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EPR STUDIES ON THE EFFECTS OF COMPLEXATION OF HEME BY HEMOPEXIN UPON ITS REACTIONS WITH ORGANIC PEROXIDES

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Hemopexin, a heme-binding serum glycoprotein, is thought to play an important role in the prevention of oxidative damage that may be catalysed by free heme. Through the use of EPR techniques, the generation of free radicals from organic hydroperoxides by heme and heme-hemopexin complexes, and the concomitant formation of high oxidation-state iron species has been studied; these species are implicated as causative agents in processes such as cardiovascular disease and carcinogenesis. From the rates of production of these species from both n-alkyl and branched hydroperoxides, it has been inferred that the dramatic reduction in the yield of oxidising species generated by heme upon its complexation with hemopexin arises from steric hindrance of the access of hydroperoxide to the bound heme.

KEY WORDS: Antioxidant, EPR, Free Radical, Heme, Hemopexin, Peroxide.

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INTRODUCTION

Free heme [iron(III) protoporphyrin(IX)], or heme bound to some proteins such as hemoglobin, leghemoglobin or myoglobin, is known to react with hydroperoxides via the reactions shown in Scheme 1; a range of hydroperoxide species may be present *in vivo* as a result of normal metabolism, via accidental or deliberate exposure (e.g. industrial uses or acne treatments) or as a result of oxidative stress forming lipid hydroperoxides.[1,2] In the first step of this process two-electron reduction of the hydroperoxide occurs resulting in the formation of the corresponding alcohol and a high-oxidation-state heme species consisting of an iron(IV)-oxo (ferryl) species and a porphyrin radical cation; the formation of these species is then followed by two sequential one-electron oxidations of two hydroperoxide molecules to generate two peroxyl radicals, with concomitant reduction of firstly the porphyrin radical cation and then the iron(IV)-oxo species to regenerate heme.[3–6] Both peroxyl radicals and high oxidation-state iron species are known to react with a range of biological substrates and have been implicated as causative agents in a range of deleterious biological processes, such as cardiovascular diseases[1,7] and carcinogenesis.[2,8,9]

Hemopexin (Hx) is a serum glycoprotein present in mammals, birds and fish at concentrations between 0.4 and 1.5 mg/ml.[10] Hx binds heme in an equimolar complex with a dissociation constant (K_d) of $<10^{-12}$ M;[10] it is also known to bind (metal-free)

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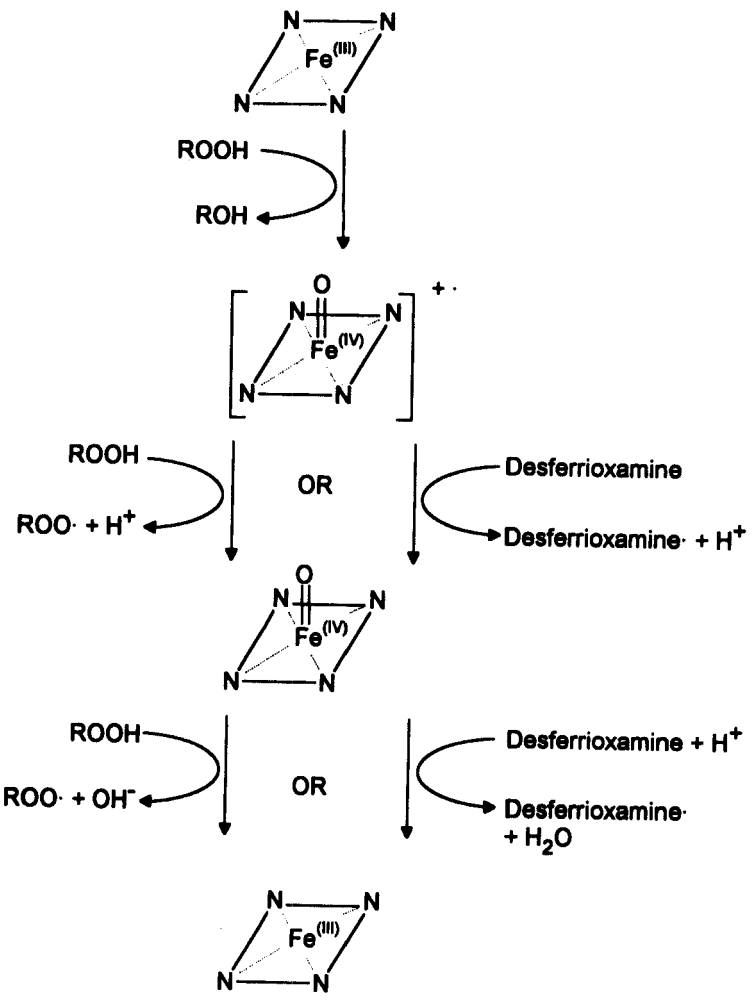

REACTION SCHEME 1 The reaction mechanism by which high oxidation-state heme and peroxyl radical species may be formed by the reactions of heme with peroxides.

porphyrins with dissociation constants of approximately 10^{-6} M.[10] In addition to its function as a heme transporting system, Hx is thought to play a key role in preventing heme-catalysed oxidative damage, though it is uncertain whether this results from *inhibition* of radical-generation or the rapid *scavenging* of radical species (formed by the reaction of heme with peroxides within the binding pocket) by the surrounding Hx protein.[11,12]

In this study, the generation of peroxyl radicals and high oxidation-state-heme species on reaction of free and Hx-bound heme with ^{n}butyl, ^{t}butyl and cumene hydroperoxides (structures in Figure 1) have been studied by EPR. The formation of peroxyl radicals has been qualitatively and semi-quantitatively measured by spin-trapping with 5,5-dimethyl-1-pyrroline-N-oxide (DMPO)[13] while the formation of

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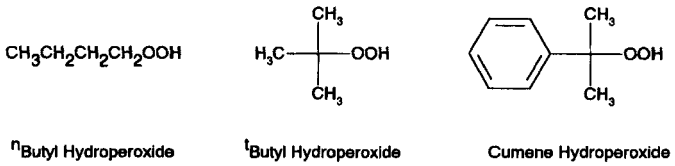

FIGURE 1 The structures of the peroxides used in this study.

high-oxidation state iron species has been determined by examination of the yield of desferrioxamine (Desferal) radicals (Des·, which can be directly determined by EPR) produced by reaction of the high-oxidation-state heme with desferrioxamine (Scheme 1).[14–17] Both DMPO-peroxyl radical adducts (DMPO-OOR·) and desferrioxamine radicals are relatively stable, allowing their characterization and quantification.[13–17]

MATERIALS AND METHODS

Cumene and ^{t}butyl hydroperoxides (cumeneOOH and ^{t}BuOOH respectively), ^{n}butyl magnesium chloride (2.0 M solution in ether), desferrioxamine (Desferal, deferrioxamine), DMPO and buffer salts were of high purity and obtained from Sigma Chemical Company. Chelex-100 resin was obtained from BioRad Ltd. All other chemicals were of high purity and obtained from BDH Ltd. Hemopexin was prepared from rat serum by the heme-agarose method;[10] its purity was >95% as judged by denaturing polyacrylamide electrophoresis (SDS-PAGE) and immunological assessment using antibodies to albumin and transferrin (the most common contaminants); it also exhibited typical absorption spectral characteristics when complexed to heme.[10] All solutions were made up in phosphate-buffered saline (0.15 M NaCl, 0.01 M sodium phosphate pH 7), treated with Chelex-100 to remove adventitious transition metal ions; DMPO was purified before use by treatment with activated charcoal. All concentrations referred to in the text are final concentrations in the reaction mixtures.

^{n}Butyl hydroperoxide (^{n}BuOOH) was synthesized from ^{n}butyl magnesium chloride by the method of Walling and Butler.[18] Purification of ^{n}BuOOH was achieved by extracting the peroxide-containing ether with one vol. of 0.1 M sodium hydroxide, followed by addition of sodium phosphate (10 mM) to the aqueous fraction and neutralization with hydrochloric acid. The aqueous layer was then extracted three times with 1 vol. of ethoxyethane; the combined organic fractions were then added to 10 mls water and the ether removed by evaporation with a stream of oxygen-free nitrogen. The resulting aqueous peroxide solution was stored in aliquots at −20°C in the dark. The ^{n}BuOOH concentration was determined by iodometric titration with thiosulphate.[19]

Reactions were carried out in microcentrifuge tubes maintained at 20°C; heme (2.5 μM), preincubated with Hx (3 μM) for 5 minutes when heme-Hx complexes were to be studied, was added to a solution of DMPO (20 mM) or Desferrioxamine (1 mM), and the reaction initiated by addition of hydroperoxide (1 mM). EPR spectra were then recorded in an aqueous sample cell using a Bruker ESP 300 spectrometer with 100 kHz modulation. All determinations are the average of three separate experiments; no radical generation was detectable under the conditions used in the absence of added heme.

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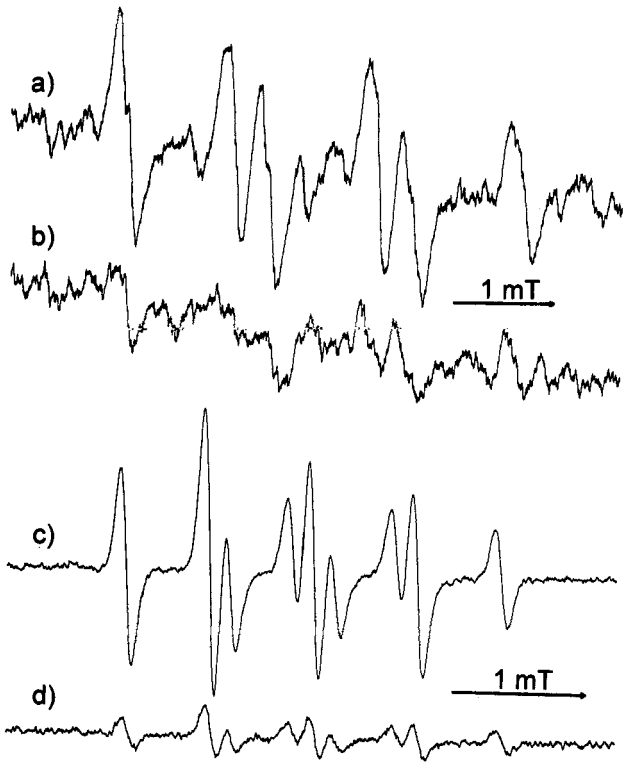

FIGURE 2 EPR Spectra of radical species obtained upon the reaction of heme (2.5 μM) with ^{t}butyl hydroperoxide (1 mM) in the presence of a) and b) DMPO (20 mM); c) and d) desferrioxamine (1 mM). Spectra a) and c) relate to the reactions of free heme; spectra b) and d) relate to the reactions of heme pre-complexed to hemopexin (3 μM for 10 minutes). Spectra were recorded at 20°C in air saturated conditions. Spectrometer settings for each pair of spectra were identical, i.e. those for a) = b) and c) = d). Signals in a) and b) assigned to the peroxyl radical adduct to DMPO; c) and d) assigned to the desferrioxamine nitroxide radical.

RESULTS AND DISCUSSION

Upon the reaction of heme with all of the peroxides studied in the presence of DMPO, isotropic EPR spectra, indicative of peroxyl radical adducts to DMPO in free solution were obtained (with hyperfine coupling constants a_N 1.46 mT, a_H 1.10, a_H 0.12 mT, as reported previously)[13]; the spectra obtained with ^{t}BuOOH, which are representative of all the peroxides, are shown in Figure 2a. Similarly in the presence of desferrioxamine, isotropic spectra indicative of the desferrioxamine nitroxide radical (a_N 0.785 and a_{2H} 0.635 mT, as reported previously)[15] were observed, Figure 2c. After preincubation of heme with a 1.2-fold molar excess of Hx, which results in complete complexation of the heme by Hx, there was a substantial reduction in yield of both the DMPO-OOR· and Des· species observed, Figures 2b and 2d.

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Kinetics of Peroxyl Radical Adduct Generation and Decay

In order to investigate the kinetics of generation of these species, the low-field lines of the DMPO-OOR· and Des· spectra were repeatedly scanned during the reaction of the heme and heme-Hx complexes with the three hydroperoxides. The intensity (height) of the observed absorption lines is directly proportional to the concentration of radicals present, and may therefore be used to determine the relative rates of formation and decay of the generated radical species. Figures 3a-d) show data for DMPO-OOR· and Des· generation from each of the three hydroperoxides catalysed by both free and Hx-bound heme. To aid comparison between these different substrates, this data has been normalised as a ratio of signal intensity at time (t) to the maximum observed signal intensity for that reaction.

The build-up and decay of the DMPO-OOR· signals observed in the cases of cumeneOOH and ^{t}BuOOH with free heme are shown in Figure 3a; the rate of formation of the peroxyl radical adducts being slightly slower for cumeneOOH than ^{t}BuOOH. In the case of ^{n}BuOOH however the maximum signal intensity was obtained within the period of time (*ca.* 45 seconds) required to fill the sample cell and tune the spectrometer,

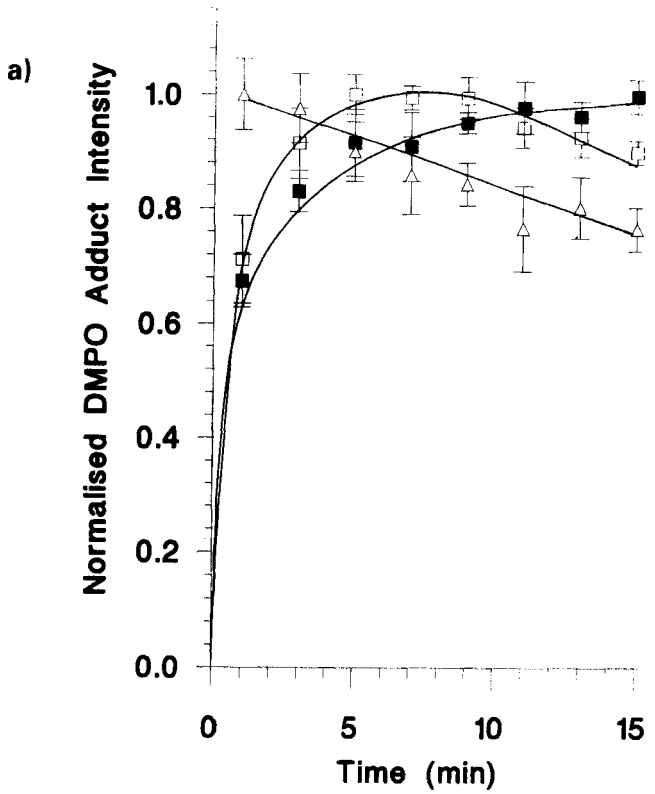

FIGURE 3 The rates of production and decay of a) and b) DMPO-peroxyl radical adducts; c) and d) desferrioxamine radical species produced from: ^{n}BuOOH (△), ^{t}BuOOH (□) and cumeneOOH (■), conditions as in text. Figures a) and c) relate to the reactions of free heme; b) and d) relate to the reactions of heme pre-complexed to hemopexin. Data is presented normalised as a ratio of the maximum signal intensity for that reaction; data shown ±1 standard deviation.

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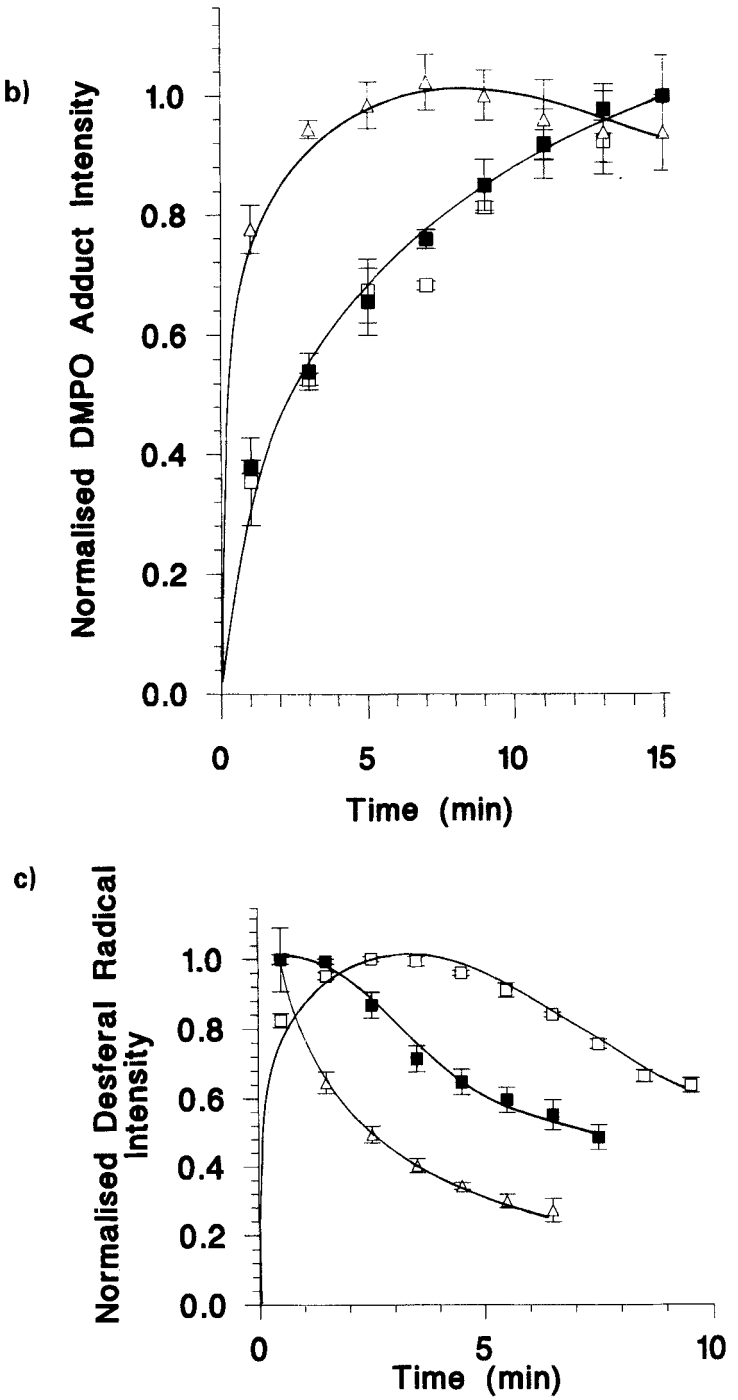

FIGURE 3 *continued*

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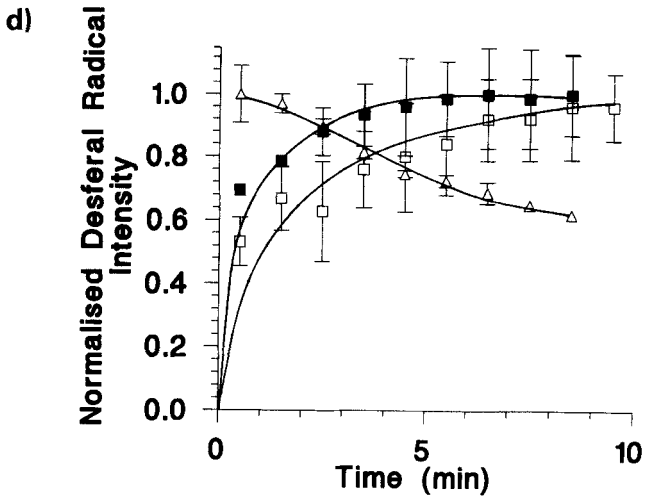

FIGURE 3 *continued*

so only the decay of the adduct species could be observed; this implies that the rate of DMPO-OOR· generation in this case is much faster than that occurring with either ^{t}BuOOH or cumeneOOH. In contrast, when the peroxides were reacted with Hx-heme complexes (Figure 3b), the rate of formation of DMPO-OOR· was, in each case, very much slower than that observed with free heme and it was therefore possible to examine the rate of formation (build-up) of DMPO-OOR· with each hydroperoxide; the rate of DMPO-OOR· formation from ^{n}BuOOH was again much more rapid than that from ^{t}BuOOH or cumeneOOH. There was a small difference between the rates of generation from ^{t}BuOOH and cumeneOOH, with the latter being faster ($p < 0.1$).

Kinetics of Desferrioxamine Radical Generation and Decay

The rate of Des· production in the case of all the peroxides with free heme was faster than the corresponding rate of DMPO-OOR· production. In the case of ^{t}BuOOH both the rate of generation and subsequent decay could be determined whereas with the other two peroxides only the decay of Des· could be observed as the maximum radical concentration was again generated during preparation of the sample and tuning of the spectrometer (Figure 3c). Upon complexation of heme with Hx, the rate of Des· production could be monitored for both ^{t}BuOOH and cumeneOOH as this was again significantly slower than in the free heme case, though with cumeneOOH being the faster. With ^{n}BuOOH only the decay of the Des· could be monitored; this occurs at a reduced rate compared to the free heme system and as the loss of this radical is likely to be via a *bimolecular* disproportionation reaction, a decrease in its rate of formation is also inferred (Figure 3d).

It can be seen that there is a significant decrease in the rates of production of both DMPO-OOR· and Des· generated from all the peroxides upon the complexation of heme by Hx. This implies that there is a corresponding decrease in the rate of generation of both the high-oxidation-state-iron species and peroxyl radicals, since the access of DMPO to peroxyl radicals and desferrioxamine to the high oxidation-state iron species in the presence of Hx is unlikely to be affected by the different peroxides. Thus it would

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seem likely that the reaction(s) of peroxide with the complexed heme are the rate-limiting step(s); that the least sterically hindered peroxide functional group (^{n}BuOOH) was also the fastest reacting (with both free and Hx-complexed heme) supports this proposition.

Further support comes from the rates of desferrioxamine radical production being faster than those of DMPO-OOR· from each peroxide, since only the first peroxide-heme reaction in Scheme 1 is necessary for the production of two Des·, while both this first and two further peroxide-heme reactions are required for the production of two peroxyl radicals. Given that peroxyl radicals react with DMPO at rate constants between 10^5 to 10^6 M^{-1} sec^{-1},[20] (*much* faster than the observed rate of DMPO-OOR· formation), the slow rate of DMPO-OOR· formation is most likely to result from the slow reaction of peroxides with Hx-complexed heme, and the decreased rate of their formation by Hx complexes is most likely to result from steric hindrance of access of the hydroperoxide to heme in the binding pocket of Hx.

Extent of Peroxyl Radical Adduct and Desferrioxamine Radical Generation

The data presented thus far has only concerned rates of formation and decay of DMPO-OOR· and Des·, and Table 1 therefore shows the total accumulated data for both these species formed from heme and peroxides, with and without Hx. It may be seen that the total amount of DMPO-OOR· generated is only significantly decreased by Hx in the case of ^{t}BuOOH, while the extent by which Des· is decreased by Hx in the case of ^{t}BuOOH is greater than that of cumeneOOH. The paradoxical *increase* in generation of Des· by Hx in the case of ^{n}BuOOH is thought to result from a decreased rate of formation causing slower bimolecular decay.

If the protective function of Hx lies in its reaction with the generated peroxyl radicals, scavenging them in a "sacrificial" fashion, it would be expected that the observed protective effect would be greater for reactions of cumeneOOH than ^{t}BuOOH due to the expected faster rates of reaction of cumeneOO· radicals than ^{t}BuOO· with Hx.[a] That the converse is observed would suggest that the major mechanism of decrease

TABLE 1
The effect of Hx complexation of heme upon total generation of DMPO-OOR· and Des· radicals over the first 9 and 6.5 minutes of reaction respectively

	^{t}BuOOH		^{n}BuOOH		CumeneOOH	
	–Hx	+Hx	–Hx	+Hx	–Hx	+Hx
Total DMPO-OOR· from t = 0 to t = 9 min (arbitrary units)	358	146	300	282	201	184
Total Des· from t = 0 to t = 6.5 min (arbitrary units)	926	128	636	782	730	468

[a]It is known that the ^{t}Bu and cumene peroxyl radicals (^{t}BuOO· and cumeneOO· respectively) react with a range of substrates at broadly similar rates, but with the former slightly slower than the latter. Thus the rate constant for the reaction of ^{t}BuOO· with 2,4,6-tri-^{t}butyl phenol to generate the corresponding phenoxyl radical is 7×10^3 M^{-1} sec^{-1} (in toluene) whereas the corresponding rate constant for cumeneOO· is 21×10^3 M^{-1} sec^{-1} (in cumene).[21] The reactions of the straight chain ^{n}butyl peroxyl radical will be quite different from the other two as this species is known to undergo rapid bimolecular disproportionation reactions (the Russel mechanism[22]) making comparison difficult.

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in radical generation from heme upon binding to Hx instead results from differences in the rates of radical *generation*, with the residues forming the heme- binding site of Hx causing substantial steric hindrance of access of peroxide to the bound heme. The heme-binding site of Hx is thought to be rather superficial in nature[12,23] with the heme being bound near the surface of the protein; however this does not preclude the observed steric hindrance of peroxide access to bound heme, although it does mean that the steric hindrance might possibly also arise from surface-bound carbohydrate moieties (Hx is extensively glycosylated)[10–12,23] in addition to amino acid residues.

The faster rates of formation of DMPO-OOR· and Des· from heme-Hx complexes for the larger cumeneOOH than ^{t}BuOOH would seem to argue against this, but it is felt that the hydrophobic interactions between heme (and also porphyrins) and the binding pocket of Hx, important in directing its binding (in addition to coordination of the 5 and 6 positions of metalloporphyrins by histidine residues)[23–26] might also favour the access of the more hydrophobic cumeneOOH into the heme-containing binding pocket of Hx and thus increase its rate of reaction. That the total extent of formation of Des· from cumene-OOH by free heme is lower than ^{t}BuOOH (presumably due to greater steric hindrance by the bulky phenyl group), yet is greater by Hx-complexed heme than ^{t}BuOOH (Table 1) supports the above proposition.

Upon the reaction of heme-Hx complexes with the peroxides in the presence of both DMPO (20 mM) and desferrioxamine (1 mM), only the Des· species was observed (data not shown). Since peroxyl radicals react faster with DMPO (rate constants 10^5 to 10^6 M^{-1} sec^{-1})[20] than with hydroxylamines (with which peroxyl radicals react at rate constants between 10^4 and 10^5 M^{-1} sec^{-1}),[27] and given the 20-fold greater concentration of DMPO, then Des· must be formed only from high-oxidation-state iron species, with the little or no formation via direct reaction with peroxyl radicals, and the reaction of desferrioxamine with the Hx-bound high-oxidation-state-heme species must be faster than that with the hydroperoxides. Thus the EPR determination of DMPO-OOR· and Des· has, in this case, allowed the separate determination of the generation of both peroxyl radical and ferryl-iron species produced by reactions of peroxides with low concentrations of heme.

CONCLUSIONS

The antioxidative effects of Hx-binding of heme would appear to function physiologically through steric-hindrance of the reaction of peroxides in the circulation with this otherwise highly-reactive ligand; the steric-effect with non-solvated membrane-bound lipid hydroperoxides or protein hydroperoxide species (both thought important *in vivo* in circulatory system oxidative stress)[1,7,12,28] would presumably be even greater. The prevention of these radical-generating reactions will therefore tend to favour their non-radical-generating metabolism by antioxidant defence enzymes (such as glutathione peroxidases) thus decreasing the *in vivo* oxidative stress caused by these compounds.

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Accepted by Professor J.V. Bannister

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