but should have increased as the DMPO concentration was increased (Figure 2). These results are in agreement with those of Allentoff et al. [32], who found that although substantial heterolytic cleavage of 4-hydroperoxy-4-methyl-2,6-di-*t*-butylcyclohexa-2,5-dien-1-one took place [reaction (1)], only a very small quantity of peroxy radicals was formed via reaction (2). Taken together, these results indicate that peroxy radical formation results from β -scission of the alkoxy radical and subsequent reaction of the methyl radical with oxygen [reactions (5) and (6)].

It has been suggested that reaction (4) might occur without a valence change of the haem iron and that an iron-bound hydroxyl radical might be formed [33]. However, the ESR spectra of methaemoglobin at 77 K showed that the signal in the $g = 6$ region, assigned to the ferric prosthetic group, decreased considerably on exposure to t-BuOOH (Figure 8). This clearly showed that iron is no longer in its initial high-spin ferric state after reaction with t-BuOOH.

Scheme 1 Proposed structure of valine radicals trapped by MNP

Further investigations showed that incubation of methaemoglobin and metmyoglobin with *t*-BuOOH also resulted in the formation of protein radicals (Figures 3 and 4). The high degree of rotational anisotropy in these immobilized signals can be removed by treatment with proteases, which results in fragmentation of the protein to relatively freely rotating peptides that give isotropic spectra [34]. With methaemoglobin a triplet with $a^N = 16.8$ G could be detected (Figure 3F). At higher methaemoglobin concentrations a second adduct with $a^N = 16.5$ G and a secondary nitrogen splitting of 2.4 G could be identified (Figure 6D). Comparison of these results with the spectra of a number of amino acids that gave tertiary carbon-centred radicals on hydrogen abstraction showed that the globin-centred radical is most probably located on a valine residue (Table 1). The spectra obtained with valine and methaemoglobin showed a strong resemblance to each other and the results indicate that two different adducts from valine were trapped (Figures 6C and 6D). The structures of these radicals are outlined in Scheme 1.

Spin trapping of species (A) by MNP will give a triplet signal, which can be assigned to the adduct with $a^N = 16.8$ G (Figure 7D). A MNP adduct of a valine radical with $a^N = 16.8$ G has also been detected in a study by Davies et al. [35], where γ -irradiated proteins and amino acids were exposed to iron in the presence of MNP. Trapping of species (B) with MNP will result in a triplet, with each line split into three by the nitrogen (Figure 7E). Exposure of metmyoglobin to *t*-BuOOH in the presence of MNP resulted in the detection of two triplets (Figure 5). One adduct had a hyperfine splitting constant of 16.8 G, as was found with methaemoglobin, which indicates that also in this case a radical located on a valine residue was trapped by MNP. The second triplet had a hyperfine splitting constant of 15.4 G, which was very similar to the triplet that could be detected after exposure of metmyoglobin to H_2O_2 (Figure 4). This adduct has recently been assigned to a tyrosyl radical; these results therefore strongly suggest that also with *t*-BuOOH a tyrosyl radical is formed in metmyoglobin [16,27].

What mechanism could be responsible for the formation of these protein radicals? Two modes of C–H activation can be observed when alkyl hydroperoxides are used as oxidants [36,37]. The first mechanism involves hydrogen abstraction by an alkoxyl radical, resulting from the homolytic cleavage [reaction (4)] of the hydroperoxide:

To investigate the involvement of alkoxyl radicals, the amino acids valine, isoleucine and leucine were exposed to alkoxyl radicals generated by methaemoglobin and BuOOH. This did not result in the formation of MNP adducts of these amino acids, which suggests that alkoxyl radicals were unable to abstract a hydrogen atom from these amino acids. Secondly, the hydrogen can also be abstracted by a metal-oxo species, resulting from

heterolytic cleavage [reaction (1)] of the O–O bond of the peroxide:

This would mean that heterolytic cleavage of the hydroperoxide by methaemoglobin and metmyoglobin results in the formation of protein-derived radicals, while concurrent homolytic cleavage of the peroxide results in peroxide-derived radicals. This concurrent heterolytic and homolytic cleavage would be in accordance with the product analysis study of Allentoff et al. [32]. Their data indicated that of the hydroperoxide that was degraded, 33 % was cleaved homolytically and 67 % was cleaved heterolytically.

The formation of protein-derived radicals by reaction (8) would be similar to the reaction of haem proteins with H_2O_2 [1–6]. It is therefore of interest that both with H_2O_2 and *t*-BuOOH a tyrosyl radical could be detected in metmyoglobin. Horse metmyoglobin contains two tyrosine residues, located at positions 103 and 146 [38]. It has been found that exposure of horse metmyoglobin to H_2O_2 resulted in covalent binding of Tyr-103 (G4) to the prosthetic haem group [14]. This suggests that the tyrosyl radical might be located on Tyr-103. This would be in agreement with the fact that exposure of methaemoglobin to *t*-BuOOH did not result in the detection of a tyrosyl radical: in human methaemoglobin the residue at position G4 is asparagine [38]. In both proteins a valine radical could be detected after exposure to *t*-BuOOH. A valine residue at position E11, which is packed against the haem ring, is present in haemoglobin [Val-62 (α -chain) or Val-67 (β -chain)] and in myoglobin (Val-68) [38]. This proximity might facilitate a reaction between reactive species generated during the reaction and a valine residue. However, further investigations are required to determine the exact location of these radicals.

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REFERENCES

- George, P. and Irvine, D. H. (1952) *Biochem. J.* **52**, 511–517
- George, P. and Irvine, D. H. (1955) *Biochem. J.* **60**, 596–604
- Gibson, J. F. and Ingram, D. J. E. (1956) *Nature (London)* **178**, 871–872
- Gibson, J. F., Ingram, D. J. E. and Nicholls, P. (1958) *Nature (London)* **181**, 1398–1399
- King, N. K. and Winfield, M. E. (1963) *J. Biol. Chem.* **238**, 1520–1528
- King, N. K., Looney, F. D. and Winfield, M. E. (1967) *Biochim. Biophys. Acta* **133**, 65–82
- Davies, M. J. (1991) *Biochim. Biophys. Acta* **1077**, 86–90
- Davies, M. J. and Puppo, A. (1992) *Biochem. J.* **281**, 197–201
- McArthur, K. M. and Davies, M. J. (1993) *Biochim. Biophys. Acta* **1202**, 173–181
- Kelman, D. J., DeGray, J. A. and Mason, R. P. (1994) *J. Biol. Chem.* **269**, 7458–7463
- Kelman, D. J. and Mason, R. P. (1992) *Free Radical Res. Commun.* **16**, 27–33
- Wilks, A. and Ortiz de Montellano, P. R. (1992) *J. Biol. Chem.* **267**, 8827–8833
- Rao, S. I., Wilks, A. and Ortiz de Montellano, P. R. (1993) *J. Biol. Chem.* **268**, 803–809
- Tew, D. and Ortiz de Montellano, P. R. (1988) *J. Biol. Chem.* **263**, 17880–17886
- Catalano, C. E., Choe, Y. S. and Ortiz de Montellano, P. R. (1989) *J. Biol. Chem.* **264**, 10534–10541
- Barr, D. P., Gunther, M. R., Deterding, L. J., Tomer, K. B. and Mason, R. P. (1996) *J. Biol. Chem.* **271**, 15498–15503
- Harada, K. and Yamazaki, I. (1987) *J. Biochem. (Tokyo)* **101**, 283–286
- Miki, H., Harada, K., Yamazaki, I., Tamura, M. and Watanabe, H. (1989) *Arch. Biochem. Biophys.* **275**, 354–362
- Barr, D. P. and Mason, R. P. (1995) *J. Biol. Chem.* **270**, 12709–12716
- Van der Zee, J., Barr, D. P. and Mason, R. P. (1996) *Free Radicals Biol. Med.* **20**, 199–206

-
- 21 Buettner, G. R. and Oberley, L. W. (1978) *Biochem. Biophys. Res. Commun.* **83**, 69–74
- 22 Buettner, G. R. (1990) *Free Radicals Res. Commun.* **10**, 11–15
- 23 Mason, R. P. (1984) *Methods Enzymol.* **105**, 416–422
- 24 Duling, D. R. (1994) *J. Magn. Res. Ser. B* **104**, 105–110
- 25 Chamulitrat, W., Takahashi, N. and Mason, R. P. (1989) *J. Biol. Chem.* **264**, 7889–7899
- 26 Rosen, G. M. and Rauckman, E. J. (1980) *Mol. Pharmacol.* **17**, 233–238
- 27 Gunther, M. R., Kelman, D. J., Corbett, J. T. and Mason, R. P. (1995) *J. Biol. Chem.* **270**, 16075–16081
- 28 Rustgi, S., Joshi, A., Moss, H. and Riesz, P. (1977) *Int. J. Radiat. Biol.* **31**, 415–440
- 29 Blumberg, W. E. (1981) *Methods Enzymol.* **76**, 312–329
- 30 Russell, G. A. (1957) *J. Am. Chem. Soc.* **79**, 3871–3877
- 31 Ingold, K. U. (1969) *Acc. Chem. Res.* **2**, 1–9
- 32 Allentoff, A. J., Bolton, J. L., Wilks, A., Thompson, J. A. and Ortiz de Montellano, P. R. (1992) *J. Am. Chem. Soc.* **114**, 9744–9749
- 33 Tappel, A. L. (1961) in *Autoxidation and Antioxidants*, vol. 1, (Lundberg, W. O., ed.), pp. 325–366, John Wiley, New York
- 34 Davies, M. J., Gilbert, B. C. and Haywood, R. M. (1991) *Free Radicals Res. Commun.* **15**, 111–127
- 35 Davies, M. J., Fu, S. L. and Dean, R. T. (1995) *Biochem. J.* **305**, 643–649
- 36 Mansuy, D., Bartoli, J.-F. and Momenteau, M. (1982) *Tetrahedron Lett.* **23**, 2781–2784
- 37 Meunier, B. (1992) *Chem. Rev.* **92**, 1411–1456
- 38 Dickerson, R. E. and Geis, I. (1983) in *Haemoglobin* (Dickerson, R. E. and Geis, I., eds.), pp. 26–60, Benjamin/Cummings, Menlo Park, CA
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