Site-specific spin trapping of tyrosine radicals in the oxidation of metmyoglobin by hydrogen peroxide

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The reaction between metmyoglobin and hydrogen peroxide produces both a ferryl-oxo heme and a globin-centred radical(s) from the two oxidizing equivalents of the hydrogen peroxide. Evidence has been presented for localization of the globin-centred radical on one tryptophan residue and tyrosines 103 and 151. When the spin-trapping agent 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) is included in the reaction mixture, a radical adduct has been detected, but the residue at which that adduct is formed has not been determined. Replacement of either tryptophans 7 and 14 or tyrosines 146 and 151 with phenylalanine has no effect on the formation of DMPO adduct in the reaction with hydrogen peroxide. When tyrosine 103 is replaced with

phenylalanine, however, only DMPOX, a product of the oxidation of the spin-trap, is detected. Tyrosine-103 is, therefore, the site of radical adduct formation with DMPO. The spin trap 2-methyl-2-nitrosopropane (MNP), however, forms radical adducts with any recombinant sperm whale metmyoglobin that contains either tyrosine 103 or 151. Detailed spectral analysis of the DMPO and MNP radical adducts of isotopically substituted tyrosine radical yield complete structural determinations. The multiple sites of trapping support a model in which the unpaired electron density is spread over a number of residues in the population of metmyoglobin molecules, at least some of which are in equilibrium with each other.

INTRODUCTION

Heme proteins such as hemoglobin and myoglobin reduce hydrogen peroxide to water with the concomitant formation of a ferryl-oxo heme and, in the case of the ferric forms of the proteins, a globin-centred free radical. The ferryl-oxo heme species is stable for hours at room temperature [1]. The globin-centred free radical undergoes subsequent chemistry, reacting with oxygen to form a peroxy radical or oxidizing external substrates including biochemical reductants, oxidizable drugs, and, potentially, other proteins [2–6]. Cross-links have been detected for sperm whale myoglobin between tyrosine 103 of one globin and tyrosine 151 of another and also between tyrosine 103 and the heme, providing evidence for unpaired electron density at both of those tyrosine residues [3,4].

The formation of the globin-centred radical in the reaction between metmyoglobin and hydrogen peroxide has been demonstrated through direct electron spin resonance (ESR) spectroscopy [7,8] and also through spin-trapping, using 3,5-dibromo-4-nitrosobenzenesulphonic acid (DBNBS) [9], 2-methyl-2-nitrosopropane (MNP) [9,10], and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) [2,11,12]. DBNBS and oxygen have been shown to trap a tryptophan-centred radical [9,13], but the residue at which DMPO forms a radical adduct has not been conclusively identified. Originally, this DMPO adduct was assigned to a peroxy radical at Tyr-103 [12,14–16], but the adduct is formed in the absence of molecular oxygen [17], and, in fact, competition

between DMPO and oxygen for radical site(s) on metmyoglobin has been demonstrated [2].

The identity of the metmyoglobin residue or residues that form(s) radical adduct(s) with DMPO has been the subject of considerable speculation in the literature [2,5,12,17,18]. The generation of site-directed mutant sperm whale metmyoglobins in which the tyrosine residues have been replaced in all combinations with phenylalanines [19] and in which either or both tryptophan residues have been replaced by phenylalanine [13] provides a tool with which the residue(s) that form the radical adduct can be determined. In the present study, the reaction between these metmyoglobins and hydrogen peroxide in the presence of DMPO has been investigated. Further information about the tyrosyl radical site(s) has been obtained using the spin trap MNP.

MATERIALS AND METHODS

Protein preparations

Horse heart myoglobin was acquired from Calbiochem (La Jolla, CA, U.S.A.) and was used without further purification. Horseradish peroxidase type VI-A (HRP) and tyrosine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The site-directed mutant sperm whale myoglobins were prepared as previously described [19,20]. Recombinant sperm whale metmyoglobins were oxidized to the ferric form using a slight excess of potassium ferricyanide and then passed over a PD-10 Sephadex

Abbreviations used: DMPO, 5,5-dimethylpyrroline *N*-oxide; DMPOX, 5,5-dimethyl-2-ketopyrrolidone-*N*-oxyl; MNP, 2-methyl-2-nitrosopropane; ESR, electron spin resonance; DBNBS, 3,5-dibromo-4-nitrosobenzenesulphonic acid; HRP, horseradish peroxidase; MNP/·Mb, the radical adduct formed between metmyoglobin and MNP; MNP-*d*₉, MNP deuterated at all nine positions of the *t*-butyl group ($\equiv$ [²H₉]MNP); ESMS, electrospray ionization mass spectrometry; MNP/·Tyr, MNP adduct of the tyrosyl radical.

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G-25 column (Pharmacia, Uppsala, Sweden). Concentrations of the recombinant metmyoglobins were determined from their visible spectra using an extinction coefficient at 630 nm of $3.3 \text{ mM}^{-1} \text{ cm}^{-1}$ [21].

Isotope labels

Tyrosines deuterated at the methylene carbon (tyrosine-3,3- d_2) and at the 2,6 and 3,5 positions of the phenol ring were purchased from Isotec, Inc. (Miami, OH, U.S.A.). Tyrosine labelled at the phenolic oxygen (50 % isotope enrichment with ^{17}O) was also from Isotec, Inc. Tyrosine deuterated at all four ring positions was from Cambridge Isotope Laboratories (Andover, MA, U.S.A.).

ESR spectroscopy

DMPO was acquired from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) and was purified by vacuum sublimation at room temperature before use. DMPO was added to the metmyoglobin solutions to a final concentration of 100 mM, and hydrogen peroxide was added to a final concentration of 0.9 times that of ferric heme. The samples were quickly transferred to a quartz ESR flat cell and placed into the TM_{110} cavity of a Bruker ESP300 ESR spectrometer, and the sample was scanned starting within 35 s of the addition of hydrogen peroxide using the following instrument parameters: modulation amplitude, 2.0 G; scan time 167 s; time constant 0.17 s; gain 1×10^5 ; microwave power 20 mW. Initiation of the magnetic field scan within 35 s of mixing was required due to the rapid decay of $\text{DMPO} \cdot \text{Mb}$ [2,11,12]. Spin-trapping experiments with MNP were done after addition of the metMb's to give a final concentration of the spin trap of 11–16.5 mM and final concentrations of the proteins between 40 and 180 μM . Reactions were initiated by the addition of hydrogen peroxide to a final concentration of 0.9 times that of the ferric heme. Instrument settings for the MNP experiments were as follows: modulation amplitude, 5.7 G ($1 \text{ G} = 10^{-4} \text{ T}$); time constant, 5.4 s; scan time 1384 s/100 G; microwave power, 20 mW.

Electrospray ionization mass spectrometry

Electrospray ionization mass spectrometry (ESMS) was performed on a triple quadrupole VG BioQ mass spectrometer (Micromass, Altrincham, U.K.). Samples were introduced into the ion source in 50 % methanol/2.5 % acetic acid solution at a flow rate of 4 $\mu\text{l}/\text{min}$. Electrospray mass spectra were acquired within a 500–1600 m/z range in a continuum mode at a rate of 20 s per scan at a resolution that afforded the m/z 998 peak from myoglobin 1.15 Da wide at half height. The electrostatic ion spray source was operated at 3.9 kV. External calibration of the mass scale was based upon multiply protonated ions derived from horse heart myoglobin (16951.4 Da).

Geometry optimization and conformational search of $\text{MNP} \cdot \text{Tyr}$ adduct

A computer model of the structure of the $\text{MNP} \cdot \text{Tyr}$ adduct was constructed using Spartan (Spartan Release 3.1, Wavefunction, Inc., Irvine, CA, U.S.A.) and optimized at its MM2 level. The optimized structure was further evaluated by conformational search using the Sybyl force field to sample all possible conformations in the energy space to ensure the lowest energy conformer was obtained.

RESULTS

When hydrogen peroxide was added to the wild-type sperm whale metmyoglobin in the presence of DMPO, the resultant ESR spectrum was consistent with formation of a partially immobilized radical adduct (Figure 1A). Formation of the ESR-active product was dependent upon the presence of DMPO, metmyoglobin and hydrogen peroxide (data not shown) [11,12]. Estimated hyperfine coupling constants for the nitrogen and the β -hydrogen of the radical adduct are $a^{\text{N}} = 13.7 \text{ G}$ and $a_{\beta}^{\text{H}} = 8.4 \text{ G}$, which are consistent with those previously reported [2,11,12]. The DMPO-radical adduct obtained in the reaction between the recombinant sperm whale myoglobin with both tryptophan

Figure 1 ESR spectra of myoglobin-derived radical adducts formed in the reaction with hydrogen peroxide in the presence of DMPO

In all cases, the concentration of DMPO was 100 mM, and scans were initiated within 35 s of the addition of hydrogen peroxide. Spectrometer settings for all scans: modulation amplitude, 2.0 G; modulation frequency, 100 kHz; centre field, 3480 G; time constant, 0.16 s; receiver gain, 1×10^5 ; microwave power, 50 mW; scan time, 168 s. Spectrum A, Recombinant sperm whale metmyoglobin (58 μM) (wild-type) + hydrogen peroxide (53 μM). Spectrum B, Recombinant sperm whale metmyoglobin (59 μM) (Trp-7 and Trp-14 replaced by phenylalanine) + hydrogen peroxide (53 μM). Spectrum C, Recombinant sperm whale metmyoglobin (148 μM) (Tyr-151 replaced by phenylalanine) + hydrogen peroxide (133 μM). Spectrum D, Recombinant sperm whale metmyoglobin (72 μM) (Tyr-146 and Tyr-151 replaced by phenylalanine) + hydrogen peroxide (65 μM). Spectrum E, Recombinant sperm whale metmyoglobin (51 μM) (Tyr-103 replaced by phenylalanine) + hydrogen peroxide (46 μM). Spectrum F, Recombinant sperm whale metmyoglobin (34 μM) (Tyr-103 and Tyr-151 replaced by phenylalanine) + hydrogen peroxide (31 μM). Spectrum F', The same as spectrum F except that the vertical scale has been multiplied by 10. Spectrum G, Recombinant sperm whale metmyoglobin (59 μM) (Tyr-103, Tyr-146, and Tyr-151 replaced by phenylalanine) + hydrogen peroxide (53 μM). Spectrum H, Recombinant sperm whale metmyoglobin (91 μM) (His-64 replaced by valine) + hydrogen peroxide (82 μM). In this and subsequent Figure note that 1 G ('Gauss') $\equiv 10^{-4} \text{ T}$ (tesla).

Figure 2 ESR spectra of the DMPO radical adduct of tyrosine oxidized by HRP

Tyrosine (6 mM) was oxidized by HRP (50 μ g/ml) and H_2O_2 (2 mM). Spectrum A, natural isotopic abundance tyrosine. Spectrum B, computer simulation of spectrum A calculated with the following parameters: $a^N = 14.2$ G, $a_\beta^H = 10.6$ G, and $a_\gamma^H = 1.3$ G; linewidth, 0.55 G. Spectrum C, tyrosine labelled at the phenolic oxygen with ^{17}O , 50% enrichment. Spectrum D, computer simulation of Spectrum C, calculated with two components, one with ^{16}O at 50% abundance with the following parameters: $a^N = 14.2$ G, $a_\beta^H = 10.6$ G, and $a_\gamma^H = 1.2$ G; linewidth 0.9 G; the second component (^{17}O , 50% isotopic abundance), $a^N = 14.2$ G, $a_\beta^H = 10.6$ G, $a_\gamma^H = 1.0$ G, and $a_\rho^{17\text{O}} = 7.5$ G; linewidth 0.8 G. Spectrum A was acquired with the following instrument parameters: scan time, 665 s; time constant, 0.65 s; modulation amplitude, 0.5 G; modulation frequency, 100 kHz; microwave power, 50 mW. Spectrum C was acquired with the following instrument parameters: scan time, 665 s; time constant, 2.6 s; modulation amplitude, 1 G; modulation frequency, 100 kHz; microwave power, 50 mW.

residues replaced by phenylalanine and hydrogen peroxide was identical to that obtained from the wild-type metmyoglobin (Figure 1B).

When the reactions of the tyrosine-to-phenylalanine mutant sperm whale myoglobins with hydrogen peroxide were studied with DMPO, however, the ESR spectra were dependent upon which tyrosine residues were retained in the proteins. When tyrosines 146 and 151 were replaced with phenylalanine, radical adducts identical to that detected from the wild-type protein were detected after reaction with hydrogen peroxide in the presence of DMPO (Figure 1, spectra C and D). The spectrum of the Tyr-151 to phenylalanine mutant was also identical to that obtained from native horse metmyoglobin, which also has a phenylalanine at residue 151, under identical conditions [2]. However, when Tyr-103 was replaced with a phenylalanine, the ESR spectrum recorded after oxidation by hydrogen peroxide in the presence of DMPO was not the same as that observed when Tyr-103 was present, and was over ten-fold weaker (Figure 1, spectra E–G). The latter spectrum (Figure 1, spectrum F) has

Figure 3 Mass spectrometric analysis of the products of the reaction between horse metmyoglobin and hydrogen peroxide in the presence of DMPO

Top: Hydrogen peroxide concentration three times that of metmyoglobin. Middle: Hydrogen peroxide concentration equal to that of metmyoglobin. Bottom: No hydrogen peroxide added.

hyperfine coupling constants $a^N = 7.2$ G and a_β^H (2H) = 3.9 G, identifying this species as the DMPO oxidation product 5,5-dimethyl-2-ketopyrrolidone-*N*-oxyl (DMPOX) [22,23].

Histidine has been proposed as a possible site of globin oxidation [7,10,20], but when the H64V protein was oxidized by hydrogen peroxide in the presence of DMPO, the resultant spectrum was identical to that observed from the wild-type protein (Figure 1, spectrum H).

The ESR spectrum of the DMPO adduct of metmyoglobin has been assigned to either a tyrosine peroxy radical [12,15–16] or a tyrosine phenoxyl radical [18] based upon its hyperfine coupling constants. Because nitron spin traps like DMPO form radical adducts on a carbon atom rather than the nitrogen that is ultimately part of the ESR-active nitroxide, coupling constants derived from the radical adduct are, in most cases, entirely derived from the spin trap. For this reason, the observed coupling constants only suggest the structure of the free radical that is trapped unless a coupling to the original radical can be resolved. Authentic DMPO/ $\cdot$ Tyr was prepared by trapping the product of the oxidation of tyrosine by HRP and H_2O_2 . The ESR spectrum of the resulting radical adduct exhibits hyperfine coupling constants determined from a spectral simulation of $a^N = 14.2$ G and $a_\beta^H = 10.6$ G and a small coupling to a γ -proton of 1.2 G (Figure 2, spectra A and B). The a^N and a_β^H values are similar to those obtained from DMPO/ $\cdot$ metMb. The small a_β^H and the resolution of a γ -proton is unprecedented in DMPO adducts of

Figure 4 ESR spectra of the MNP- d_9 radical adduct of horse metmyoglobin formed in its reaction with hydrogen peroxide

Spectrum A, horse metmyoglobin (500 μ M) + perdeuterated MNP (22 mM) + hydrogen peroxide (500 μ M). Spectrum B, the sample from spectrum A after a 30-min incubation at room temperature with Pronase (10 mg/ml, final); the scale of this spectrum is 0.1 times that of Spectrum A. Spectrum C, solid line, a higher resolution spectrum of the low-field line of spectrum B; broken line, computer simulation of the low-field line in spectrum B calculated with superhyperfine couplings to one atom with a spin of 1/2 of 1.1 G and to two identical atoms with nuclear spin 1/2 of 0.66 G, as previously reported [24]. Instrument settings for spectra A and B: modulation amplitude, 1 G; time constant, 1.3 s; scan time, 1342 s/100 G; receiver gain, 1×10^5 (spectrum A), 1×10^4 (spectrum B); microwave power, 20 mW. Instrument settings for spectrum C: modulation amplitude, 0.14 G; time constant, 5.4 s; scan time, 2684 s/12 G; receiver gain, 1×10^5 ; microwave power, 20 mW.

carbon-centred radicals. Not surprisingly, when the experiment was repeated using tyrosine labelled at the 6 ring positions with ^{13}C , no hyperfine couplings to ^{13}C were detected (data not shown). However, when tyrosine labelled with ^{17}O (which has a nuclear spin of 5/2) on the phenolic oxygen was used, a large number of additional lines were resolved (Figure 2, spectrum C). The experimental spectrum was simulated with $a^{\text{N}} = 14.2$ G, $a_{\beta}^{\text{H}} = 10.6$ G, $a_{\gamma}^{\text{H}} = 1.2$ G, and $a^{17\text{O}} = 7.5$ G and with the $^{16}\text{O}/^{17}\text{O}$ ratio of the starting material (Figure 2, spectrum D), demonstrating that the observed additional coupling is to the phenolic oxygen of the amino acid. This result proves that the tyrosine radical adduct DMPO/ $\cdot\text{OTyr}$ is formed by trapping phenolic oxygen, which demonstrates that the tyrosine-derived radical is a phenoxyl radical.

To confirm that the observed radical adduct spectra were the result of trapping a tyrosine-centred radical rather than a tyrosine-peroxyl radical, as has been suggested [12,14–16], the DMPO/ $\cdot\text{Mb}$ adduct from horse heart myoglobin was subjected to mass spectrometric analysis. As shown in Figure 3, addition of hydrogen peroxide to the protein in the presence of DMPO

Figure 5 Superhyperfine ESR spectra of the MNP- d_9 adducts of isotopically labelled tyrosines

Spectra were acquired using the instrument parameters described for Figure 4, spectrum C. The lower line for each spectrum is the computer simulation calculated using the hyperfine coupling constants shown. Spectrum A, natural abundance tyrosine. Spectrum B, tyrosine labelled at the 2 and 6 positions of the phenol ring with deuterium. Spectrum C, tyrosine labelled at the methylene carbon with deuterium. Spectrum D, tyrosine labelled at the 3 and 5 positions of the phenol ring with deuterium. Spectrum E, tyrosine labelled at the 2, 3, 5 and 6 positions of the phenol ring with deuterium. Spectrum F, tyrosine labelled at the phenolic oxygen with ^{17}O in 45% of the molecules.

resulted in an increase in mass by 113, consistent with the addition of a single DMPO to the protein, but not consistent with the addition of DMPO and two oxygen atoms as would be the case if an amino acid-derived peroxy radical had been trapped.

Radical adducts of globin-derived tyrosyl radicals are also reportedly formed with the spin-trap MNP [10,24]. The horse metmyoglobin-derived free radical was trapped with perdeuterated MNP and subsequently degraded into free amino acids and small peptides using Pronase, resulting in the conversion of the spectrum of the immobilized nitroxide into an isotropic 3-line spectrum with $a^{\text{N}} = 15.5$ G, as previously reported for cytochrome c (Figure 4, spectra A and B; ref. [24]). When the low-field line of the isotropic triplet was scanned for superhyperfine structure, the spectrum shown in Figure 4C was detected. The superhyperfine spectrum detected has been shown to arise from a tyrosyl radical adduct by comparison with authentic MNP- d_9 / $\cdot\text{Tyr}$ that was synthesized by oxidation of the free amino acid

Recombinant sperm whale Mb + H₂O₂ + MNP

Figure 6 ESR spectra of the radical adducts formed between recombinant metmyoglobins and MNP after reaction with hydrogen peroxide

Spectrum A, recombinant sperm whale metmyoglobin (119 μ M) (wild-type) + hydrogen peroxide (107 μ M) + MNP (16.5 mM). Spectrum B, recombinant sperm whale metmyoglobin (61 μ M) (Trp-7 and Trp-14 replaced by phenylalanine) + hydrogen peroxide (55 μ M) + MNP (11 mM). Spectrum C, recombinant metmyoglobin (75 μ M) (Tyr-151 replaced by phenylalanine) + hydrogen peroxide (67.5 μ M) + MNP (16.5 mM). Spectrum D, recombinant metmyoglobin (83 μ M) (Tyr-103 replaced with phenylalanine) + hydrogen peroxide (75 μ M) + MNP (16.5 mM). Spectrum E, recombinant metmyoglobin (67 μ M) (Tyr-103 and Tyr-151 replaced by phenylalanines) + hydrogen peroxide (60 μ M) + MNP (16.5 mM). Spectrum F, recombinant metmyoglobin (140 μ M) (Tyr-103, Tyr-146 and Tyr-151 replaced by phenylalanine) + hydrogen peroxide (126 μ M) + MNP (16.5 mM). Instrument conditions for all scans were as follows: modulation amplitude, 5.7 G; time constant, 5.4 s; receiver gain, 1×10^3 ; scan time, 1342 s/100 G; microwave power, 20 mW.

by horseradish peroxidase [24]. The superhyperfine structure of the tyrosine radical adduct was simulated with coupling to two equivalent and 1 non-equivalent protons [24], consistent with adduct formation at C-1 (the ring carbon next to the amino acid side chain).

Previous experiments using ¹³C-labelled tyrosine proved that the radical adduct was formed at either C-1 or C-4 of the phenoxyl ring, either of which would be a site that had not been previously shown to be reactive in protein radicals [24]. To differentiate between C-1 and C-4, MNP-d₉/·Tyr were prepared using selectively deuterated tyrosines. The radical adduct formed from tyrosine deuterated at the 2 and 6 positions of the ring had a superhyperfine spectrum consisting of a doublet with $a^H = 0.7$ G (Figure 5, spectrum B). The spectrum obtained from the MNP-d₉ adduct of tyrosine deuterated at the methylene carbon, however, was identical to the spectrum from normal tyrosine (Figure 5, spectrum C). Deuteration at the 3 and 5 positions of the ring resulted in a four-line superhyperfine pattern, with couplings to two protons with $a^H = 1.05$ G and $a^H = 0.55$ G, respectively (Figure 5, spectrum D). Deuteration at all four ring protons resulted in the simplification of the spectrum to a single line (Figure 5, spectrum E), confirming that all three protons with resolvable superhyperfine couplings are on the ring. When

the superhyperfine experiment was repeated with tyrosine labelled at the phenolic oxygen with ¹⁷O, no additional superhyperfine lines were detected (Figure 5, spectrum F), which eliminates C-4 as a possible site of radical adduct formation.

The result of the experiment with the ring-3,5 dideuterated tyrosine demonstrated that the superhyperfine structure arose from three non-equivalent ring protons. When the spectra from the deuterated tyrosines were simulated using a nuclear spin of 1 and hyperfine coupling constants $a^D = (\gamma_D/\gamma_H)a^H = 0.153 a^H$ for the deuterium atoms, a completely self-consistent set of simulations was obtained, with $a^{H(2 \text{ or } 6)} = 1.05$ G, $a^{H(6 \text{ or } 2)} = 0.55$ G, and $a^{H(3 \text{ or } 5)} = 0.70$ G (Figure 5). Prediction of the molecular structure of MNP/·Tyr using the Spartan molecular modelling program indicated a short (2.4 Å) hydrogen bond between the nitroxide oxygen and a proton on the α -amine, completing a seven-membered ring (data not shown). The hydrogen bond-induced ring locks the asymmetric carbon in a fixed position preventing free rotation of the phenoxyl ring, thereby inducing inequivalence of the 2 and 6 protons. Furthermore, the rigid structure of the adduct accounts for the detection of the unusual long-range hyperfine coupling to the 3 or 5 proton, which is three bonds away from the nitroxide nitrogen [25].

In order to determine which tyrosine residue in oxidized sperm whale myoglobin forms a radical adduct with MNP, the recombinant proteins were reacted with hydrogen peroxide in the presence of the spin trap. Protein-derived radical adducts were detected in the wild-type sperm whale metMb (Figure 6A), when both tryptophan residues were replaced with phenylalanine (Figure 6B), and when either Tyr-103 or Tyr-151 was replaced by phenylalanine (Figure 6C and 6D). However, when both Tyr-103 and Tyr-151 were replaced by phenylalanine, no radical adduct was detected (Figure 6E and 6F). This result indicates that radical adducts with MNP are formed at both tyrosines 103 and 151, but not tyrosine 146 of the sperm whale protein.

DISCUSSION

When hydrogen peroxide reacts with metmyoglobin, the heme is oxidized to the ferryl state and globin-centred radical(s) are formed. The hydrogen peroxide could be reduced either by one or two electrons, with the formation of either ferryl heme and a hydroxyl radical or a higher oxidation state of the heme. Free hydroxyl radical has never been detected in this system [12,26], leaving globin radical formation by reduction of the hypervalent heme complex (formally Fe⁵⁺) the more likely pathway.

The ESR spectra acquired from the DMPO experiments are consistent with the formation of a partially immobilized nitroxide by trapping of a radical at Tyr-103. This is consistent with previously reported data in which iodination of Tyr-103 prevents radical adduct formation [14,18]. Controversy has also existed over the nature of the radical that is trapped, with adduct formation to a tyrosine peroxy radical being proposed [12,15–16] and disputed [2,17]. The ¹⁷O hyperfine coupling (Figure 2) and the mass spectrometric data (Figure 3) provide conclusive evidence for the trapping of a tyrosine phenoxyl radical rather than a tyrosine peroxy radical.

In the absence of Tyr-103, a very weak spectrum of the DMPO oxidation product identified as DMPOX was detected (Figure 1, spectra E–G). This product arises either by decomposition of a previously formed radical adduct such as that of a peroxy radical [27], or by the direct oxidation of DMPO [28]. The most straightforward interpretation of the detection of DMPOX in the

Scheme 1 Summary of the currently known reactions of several amino acid residue free radical sites formed in the metmyoglobin/hydrogen peroxide system

Symbols used: P, porphyrin; Fe^{n+} , the heme iron in the indicated oxidation state; $Fe^{4+}=O$, the ferryl-oxo heme complex. Lines without arrows between two elements indicate formation of a covalent bond, i.e. the line between P and Tyr 103 at the bottom indicates a covalent crosslink between the porphyrin and the tyrosine.

mutant proteins lacking Tyr-103 is that in those mutants, oxidation of the spin trap via the peroxidase activity of metmyoglobin is favoured over the formation of a radical adduct at another site. Both Tyr-151 and Trp-14 have been shown to form free radicals that can be detected by other methods [4,8,13], and the Tyr-151 phenoxyl radical forms an adduct with MNP (Figure 6). It must be remembered that the inability to detect a radical adduct does not preclude its formation. An important limiting factor in the detection of radical adducts is their persistence, which is determined not by their relative rates of formation, but rather by their decay to diamagnetic products [28,29].

The identification of the phenolic oxygen as the site of radical adduct formation with DMPO (Figure 2) and C-1 of the phenolic ring as the site of reaction with MNP (Figure 5) indicates that all of the sites on the tyrosyl radical known to have significant electron density in tyrosyl radicals (the oxygen, C-3/C-5, and C-1) [30,31] are chemically reactive. The reactivity of C-3/C-5 has been demonstrated in the formation of dityrosine crosslinks [3].

It is clear from a number of studies that at least some free electron population is present at a number of different sites on the globin and that which site is detected depends upon the detection method. Free electron population has been proven at Tyr-103 with site-specific spin trapping with DMPO, in accordance with previous results [3,4,10]. Free electron population has also been shown at Tyr-151 by site-specific spin trapping with MNP as is consistent with previous results [3,8]. Finally, free electron population has been demonstrated at Trp-14 with site-specific direct ESR [13]. Formation of an MNP radical adduct has been suggested at Lys-42 by mass spectroscopic evidence

[10], but that adduct has not been observed with ESR spectroscopy.

The nearness of Tyr-103 to the porphyrin suggests that it is the initial site of globin oxidation with subsequent electron transfer from the other residues resulting in their oxidation. Electron transfer between residues in the metmyoglobin system is suggested by the reported competition between O_2 and DMPO for oxidized globin, since these compounds have been shown to react at Trp-14 [13] and Tyr-103, respectively. Tyrosine and tryptophan have nearly identical oxidation potentials [29,32], and electron transfer has been observed between tyrosines and tryptophans in lysozyme and other proteins [33,34]. However, that hypothesis is not consistent with other data. Mutant myoglobins lacking Tyr-103 residue form the Trp-14-centred peroxy radical with an intensity similar to that seen for the wild-type protein [13], suggesting that no modification of the electron-transfer pathway from Trp-14 to the heme is caused by the Tyr-103 mutation. Furthermore, the MNP-radical adduct is formed with a radical at Tyr-151 even in the absence of Tyr-103.

A more likely hypothesis is that a number of globin amino acid residues reduce the initial hypervalent state, with subsequent electron transfer occurring between the sites. No prediction of the distribution of the unpaired electron among the various sites within myoglobin is possible from the available data because each trapping agent (DMPO, MNP, DBNBS, O_2 , and myoglobin-) reacts with each residue radical at a different rate, with the subsequent decay also occurring at different rates. A summary of the accumulated data identifying the free radical sites of metmyoglobin is shown in Scheme 1. The reported enhancement

of protein tyrosyl-tyrosyl oligomerization by inclusion of styrene, which is epoxidized by the Trp-14 peroxy radical, has been interpreted to indicate that Trp-14 is not in equilibrium with one or more of the tyrosyl radical sites [35]. It may be that not all residues that form radicals undergo electron transfer.

In conclusion, the site at which DMPO adduct formation occurs in the metmyoglobin/hydrogen peroxide system has been identified to be Tyr-103. The reasons for this site-specificity remain unclear but likely result from differences in radical adduct formation and/or instability. For instance, Tyr-146 may be oxidized to a free radical but be untrappable because it is buried in the protein and is, therefore, inaccessible for steric reasons. On the other hand, Tyr-151 has been shown to be oxidized to a tyrosyl radical by direct ESR investigations, by trapping with MNP, and by the detection of crosslinking involving that residue. Since it is on the periphery of the protein, the absence of detectable DMPO radical adduct formation at this site must reflect instability of DMPO/ $\cdot$ Tyr-151. Our data support a model in which a number of sites serve as the reductant for the initially oxidized heme, with the site that is detected experimentally being primarily determined by a number of kinetic and steric factors.

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