Free Rad. Res. Comms., Vol. 16, No. 1, pp. 27–33
Reprints available directly from the publisher
Photocopying permitted by license only

THE MYOGLOBIN-DERIVED RADICAL FORMED ON REACTION OF METMYOGLOBIN WITH HYDROGEN PEROXIDE IS NOT A TYROSINE PEROXYL RADICAL

DAVID J. KELMAN‡ and RONALD P. MASON

Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709, USA

(Received April 29, 1991; in final form October 24, 1991)

The reductive cleavage of hydrogen peroxide by metmyoglobin produces a protein-derived, motionally restricted free radical detectable by the spin-trapping EPR technique. In order to determine if the detected radical was a peroxyl radical, $^{17}O_2$ and anoxic conditions were employed. The EPR spectra of the metmyoglobin-derived radical adduct detected under nitrogen incubations were identical to those of the oxygenated systems in both intensity and form. No additional hyperfine couplings were detected in the EPR spectrum when $^{17}O_2$ was used. Both of these results indicate that a peroxyl radical derived from molecular oxygen was not found. Additionally, spectra of spin trapped metmyoglobin from four different mammalian species were examined. No significant difference was seen among any of the species, even though one of the species, sperm whale, has one more tyrosine residue than the others.

KEY WORDS: EPR, metmyoglobin, spin trapping, peroxyl radical, hydrogen peroxide.

INTRODUCTION

The peroxidase activity of metmyoglobin has been known for some time. Early interest was centered around identifying the nature of two oxidative equivalents necessary for the catalysis.[1–4] Later, interest was renewed when evidence showed that hemoproteins which possess peroxidase activity can initiate lipid peroxidation and, hence, be involved in a number of human diseases as well as the aging process.[5–7] From both the older and more recent EPR experiments, the protein radical is thought to be centered on a tyrosine residue.[2–4,8] Recently, it has been suggested that molecular oxygen plays an important role is this peroxidase activity.[9] Furthermore, it has been proposed that the radical that is trapped by the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) is a peroxyl radical derived from molecular oxygen which had reacted with a tyrosyl radical of the globin[10,11]. However, the reaction of molecular oxygen and tyrosyl radical has been shown to be a very slow process relative to the dimerization of tyrosyl radical.[12,13] The purpose of this study was to determine whether the free radical trapped by DMPO is a peroxyl radical derived from metmyoglobin, as has been proposed.[10,11] To test this, three sets of experiments were performed to determine the effects of oxygen on the metmyoglobin-hydrogen peroxide system. The first experiment was to compare the spectra obtained under

‡To whom the correspondence should be addressed.

oxygenated and anoxic conditions. If molecular oxygen is essential for formation of the radical, then little or no signal would be detected in the anoxic incubations. In the second experiment, the metmyoglobin-hydrogen peroxide reaction was carried out in the presence of $^{17}O_2$. If a peroxyl radical adduct were formed, additional hyperfine couplings from the isotopically labelled oxygen should be observed.[13] Third, oxygen uptake was also determined for the system, as formation of a peroxyl radical utilizing molecular oxygen would require oxygen consumption. In addition, the possibility that metmyoglobin from different mammalian species would give distinguishably different EPR spectra of their DMPO radical adducts was investigated. It is known that horse, dog and sheep metmyoglobin have but two tyrosine residues, while sperm whale metmyoglobin has a third tyrosine located on the globin surface.[12] If the signal detected by spin trapping with DMPO were indeed due to a tyrosyl radical, there might be a difference between the spectrum from sperm whale metmyoglobin and that of other species due to the greater motional freedom of the third tyrosine of the sperm whale metmyoglobin.

MATERIALS AND METHODS

Horse heart myoglobin, recombinant sperm whale metmyoglobin, sheep skeletal muscle metmyoglobin, dog skeletal muscle metmyoglobin, D-glucose, DMPO and hydrogen peroxide were obtained from Sigma. Glucose oxidase (250 units/mg, 1 unit converts 1 μmole glucose to product in 1 minute at 25°C and pH 7) was purchased from Boehringer Mannheim (Indianapolis, IN). O_2 enriched 51% with $^{17}O_2$ was obtained from ICON Services Inc. (Summit, NJ). The DMPO was further purified before use by vacuum distillation.

All EPR spectra were recorded in an aqueous flat cell at room temperature on a Varian E-109 spectrometer equipped with a TM_{110} cavity. Anoxic and $^{17}O_2$ experiments were initiated by the addition of hydrogen peroxide and aspirated into the flat cell by use of a rapid sampling device.[14] Oxygen uptake measurements were made at room temperature with a Yellow Springs Instrument Company oxygen monitor equipped with a Clark electrode (Model 53).

All incubations contained 100 mM sodium phosphate buffer and 1 mM DTPA to give a final pH of 7.0 and a final volume of 2 ml. The concentration of metmyoglobin was 266 μM, the hydrogen peroxide was 200 μM and that of the DMPO was 45 mM. An anoxic incubation was obtained by bubbling with nitrogen for five minutes in a closed-septum vial. The anoxic incubations containing glucose oxidase (1 mg/ml, 250 U/ml) and glucose (60 mM), an oxygen purging system, were bubbled with nitrogen prior to the addition of glucose. The incubation was allowed to preincubate for 30 seconds so that any remaining molecular oxygen would be consumed, after which the hydrogen peroxide was added. The experiments with $^{17}O_2$ were performed by first bubbling with nitrogen gas in a vial closed with a rubber septum for five minutes in order to eliminate as much atmospheric $^{16}O_2$ as possible, then bubbled with $^{17}O_2$ for 3 minutes. Control incubations for both the anoxic and $^{17}O_2$ experiments were bubbled with normal oxygen for five minutes.

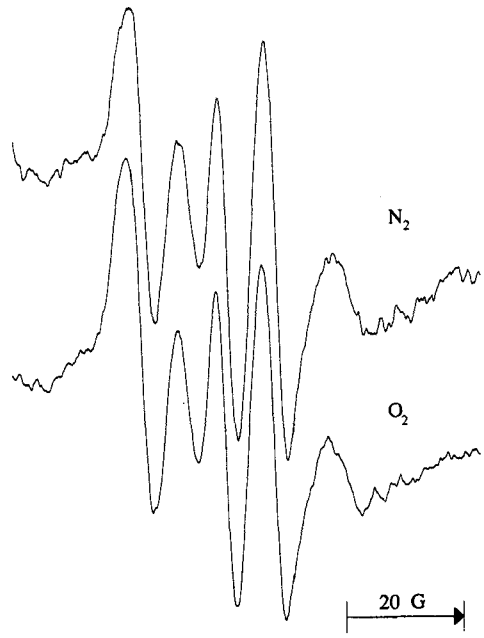

FIGURE 1 The top spectrum is obtained from an incubation of metmyoglobin, DMPO and hydrogen peroxide bubbled with nitrogen gas for five minutes before the addition of the hydrogen peroxide. The lower spectrum is a control experiment where oxygen gas was bubbled for five minutes before initiation with hydrogen peroxide. Instrument settings were: microwave power, 40 mW; modulation amplitude, 2.0 G; receiver gain 1.6×10^4; scan time, 4 min.; and scan range, 80 G.

RESULTS

Anoxic Incubations

The oxygenated and anoxic incubations both gave the five-line spectrum (Figure 1) characteristic of DMPO/metmyoglobin radical adduct.[10,15,16] The spectrum is broad and indicative of a partially immobilized species, as would be expected of a protein-bound radical adduct. Davies postulated that this species is a peroxyl radical, based upon hyperfine parameters of $a^N = 14.5$ G and $a^H_\beta = 8.3$ G.[10] However, it is known that steric factors due to the trapped radical strongly affect the hyperfine coupling constant of the β hydrogen of a DMPO radical adduct.[17] Given the size of the radical trapped by DMPO in this case, it would be difficult to assign these parameters to any particular species. The amplitude of the spectra revealed that there was no significant difference between the anoxic and oxygenated systems, $p > 0.90$ ($n = 5$). No reproducible differences in the shape of the spectra or intensity of any particular lines could be observed (Figure 1).

$^{17}O_2$ Incubations

The $^{17}O_2$ incubations produced results similar to the $^{16}O_2$ trials (Figure 2A, B). Since ^{17}O has a nuclear spin of 5/2, a peroxyl radical adduct would give a six-line signal for every line present in the original experiment. Although many lines would be overlapped

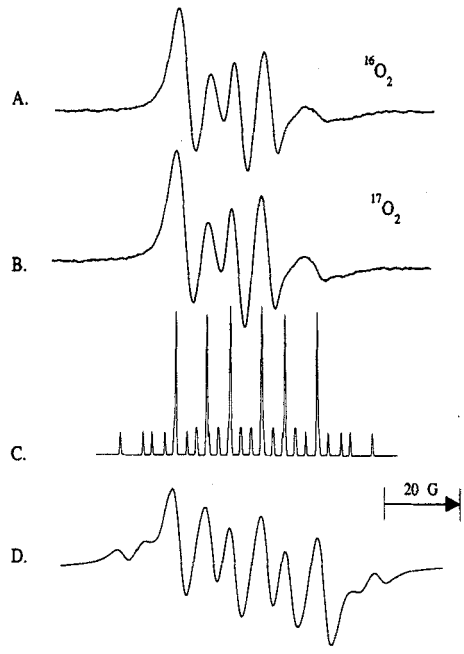

FIGURE 2 (A) Spectrum from incubation of metmyoglobin, DMPO and hydrogen peroxide after bubbling with $^{16}O_2$ for 3 minutes. (B) Same as A, but bubbled with 51% enriched $^{17}O_2$ for 3 minutes. (C) Stick diagram simulation of radical adduct containing 51% enriched $^{17}O_2$ (I = 5/2) with a^N = 14.5 G, a_β^H = 8.3 G and $a^{17}O$ = 5.9 G. (D) Simulation using same parameters as in C, with 3 G line width and 100% Lorentzian line shape.

and unresolved, several lines should be evident in the low field portion of the spectrum.[18] The epoxidizing species of metmyoglobin, proposed to be a peroxyl radical, has been demonstrated to be derived from molecular oxygen rather than from hydrogen peroxide.[9] If the radical species trapped was a peroxyl radical, and given the 51% $^{17}O_2$ enrichment, then the low field portion of the spectrum would resemble the low field portion of the simulation shown in Figure 2D. However, no additional hyperfine couplings in this region were observed (Figure 2B). In the hypothetical stick diagram (Figure 2C) and simulation, $a^{17}O$ was assumed to equal that of DMPO/$^{\cdot 17}$OOH.[18]

Species Differences

The spectra obtained from horse, sperm whale, sheep and dog metmyoglobins were remarkably similar (Figure 3). There is no noticeable difference between the EPR spectra of different metmyoglobin species in either intensity or shape. This is interesting in light of the findings by direct EPR studies that there are two distinguishable tyrosine-derived radical species present in the oxidized sperm whale metmyoglobin, but only one from horse metmyoglobin.[8] That there is no difference between horse, dog and sheep is not surprising, as the myoglobins of those species have only two tyrosines at positions 103 and 146. Structural determination has shown that sperm whale metmyoglobin has three tyrosines, the two at 103 and 146 as well as one at

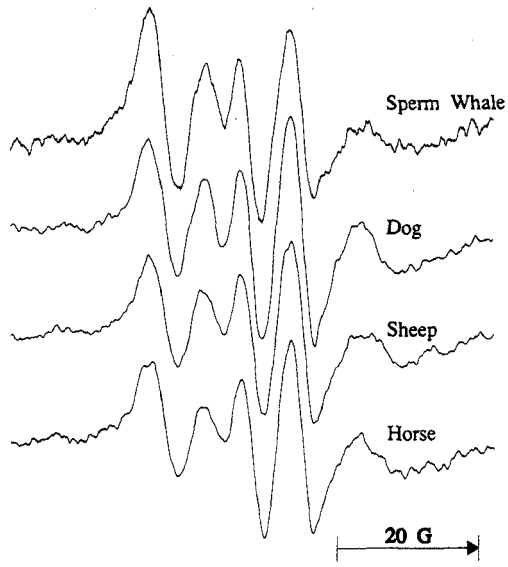

FIGURE 3 Spectra from incubations of metmyoglobin from sperm whale, dog, sheep and horse.

position 151.[19] This third tyrosine is at the surface of the globin[12] and thus is accessible to the spin trap. Miki *et al.*[8] demonstrated that the tyrosine at position 151 of sperm whale metmyoglobin does generate a distinguishable free radical upon reaction of the protein with hydrogen peroxide, and that the signal can be removed by the addition of tetranitromethane, which selectively removes tyrosine 151. Hence, the fact that the same signal that is seen from horse metmyoglobin, which does not possess a tyrosine at position 151, and sperm whale metmyoglobin calls into question whether the species that is trapped by DMPO is, in fact, a tyrosyl radical at all. No direct evidence that the metmyoglobin-derived radical adduct is due to the trapping of a tyrosyl has been presented as yet.

Oxygen Uptake

Metmyoglobin has a considerable catalase-like activity,[9] generating molecular oxygen in the presence of hydrogen peroxide (Figure 4A). The oxygen uptake studies under anoxic conditions (Figure 4B) demonstrate that not only is oxygen evolved from this system, but is also consumed as would be consistent with peroxyl radical formation of some type. In order to ensure that the anoxic EPR experiments were as oxygen-free as possible, glucose oxidase and glucose were added. Glucose oxidase oxidizes D-glucose to D-gluconic acid, while concomitantly reducing O_2 to H_2O_2. Adding glucose oxidase and D-glucose to an incubation of metmyoglobin and hydrogen peroxide removes all detectable molecular oxygen from the solution (Figure 4C). Even when additional hydrogen peroxide is added, there is no net molecular oxygen produced. When glucose oxidase and glucose were added to incubations that had already been bubbled with nitrogen, comparison of the EPR spectra from these incubations against those from an oxygenated system showed no significant difference in the peak-to-peak amplitudes, $p > 0.3$ ($n = 6$).

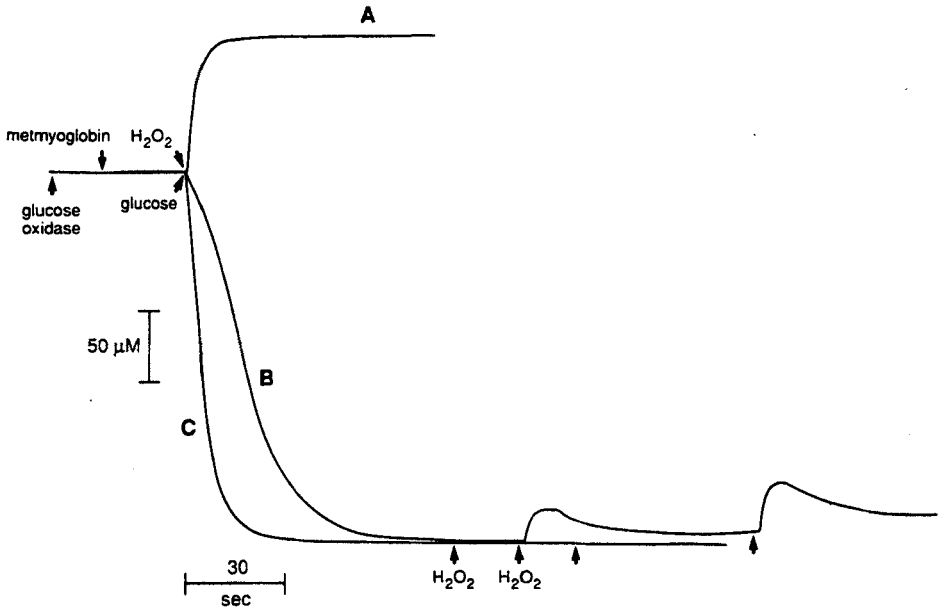

FIGURE 4 Oxygen consumption graphs of metmyoglobin incubations. (A) Metmyoglobin and hydrogen peroxide alone. (B) Same as A, but hydrogen peroxide was added after bubbling with N_2. (C) Same as A, but hydrogen peroxide was added after the incubation has been made anoxic by the addition of glucose oxidase and glucose. Note that in C, the incubation remains anoxic even after the addition of hydrogen peroxide, while in B there is detectable oxygen evolution after the addition of hydrogen peroxide.

DISCUSSION

The anoxic incubations, especially those in the presence of the glucose oxidase-glucose system, support the contention that the radical is not a peroxyl radical derived from molecular oxygen. Water, at room temperature and standard atmospheric pressure, contains oxygen of approximately 268 μM. Vanderkooi *et al.*[20] have demonstrated that the glucose oxidase-glucose system is capable of lowering the concentration of dissolved oxygen to less than 10 nM. Although catalase is a contaminant of glucose oxidase (2–3 units/mg glucose oxidase) and metmyoglobin itself possesses a catalase-like activity, hydrogen peroxide levels are maintained by glucose oxidase, which generates hydrogen peroxide concomitantly with the oxidation of glucose and the consumption of molecular oxygen. The low concentration of molecular oxygen in our anoxic incubations implies that a peroxyl radical derived from molecular oxygen was not trapped by DMPO. Since a nitrogen-centered radical would probably give a resolvable hyperfine coupling from the nitrogen, and metmyoglobin does not contain sulphydryl groups, a carbon-centered radical is most likely the metmyoglobin-derived species trapped by DMPO, but the structure of the species is yet to be determined.

References

1. J.F. Gibson, D.J.E. Ingram and P. Nicholls (1958) Free Radical produced in the Reaction of Metmyoglobin with Hydrogen Peroxide. *Nature*, **181,** 1398–1399.
2. N.K. King and M.E. Winfield (1963) The Mechanism of Metmyoglobin Oxidation. *Journal of Biological Chemistry*, **238,** 1520–1528.
3. N.K. King, F.D. Looney and M.E. Winfield (1967) Amino Acid Free Radicals in Oxidised Metmyoglobin. *Biochimica et Biophysica Acta*, **133,** 65–82.

4. K. Harada and I. Yamazaki (1987) Electron Spin Resonance of Free Radicals Formed in the Reaction of Metmyoglobins with Ethylhydroperoxide. *Journal of Biochemistry*, **101,** 283–286.
5. D. Galaris, A Sevanian, E. Cadenas and P. Hochstein (1990) Ferrylmyoglobin-Catalyzed Linoleic Acid Peroxidation. *Archives of Biochemistry and Biophysics*, **281,** 163–169.
6. J.M.C. Gutteridge and B. Halliwell (1990) The Measurement and Mechanism of Lipid Peroxidation in Biological Systems. *Trends in Biochemical Science*, **15,** 129–135.
7. A.J. Sinclair, A.H. Barnett and J. Lunec (1990) Free Radicals and Antioxidant Systems in Health and Disease. *British Journal of Hospital Medicine*, **43,** 334–344.
8. H. Miki, K. Harada, I. Yamazaki, M. Tamura and H. Watanabe (1989) Electron Spin Resonance Spectrum of Tyr-151 Free Radical Formed in Reactions of Sperm Whale Metmyoglobin with Ethyl Hydroperoxide and Potassium Irridate. *Archives of Biochemistry and Biophysics*, **275,** 354–362.
9. P.R. Ortiz de Montellano and C.E. Catalano (1985) Epoxidation of Styrene by Hemoglobin and Myoglobin. *Journal of Biological Chemistry*, **260,** 9265–9271.
10. M.J. Davies (1990) Detection of Metmyoglobin-Derived Radicals on Reaction of Metmyoglobin with Hydrogen Peroxide and Other Peroxidic Compounds. *Free Radical Research Communications*, **10,** 361–370.
11. M.J. Davies (1991) Identification of a Globin Free Radical in Equine Myoglobin Treated with Peroxides. *Biochimica et Biophysica Acta*, **1077,** 86–90.
12. D. Tew and P.R. Ortiz de Montellano (1988) The Myoglobin Protein Radical. *Journal of Biological Chemistry*, **263,** 17880–17886.
13. E.P.L. Hunter, M.F. Desrosiers and M.G. Simic (1989) The Effect of Oxygen, Antioxidants, and Superoxide Radical on Tyrosine Phenoxyl Radical Dimerization. *Free Radicals in Biology & Medicine*, **6,** 581–585.
14. R.P. Mason (1984) Assay of *in Situ* Radicals by Electron Spin Resonance. *Methods in Enzymology*, **105,** 416–422.
15. S.-J. Hong and L.H. Piette (1989) Electron Spin Resonance Studies of Spin-Trapped Free Radicals Produced by Reaction of Metmyoglobins with Hydrogen Peroxide. *Korean Biochemistry Journal*, **22,** 196–201.
16. Y. Xu, A. Asghar, J.I. Gray, A.M. Pearson, A. Haug and E.A. Grulke (1990) ESR Spin-Trapping Studies of Free Radicals Generated by Hydrogen Peroxide Activation of Metmyoglobin. *Journal of Agriculture and Food Chemistry*, **38,** 1494–1497.
17. E.G. Janzen (1971) Spin Trapping. *Accounts in Chemical Research*, **4,** 31–40.
18. C. Mottley, H. Connor and R.P. Mason (1986) [^{17}O]-Oxygen Hyperfine Structure for the Hydroxyl and Superoxide Radical Adducts of the Spin Traps DMPO, PBN and 4-POBN. *Biochemistry and Biophysics Research Communications*, **141,** 622–628.
19. T. Takano (1977) Structure of Myoglobin Refined at 2-0 Å Resolution. I. Crystallographic Refinement of Metmyoglobin from Sperm Whale. *Journal of Molecular Biology*, **110,** 537–568.
20. J.M. Vanderkooi, G. Maniara, T.J. Green and D.F. Wilson (1987) An Optical Method for Measurement of Dioxygen Concentration Based upon Quenching of Phosphorescence. *Journal of Biological Chemistry*, **262,** 5476–5482.

Accepted by Dr. E. Janzen