Peroxidation of a Specific Tryptophan of Metmyoglobin by Hydrogen Peroxide*

Janice A. DeGray, Michael R. Gunther†, Richard Tschirret-Guth§, Paul R. Ortiz de Montellano¶, and Ronald P. Mason

From the Laboratory of Pharmacology and Chemistry, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina 27709 and the Departments of Pharmaceutical Chemistry and Pharmacology, University of California, San Francisco, California 94143-0446

Globin-centered radicals at tyrosine and tryptophan residues and a peroxyl radical at an unknown location have been reported previously as products of the reaction of metmyoglobin with hydrogen peroxide. The peroxyl radical is shown here to be localized on tryptophan through the use of recombinant sperm whale myoglobin labeled with 13C at the indole ring C-3. Peroxyl radical formation was not prevented by site-directed mutations that replaced all three tyrosines, the distal histidine, or tryptophan 7 with non-oxidizable residues. In contrast, mutation of tryptophan 14 prevents peroxyl radical formation, implicating tryptophan 14 as the specific site of the peroxidation.

Hydrogen peroxide is the most stable of the reactive species produced by the partial reduction of molecular oxygen. It has been shown to have adverse effects upon the heart (1–3), and its production has been clearly demonstrated when a previously ischemic heart was reperfused with oxygen-containing perfusate (4). Possible targets for acute damage by hydrogen peroxide in the reperfused heart include membrane lipids, which maintain the structural integrity of the cells, and the proteins that are responsible for all cellular functions.

Oxidative damage to proteins has been clearly demonstrated in reactions with peroxides (5). Such oxidative damage has been detected in the form of a greatly increased carbonyl content of the proteins when exposed to peroxides in the presence of transition metal ions (6). Oxidative protein damage that is independent of free transition metal ions, which are scarce in vivo, is potentially of equal importance. However, despite the growing body of evidence for this type of oxidative damage (7–9), the specific sites at which the damage occurs have not been identified. It is likely that within the relatively ordered structures of proteins there are specific sites that are more susceptible to oxidative damage than others.

Myoglobin, a protein present at near millimolar concentrations in cardiac myocytes, undergoes a relatively well studied reaction with hydrogen peroxide, a reaction that does not require catalysis by trace transition metals. Hydrogen peroxide oxidizes ferrous myoglobin (10, 11) and metmyoglobin to form an oxy-ferryl intermediate (12). The second oxidizing equivalent required for the reduction of hydrogen peroxide to water by metmyoglobin can be accounted for by the oxidation of the globin structure, resulting in the formation of a protein-centered radical (13, 14). Subsequent studies have demonstrated that the globin-centered radical reacts with oxygen to form a peroxyl radical (15) and have shown that a radical centered on a tryptophan residue can be spin-trapped (7). However, tyrosine-centered radicals have also been shown to be formed by ESR (16) and protein cross-linking studies (17, 18). Despite evidence that the peroxyl radical is not centered on a tyrosine residue (19), the residue on which it is located is still unknown. In the present study we have prepared site-directed mutants of sperm whale myoglobin in which the two conserved tryptophan residues have been selectively labeled with 13C or replaced singly or doubly with phenylalanine. We have examined the reactions of these mutant proteins with hydrogen peroxide using ESR spectroscopy to determine the site of peroxyl radical formation.

MATERIALS AND METHODS

Site-directed Mutant Protein Preparation and Expression—L-[indole-3-13C]Tryptophan-labeled myoglobin was prepared by transformation of Escherichia coli strain K125P044re/EA1, thr-1 trp/Tn10) (20) with pUC18-Mb DNA (19). Myoglobin was expressed by overnight fermentation at 37 °C in Moore’s medium supplemented with d-aminolevulinic acid (50 μM), ampicillin (200 μg/ml), biotin (2 μg/ml), nicotinamide (2 μg/ml), riboflavin (0.2 μg/ml), thiamine (2 μg/ml), L-[3-13C]tryptophan (37 μg/ml) (Cambridge Isotope Laboratories), and all 17 nonaromatic amino acids (400 μg/ml each). After 16 h, the medium was further supplemented with a carbon source (glucose), and the fermentation was allowed to proceed for an additional 6 h. The protein was then purified as described (19). Site-directed mutations were constructed by cassette mutagenesis as described previously (19, 21). For the W7F mutant, the cassette was designed to include the phenylalanine substitution and a new silent EcoRI restriction site for rapid screening and was inserted between the NdeI and SpeI restriction sites. For the W14F mutant, the cassette was designed to include the phenylalanine substitution and a new DdeI restriction site and was inserted between the SpeI and Sfl restriction sites. The W7F/W14F double mutant was constructed using the W7F mutant plasmid as vector and the cassette designed for the construction of the W14F mutant. All the mutant plasmids were sequenced to confirm the codon changes. The mutant proteins were purified as described for the wild-type protein (19, 21). In all of the myoglobin tyrosine and tryptophan mutants examined, the UV-visible spectra observed were nearly identical to those of the native protein in both the ferrous dioxy and ferric oxidation states, indicating that any changes to the structure surrounding the heme iron were minimal (data not shown).

ESR Spectroscopy—All of the bacterially expressed myoglobins were oxidized to the met form by addition of a slight excess of KFe(CN)6, and were passed over a prepoured Sephadex G-25 column and eluted with 50 mM sodium phosphate buffer, pH 7.4. Metmyoglobin concentrations were determined from their visible spectra using an extinction coefficient of 3.3 molar−1 cm−1 (22). Samples were prepared for ESR experiments by addition of hydrogen peroxide to a final concentration of 0.9 times that of the ferric heme of the myoglobin and were frozen within 8 s in liquid nitrogen. Spectra were recorded at 77 K in a fingertip Dewar inserted into the TM110 cavity of a Bruker ESP 300 ESR spec-

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‡ To whom correspondence should be addressed.

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In previous attempts to determine the site of the protein-centered radical, site-directed mutant proteins were prepared in which one, two, or all three of the tyrosine residues were replaced with phenylalalanines and in which the distal histidine, which is very near the heme iron center, was replaced by a valine, and their ESR spectra were recorded (19, 21). Spectral simulations were calculated using the reported parameters with the Powfit program in the NIEHS public EPR software package, which is available over the Internet.

RESULTS AND DISCUSSION

In previous attempts to determine the site of the protein-centered radical, site-directed mutant proteins were prepared in which one, two, or all three of the tyrosine residues were replaced with phenylalalanines and in which the distal histidine, which is very near the heme iron center, was replaced by a valine, and their ESR spectra were recorded (19, 21). The reported spectra, however, were not uniformly consistent with the simulated spectrum for a peroxyl radical (15, 25). In order to demonstrate that the detected free radical in those proteins is a peroxyl radical, we have repeated the earlier experiments. As shown in Fig. 1, the ESR spectrum obtained for the site-directed mutant peroxyl radical in which the distal histidine has been replaced by valine (Fig. 1, spectrum D) also gives rise to a spectrum in which the low-field feature ($g = 2.037$) characteristic of a peroxyl radical is present, albeit weak, indicating that part of the signal detected in the distal histidine mutant is from a peroxyl radical. The relative intensities in the $g = 2.00$ and $g = 2.03$ regions of the spectrum obtained after oxidation of the H64V protein indicate that the spectrum is a composite of the spectra from a peroxyl radical and a second, more abundant free radical whose spectrum is more isotropic and relatively featureless as was confirmed by computer simulation (Fig. 1, spectra E–G). Relatively isotropic spectra similar to that of the H64V spectrum are observed from all of the recombinant myoglobins after they have been exposed to hydrogen peroxide in an anaerobic environment (data not shown). These results show that the majority of the peroxyl radical signal comes from residues other than the tyrosines and distal histidine.

Two experiments were performed to test the recent suggestion (7) that the globin-derived peroxyl radical is centered on a tryptophan residue. In the first experiment, the wild-type sperm whale myoglobin labeled with $^{13}$C (I = ½) at C-3 of the indole ring of tryptophan was incubated with hydrogen peroxide. The resulting ESR spectrum reveals additional hyperfine coupling to a nucleus with a spin of ½ in both the low-field extrema and the $g = 2.00$ region (Fig. 2, spectra A, A′, B, B′). The spectrum acquired from the $^{13}$C-labeled protein was simulated using the same $g$-tensor with additional hyperfine coupling to one nucleus with spin ½. The hyperfine coupling was nearly isotropic ($A_x = 4.1$, $A_y = 3.9$, and $A_z = 5.6$ G), as would be expected for an atom beta to the site of maximum spin density. This result is totally consistent with the formation of the peroxyl radical at C-3 of the indole ring of a tryptophan residue in myoglobin. Simulations of the low-field line of the $^{13}$C Mb spectrum that were calculated assuming a combination of $^{12}$C- and $^{13}$C-centered peroxyl radicals indicated that a maximum of 8% of the signal was centered on $^{13}$C with the rest coming from the labeled carbon (data not shown).

The second experiment involved oxidation of the site-directed mutant proteins in which either or both tryptophan residues were replaced with phenylalanine. Addition of hydrogen peroxide at a concentration equal to that of the heme iron to the tryptophan mutants had an unexpected result. When Trp-7 was replaced by phenylalanine, the resulting ESR spectrum was characteristic of a peroxyl radical (Fig. 2, spectra A and D). However, when Trp-14 was replaced by a phenylalanine, regardless of whether or not Trp-7 was present, the signal for a peroxyl radical was replaced by a singlet at $g = 2.004 ± 0.001$ (Fig. 2, spectrum E). This unassigned singlet is very similar to the spectrum acquired from all of the recombinant proteins studied under anaerobic conditions (data not shown). This result indicates that Trp-14 is absolutely required for the formation of a detectable protein-centered peroxyl radical.

Since there is very little evidence for a $^{12}$C-centered peroxyl radical in the low-field feature of the ESR spectrum of the $^{13}$C-labeled protein (Fig. 2, B′ compared with A′), the peroxyl radical observed is primarily, if not exclusively, centered on a tryptophan residue. Considering the $^{13}$C labeling result, there are two primary interpretations available for the data from the site-directed Trp-14 mutant proteins. First, both tryptophan residues form peroxyl radicals, but Trp-14 is critically involved in the transfer of electrons from Trp-7 to the oxidized heme center that is the immediate product of the reaction between metmyoglobin and hydrogen peroxide. In the second hypothesis, only Trp-14 forms the detected peroxyl radical.

The first hypothesis implies that the flow of electrons from
Trp-7 to the oxidized heme is modified by the replacement of Trp-14 by phenylalanine. Such modification could arise from a minor structural change that coincidentally changes the pathway from Trp-7 to the heme. An example of this is provided by the H64V myoglobin mutant. The ESR spectrum of that protein contains elements of the peroxyl radical as a minor species, which indicates that even major structural changes in the vicinity of the heme pocket such as those caused by the H64V mutation (24) do not completely eliminate the electron transfer pathway from the tryptophan residues. Since no detectable peroxyl radical is found in the Trp-14 mutants, even though they provide significantly greater overall spectral intensity when prepared at the same concentration as the H64V protein (data not shown), the structural change hypothesis seems unlikely.

Formation of peroxyl radicals on both tryptophan residues with detection of only the Trp-14 peroxyl radical could also arise from a great difference in the stability of the radical between the two sites. In that case, the globin structure could selectively stabilize the peroxyl radical at Trp-14 or selectively destabilize it at Trp-7. The half-life of the peroxyl radical formed on horse metMb has been shown to be 7 s (15). This measured half-life is very similar to that calculated for peroxyl radicals in aqueous solutions (25). Nevertheless, the formation of a peroxyl radical on Trp-7 too unstable to be detected cannot be discounted by the current data.

The second hypothesis is that the peroxyl radical is formed only on Trp-14. This implies that the transfer of electrons from Trp-7 to the heme is kinetically slow. Electron transfer studies involving myoglobins modified by attachment to the surface histidine residues of ruthenium complexes have demonstrated that there is no difficulty in electron transfer from the heme to any portion of the surface of myoglobin (26), indicating that the slightly greater distance from the heme iron to Trp-7 than to Trp-14 could not prevent electron transfer from occurring. Several studies have shown that the relative orientation of the electron donor and electron acceptor in multisite electron transfer proteins greatly affects the rates of the electron transfer (27–29). In particular, electron transfer is greatly slowed when the electron donor is orthogonal to the acceptor (29). Examination of the crystal structure of sperm whale metmyoglobin for the orientations of the two tryptophan residues relative to the heme iron shows that Trp-14 is nearly coplanar with the unsaturated heme system, while Trp-7 is nearly orthogonal to both Trp-14 and to the heme (Fig. 3; Ref. 30). This orientation, with the resulting alignment of the p-orbitals, suggests that Trp-14 would be the more likely reductant of the

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**Fig. 2.** ESR spectra of free radicals formed in site-directed tryptophan mutants of sperm whale metmyoglobin. Spectrum A, recombinant wild-type sperm whale myoglobin (80 μM) prepared as discussed in Fig. 1. Spectrum A’, an expanded scan of the low-field extrema of the peroxyl radical observed in the spectrum of the wild-type sperm whale metmyoglobin after reaction with hydrogen peroxide. Spectrum B, wild-type sperm whale metmyoglobin selectively labeled with 13C at C-3 of the indole rings of the two tryptophan residues (80 μM) after reaction with hydrogen peroxide. Spectrum B’, an expanded scale spectrum of the low-field extrema of the 13C-labeled myoglobin after reaction with hydrogen peroxide. Note the clearly observed minimum in the low-field extrema due to partial resolution of (I = 1/2). Spectrum C, computer simulation of spectrum B (13C-labeled Mb) calculated with the following parameters: g_x = 2.037, g_y = 2.009, and g_z = 2.003 and the hyperfine coupling tensor for the 13C atom: A_x = 4.53, A_y = 4.15, A_z = 5.89 G. Spectrum C’, computer simulation of the low-field line shown in spectrum B’ calculated using the same parameters as used for spectrum C. Spectrum D, recombinant sperm whale metmyoglobin W7F (127 μM) after reaction with hydrogen peroxide. The scale in this spectrum is 0.1 times that in the other spectra. Spectrum E, the spectrum obtained from recombinant sperm whale W14F (127 μM) after reaction with hydrogen peroxide. Note the total absence of the low-field extrema characteristic of a peroxyl free radical.

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**Fig. 3.** Relative orientations of the tryptophan residues and the heme in the crystal structure of sperm whale metmyoglobin. The image was derived from the crystal structure (30). The heme center is red and green, and the two tryptophan residues are yellow. The van der Waals surface of the remainder of the protein is shown in the white dots. This view was selected to emphasize the orthogonal arrangement of Trp-7 to the nearly coplanar Trp-14 and heme.
heme. Interestingly, the relative orientations of the tyrosine residues (Tyr-103 and Tyr-151) that have been shown to be oxidized to phenoxyl radicals by ESR (16) and by cross-linking studies (17, 18) are also roughly coplanar to the heme (data not shown).

The present results provide evidence for peroxidation of a specific site of metmyoglobin during its reaction with hydrogen peroxide. The specificity of the peroxidation demonstrates that the protein structure can have a significant effect upon the sites that can be oxidatively damaged by more discriminating oxidants than hydroxyl radical, which will damage virtually any biological material that it encounters. The data strongly suggest that only one site on sperm whale metmyoglobin forms a detectable peroxyl radical, and that site is Trp-14. The basis for this specificity cannot be unambiguously determined from the present data, but the relative orientations of the heme and the indole rings of the two tryptophan residues may promote, through kinetic control, exclusive radical formation on Trp-14.

REFERENCES

1. Okabe, E., Takahashi, S., Norisue, M., Manson, N. H., Kukreja, R. C., Hess, M. L., and Ito, H. (1993) *Eur. J. Pharmacol.* 248, 33–39
2. Huang, H-S., Stahl, G. L., and Longhurst, J. C. (1995) *Am. J. Physiol.* 268, H2114–H2124
3. Huang, H-S., Pan, H-L., Stahl, G. L., and Longhurst, J. C. (1995) *Am. J. Physiol.* 269, H888–H901
4. Slezak, J., Tribulova, N., Pristacova, J., Uhrík, B., Thomas, T., Khaper, N., Kaul, N., and Singal, P. K. (1995) *Am. J. Pathol.* 147, 772–781
5. Stadtman, E. R. (1990) *Free Radical Biol. Med.* 9, 315–325
6. Fucci, L., Oliver, C. N., Coon, M. J., and Stadtman, E. R. (1983) *Proc. Natl. Acad. Sci. U. S. A.* 80, 1521–1525
7. Gunther, M. R., Kelman, D. J., Corbett, J. T., and Mason, R. P. (1995) *J. Biol. Chem.* 270, 16075–16081
8. Barr, D. P., Gunther, M. R., Deterding, L. J., Tomer, K. B., and Mason, R. P. (1996) *J. Biol. Chem.* 271, 15498–15503
9. Svistunenko, D. A., Patel, R. P., and Wilson, M. T. (1996) *Free Radical Res.* 24, 269–280
10. Whitburn, K. D. (1987) *Arch. Biochem. Biophys.* 253, 419–430
11. Yusa, K., and Shikama, K. (1987) *Biochemistry* 26, 6684–6688
12. George, P., and Irving, D. H. (1952) *Biochem. J.* 52, 511–517
13. Gibson, J. F., Ingram, D. J. E., and Nicholls, P. (1958) *Nature* 181, 1398–1399
14. King, N. R., and Winfield, M. E. (1963) *J. Biol. Chem.* 238, 1520–1528
15. Kelman, D. J., DeGray, J. A., and Mason, R. P. (1994) *J. Biol. Chem.* 269, 7458–7463
16. Miki, H., Harada, K., Yamazaki, I., Tamura, M., and Watanabe, H. (1989) *Arch. Biochem. Biophys.* 275, 354–362
17. Tew, D., and Ortiz de Montellano, P. R. (1988) *J. Biol. Chem.* 263, 17880–17886
18. Catalano, C. E., Choe, Y. S., and Ortiz de Montellano, P. R. (1989) *J. Biol. Chem.* 264, 10554–10561
19. Wilks, A., and Ortiz de Montellano, P. R. (1992) *J. Biol. Chem.* 267, 8827–8833
20. Low, B. (1968) *Proc. Natl. Acad. Sci. U. S. A.* 60, 160–167
21. Rao, S. I., Wilks, A., and Ortiz de Montellano, P. R. (1993) *J. Biol. Chem.* 268, 803–809
22. Antonini, E., and Brunori, M. (1971) *Hemoglobin and Myoglobin in their Reactions with Ligands*, North-Holland Publishing Co., Amsterdam
23. Kevan, L., and Schlick, S. (1986) *J. Phys. Chem.* 90, 2008–2007
24. Rizzi, M., Bolognesi, M., Coda, A., Cutruzzola, F., Allocatelli, C. T., Brancacchio, A., and Brunori, M. (1993) *FEBS Lett.* 320, 13–16
25. Pryor, W. A. (1986) *Annu. Rev. Physiol.* 48, 657–667
26. Mayo, S. L., Ellis, W. R., Jr., Crutchley, R. J., and Gray, H. B. (1986) *Science* 233, 948–952
27. Siders, P., Cave, R. J., and Marcus, R. A. (1984) *J. Chem. Phys.* 81, 5613–5624
28. Cave, R. J., Klippenstein, S. J., and Marcus, R. A. (1986) *J. Chem. Phys.* 84, 3089–3096
29. Makinen, M. W., Schichman, S. A., Hill, S. C., and Gray, H. B. (1983) *Science* 222, 929–931
30. Watson, H. C. (1969) *Prog. Stereochem.* 4, 299–333