Trapping and LC−MS Identification of Protein Radicals Formed in the Horse Heart Metmyoglobin−H_2O_2 Reaction

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Metmyoglobin (metMb) reacts with H_2O_2 to form an oxyferryl (Fe^{IV}=O) heme and unstable protein radical species. Although the latter have been under investigation for over 40 years, there is still debate as to the sites of radical formation.[1] Both the free radicals formed and their spin adducts have been detected by EPR,[1] but the signals have not been unambiguously assigned to specific residues.[1a,b] Since protein radicals appear to play key roles in redox catalysis and redox signaling,[2] we are investigating their locations in proteins using on-line LC−MS analysis of their spin adducts. We report here the identities of the protein-based radicals trapped in horse heart Mb following its reaction with H_2O_2 under a variety of experimental conditions.

Mb and the spin trap 2-methyl-2-nitrosopropane (MNP)[3] were mixed under normoxic conditions to give a solution of 200 μM metMb and 18.4 mM MNP in 50 mM NaPi buffer (pH 7.4). This was reacted with 1−10 molar equiv (1−10×) of H_2O_2 for 5 min,[4] and excess H_2O_2 removed with bovine catalase (Sigma). Analysis of the desalted metMb−H_2O_2−MNP reaction products by direct infusion into the electrospray (ES) source of the mass spectrometer[5] showed that a fraction of the protein (10−15% with 1× H_2O_2 and 20−30% with 2× H_2O_2) had an increased mass of 86 Da over untreated Mb, corresponding to the formation of MNP spin adducts. Tryptic digests, however, contained exclusively native Mb peptides, indicating that the spin adducts are unstable under the peptide mapping conditions.[6]

The Fe^{IV}=O heme and spin adducts were reduced with excess ascorbic acid and acidified to pH 2 with HCl. The products were separated by RP-HPLC, and the mass spectrum of the protein peak revealed that the reduced MNP adducts (R−MNP−Mb) have a mass of 16950 + 71 Da vs 16950 + 86 Da for the spin adducts.[5] The R−MNP−Mb derivatives yielded modified peptides, which were observed when the following controls and samples were subjected to tryptic digestion and on-line LC−MS analysis: (1) Mb, (2) Mb + 5× H_2O_2, (3) Mb + MNP, (4) Mb + 1× H_2O_2 + MNP, (5) Mb + 2× H_2O_2 + MNP, (6) Mb + 5× H_2O_2 + MNP, (7) Mb + 10× H_2O_2 + MNP. The controls (1−3) yielded only native tryptic peptides (Figure 1A), while samples 4−7 possessed a new peak (denoted by an asterisk in Figure 1B) corresponding to peptide 103−118 plus 71 Da. The intensity of this peak leveled off at 5× H_2O_2, and sequencing by collision-induced dissociation (CID) (Figure 2) revealed Tyr103 to be the site of modification. Two minor peaks (denoted by a circled asterisk in Figure 1B) were also present in the spectra of samples 4−7 but not in the controls. CID[8] provided partial sequences, allowing the peaks to be assigned to peptides 32−45 and 32−47. Peptide 32−47 was

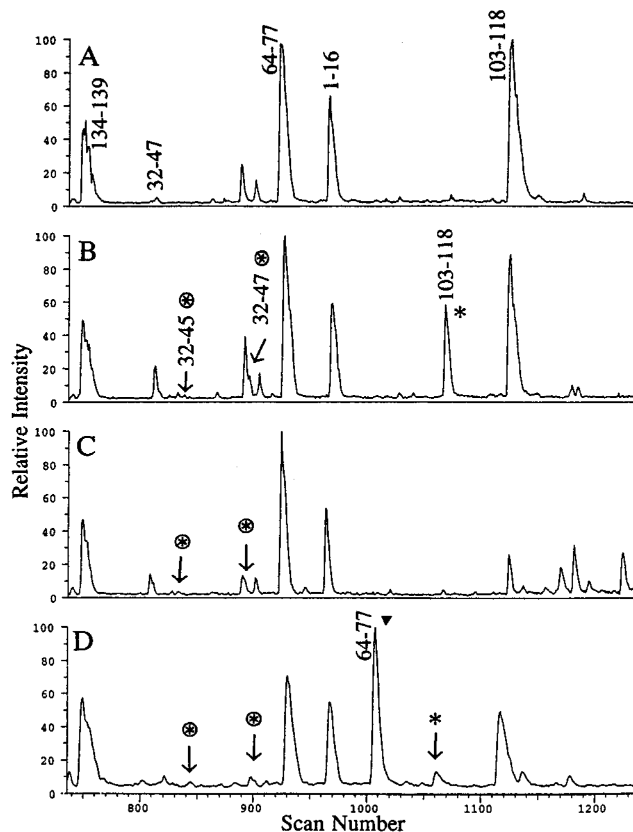

Figure 1. Peptide mass maps of horse heart myoglobin. (A) Tryptic digestion following incubation of metMb with 18.4 mM MNP (pH 7.4) in the absence of H_2O_2 yields only native Mb peptides. (B) Reaction of metMb with 2× H_2O_2 and 18.4 mM MNP (pH 7.4) followed by ascorbate reduction gives rise to ~30% peptide 103−118 with an increased mass of 71 Da (*) (*m/z* 1955) and to peptides 32−45 and 32−47 with decreased masses of 17 Da (circled asterisk) (*m/z* 1645 and 1919). (C) Iodination of Mb[10] prior to reaction with 2× H_2O_2 and 18.4 mM MNP (pH 7.4) results in loss of MNP-modified peptide 103−118 (*), and MNP-modified peptides 32−45 and 32−47 (circled asterisk) appear slightly more prominent. The new peaks above scan number 1170 contain iodinated peptide 103−118. (D) Same reaction as in (B) carried out at pH 5.0. Approximately 30% of peptide 64−77 has increased in mass by 40 Da (▼) (*m/z* 1419), and MNP-modified peptides 103−118 (*), and 32−45 and 32−47 (circled asterisk) are also seen in the map.

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(3) A 1 mM stock horse heart metMb (Sigma) solution was prepared in 50 mM NaPi (pH 7.4) buffer treated with Chelex resin (BioRad) followed by the addition of diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid (DETPA; ICN) to a final concentration of 50 μM. A stock MNP (Aldrich) solution (23 mM) was prepared from the MNP dimer in the same buffer as described previously (Makino, K.; Moriya, F.; Hatano, H. *J. Chromatogr.* **1985**, *332*, 71).

(4) The UV/vis absorption spectra of the metMb−H_2O_2 reaction products were identical in the presence and absence of excess MNP, indicating that the latter does not react with the Fe^{IV}=O heme center.

(5) The reaction mixture (~20 μg of protein) was injected onto a Hamilton PRP-∞ HPLC column (4.6 × 30 mm) and the protein separated from its heme and buffer salts with a 20−65% CH_3CN gradient in 0.1% TFA at 2 mL/min over 10 min. The protein peak was lyophilized and <1 μg resuspended in 50 μL of 1:1 methanol/H_2O with 0.5% acetic acid and infused into the ES source of a Finnigan SSQ mass spectrometer at a flow rate of 1−2 μL/min. Peaks at *m/z* 19 650 and 17 036 in the mass spectrum were assigned to apoMb and its MNP spin adduct.

(6) The ApoMb (~20 μg) samples were digested with 1:50 (w/w) trypsin at 25 °C for 8 h in 50 mM Tris buffer, pH 8.0. The digests were acidified to pH 2 with HCl, and 2−4 μg was separated on a Vydac microbore C18 (1 × 300 mm) column with a 3−55% CH_3CN gradient in 0.05% TFA at 40 μL/min over 120 min.

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(8) CID sequencing was also performed on the SSQ in the capillary/skimmer region with the capillary at 90 mV and the tube lens at 145 mV. The +1, +2, and +3 ions simultaneously fragmented in this region and were analyzed in Q1.

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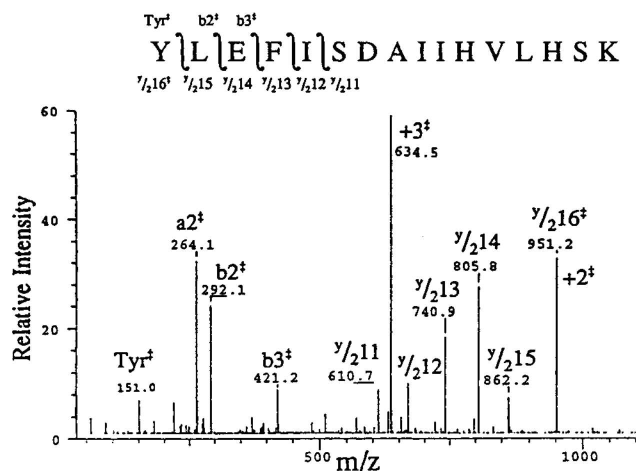

Figure 2. CID sequencing of MNP-modified peptide 103−118. The peptide was purified from the tryptic digest,[6] lyophilized, resuspended in 1:1 methanol/H_2O + 0.05% acetic acid, and directly infused at ∼1.5 μL/min into the ES source of a Finnigan TSQ 7000 triple quadrupole MS. The +3 ion at *m/z* 653.2 was selected with Q1 and fragmented by CID in the collision cell (Q2) with Ar gas at 2.5 mTorr and a collision offset voltage of −22 eV (laboratory frame of reference). The fragment ions were analyzed in Q3. Masses of the major *a*, *b*, and y^2 (doubly-charged) fragment ions[7] are given. The R−MNP−Tyr moiety is cleaved during CID, and fragment ions containing the altered Tyr103 residue (+15 Da) are denoted by a double dagger.

further digested with V8 protease,[9] and CID of the resulting peptides showed that residue 42 (Lys) was 17 Da lighter than in untreated Mb.

Iodination of Mb at Tyr103 was reported to alter the radical signal observed by EPR.[1b] Hence, following iodination[10] the species trapped by MNP were determined by peptide mass mapping (Figure 1C). A radical was no longer found to be trapped at Tyr103, but peaks containing modified peptides 32−45 and 32−47 were consistently 30−40% more abundant in the iodinated samples.

MNP spin trapping experiments were also carried out at pH 5.0, since the radicals formed in the metMb−H_2O_2 reaction exhibit different EPR and UV/vis spectra at acidic pH values.[11] Modification of Tyr103 and Lys42 was again observed, and an additional intense peak (▼) appeared in the map (Figure 1D), which was shown to be peptide 64−77 with an increased mass of +40 by CID sequencing. Peptide 64−77 was further digested with pepsin,[12] and CID of peptide 64−69 unambiguously demonstrated that His64 was modified. Hence, MNP, which is primarily a carbon-based spin trap,[13] reacts with Tyr, Lys, and His radicals in Mb, but the reduced R−MNP derivatives exhibit different peptide mass mapping behavior, a subject under further investigation.

The metMb−H_2O_2 reaction is >99% complete within 20 s in the presence of 10× (2 mM) peroxide ($k \approx 200\ M^{-1}\ s^{-1}$),[14] but the maximum MNP−Mb spin adduct yield was ∼50% at pH 7.4. Extrapolation of the EPR signal intensity to time zero indicated that the radical concentration was ∼50% that of the globin,[15] suggesting that MNP efficiently traps the EPR-

(9) Digestion was performed with 1:50 (w/w) V8 endoproteinase Glu-C (Sigma) at 25 °C for 4 h in 50 mM NH_3HCO_3, pH 7.8.

(10) Iodination was performed as reported (Parker, C. W. *Methods Enzymol.* **1990**, *182*, 729) by titrating 200 μM Mb in 0.1 M sodium borate (pH 9.0) with ICl and monitoring the extent of iodination by LC−MS. Figure 1C shows that Tyr103 was singly and doubly iodinated with little unmodified Tyr103 remaining.

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(12) Digestion was performed with 1:50 (w/w) pepsin (Sigma) in 3 mM HCl at 25 °C for 4 h.

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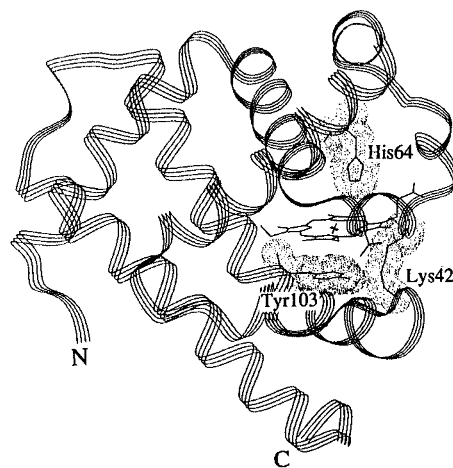

Figure 3. Sites of MNP spin adduct formation (depicted by their van der Waals radii) in horse heart Mb and the heme superimposed on the Cα backbone.[19] Tyr103 and Lys42, the sites of radical trapping at pH 7.4, are solvent exposed and form a triangle with the heme edge. The distal His64 above the heme is a site of radical trapping at pH 5.0.

detectable protein-based radicals generated in the metMb−H_2O_2 reaction. Figure 3 shows the location of the radical species identified by LC−MS. Tyr103 is in van der Waals contact with the heme edge and solvent exposed, so trapping a radical on Tyr103 should be facile. Lys42 is also solvent exposed and adjacent to Tyr103. Efficient spin trapping at the distal His64 at pH 5.0, which gives rise to the intense peak (▼) in Figure 1D, may be due to opening of the heme pocket at low pH,[16] allowing MNP better access to the distal cavity.

The present trapping results are consistent with the assignment of the EPR signals to radicals centered on Tyr103 and additional residues.[1b] Furthermore, radical formation on non-Tyr residues has been confirmed, since sperm whale metMb mutants devoid of Tyr exhibit EPR signals arising from protein-based radicals on oxidation with H_2O_2.[1c] The LC−MS data in Figure 1 are, however, inconsistent with the proposed[1a] MNP spin adduct formation at Trp7 and/or Trp14 of horse heart Mb, since peptide 1−16 is not modified under the conditions examined here. More recent analysis of the EPR data reveal that MNP spin adducts are most likely formed on Tyr residues in both Mb and cytochrome *c*.[17] It has also been proposed that Tyr103 is cross-linked to the heme in H_2O_2-oxidized horse heart Mb,[18] but this is unlikely since LC−MS revealed that globin modification does *not* occur in the absence of MNP.

Clearly, whole-protein EPR data acquired in the presence or absence of spin traps cannot provide unambiguous assignments for protein radical signals.[1] However, conversion of the spin adducts to more stable diamagnetic species permits peptide mass mapping and the identification of *each* of the spin-trapped species. A further advantage of coupling spin trapping with LC−MS is that picomole quantities of sample can be analyzed. With the biological roles of redox-active amino acid residues becoming more apparent,[2] the identification of protein-based radicals is of increasing importance and interest.

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