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Application of Radiation and Electron Spin Resonance Spectroscopy to the Study of Ferryl Myoglobin

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Addition of an excess of hydrogen peroxide to aqueous metmyoglobin (Fe^{3+}—OH_2) followed by rapid freezing gave a species having no detectable e.s.r. features assignable to iron. However, on exposure to ^{60}Co γ-rays at 77 K, e.s.r. features characteristic of a low-spin Fe^{III} centre grew-in. On annealing above 77 K a modified centre with a larger spread of g values was identified as hydroxy-myoglobin. On further annealing this finally changed to normal high-spin Hb^+ characterised by an intense feature at $g = 6$. These results strongly support the 'ferryl' structure, (FeO^{2+}) previously assigned to the major product formed from hydrogen peroxide.

We were unable to detect e.s.r. features assignable to the expected (Fe^{3+}—$OO\bar{H}$) intermediate even at relatively low H_2O_2 concentrations. This species has been previously identified by e.s.r. spectroscopy as an intermediate in the formation of ($Fe^{3+}H_2O$) following electron addition to oxymyoglobin ($Fe^{2+}O_2$). This result shows that, although the hydroperoxide derivative, (Fe^{3+}—$OO\bar{H}$), is probably an essential intermediate in the route to the ferryl derivative, it is never present in concentrations high enough to be detected by e.s.r. spectroscopy.

It is well established that various ferro-proteins such as horseradish peroxidase (Hrp), cytochrome oxidase and cytochrome peroxidase, can be oxidized to states which correspond formally to Fe^{IV} (Hrp compound II) and 'Fe^{V}' (Hrp compound I) oxidation levels.[1,2] These valence states are not unprecedented since, for example, the ferrate ion (FeO_4^{2-}), containing $Fe^{VI}(d^2)$, is quite stable.[3] Current opinion seems to be that the Fe^{IV} derivative is an oxo-complex comparable to vanadyl (VO^{2+}) whilst the Fe^{V} derivative is a similar complex, the extra electron being lost from the porphin π-system ($P^{\cdot+}FeO^{2+}$).[4,5]

Metmyoglobin (Fe^{3+}) also reacts with hydrogen peroxide to give a relatively stable species having ferryl-like properties (FeO^{2+}), although there is still some controversy regarding structure, and some prefer to describe these centres as myoglobin peroxide.[6,7]

There is a link between these room-temperature studies involving met-derivatives and hydrogen peroxide and our work on electron addition to oxymyoglobin and oxyhaemoglobin.[8–12] Here the electron adduct, usually written as (FeO_2^-), formed at 77 K, is thought to protonate on annealing, giving the low-spin (Fe^{3+}—O_2H^-) derivative, which then loses HO_2^- (or H_2O_2) to form the normal high-spin aquo derivative. The (Fe^{3+}—O_2H^-) complex is a reasonable intermediate in the reaction between met-derivatives and hydrogen peroxide, and since it has a well defined e.s.r. spectrum, one of our aims was to see if we could detect this as an intermediate in the formation of the ferryl species.

Another aim was to use the low-temperature radiolysis procedure to study electron addition to the ferryl derivatives. After our work was completed, we discovered that a somewhat similar study, using glycol solutions, was carried out by Gasyna.[13] This work is strongly supported by our present study. Gasyna has also described his e.s.r. studies of electron addition to oxymyoglobin and has reported changes in the u.v.–visible spectra.[14] These results are discussed and utilised below.

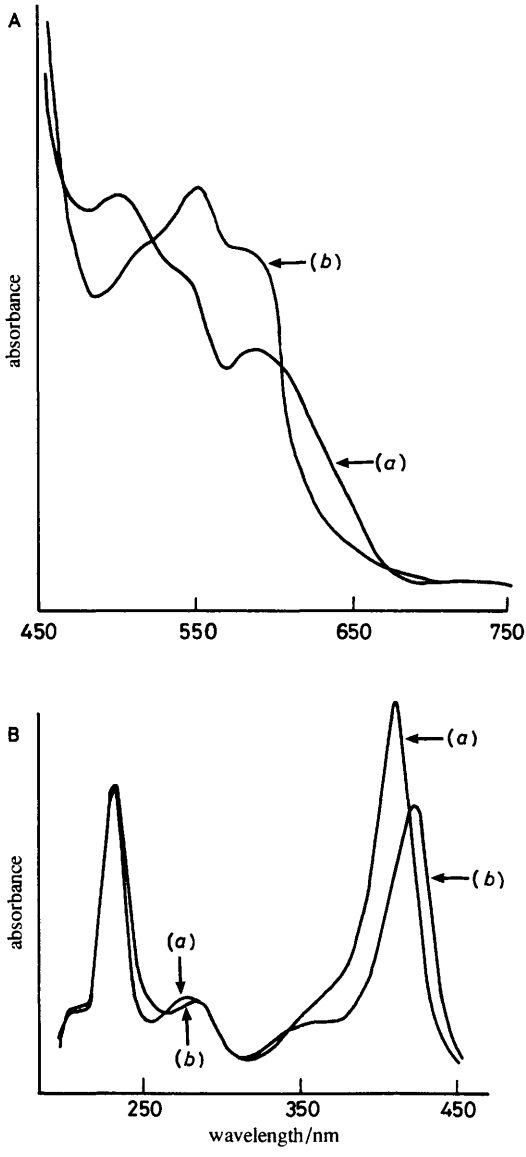

Fig. 1. Visible (A) and u.v. (B) spectra for aqueous metmyoglobin (pH 9.0) (*a*) and after the addition of excess hydrogen peroxide (*b*) showing features assigned to the ferryl derivative $(FeO)^{2+}$.

Experimental

Sperm-whale myoglobin was obtained from Sigma Chemical Company, usually in the oxidised (met) form. To obtain pure metmyoglobin, a solution of the myoglobin was oxidised by a two-fold excess of tripotassium hexacyanoferrate(III). The resulting mixture was dialysed overnight against the appropriate buffer. Solutions of oxyhaemoglobin and methaemoglobin were obtained as described in previous communications.[9-11]

Concentrations of haemoglobin and myoglobin solutions were routinely determined

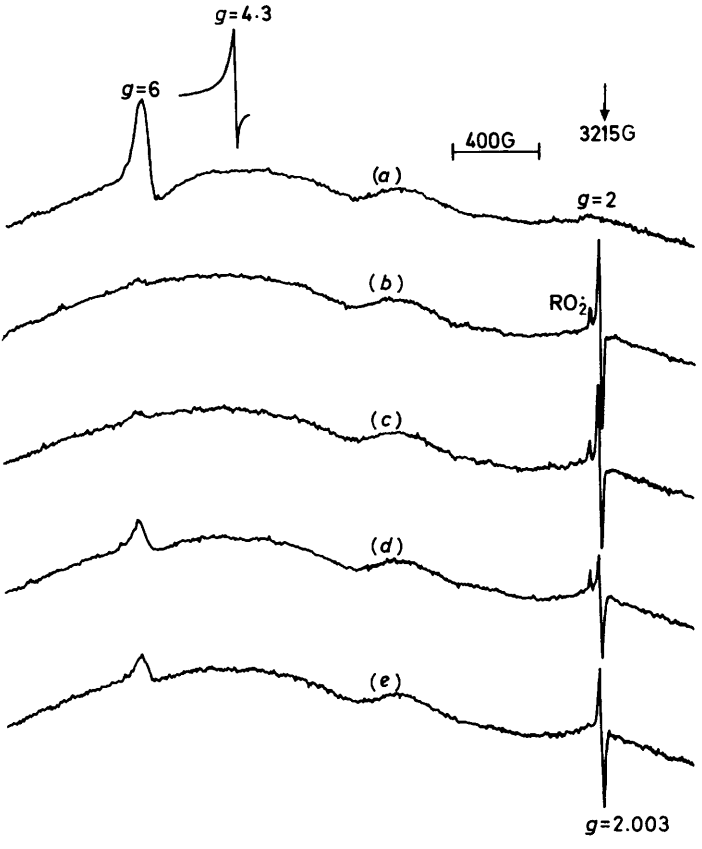

Fig. 2. First-derivative X-band e.s.r. spectra for metmyoglobin solutions at pH 7.4, after addition of dilute hydrogen peroxide and freezing to 77 K after various time intervals: (*a*) no H_2O_2, (*b*) 20 s, (*c*) 2 min, (*d*) 4 min, (*e*) 15 min. The spectra show loss of Fe^{III} and the absence of other Fe-signals; α shows the $g = 4.3$ transition for a small sample of iron(III) acetonylacetonate used for standardisation. The $g = 6$ signal is due to Fe^{III} and the $g = 2$ signal is due to a low yield of organic radicals.

by measuring the optical density of the met-cyanide complex at 540 nm. The molar extinction coefficients used were 4.6×10^4 mol^{-1} dm^{-3} for haemoglobin tetramer and 1.15×10^4 mol^{-1} dm^{-3} for myoglobin, according to Drabkin.[15]

Hydrogen peroxide solutions were freshly prepared prior to each experiment. The concentration of each solution was determined titrimetrically against standardised solutions of potassium tetraoxo-manganate(VII);

$$2MnO_4^- + 5H_2O_2 + 6H^+ \rightarrow 2Mn^{2+} + 8H_2O + 5O_2.$$

Calculated volumes of hydrogen peroxide were added to each of the protein solutions to give [protein]:[peroxide] molar ratios of 1:10. After each addition the reaction mixture was rapidly frozen in liquid nitrogen. Reaction times were estimated as the interval between mixing of reactants and freezing of mixtures.

γ-Radiation and e.s.r. recordings were performed as described in ref. (16). Electronic spectra were recorded on a Perkin-Elmer 340 spectrophotometer at 298 K.

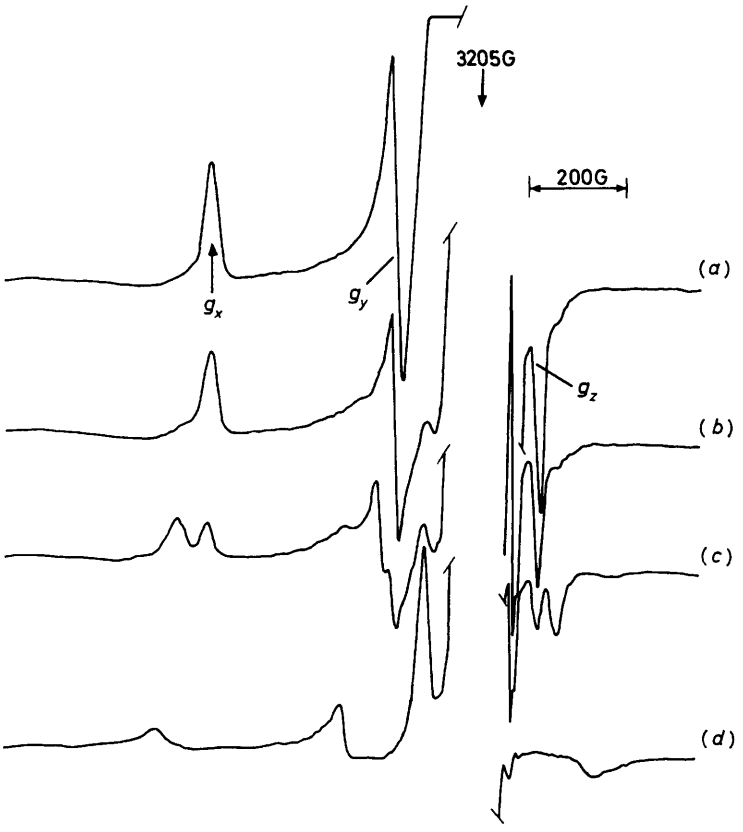

Fig. 3. First-derivative X-band e.s.r. spectra for the ferryl derivative after exposure of an aqueous solution of ferryl myoglobin to ^{60}Co γ-rays at 77 K showing e.s.r. spectra assigned to the electron-addition species, $(FeO)^+$ (*a*) and new centres derived from FeO^+ on warming to 135 K (*b*) 155 K (*c*) showing the gain of a second species, and 180 K (*d*) showing the formation of the normal low-spin Fe^{III} OH^- derivative. The central lines are due to solvent radicals and organic radicals.

Results and Discussion

Optical results obtained under various conditions are given in fig. 1. These agree with those reported by Kobayashi and Hayashi,[17] Halliwell and co-workers[18–20] and Aviram *et al.*[21] but differ somewhat from those given by Gasyna.[13,14] However, he used 50% aqueous glycol at 77 K, so differences are to be expected as a result of interaction between the protein and the alcohol.[22] The solutions thought to contain ferryl myoglobin are remarkably stable in the alkaline pH region ($\gtrsim 7.4$), but decompose slowly in more acidic solutions (pH < 6.0).

On freezing to 77 K following rapid mixing of metmyoglobin and hydrogen peroxide ($\geqslant$ 4-fold excess generally), the e.s.r. spectra showed the complete absence of high- or low-spin Fe^{III} centres, but an appreciable signal at $g = 2.004$. Also, in some cases, a parallel feature at $g_{\parallel} = 2.034$ was apparent [fig. 2(*a*)]. This is assigned to $RO_2^{\cdot}$ radicals.

On exposure to ^{60}Co γ-rays at 77 K, there was efficient generation of a set of features (g_x, g_y and g_z in fig. 3) which are characteristic of a low-spin Fe^{III} complex. We have established for a range of proteins that electrons generated by ionizing radiation can migrate within proteins until they are captured by centres with electron affinities greater than those of the amide units of the protein itself.[23] However, the electron-loss centres

Table 1. E.s.r. parameters for the $(FeO)^{+}$ centre obtained from $(FeO)^{2+}$ by radiolysis

T/K	g_x	g_y	g_z	g_{av}	Δg
	pH 8.0, 50% E/G				
77	2.41	2.11	1.95	2.16	0.46
ca. 120	2.47	2.12	1.93	2.17	0.54
ca. 155	2.57	2.17	1.85	2.20	0.72
ca. 180	—	—	—	—	—
	pH 9.0, 14% E/G				
77	2.43	2.12	1.93	2.16	0.50
ca. 155	2.49	2.14	1.91	2.18	0.58
ca. 180	2.55	2.20	1.88	2.21	0.67
ca. 200	2.58	2.20	1.86	2.21	0.72

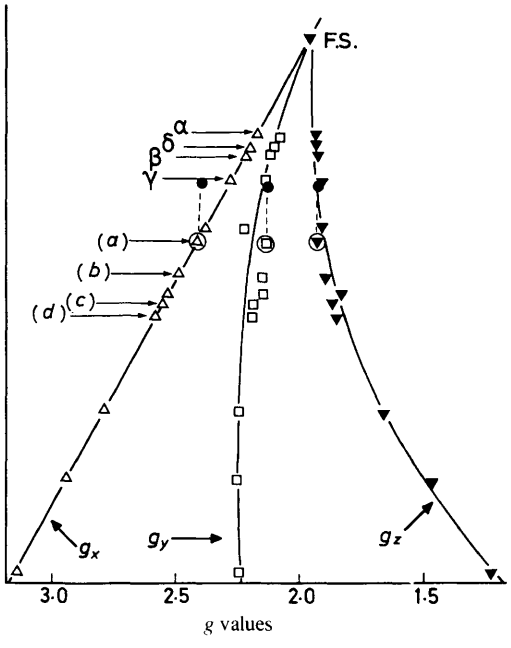

Fig. 4. Trends in the g values for a wide range of low-spin Fe^{III} complexes including FeO_2^- and the ferryl-derived species (*a*)–(*d*). The filled circles for the FeO^+ derivative (*a*) show the enhanced value for g_x obtained if g_y and g_z are constrained to the lines shown. The species, α, β, γ and δ are those formed from electron addition to oxyhaemoglobin.

do not seem to be mobile, reacting preferentially to give amido-radicals. We therefore conclude that the new centres must be formed by electron capture at iron. The rapid linear growth of these Fe^{III} centres clearly establishes that they are formed from an Fe^{IV} unit, and that there are only few protein molecules containing porphyrin cations ($P^{\bullet+}$), since these would be expected to react with electrons preferentially, leaving the Fe^{IV} units largely unaffected.

The e.s.r. parameters for the primary reduction species, herein referred to as (*a*), (*b*), (*c*) and (*d*), are given in table 1 and are compared with those for other low-spin Fe^{III} derivatives in fig. 4. This plot is arbitrarily drawn to display g_{max} (g_x) in a linear fashion.

This then determines the form of the g_y and g_z trends. This plot confirms that the new species is a typical low-spin Fe^{III} complex (see below).

On annealing, a second form of the Fe^{III} centre is formed from the primary centre, and later, a third species grows in [fig. 2(*c*)]. The last is clearly the hydroxy-met derivative, (Fe^{3+}—OH^-). Thus the trends on annealing closely resemble those found for the (FeO_2^-) centres.[9-12] We suggest that the initial species is the unstable electron adduct, FeO^+, retaining the strongly bound oxo-ligand. This forms a hydrogen bond on annealing before becoming protonated to give the (Fe^{3+}—OH^-) low-spin complex. Finally, a second protonation occurs and normal high-spin methaemoglobin or myoglobin is formed.

Further support for the ferryl structure of the Fe^{IV} species comes from the form of the *g* tensor for the primary electron adduct. If the g_x value is included on the linear correlation of fig. 4, the g_y feature is well removed from the correlation. Alternatively, if the g_y feature is placed on the line, g_x is seen to be unexpectedly large. The latter situation accords well with expectation for the (FeO^+) formulation. There are two controlling factors: one is that spin delocalisation onto oxygen will reduce the contribution of iron to all three *g* values, as postulated for the FeO_2^- centre.[9-12] The other is that because of the near-equivalence of the two π^* orbitals, formally represented as d_{xz} and d_{xy}, orbital angular momentum around the *x* axis should be somewhat enhanced, relative to either FeO_2^- units or to Fe^{III}—OH^- units. This nicely accounts for the abnormal shift in g_x indicated in fig. 4. We conclude that the small magnitudes of the *g* shifts are due to π-spin-delocalisation onto the oxo-ligand and the unusually large difference between g_x and g_y arise because of the high symmetry of the FeO unit along the *x* axis.

Thus all these results strongly favour the FeO^{2+} formulation for the 'peroxide complex'. By analogy with nomenclature for other mono-oxoderivatives (vanadyl, molybdyl *etc.*), we favour the name 'ferryl' for this species rather than such names as 'myoglobin peroxide' which seem to be currently favoured.[6,7] The species is clearly *not* a peroxide in the chemically accepted sense.

Aspects of Structure

In addition to the evidence given above, there is evidence from Raman[24] and EXAFS[6] studies in support of the ferryl structure for the compound formed by hydrogen peroxide. The Raman studies show the presence of a strong Fe—O bond and the absence of a peroxide linkage. The EXAFS studies confirm the presence of a short Fe—O bond, in line with expectation for the ferryl unit.

Aspects of Mechanism

The electron/proton pathway linking the met and oxy forms established in our previous radiation studies[9-12] and those of others[1,14,25] is:

$$\underset{(\text{met})}{Fe^{III}} + HO_2^- \rightleftharpoons F^{III}O_2H^- \overset{(+H^+)}{\rightleftharpoons} FeO_2^- \overset{(+e^-)}{\longleftarrow} \underset{(\text{oxy})}{FeO_2} \quad . \qquad \text{(I)}$$

This work established the relative stability of the $Fe^{III}O_2H^-$ complex and its strong tendency to decompose into normal methaemoglobin rather than to become converted into a ferryl derivative. Indeed, it seems that this conversion is almost quantitative.[16] This accords with our inability to generate the ($Fe^{III}O_2H^-$) complex in our present experiments with hydrogen peroxide. However, had (Fe^{III}—O_2H^-) been unable to react in any other way, some should surely have been formed with excess H_2O_2. Clearly the

ferryl pathway removes (Fe^{III}—O_2H^-) (or the H_2O_2 complex) so rapidly that there is never enough for e.s.r. detection. If we assume that complexation occurs prior to reaction, we can write two alternative unimolecular pathways:

$$(Fe^{III}—O_2H^-)^{2+} \rightarrow (Fe^{IV}O^{2-})^{2+} \text{ (}i.e.\ FeO^{2+}\text{)} + {}^{\bullet}OH \quad (1)$$

$$(Fe^{III}—O_2H^-)^{2+} \rightarrow (P^{\bullet+}, Fe^{IV}O^{2-})^{3+} \text{ (}i.e.\ P^{\bullet+}FeO^{2+}\text{)} + OH^-. \quad (2)$$

The former is improbable because of the high-energy $^{\bullet}OH$ radical, whilst the latter is certainly not the major product, although its analogue in the peroxidases is relatively stable.

Since an excess of H_2O_2 is required for these reactions, it is possible that the initial (Fe^{III}—O_2H^-) species reacts with a second H_2O_2 molecule, as in

$$(Fe^{III}—O_2H^-)^{2+} + H_2O_2 \rightarrow (Fe^{IV}O^{2-})^{2+} + HO_2^{\bullet} + H_2O. \quad (3)$$

This reaction explains the requirement for an excess of H_2O_2 and overcomes the need to formulate $^{\bullet}OH$ radical formation. Thus reaction (3) is probably favoured. If reaction (2) occurs, further reaction with H_2O_2 could destroy the porphyrin cation centre:

$$(P^{\bullet+}, Fe^{IV}O^{2-})^{3+} + H_2O_2 \rightarrow (Fe^{IV}O^{2-})^{2+} + HO_2^{\bullet} + (H^+). \quad (4)$$

Both reactions (3) and (4) require the formation of $HO_2^{\bullet}$, which at normal pH values will largely be converted into $O_2^{\bullet-}$. This is a fairly stable entity and is likely to escape from the protein pocket into free solution. It is expected to react with methaemoglobin to form the oxy-derivative:

$$Fe^{III} + O_2^- \rightarrow Fe^{II}O_2. \quad (5)$$

We have not obtained evidence for any extensive conversion into FeO_2, but this does not rule out reaction (5) since the $Fe^{II}O_2$ and Fe^{II} forms of haemoglobin and myoglobin are also oxidized to the ferryl derivative by hydrogen peroxide. Indeed, these reactions, which can be represented as

$$[Fe^{II}] + H_2O_2 \rightarrow [FeO^{2+}] + H_2O \quad (6)$$

$$[Fe^{II}O_2] + H_2O_2 \rightarrow [FeO^{2+}] + H_2O + O_2 \quad (7)$$

are actually faster than the reaction with the met-derivatives, with reaction (6) faster than reaction (7).[18,26] These reactions explain, in principle, why the ferryl derivative is initially the dominant species in all these reactions.

It has been suggested that hydrated electrons add to oxymyoglobin to give the ferryl derivative.[17] Given that the formulation [FeO^{2+}] is correct, this cannot be a direct reaction, despite the claim that it is a genuine one-electron reaction without the involvement of intermediates. Proof that the ferryl species is formed rests upon difference u.v.–visible spectra [fig. 4(*a*)]. However, although this difference spectra are compatible with the concept of conversion of the FeO_2 species to ferryl, they are also compatible with conversion to FeO_2^- units. These reactions are extremely rapid, with second-order rate constants of *ca.* 4×10^{10} dm^3 mol^{-1} s^{-1}, and we therefore suggest that simple electron addition to give the FeO_2^- centre is chemically more reasonable than conversion into the ferryl unit. Both e.s.r. and u.v.–visible studies confirm that this is the primary step at 77 K.[9-12] Gasyna has reported the optical spectrum for FeO_2^- and we have computed a difference spectrum for those for FeO_2 and FeO_2^-. The agreement with experiment is just as satisfactory as that obtained using the spectrum for the ferryl derivative,[17] and we conclude that the species detected by Kobayashi and Hayashi is probably FeO_2^- rather than ferryl.

There has been much discussion about the possibility of deoxymyoglobin or haemoglobin reacting with H_2O_2 in the manner postulated for free Fe^{2+} (the Fenton reaction). Sadrzadeh *et al.*[19] reported that intact haemoglobin can generate $^{\bullet}OH$ from

H_2O_2, whereas Halliwell and Gutteridge[18] found that although *intact* haemoglobin does not give detectable $^{\bullet}OH$ radicals, the protein is broken down by H_2O_2, liberating iron ions which can then generate $^{\bullet}OH$ radicals from H_2O_2. Recently Puppo and Halliwell[20] have confirmed that H_2O_2 degrades methaemoglobin, thereby releasing iron which then reacts with H_2O_2 giving $^{\bullet}OH$. This is detected by its ability to attack both deoxyribose and phenylalanine. Curiously, oxyhaemoglobin seems to differ in that the 'reactive species' formed is less reactive than ''$^{\bullet}OH$' towards some substrates but more reactive towards others. As discussed in ref. (20), it is difficult to see how any of these bulky substrates could enter the haem pocket and react directly with, say, the ferryl unit. It is also hard to understand how any species other than $^{\bullet}OH$ could escape the haem pocket and be able to mimic $^{\bullet}OH$ in certain respects. As we imply above, $HO_2^{\bullet}$ and $O_2^{\bullet-}$ could well escape, but these are not normally sufficiently reactive to mimic $^{\bullet}OH$ radicals.

Our results confirm that ferryl myoglobin is a major product when all three derivatives (met-, deoxy- and oxy-) react with hydrogen peroxide. The results of others show clearly that there is some degradation of the globin units under these conditions, although we were unable to detect haemichrome formation. These degradation reactions could well make the iron available for reaction with substrates, but the distinction between methaemoglobin and oxyhaemoglobin remains most puzzling.

It is important to note that our attempts to repeat these results using methaemoglobin have so far failed. In view of the similarity between the iron complexes in metmyoglobin and methaemoglobin the argument that both should form the ferryl derivative is strong. However, the reaction path is clearly complex, and possibly a different route is taken for the latter protein. Differences in site accessibility may be involved.

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