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Direct detection of a globin-derived radical in leghaemoglobin treated with peroxides

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The root nodules of leguminous plants contain an oxygen-carrying protein which is somewhat similar to myoglobin. Reaction of the Fe^{3+} form of this protein (metleghaemoglobin; MetLb) with H_2O_2 is known to generate a ferryl [iron(IV)-oxo] species. This intermediate, which is analogous to Compound II of peroxidases and ferryl myoglobin, is one oxidizing equivalent above the initial level. In the present study it is shown that the second oxidizing equivalent from the peroxide is rapidly transferred into the surrounding protein, generating a protein radical which has been detected by e.p.r. spectroscopy; this reaction is analogous to that observed with metmyoglobin. An identical protein-derived species is observed with all three forms of MetLb tested (a, c_1, c_3) and with a number of other hydroperoxides and two-electron oxidants. This latter result, the observation that the concentration of this species is not affected by certain hydroxyl-radical scavengers, and the loss of the radical when the oxy or deoxy forms are used, demonstrate that this species is formed by electron transfer within the protein rather than by the generation and subsequent reaction of hydroxyl radicals (and related species from the other hydroperoxides). The e.p.r. signal of this species, which decays rapidly with a half-life of approx. 40 s, is consistent with the formation of a sterically constrained tyrosine-derived phenoxyl radical; protein-iodination experiments lend support to this assignment. Reaction between the radical and a number of other compounds has been observed, demonstrating that it is at least partially exposed on the surface of the protein. Analysis of the protein structure suggest that the radical may be centred on a tyrosine residue present at position 132 in the protein; this residue is close to the haem prosthetic group, which would facilitate rapid electron transfer.

INTRODUCTION

Leghaemoglobins (Lb), a closely related family of monomeric proteins of M_r approx. 16000, are found in the active nitrogen-fixing root nodules of legumes [1–3]. Their major function *in vivo* appears to be the transport of oxygen to the bacteroids [4]. As with the other oxygen-carrying haemoproteins, the prosthetic group is protohaem (iron protoporphyrin IX) [5], and this exists mainly in reduced (Fe^{2+}) form [6]. This form has an exceptionally high oxygen affinity, although owing to the low [O_2] *in vivo*, only about 20% is oxygenated under steady-state conditions to give oxyleghaemoglobin (OxyLb) [3]. OxyLb can undergo a slow autoxidation to the Fe^{3+} form (MetLb) with the concomitant production of superoxide radical ($O_2^{-\cdot}$) (and hence H_2O_2 by disproportionation; [7]).

A considerable number of structural studies have been carried out on these proteins, and it has been shown that there is extensive identity with the sequence and structures of mammalian haem proteins and, in particular, myoglobins [2,8–10]. A number of workers have also demonstrated that there are also certain similarities between leghaemoglobins and myoglobins in their reactions with H_2O_2 and related compounds [11–15]. Thus both types of protein, when in the reduced (Fe^{2+}) form, react with H_2O_2 to give a high-oxidation-state species where the formal oxidation state is 4+; this intermediate is often called the 'ferryl' form. A similar species can also be generated from the Fe^{3+} state with identical reagents.

In the case of metmyoglobin (MetMb) it has been demonstrated that this ferryl species is one oxidizing equivalent above the Fe^{3+} state [16,17] and that the second oxidizing equivalent is rapidly transferred into the surrounding globin, generating a protein radical [17–19]. In equine MetMb this species has been demonstrated to be a tyrosine phenoxyl radical present at position 103 in the protein [20]. Subsequent reaction of this radical with oxygen is believed to generate a peroxyl radical [20,21], and recent studies have demonstrated that this latter species (but not the phenoxyl radical) can initiate membrane damage (E. S. R. Newman, C. A. Rice-Evans & M. J. Davies, unpublished work). Considerably less work has been carried out on the possibility of analogous processes with leghaemoglobins, though it is known that the species formed on reaction of Lb with H_2O_2 has pseudoperoxidatic activity [12,13] (a method commonly used to locate Lb in root nodules; [22,23]), can oxidize dimethyl sulphoxide (though not through the generation of 'free' hydroxyl radicals; [15]) and reacts (through some unknown pathway) with the protein, resulting in its inactivation [13]. In the present study it is shown that, as with MetMb, a globin radical is generated on reaction with H_2O_2 and related compounds. This species has been identified and some of its reactions investigated.

EXPERIMENTAL

Soybeans were grown in a glasshouse and the leghaemoglobins (types a, c_1 and c_3) purified from the root nodules as described previously [24,25]. MetLb was prepared by oxidation with ferricyanide, followed by chromatography on a Sephadex G-15 column. OxyLb was prepared by dithionite reduction, gassing with O_2 and separation on an identical column. Ferrous Lb was prepared as described previously [11]. All other chemicals were commercial samples of high purity and used as supplied with the exception of the spin trap, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), which was purified as previously [26]. The e.p.r. experiments were carried out as described in [20,21].

Abbreviations used: Lb, leghaemoglobin; MetLb, metleghaemoglobin; OxyLb, oxyleghaemoglobin; $O_2^{-\cdot}$, superoxide radical; MetMb, metmyoglobin; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; $OH^{\cdot}$, hydroxy radical; DMPOX, 5,5-dimethyl-2-oxopyrrolidino-1-oxyl radical; ^tBuOOH, t-butyl hydroperoxide; $^tBuOO^{\cdot}$, t-butyl peroxyl radical; MetHb, methaemoglobin.

RESULTS

Stopped-flow experiments

Rapid mixing of air-saturated solutions of $MetLb_a$ (250 μM in 50 mM-phosphate buffer, pH 7.4) with H_2O_2 (250 μM) using a stopped-flow system within the cavity of a Bruker e.p.r. spectrometer led to the immediate observation of a broad multiplet e.p.r. signal (Fig. 1). Omission of either reagent resulted in the loss of this species. Rapid scanning of the magnetic field was necessary for the observation of this signal, as it decays rapidly. In experiments where the magnetic field was set to that of the second, low-field, peak of the multiplet signal, and the intensity of this peak (which is directly proportional to the radical concentration) recorded with time after mixing, a half-life of approx. 40 s was obtained for this species (Fig. 2). Repetition of these experiments with $MetLb_{c1}$ or $MetLb_{c3}$ in place of $MetLb_a$, gave identical results, whereas use of either the oxy (Fe^{2+}-O_2) or deoxy (Fe^{2+}) forms gave only very weak signals.

Altering the concentration of H_2O_2 to 500 μM (i.e. peroxide/haem ratio 2:1) resulted in an increase in the intensity of the radical signal (as measured by peak-to-trough line heights); further increases, however, resulted in a decrease in the radical concentration (Fig. 3), and with very high ratios (peroxide/haem > 50) this signal was almost completely lost. These results suggest that this radical species undergoes a further reaction with excess H_2O_2. Similar behaviour was observed with a large number of other hydroperoxides and powerful oxidants. Thus a signal identical with that detected with H_2O_2 was obtained with t-butyl, cumene or ethyl hydroperoxides, iodosylbenzene or peracetic acid [$CH_3C(O)OOH$] when these were used at an oxidant/haem ratio of 2:1. With the first three of these substrates increasing the hydroperoxide concentration to give a peroxide/haem ratio of 20:1 resulted in the complete loss of this signal. This occurs at lower peroxide/haem ratios than with H_2O_2, and it is again indicative of a further reaction between the observed radical and excess oxidant. With t-butyl hydroperoxide at these high ratios, a further weak signal was observed at lower magnetic-field values (g 2.014).

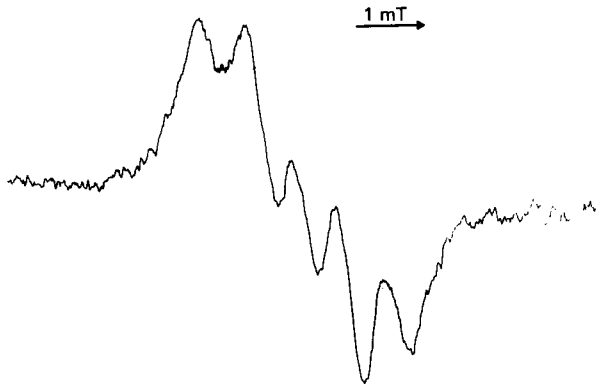


Fig. 1. E.p.r. spectrum observed immediately after mixing of $MetLb_a$ (250 μM) with H_2O_2 (250 μM) at pH 7.4

The reaction was studied by using a two-way stopped-flow system inserted into the cavity of the e.p.r. spectrometer. The signal is assigned to a sterically constrained tyrosine phenoxyl radical (probably at position 132 in the protein) with a_H 1.99, a_{2H} 0.70 mT and g 2.0044.

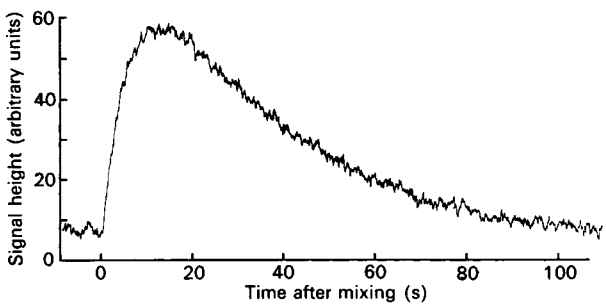


Fig. 2. Build-up and decay of the signal assigned to the tyrosine phenoxyl radical

The reaction system was as in Fig. 1.

Substitution of OxyLb for MetLb in experiments where the oxidant/haem ratio was 2:1 resulted in a dramatic decrease in the intensity of the multiplet signal in all cases, though this was less pronounced with iodosylbenzene than with the other oxidants. With iodosylbenzene the signal was of sufficient intensity to allow its build-up and decay to be monitored; both of these occur at lower rates than with the Met derivative.

In an effort to obtain more information as to the nature of this species and its mechanism of formation, the effects of a number of potential inhibitors and scavengers were studied. Pretreatment of MetLb (125 μM) with either KCN (2.5 mM), NaN_3 (2.5 mM) or nicotinic acid (2.5 mM), before reaction with H_2O_2 (250 μM) resulted in the complete loss of the multiplet signal; lower concentrations of nicotinic acid (500 μM) gave only partial inhibition (32%). The results obtained with these ligands, which are all known to bind strongly to the haem iron atom [3,27,28], demonstrate that the radical species arises via a reaction occurring at the haem prosthetic group.

Inclusion of 2-deoxyribose (6.6 mM) in a similar reaction system did not have any significant effect on the intensity of the radical signal, whereas addition of 8.4 mM-salicylate, 500 μM-desferal (desferrioxamine), 500 μM-ascorbic acid, 5 mM-Trolox C, 1.25 mM-cysteine, or 10 mM-thiourea resulted in the complete loss of this species. In some of these systems other radical species derived from the added compound were observed. Thus with desferal the desferal nitroxide radical was detected, with ascorbic acid the ascorbyl radical and, from Trolox C the Trolox C phenoxyl radical; each of these species had e.p.r. parameters identical with those determined previously [29,30]. The addition of 1.25 mM-GSH produced only partial inhibition (74%), suggesting that the size of the reducing agent may be an important factor in determining access to the site of the radical.

In order to obtain further information as to the identity of this radical species and, in particular, to determine whether, like the myoglobins, this species is a tyrosine phenoxyl radical, studies have been carried out with $MetLb_a$ chemically modified with KI_3; such a process results in the iodination of the tyrosine residues. Use of this iodinated $MetLb_a$ in place of untreated $MetLb_a$ results in the complete loss of the multiplet signal. This is consistent with the radical species being a tyrosine phenoxyl radical, as it has been previously demonstrated that the incorporation of iodine substituents into the phenol ring results in the loss of the e.p.r. signal, owing to the severe line-broadening induced by the interaction of the unpaired electron with the iodine atoms [20,31]. It does not, however, provide any information as to which one of the three tyrosine residues present in Lb_a [10,32] it is derived from.

In the light of these observations it is possible to suggest an analysis for the observed multiplet signal. This signal is believed to consist of a large doublet splitting from a single proton of approx. 1.99 mT, a smaller triplet splitting from two protons of approx. 0.70 mT and has a g-value of 2.0044. This splitting

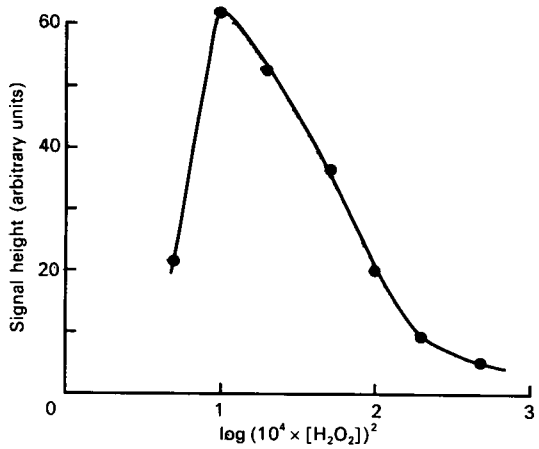


Fig. 3. Dependence of the signal intensity of the tyrosine phenoxyl radical on the concentration of H_2O_2 (with [$MetLb_a$] 500 μM)

pattern, which is what would be expected for a phenoxyl radical, is, however, significantly distorted by two factors: firstly the rapid decay of the signal as it is being recorded and, secondly, the fact that the signal is markedly anisotropic, which results in the broadening of the higher field lines; both of these factors result in the pattern of line intensities not being in the expected proportion 1:2:1:1:2:1. This anisotropy is believed to be due to the restricted motion of the radical species and a low rate of tumbling of the molecule, rather than changes in the solvent viscosity.

Spin-trapping experiments

Further information about the radical species generated during the reaction of MetLb with H_2O_2 and related peroxides has been obtained from experiments carried out in the presence of the spin trap DMPO. In particular it was of interest to determine whether further radical species are generated from the peroxides or the protein which might not be observed in the direct stopped-flow experiments owing to their reactivity and hence very short lifetimes. This particular trap was chosen for these studies as it is known that it will give observable adducts (i.e. the adducts have lifetimes greater than the time required to record their spectra) with a wide variety of different radicals, including species such as hydroxyl radical (HO$^\cdot$), superoxide radical ($O_2^{-\cdot}$), $RO_2^\cdot$, RO$^\cdot$, and R$^\cdot$, which might be formed in these reactions, and that the spectra of these various adducts are easily distinguished from each other (i.e. they have distinctive, and in most cases unique, hyperfine coupling constants [33,34]).

Reaction of $MetLb_a$ (225 μM) with equimolar H_2O_2 in the presence of 25 mM-DMPO in air-saturated 50 mM-phosphate buffer, pH 7.4, resulted in the observation, immediately after mixing, of an e.p.r. signal. This signal, which was only observed when all components of the reaction mixture were present, is characterized by a low a_N value (0.72 mT) and a further triplet splitting from *two* hydrogens atoms (a_{2H} 0.41 mT) and is assigned to the well-known oxidation product of the spin trap, the 5,5'-dimethyl-2-oxopyrrolidino-1-oxyl radical (DMPOX) [34]; identical signals were observed with $MetLb_{c1}$ and $MetLb_{c3}$. No additional species were observed. Repetition of these experiments with MetLb concentrations in the range 10–225 μM and H_2O_2 concentrations in the range 225 μM–10 mM gave only this species; the intensity (concentration) of this oxidation product did, however, decrease as the peroxide/haem ratio was raised above 1:1.

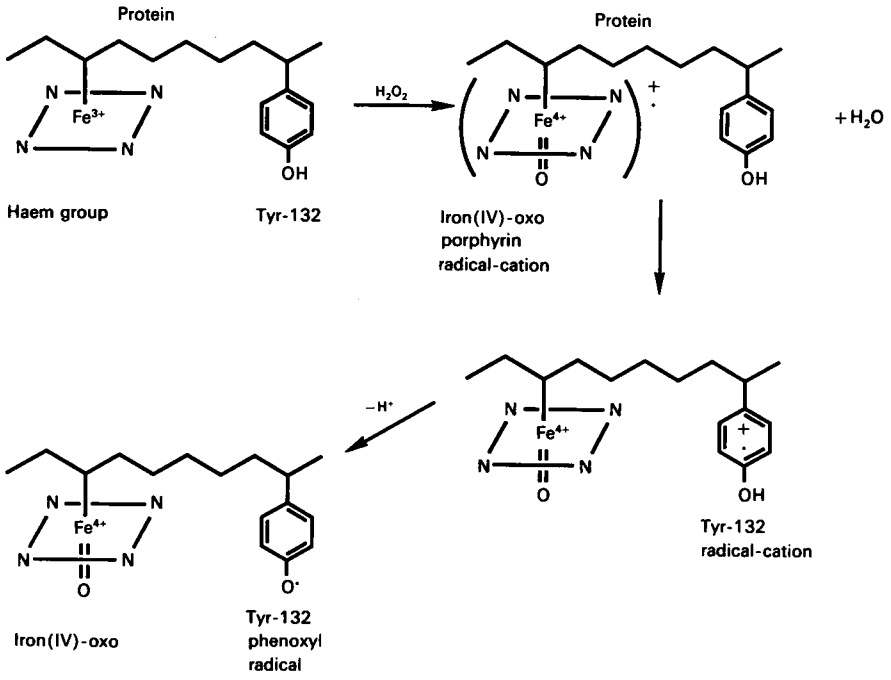
Addition of the various organic compounds used in the stopped-flow experiments (see above) led in some cases to alterations in the observed signals. Thus addition of 2-deoxyribose (6.6 mM) to a standard 250 μM-$MetLb_a$, 500 μM-H_2O_2, 25 mM-DMPO system, did not give any other signals apart from DMPOX, whereas inclusion of 5 mM-Trolox C gave only a strong sigal from the corresponding Trolox C phenoxyl radical, 500 μM-desferal gave only the desferal nitroxide radical, 500 μM-ascorbic acid gave only the ascorbyl radical, and inclusion of 1.25 μM-cysteine or GSH resulted in the loss of the DMPOX signal and the detection of the corresponding thiyl radical adducts to the spin trap (with parameters identical with these determined previously [35]). In none of these experiments were any signals which might be assigned to either a peroxide-derived species (such as HO$^\cdot$ or $O_2^{-\cdot}$), or a protein-derived radical, observed.

In contrast, when t-butyl hydroperoxide (^{t}BuOOH) was used in place of H_2O_2 in the standard system described above, weak signals from peroxide-derived radicals were observed, though only at very large excesses of peroxide. Thus when ^{t}BuOOH was used at a 1:1 ratio with the haem protein, a strong DMPOX signal was detected, but as the peroxide/haem ratio was increased the intensity of this species decreased, and at concentrations of ^{t}BuOOH greater than approx. 10 mM further signals, which can be assigned to the t-butyl peroxyl radical (^{t}BuOO$^\cdot$) adduct to the spin trap (with parameters identical with those determined previously [33,36]), were observed and the DMPOX signal was very weak. No protein-derived species were detected under any of these conditions.

DISCUSSION

The relatively broad anisotropic e.p.r. signal observed on reaction of $MetLb_a$ with H_2O_2 in the absence of other reagents is consistent with the generation of a novel radical species situated somewhere in the surrounding globin. Similar signals are observed with both $MetLb_{c1}$ and $MetLb_{c3}$, suggesting that all three haem proteins react in an identical manner and that the same radical species is produced in each system; it must therefore arise from a conserved amino acid. Replacement of the met proteins with the oxy forms results in a dramatic decrease in the intensity (and hence concentration) of this species, suggesting that this species is formed via the initial generation of a high-oxidation-state species which is two oxidizing equivalents above the Fe^{3+} state rather than via a further reaction of the iron(IV)-oxo species which is known to be formed on reaction of the oxy or deoxy forms with H_2O_2 [11]. Similar behaviour is observed with equine Mb [20]. These observations suggest that the radical is generated by electron transfer from the globin to an initially generated iron(IV)-oxo porphyrin radical-cation intermediate, resulting in the formation of an iron(IV)-oxo species and a protein radical (see Scheme 1). Previous studies have demonstrated the generation of the iron (IV)-oxo species by optical spectroscopy [11,12], but the latter protein radical species has not been detected until now. The low rate of formation of the protein radical with the oxy form is believed to arise via initial formation of the met form (a process observed in previous work [7]) and subsequent reaction of that form with excess peroxide via the above pathway.

The e.p.r. parameters of the observed signal, its lifetime, and the fact that this species is not observed with the iodinated protein, are all consistent with the radical being a tyrosine phenoxyl radical. The e.p.r. parameters of this species determined in these experiments (a_H 1.99, a_{2H} 0.70 mT, g 2.0044) are similar to those previously determined for the tyrosine phenoxyl radical in equine MetMb (a_H 2.26, a_{2H} 0.75 mT, g 2.0044; [20]), and similar large doublet splittings have been determined for the tyrosine-derived radicals in both ribonucleotide reductase



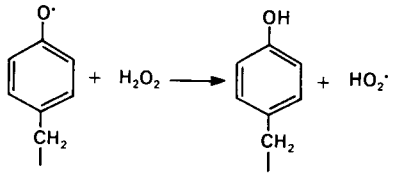
Scheme 1. Proposed reaction pathway for the formation of tyrosine phenoxyl radical on reaction of MetLb with H_2O_2

(1.88 mT; [37]) and Photosystem II (2.72 mT; [38]). The large doublet splitting is assigned to the interaction of the unpaired electron with *one* of the $-CH_2-$ protons of the side chain, and the smaller triplet splitting to interaction with the two *ortho* hydrogen atoms on the ring. As with the MetMb species, the splittings from the two *meta* ring protons (expected to be of approx. 0.15 mT; [39]) and the other $-CH_2-$ proton on the side chain (calculated, as previously [20], as being approx. 0.03 mT by use of a standard $a_\beta = B\cos^2\theta$ relationship) are less than the observed linewidth and hence unresolved. This analysis suggests that the protein structure constrains the phenolic ring very dramatically, and this is borne out by the anisotropic nature of the e.p.r. signal, which is consistent with both slow rotational motion of the phenoxyl radical and slow tumbling of the haem protein.

All three of the soybean Lbs used in the present study contain three tyrosine residues at positions 25, 30 and 132 (using the numbering system in [32]; the last of these residues is numbered 133 in [10]). The first two of these are present in the B-helix, with residue 30 being on the edge of the haem pocket. This latter residue is thought to be in contact with any large charged ligands present in the sixth co-ordination site; thus it is thought that it might hydrogen-bond to the carboxylate group of nicotinic acid in the $MetLb_a$–nicotinic acid complex [10]. The third tyrosine residue (132) is present in the H-helix; this is in close contact with the edge of the haem ring and, in particular, the vinyl group of pyrrole ring 1 [10]. The short distance between this residue and the haem would suggest that it is the most likely site for the phenoxyl radical, though it is impossible to exclude the possibility that the radical is centred on residue 30, which is also reasonably close to the haem and hence to the oxygen of the iron(IV)-oxo species. Further studies are needed to clarify this point and, in particular, examination of similar reactions with other Lbs such as that of lupin (which has Tyr-132 conserved, but lacks that at position 30, which is a phenylalanine in this form; [10]), may provide valuable information.

The results obtained with the various added organic compounds provide further information as to the mechanism of the reaction of the haem proteins with peroxides and the reactivity of the intermediate species. The lack of any observable reaction of 2-deoxyribose with the phenoxyl radical and absence of any $HO^\bullet$ or 2-deoxyribose-derived radical adducts in the spin-trapping experiments confirms that hydroxyl radicals are not being generated in this system, and that no reaction occurs between any of the postulated intermediates and 2-deoxyribose (which would lead to 2-deoxyribose-derived radicals and sugar degradation; the latter process has, however, been previously observed at high peroxide/haem ratios, where haem degradation and iron release occurs [40]). However, reaction has been detected between the phenoxyl radical and salicylate, thiourea, Trolox C, desferal, ascorbic acid, cysteine and glutathione; these results suggest that the phenoxyl radical is reasonably accessible to species in the bulk solution and is not completely buried within the protein, though the observation that glutathione is less inhibitory than cysteine at identical concentrations suggests that access to the phenoxyl radical is constrained to a certain extent. Previous studies have shown that reaction of H_2O_2 with $MetLb_a$ or $MetLb_c$ results in the inactivation of the protein [13] whereas generation of the iron(IV)-oxo species from the Fe^{2+} state (i.e. without the formation of the globin radical) does not give the oxygenated protein [11]; this information is consistent with the phenoxyl species playing an important role in inactivation of the protein, possibly via a haem to protein cross-link. A haem-to-tyrosine cross-link has been observed in Mb treated with H_2O_2 [41], and if the radical is indeed situated at tyrosine-132 in the MetLb proteins, a similar event may occur. In this context it would be expected that the above compounds, which react with the phenoxyl radical, might protect against inactivation and degradation; previous reports have shown that ascorbic acid can slow down the destruction of Lbs in root nodules [42] and that salicylate, thiourea or desferal can inhibit haem degradation [40], suggesting that such reactions may be of importance. The radical also appears to undergo further reaction with excess H_2O_2, as the intensity of this signal decreases with increasing H_2O_2 concentration (see Fig. 3); similar behaviour has been

observed with the phenoxyl radical from MetMb [20], and this behaviour is likewise assigned to reaction (1):



(1)

Such a reaction must, however, be relatively slow, as no evidence was obtained in the spin-trapping experiments for the production of $HO_2^{\cdot}/O_2^{\cdot-}$; this is not, however, altogether surprising, as the rates of trapping of these radicals by DMPO is also known to be very slow [43]. Similar behaviour is observed with other hydroperoxides, though these analogous reactions appear to be somewhat faster as the phenoxyl-radical signal disappeared at lower peroxide/haem ratios. With 'BuOOH, weak signals from peroxyl and alkoxyl radical adducts (believed to arise from 'BuOO· and 'BuO· respectively) were observed in the spin-trapping experiments, and weak signals from what is believed to be 'BuOO· (with g 2.014) in direct experiments at very high 'BuOOH/haem ratios, providing support for the above reaction. The 'BuO· adducts observed in the spin-trapping experiments are thought to arise via a secondary reaction as a result of decomposition of the peroxyl adduct; this is in accord with previous observations [33].

The behaviour of these haem proteins in the presence of high concentrations of alkyl hydroperoxides (e.g. 'BuOOH and cumene hydroperoxide) is somewhat different from that observed with MetMb and methaemoglobin (MetHb), where high concentrations of peroxyl radicals are observed in both direct and spin-trapping experiments [33,44]; this radical generation has been suggested to arise via a further reaction of the iron(IV)-oxo species with excess peroxide (reaction 2):

$$Fe^{4+}{=}O + {}^{t}BuOOH \rightarrow Fe^{3+} + HO^{-} + {}^{t}BuOO^{\cdot} \qquad (2)$$

though a minor contribution from reactions analogous to (1) cannot be ruled out.

This change in behaviour is in agreement with previous optical studies on the reactivity of the iron(IV)-oxo species in Lbs; this species is known to be very stable, with little change being observed over a period of 12 h at room temperature [11]. The reason for this change is not clear at the present moment, though it appears as if this Lb-derived species is not completely unreactive, since it has been previously suggested [45] that the production of DMPOX from DMPO (a process which appears to occur reasonably rapidly) is a reaction of the Fe^{4+}-oxo species. It would appear from these results that this reaction competes with reaction with excess peroxide and that the relative rates of these two processes in leghaemoglobins and in MetMb or MetHb are different.

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