

Reaction of Myoglobin with Hydrogen Peroxide Forms a Peroxyl Radical Which Oxidizes Substrates*

(Received for publication, September 20, 1993, and in revised form, December 16, 1993)

David J. Kelman^{‡§¶}, Janice A. DeGray[‡] and Ronald P. Mason^{‡§}

From the [‡]Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709 and the [§]Curriculum in Toxicology, University of North Carolina, Chapel Hill, North Carolina 27599

Evidence is presented that the radical observed upon reaction of myoglobin with hydrogen peroxide is a peroxyl radical. Simulation of this spectrum gives principal values for the *g* tensor of $g_x = 2.0357$, $g_y = 2.0082$, and $g_z = 2.0016$, which are consistent with those of a peroxyl radical. Use of molecular oxygen isotopically labeled with ¹⁷O confirmed that the radical observed was a peroxyl radical. Removal of oxygen from the incubation by use of glucose and glucose oxidase revealed two radicals, one at $g_{iso} = 2.0028$ and the other at $g_{iso} = 2.0073$. Addition of various amounts of the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide revealed that the spin trap and oxygen compete for the same radical site. Four model substrates, glutathione, styrene, arachidonic acid and linoleic acid, were individually added to both the aerobic and anoxic systems. Glutathione reacted with the peroxyl radical, reducing its intensity by 98%, and entirely eliminated the $g_{iso} = 2.0028$ line from the spectrum of the anoxic incubation. Styrene, arachidonic acid and linoleic acid reacted with the peroxyl radical, reducing its amplitude by 84, 57, and 35%, respectively, but did not decrease the amplitude of either radical species in the anoxic incubation. The $g_{iso} = 2.0028$ species detected in the anoxic incubation appears to be the original radical site to which molecular oxygen binds to form the peroxyl radical. This myoglobin-derived peroxyl radical species is responsible for the advent of lipid peroxidation as proposed in ischemia/reperfusion injury, as well as other reactions, as exemplified by the O₂-dependent epoxidation of styrene.

There has been increasing interest in the identification of the radical species that forms on metmyoglobin upon exposure to hydrogen peroxide (1–10). This interest is stimulated by evidence that this reaction might be of importance in elucidating the origin of the lipid peroxidation and cell damage resulting from ischemia/reperfusion of the heart (11–15). While myoglobin is not primarily a catalytic protein, it has been demonstrated to possess peroxidase-like activity (1, 2, 16–18). Similar to classical peroxidases such as horseradish peroxidase, myoglobin reductively cleaves hydrogen peroxide, retaining one of the oxidizing equivalents on the iron of the heme in the form of an oxo-ferryl species (Fe^{IV}=O). However, the second oxidizing equivalent is located on the globin as a free radical (19–21), whose structure has been the subject of speculation since 1958 (18). This is different from the classical horseradish peroxidase, where the second oxidizing equivalent is the porphyrin IX cat-

ion radical (22). The nature of the metmyoglobin radical site has been proposed to be a tyrosine-based peroxyl radical using spin trapping (2, 8); however, this assignment has been contested (3, 9). Utilizing direct EPR¹ spectroscopy, we demonstrated that a peroxyl radical is formed, and that this species is derived from the reaction of molecular oxygen with a globin free radical, which is not a tyrosyl radical. Both free radicals oxidize cellular constituents, although with different specificities.

MATERIALS AND METHODS

Horse heart myoglobin, D-glucose, DMPO, glutathione, and hydrogen peroxide were obtained from Sigma. Glucose oxidase (250 units/mg, 1 unit converts 1 mmol of glucose to product in 1 min at 25 °C and pH 7) was purchased from Boehringer Mannheim. O₂ enriched 37% with ¹⁷O was obtained from ICON Services Inc. (Summit, NJ). Styrene was obtained from Aldrich. Arachidonic and linoleic acids were obtained from Nu Chek (Elysian, MN). The DMPO was further purified before use by vacuum distillation at room temperature.

All EPR spectra were recorded at liquid nitrogen temperature using a fingertip liquid nitrogen Dewar flask (Wilmad, Buena, NJ) on a Bruker ESP300 spectrometer equipped with a TM₁₁₀ cavity. All incubations were 532 μM metmyoglobin in 0.1 M sodium phosphate buffer, pH 7.4, and initiated with 400 μM hydrogen peroxide. EPR spectral simulations were performed using the powder pattern simulation program POW, part of the Brown University Powder Pattern package (23, 24).

Aerobic and Anoxic Incubations—The aerobic system was initiated with the addition of hydrogen peroxide and frozen in liquid nitrogen within 10 s after the initiation of the reaction. The anoxic system was achieved as follows. Metmyoglobin solution was placed in a 20-ml vial, and a stream of nitrogen gas was passed on to the solution without bubbling by use of a Pasteur pipette. The oxygen-consuming system of glucose (15 mM) and glucose oxidase (50 units/ml) was added, hydrogen peroxide was added immediately afterward, and the incubation was frozen in liquid nitrogen within 10 s. In order to accurately determine the values of the *g* tensor for each species, a *g* standard of Cr³⁺ in MgO was employed ($g_{iso} = 1.9800 \pm 0.0006$) (25).

Time Dependence—The stability of the radicals formed in both the aerobic and anoxic conditions was explored. Incubations were prepared as above, and times between initiation of the reaction with hydrogen peroxide and freezing in liquid nitrogen were varied.

DMPO Competition—DMPO was added at various concentrations to both aerobic and anoxic incubations prior to the addition of the hydrogen peroxide. These incubations were then frozen in liquid nitrogen within 10 s.

Isotopic Substitution with ¹⁷O—Myoglobin in buffer was blown with nitrogen gas as described above. Oxygen gas enriched 37% with ¹⁷O was bubbled into the incubation for 3 min. Hydrogen peroxide was added to initiate the reaction, and the incubation was frozen immediately in liquid nitrogen.

Reaction with Glutathione—Hydrogen peroxide was added to myoglobin in buffer, glutathione (2 mM) was added immediately afterward, and the incubation was frozen immediately in liquid nitrogen. The anoxic incubations were treated as above.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed.

¹ The abbreviations used are: EPR, electron paramagnetic resonance; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide.

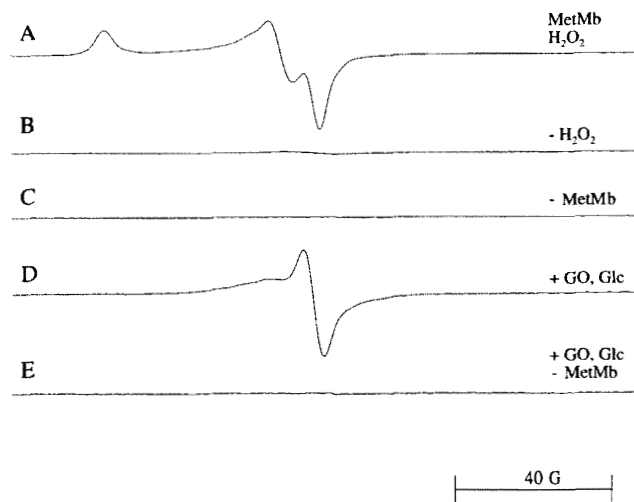


FIG. 1. Aerobic and anoxic metmyoglobin (*MetMb*) incubations. A, EPR spectrum of the 532 μM metmyoglobin (*MetMb*) and 400 μM hydrogen peroxide system. B and C, without either hydrogen peroxide (B) or metmyoglobin (C), no signal is detected. D, with the addition of glucose oxidase (GO) and glucose, a different spectrum results. E, the anoxic spectrum is also dependent on the presence of metmyoglobin. Spectrometer conditions were as follows: gain, 1.0×10^5 ; modulation amplitude, 2.0 gauss; power, 2.0 milliwatts; time constant, 655 ms; scan time, 335 s; scan range, 160 gauss.

Reaction with Styrene—Styrene (10 mM) was added to myoglobin in buffer, and the incubation was initiated with the addition of hydrogen peroxide, then frozen immediately in liquid nitrogen. Similarly, anoxic incubations made with glucose and glucose oxidase, as described above, were treated with styrene.

Reaction with Arachidonic and Linoleic Acids—Arachidonic acid (82 mM) or linoleic acid (80 mM) in absolute ethanol was pipetted into a test tube to coat the bottom of the tube. The ethanol was then evaporated with a stream of nitrogen gas. Myoglobin in buffer was added and the solution vortexed for 30 s. The final concentration of the fatty acids was 400 μM . Hydrogen peroxide was added to initiate the reaction, and the incubation frozen immediately in liquid nitrogen. Anoxic incubations were treated similarly.

RESULTS

Aerobic and Anoxic Systems—Fig. 1 shows both the aerobic and anoxic incubations with their controls. A three-line anisotropic spectrum is observed in the complete aerobic system (Fig. 1A). This spectrum is not observed if either the hydrogen peroxide or the metmyoglobin is omitted. Forcing the system to be anoxic by use of a stream of nitrogen gas and addition of glucose and glucose oxidase results in the disappearance of the anisotropic spectrum, which was replaced by two isotropic spectra due to species with different g values (Fig. 1D). The broader line has a g value of 2.0073, whereas the upfield line has a g value of 2.0028 and a peak-to-trough linewidth of 5 gauss. This spectrum is not observed if metmyoglobin is eliminated from the incubation (Fig. 1E). This demonstrates that formation of the species detected in the complete incubation requires the presence of all the components. In addition, it is shown that the species observed in the aerobic system is indeed dependent on the presence of oxygen.

In order to determine the kinetic properties of the radicals detected, incubations of both the aerobic and anoxic systems were initiated with hydrogen peroxide and the time before freezing the sample was varied. Fig. 2 shows the time dependence of the radical species in the aerobic and anoxic systems. As the time between initiation and cessation of the reaction increases, the intensity of the aerobic species decreases with an apparent half-life of 7 s, as determined by kinetic analysis of the low field extrema. In the anoxic system, the sharper, higher

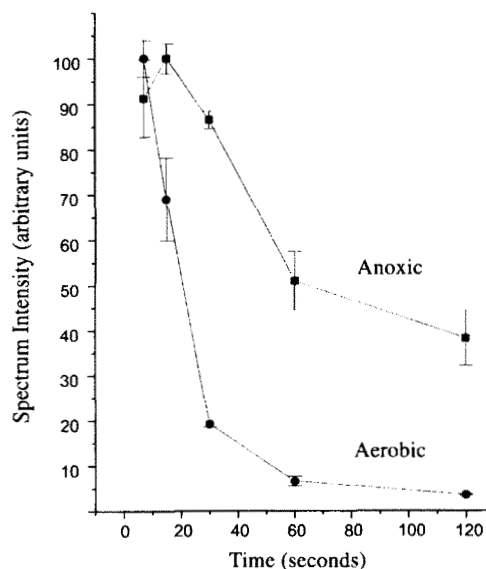


FIG. 2. Timed incubations of myoglobin and hydrogen peroxide for aerobic (●) and anoxic (■) incubations. The aerobic species decays with an apparent half-life of 7 s, while the anoxic species decays with an apparent half-life of 45 s.

field line increases initially, then decreases after 30 s with an apparent half-life of 45 s.

Fig. 3 shows the spectrum from the aerobic system with its simulation. It was assumed that only one principal radical species was present, with any other species being too low in concentration to perturb the simulation. The principal values of the g tensor were calculated to be $g_x = 2.0357$, $g_y = 2.0082$, and $g_z = 2.0016$ by comparison to the upfield Cr^{3+} standard. The dependence on oxygen and the values of the g tensor are indicative of a peroxyl radical (26).

DMPO Competition—Fig. 4 demonstrates the effect of the addition of varying concentrations of DMPO to the aerobic system. When the concentration of DMPO is 5 mM or higher, there is little or no evidence of the species observed in the absence of DMPO. As the concentration of DMPO decreases, the peroxyl radical becomes more evident. At 500 μM DMPO, the radical adduct is virtually absent. This indicates that DMPO is competing with oxygen for a radical site on the globin.

Fig. 5 shows the results of adding varying concentrations of DMPO to the anoxic system. Similar to the aerobic system, only the radical adduct, which has the same spectrum, is detected at the higher concentrations of the spin trap. However, unlike the aerobic system, presence of the radical adduct can be observed at concentrations as low as 50 μM . Without oxygen present, DMPO does not have to compete for the radical site, and therefore the radical adduct can be detected at much lower concentrations of DMPO.

Fig. 6 demonstrates that the radical adduct detected at low temperature is the same as that previously detected at room temperature (9). An incubation of metmyoglobin, hydrogen peroxide, and DMPO was frozen, and the spectrum was recorded (Fig. 6A). The parallel nitrogen hyperfine coupling constant, a_{\parallel}^{N} , was determined to be 32.5 gauss. The sample was allowed to thaw into a flat cell, and the room temperature spectrum was then recorded (Fig. 6B). The nitrogen hyperfine coupling constant ($a_{\parallel}^{\text{N}} = 14.5$ gauss) and the β -hydrogen hyperfine coupling constant ($a_{\beta}^{\text{H}} = 8.2$ gauss) are similar to the reported values (9).

Isotopic Substitution with ^{17}O —A powerful method in determining the structure of a radical is the use of isotopic substitution. Fig. 7 shows the experimental results of the substitution of air by molecular oxygen enriched with 37% ^{17}O after a

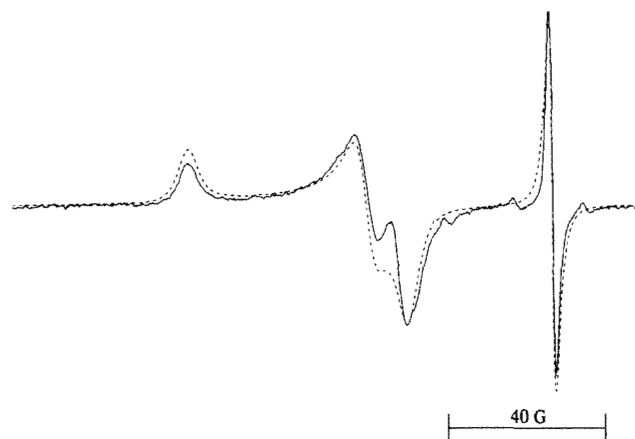


FIG. 3. Experimental (—) and simulated (---) EPR spectrum from metmyoglobin and hydrogen peroxide incubation. The sharp line at high field is a Cr^{3+} g standard. The values of the g tensor of the simulation are $g_x = 2.0357$, $g_y = 2.0082$, and $g_z = 2.0016$. Experimental conditions were the same as those in Fig. 1.

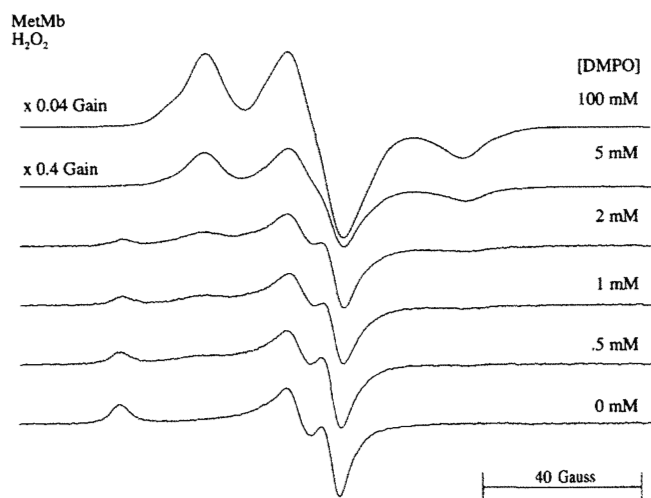


FIG. 4. Addition of various concentrations of the spin trap, DMPO, to aerobic metmyoglobin (*MetMb*) incubations. As the concentration of DMPO decreases, more of the species detected in the spin trap's absence is detected. Spectrometer conditions were the same as those in Fig. 1, except that the gain for the 100 mM incubation was 4.0×10^3 , the gain for 5 mM was 4.0×10^4 , and the gain for the remainder was 1.0×10^5 .

nitrogen gas purge. Additional hyperfine structure due to the 5/2 nuclear spin of the ^{17}O can be seen in Fig. 7A. Two sets of hyperfine structure arise from the fact that the majority of the ^{17}O is in the form of $^{17}\text{O}^{16}\text{O}$, and the hyperfine coupling constant depends on which oxygen the unpaired electron resides, which in turn depends upon which oxygen reacted with the original radical site (24). A third set of hyperfine coupling from peroxy radicals formed by molecular oxygen comprised of $^{17}\text{O}^{17}\text{O}$, which makes up 12% of the peroxy radical observed, can be detected at $\times 40$ gain (Fig. 7B). While one would expect that 40% of the peroxy radical would be from $^{16}\text{O}^{16}\text{O}$ due to the isotopic ratio of oxygen in the gas used, there is actually a higher percentage due to the catalase-like activity of myoglobin. Myoglobin generates molecular oxygen from hydrogen peroxide as determined by oxygraph measurements (9); thus, a portion of the added hydrogen peroxide will be consumed in this manner. This leads to a percentage of the peroxy radical being formed from $^{16}\text{O}^{16}\text{O}$ higher than 40%. Corresponding lower percentages of ^{17}O containing peroxy radicals are found in the simulation. The percentages of each type of peroxy species, as

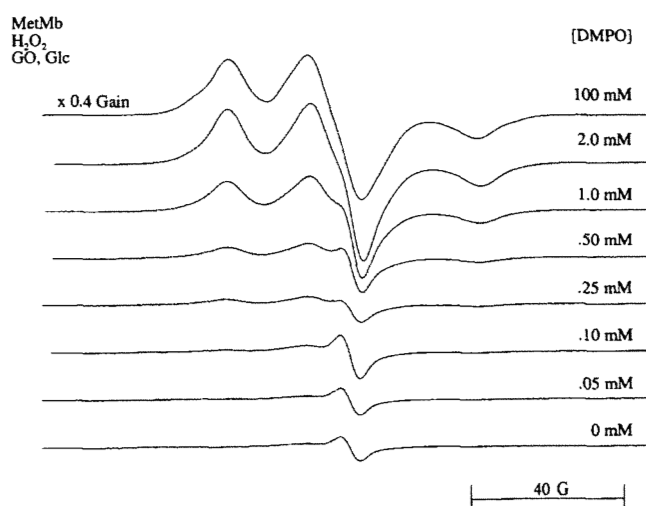


FIG. 5. Addition of various concentrations of the spin trap, DMPO, to anoxic metmyoglobin (*MetMb*) incubations. Identical to Fig. 4, except that the incubations were made anoxic with glucose and glucose oxidase (*GO*) before addition of DMPO and hydrogen peroxide. The DMPO radical adduct can be detected at a much lower concentration of DMPO than in the aerobic system. Spectrometer conditions were similar to Fig. 4, except that gain for 100 mM was 2.0×10^4 and all other gains were 5.0×10^4 .

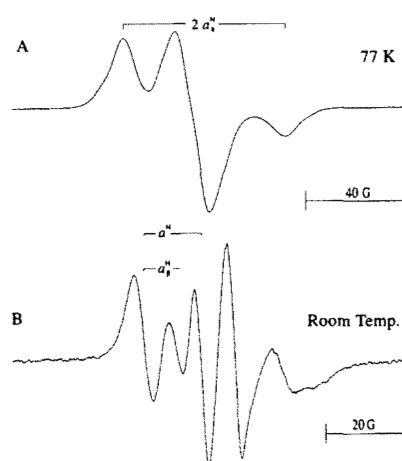


FIG. 6. 77 K and room temperature spectra of the DMPO-myoglobin radical adduct. A, the spectrum from a frozen incubation of DMPO, myoglobin, and hydrogen peroxide yielded $a_N^{\bullet} = 32.5$ gauss. B, the spectrum of the same sample after it had been thawed to room temperature yielded $a_N^{\bullet} = 14.5$ gauss, $a_B^{\text{H}} = 8.2$ gauss. Spectrometer parameters for the frozen sample were the same as in Fig. 4. Spectrometer parameters for the room temperature spectrum were as follows: gain, 6.3×10^4 ; modulation amplitude, 1.0 gauss; power, 20.0 milliwatts; time constant, 1.3 s; scan time, 335 s; scan range, 100 gauss.

determined by the simulation, were $^{16}\text{O}^{16}\text{O}^{\bullet}$ (49.0%), $^{17}\text{O}^{16}\text{O}^{\bullet}$ (19.5%), $^{16}\text{O}^{17}\text{O}^{\bullet}$ (19.5%), and $^{17}\text{O}^{17}\text{O}^{\bullet}$ (12.0%).

Reaction with Glutathione—Fig. 8 shows the results of the addition of 2 mM glutathione to both the aerobic and anoxic systems. In the aerobic system, the signal intensity is decreased by 98% ($n = 4$, $p < 0.00005$) (Fig. 8B). In the absence of oxygen, the sharp line at $g_{\text{iso}} = 2.0028$ disappears entirely, leaving the broad line at $g_{\text{iso}} = 2.0073$ (Fig. 8D).

Reaction with Styrene—The effect of adding styrene to the incubations is seen in Fig. 9. When styrene is added to the aerobic system, the amplitude of the peroxy spectrum is reduced by 84%, as determined by the signal intensity of the low field extrema ($n = 3$, $p < 0.00005$), and a radical with less anisotropy is formed (Fig. 9B). No signal was observed with styrene and hydrogen peroxide alone. In the absence of oxygen,

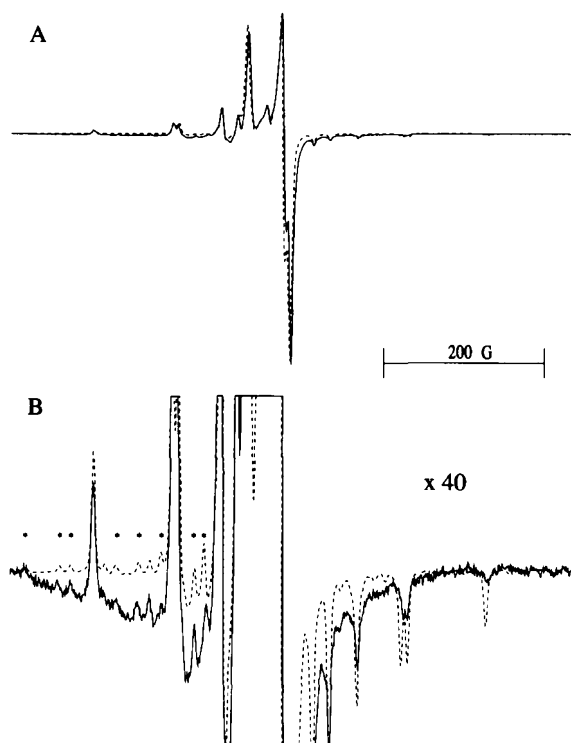


FIG. 7. Experimental (—) and simulated (---) myoglobin and hydrogen peroxide incubation containing molecular oxygen 37% enriched with ^{17}O . A, simulation at nominal scale. The hyperfine coupling of the additional features present due to ^{17}O indicates that the spectrum is from a peroxyl radical. B, repeat of spectrum and simulation at $\times 40$ scale. The peaks marked by asterisks are those that are derived from peroxyl radicals containing two ^{17}O atoms. Parallel hyperfine coupling constants for the two mixed isotope species, $^{-17}\text{O}^{16}\text{O}$ and $^{-16}\text{O}^{17}\text{O}$, were 55 and 97 gauss, respectively. Spectrometer conditions: gain, 1.0×10^6 ; modulation amplitude, 2.0 gauss; power, 2.0 milliwatts; time constant, 2.6 s; scan time, 46 min; scan range, 700 gauss.

there is no significant decrease in the signal intensity. This indicates that styrene is reacting with the peroxyl radical rather than with the original radical site.

Reaction with Arachidonic and Linoleic Acids—Fig. 10 shows that the addition of arachidonic acid to the incubation results in a decrease in the peroxyl radical. Arachidonic acid reduced the spectrum amplitude by 57% ($n = 4$, $p < 0.005$). Similarly, linoleic acid reduced the spectrum amplitude by 35% ($n = 4$, $p < 0.05$) (data not shown). There was no significant difference in the signal intensities from the anoxic incubations.

DISCUSSION

The g tensor for the species observed in the aerobic system closely matches those observed for peroxyl free radical species (26). It is unclear as to why the g_z value is lower than the expected value of 2.0023, though this may be due to the uncertainty in the g standard or an effect of the paramagnetic heme. The addition of glucose and glucose oxidase causes a dramatic change in the spectrum of myoglobin oxidized with hydrogen peroxide. Glucose oxidase oxidizes D-glucose to D-gluconic acid, consuming a molecule of oxygen concomitantly. Studies have shown that this system can decrease the oxygen content of an aqueous solution to 10 nM (27).

Binding of DMPO to the radical site produces a spin trap radical adduct, while the binding of oxygen produces the peroxyl radical. Kelman and Mason (9) have shown that the radical that is trapped by DMPO is not a peroxyl radical. The radical adduct detected at low temperature is the same species previously detected at room temperature, as shown by the

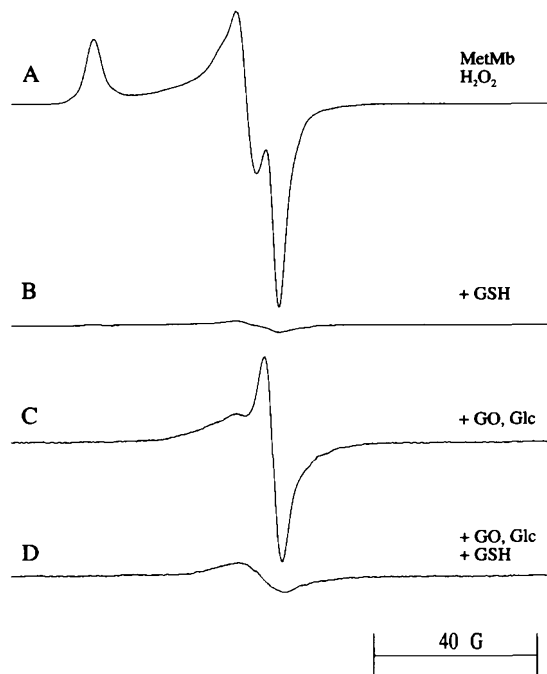


FIG. 8. Metmyoglobin (*MetMb*) incubations and the addition of 2 mM glutathione. A, without glutathione. B, GSH reduces the signal intensity by 98%. C, same as A except that the incubation was made anoxic prior to initiation of the reaction by the addition of glucose and glucose oxidase (GO). D, with the addition of GSH, the line at $g_{100} = 2.0028$ disappears entirely, leaving a broad line at $g_{100} = 2.0073$. Spectrometer conditions were as in Fig. 1, except that gain is 8.0×10^4 .

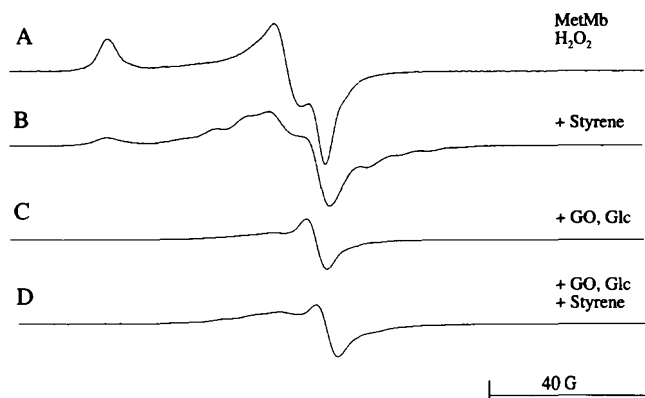


FIG. 9. Metmyoglobin (*MetMb*) incubations and the addition of 10 mM styrene. A, aerobic incubation without styrene. B, aerobic incubation containing 10 mM styrene. C and D are identical to A and B, respectively, except that the incubations were made anoxic by use of glucose and glucose oxidase (GO). There is an 84% decrease in the intensity of the peroxyl radical, but no significant decrease of the species in the anoxic incubation. Spectrometer conditions were the same as Fig. 8.

thawing experiment. Immobilization of the radical adduct at 77 K broadens the spectrum (Fig. 6A), hence obscuring the hydrogen hyperfine coupling apparent in the room temperature spectrum (Fig. 6B).

Fig. 7 clearly demonstrates that the radical detected directly with EPR is a peroxyl species. The parallel ^{17}O hyperfine coupling constants, 97 and 55 gauss, are consistent with those found by Sevilla and co-workers (26) for a carbon-centered radical formed by x-irradiation, with the 55-gauss hyperfine coupling being due to the oxygen bound to a carbon of the myoglobin. The two different sets of coupling constants arise from the two different configurations that can occur with a molecule of oxygen containing one each of the two isotopes. Thus there will

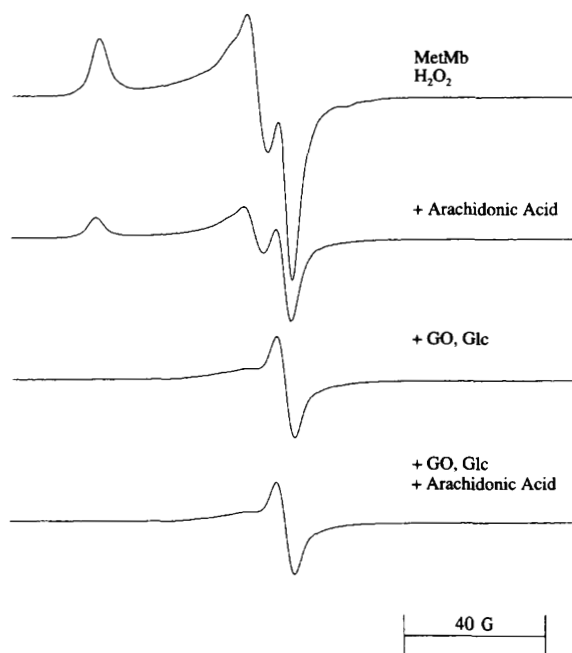
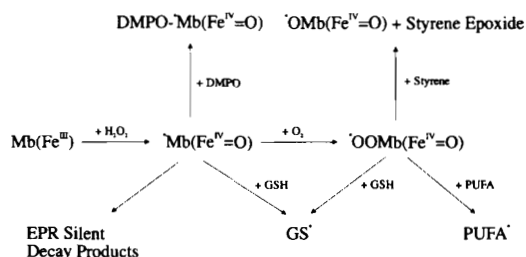


FIG. 10. Addition of 400 μM arachidonic acid to both aerobic and anoxic metmyoglobin (*MetMb*) incubations. There is a 57% decrease in the intensity of the peroxy radical, but no significant decrease of the species in the anoxic incubation. Spectrometer conditions were the same as Fig. 8.

be a different coupling constant depending on whether the ^{17}O or the ^{16}O is the site of the unpaired electron. In the case of the molecule of oxygen containing two atoms of ^{17}O , each of the lines separated by 97 gauss would in turn be split by each of the lines separated by 55 gauss, leading to a multi-line spectrum. While not all these lines can be identified in the spectrum observed (Fig. 7B), enough of these lines are visible to confirm the presence of both oxygen atoms in the same radical.

Fig. 8 shows that glutathione is a very efficient scavenger of both of the radicals that form on myoglobin (or a precursor of these radicals). Within seconds, glutathione decreases the peroxy radical concentration nearly 2 orders of magnitude. Romero *et al.* (28) have trapped the glutathione thiyl radicals with DMPO after the addition of glutathione to myoglobin and hydrogen peroxide. The authors added hydrogen peroxide to myoglobin, then added catalase 1 min later in order to eliminate excess hydrogen peroxide. Glutathione was added subsequent to the catalase. Given the lifetime of the myoglobin peroxy radical, it is likely that only a small portion of the glutathione radicals were derived from the reaction of glutathione with this peroxy radical, and that the majority of the radicals were generated through oxidation by the ferryl heme (Compound II). The anoxic incubation with glutathione shows that treatment of myoglobin with hydrogen peroxide actually results in the formation of two distinct radicals. One radical, with $g_{\text{iso}} = 2.0028$, is readily reducible by glutathione, as this signal disappears entirely when glutathione is added to the incubation. The other species, at $g_{\text{iso}} = 2.0073$, does not react with glutathione. This species was detected in incubations with glutathione as long as 5 min before freezing, with no reduction in signal intensity (data not shown). This may be due to either the radical being inaccessible to glutathione, or the radical being very stable and thus unreactive.

Styrene appears to react with the peroxy radical, but not with the original radical site, as there is little decrease in the spectrum amplitude in the absence of oxygen. It has been shown by Ortiz de Montellano and Catalano (16) that the ad-



SCHEME 1. The fate of the radical formed on metmyoglobin (*MetMb*) treated with hydrogen peroxide in the presence of oxygen, reduced glutathione, styrene, and polyunsaturated fatty acids (PUFA).

dition of styrene to myoglobin and hydrogen peroxide will result in the formation of styrene oxide, with 78% of the oxygen added to the styrene oxide derived from molecular oxygen. Rao *et al.* (4) demonstrated that when oxygen was added to *cis*- β -methylstyrene, it could do so in such a manner that the stereochemistry was not preserved, resulting in *trans*- β -methylstyrene oxide. Although the ferryl iron of the myoglobin did oxidize *cis*- β -methylstyrene under these conditions, it preserved the stereochemistry of the molecule, resulting in *cis*- β -methylstyrene oxide. These results showed that oxygen was being added to *cis*- β -methylstyrene by myoglobin from both the ferryl iron and another site. Given that the reaction of a methylenyl carbon with a peroxy radical will produce an epoxide (29), it is likely that the peroxy radical reported here is the site at which the epoxidation reaction is occurring.

The addition of either arachidonic or linoleic acids, like styrene, decreases the amount of peroxy radical present, but they do not react with the original radical site. Lipid peroxidation due to the exposure to myoglobin and hydrogen peroxide has been reported (11, 14); however, evidence that the hydrogen extraction was due to interaction with a peroxy radical located on myoglobin had not been previously demonstrated. The peroxy radical formed on myoglobin upon reaction with hydrogen peroxide is the likely site that initiates lipid peroxidation proposed to result from ischemia/reperfusion of the heart.

The identity of the original radical site, characterized by $g_{\text{iso}} = 2.0028$, is not yet resolved. Wilks and Ortiz de Montellano used site-directed mutagenesis to replace one, two, or all three tyrosine residues from sperm whale myoglobin without detecting a change in the EPR spectrum (3). The authors postulated that the radical was therefore not based upon a tyrosine residue. However, since the radical species detected by these authors was a peroxy radical, there would be no noticeable change in the spectrum if the peroxy radical formed on different residues in different mutants. Peroxy radicals can form on different residues, yet have similar g values (26, 30). However, the initial radical site is indeed not a tyrosyl radical, based on spectroscopic grounds. DeGray *et al.* (31) have shown that the linewidth of the immobilized tyrosyl radical is at least on the order of 20 gauss, much broader than the 5-gauss linewidth of the anoxic species, confirming that the original radical site is not a tyrosyl radical. Given an apparent lack of hyperfine coupling, this radical may be a tertiary carbon-centered radical, as would be consistent with its g value and the absence of nearby nuclei with nuclear spin. The relative stability of the myoglobin peroxy is also consistent with the known stability of tertiary peroxy radicals, which are known to be much more persistent than either primary or secondary peroxy radicals (29). However, an assignment to a particular residue cannot be made from these data.

CONCLUSION

A summary for these reactions is shown in Scheme 1. In the absence of DMPO, the radical formed reacts with molecular oxygen to form a peroxyl radical and then decays to an EPR silent species. In the presence of DMPO, the spin trap competes with molecular oxygen for the radical site. In the absence of molecular oxygen, DMPO binds to the radical site without competition, allowing the radical adduct to be observed with much lower concentrations of the spin trap. The addition of glutathione demonstrated that the peroxyl species is readily accessible to molecules in solution and that the narrow, single-line species detected in the anoxic incubation is likely the original radical site. Use of styrene, arachidonic acid, and linoleic acid showed that the myoglobin peroxyl radical reacts in a manner typical of smaller peroxyl radicals. This indicates that myoglobin-derived radicals are potential sources of damage to cellular constituents such as membranes and protein sulfhydryl groups.

REFERENCES

1. Arduini, A., Mancinelli, G., Radatti, G. L., Damonti, W., Hochstein, P., and Cadenas, E. (1992) *Free Radical Biol. Med.* **13**, 449–454
2. Davies, M. J. (1991) *Biochim. Biophys. Acta* **1077**, 86–90
3. Wilks, A., and Ortiz de Montellano, P. R. (1992) *J. Biol. Chem.* **267**, 8827–8833
4. Rao, S. I., Wilks, A., and Ortiz de Montellano, P. R. (1993) *J. Biol. Chem.* **268**, 803–809
5. Xu, Y., Asghar, A., Gray, J. I., Pearson, A. M., Haug, A., and Grulke, E. A. (1990) *J. Agric. Food Chem.* **38**, 1494–1497
6. Hong, S.-J., and Piette, L. H. (1989) *Korean Biochem. J.* **22**, 196–201
7. Tew, D., and Ortiz de Montellano, P. R. (1988) *J. Biol. Chem.* **263**, 17880–17886
8. Davies, M. J. (1990) *Free Radical Res. Commun.* **10**, 361–370
9. Kelman, D. J., and Mason, R. P. (1992) *Free Radical Res. Commun.* **16**, 27–33
10. Miki, H., Harada, K., Yamazaki, I., Tamura, M., and Watanabe, H. (1989) *Arch. Biochem. Biophys.* **275**, 354–362
11. Galaris, D., Sevanian, A., Cadenas, E., and Hochstein, P. (1990) *Arch. Biochem. Biophys.* **281**, 163–169
12. Gutteridge, J. M. C., and Halliwell, B. (1990) *Trends Biochem. Sci.* **15**, 129–135
13. Sinclair, A. J., Barnett, A. H., and Lunec, J. (1990) *Br. J. Hosp. Med.* **43**, 334–344
14. Grisham, M. B. (1985) *J. Free Radicals Biol. Med.* **1**, 227–232
15. Ambrosio, G., Santoro, G., Tritto, I., Elia, P. P., Duilio, C., Basso, A., Scognamiglio, A., and Chiariello, M. (1992) *Am. J. Physiol.* **262**, H23–H30
16. Ortiz de Montellano, P. R., and Catalano, C. E. (1985) *J. Biol. Chem.* **260**, 9265–9271
17. Catalano, C. E., Choe, Y. S., and Ortiz de Montellano, P. R. (1989) *J. Biol. Chem.* **264**, 10534–10541
18. Gibson, J. F., Ingram, D. J. E., and Nicholls, P. (1958) *Nature* **181**, 1398–1399
19. Harada, K., and Yamazaki, I. (1987) *J. Biochem. (Tokyo)* **101**, 283–286
20. King, N. K., and Winfield, M. E. (1963) *J. Biol. Chem.* **238**, 1520–1528
21. King, N. K., Looney, F. D., and Winfield, M. E. (1967) *Biochim. Biophys. Acta* **133**, 65–82
22. Roberts, J. E., Hoffman, B. M., Rutter, R., and Hager, L. P. (1981) *J. Biol. Chem.* **256**, 2118–2121
23. DeGray, J. A. (1987) *Computer Simulations as an Aid to ESR Powder Pattern Analysis of Some Organocobalt and Organopalladium Compounds*. Ph.D. thesis, Brown University, Providence, RI
24. Rieger, P. H. (1982) *J. Magn. Reson.* **50**, 485–489
25. Low, W. (1957) *Phys. Rev.* **105**, 801–805
26. Sevilla, M. D., Becker, D., and Yan, M. (1990) *Int. J. Radiat. Biol.* **57**, 65–81
27. Vanderkooi, J. M., Maniara, G., Green, T. J., and Wilson, D. F. (1987) *J. Biol. Chem.* **262**, 5476–5482
28. Romero, F. J., Ordoñez, I., Arduini, A., and Cadenas, E. (1992) *J. Biol. Chem.* **267**, 1680–1688
29. Ingold, K. U. (1969) *Acc. Chem. Res.* **2**, 1–9
30. Dimmey, L. J., and Gordy, W. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77**, 343–346
31. DeGray, J. A., Lassmann, G., Curtis, J. F., Kennedy, T. A., Marnett, L. J., Eling, T. E., and Mason, R. P. (1992) *J. Biol. Chem.* **267**, 23583–23588