Identification of Free Radicals on Hemoglobin from its Self-peroxidation Using Mass Spectrometry and Immuno-spin Trapping

OBSERVATION OF A HISTIDINYL RADICAL*

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In an effort to understand the mechanism of radical formation on heme proteins, the formation of radicals on hemoglobin was initiated by reaction with hydrogen peroxide in the presence of the spin trap 5,5-dimethyl-1-pyrroline N-oxide (DMPO). The DMPO nitrotrone adducts were analyzed by mass spectrometry (MS) and immuno-spin trapping. The spin-trapped protein adducts were subsequently subjected to tryptic digestion and MS analyses. When hemoglobin was reacted with hydrogen peroxide (H₂O₂) in the presence of DMPO, a DMPO nitrotrone adduct could be detected by immuno-spin trapping. To verify that DMPO adducts of the protein free radicals had been formed, the reaction mixtures were analyzed by flow injection electrospray ionization mass spectrometry (ESI/MS). The ESI mass spectrum of the hemoglobin/H₂O₂/DMPO sample shows one adduct each on both the α chain and the β chain of hemoglobin which corresponds in mass to the addition of one DMPO molecule. The nature of the radicals formed on hemoglobin was explored using proteolysis techniques followed by liquid chromatography/mass spectrometry (LC/MS) and tandem mass spectrometry (MS/MS) analyses. The following sites of DMPO addition were identified on heme protein: Cys-93 of the β chain, and Tyr-42, Tyr-24, and His-20 of the α chain. Because of the pi-pi interaction of Tyr-24 and His-20, the unpaired electron is apparently delocalized on both the tyrosine and histidine residue (pi-pi stacked pair radical).

The role of free radicals in the pathogenesis of human disease has led to an increased interest in the study of free radicals and their reactions. Reactive oxygen metabolites can interact with cellular constituents, including DNA/RNA, proteins, and unsaturated lipids (1–4). Previous studies have suggested that hemoproteins may be involved in redox reactions which contribute to tissue and/or organ damage via reaction with hydrogen peroxide (5–7). A recent study has reported evidence for the association of nitrotyrosine (which forms via tyrosyl radical) and coronary artery disease (8). To understand the mechanisms of these reactions and their contributions to human diseases, it is important to determine the nature of heme protein radical intermediates involved in these processes.

Protein-centered radicals have traditionally been studied through either direct electron spin resonance or by spin trapping (9). Because most radicals are short-lived (i.e., micro-seconds to minutes), the spin trapping approach has been more widely used. With the spin trapping approach, protein radicals react with a spin trap molecule, such as 2-methyl-2-nitrosopropane or 5,5-dimethyl-1-pyrroline N-oxide (DMPO), thereby making the radical more stable and, consequently, more long-lived.

To take advantage of these more stable, trapped adducts, our research group recently reported the development of an antibody with specificity for the nitrotrone adduct, which is the final product of trapping radicals with DMPO (10). For this work, antibodies were raised against a DMPO derivative in which an octanoic acid side chain was conjugated to ovalbumin. Using this DMPO-specific antibody, we have demonstrated that the predominant protein radical formed in rat heart supernatant by hydrogen peroxide is myoglobin-derived (10).

Assignment of the specific amino acid residue or residues that form radical adducts with the spin trap molecule is difficult with ESR or immunostaining. Consequently, researchers have used other biochemical techniques, such as site-directed mutagenesis and amino acid derivatization, to address these questions. These methods, however, suffer from potential drawbacks, such as the protein’s stability and conformation can be perturbed by mutations and the formation of the amino acid radical and transfer of the radical is expected to be affected. Recently, mass spectrometry has been utilized to study protein free radicals (11–15). The spin trap molecules form a covalent bond with the protein resulting in a new nitrotrone radical, which may be stable under electrospray ionization mass spectrometry conditions. The one-electron oxidation product of this nitrotrone is a stable nitrotrone with a covalent bond between DMPO and the original free radical (Scheme 1). In addition, the use of peptide mapping methodologies with mass spectrometry allows for the unequivocal assignment of the trapped radical on the protein. We are, therefore, using a combination of peptide mapping by mass spectrometry and the immuno-spin trapping technique to investigate the formation and structures of the protein radicals generated on heme-containing proteins.

In a previous report using the immuno-spin trapping approach (16), we demonstrated that hemoglobin reacted with H₂O₂ in the presence of DMPO and suggested that the tyrosine

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¶ The abbreviations used are: DMPO, 5,5-dimethyl-1-pyrroline N-oxide; Hb, hemoglobin; oxyHb, oxyferrous Hb; DTPA, diethylene-triamine pentaacetic acid; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; ESI, electrospray ionization mass spectrometry; LC, liquid chromatography; MS, mass spectrometry.
residues and possibly a cysteine residue of hemoglobin are the sites of formation of the radical-derived nitro oxide adducts. The exposure of hemoproteins, such as hemoglobin (Hb) and myoglobin (Mb), to hydrogen peroxide has been shown to initially produce a porphyrin cation radical and ferryl ion. The porphyrin cation radical oxidizes one or more residues to form globin-centered radical(s) (16–21). The exact site of this radical in hemoglobin has been speculated to be Tyr-42 on the α chain, based on x-ray data and the inhibition of the radical adduct signal by iodination of the tyrosine (18). Maples et al. (19) reported the detection of a peroxide-dependent hemoglobin-thiyl radical in rat and human hemoglobin and concluded, based on ESR parameters and the effect of thiol blocking agents, that the radical adduct was a thyl radical adduct.

In the presence of a spin trap, such as DMPO, the Hb-centered radical(s) yield a nitroxide radical adduct(s), which decays on the order of 1 min (22). This decay is the result of the oxidation of the nitroxide radical adduct to the corresponding globin radical-derived nitro oxide adduct by the ferryl moiety (Scheme 1). The globin radical-derived nitro oxide adduct can be investigated by immuno-spin trapping (16). In the absence of a spin trap, the globin-centered radical(s) can decay by formation of cross links (23), such as dityrosine (24) and possibly other cross-links.

To identify the precise amino acid residues trapped by the DMPO, we have utilized mass spectrometry-based sequencing. This approach includes proteolysis of the hemoglobin-derived DMPO adducts by trypsin, followed by MS peptide mapping, and finally MS/MS analyses of the peptides. Using this approach, we have identified the specific location of the DMPO adducts on hemoglobin and report our results here.

EXPERIMENTAL PROCEDURES

Materials—Pure human oxyferrous Hb (oxyHb) was a kind gift of Apex Biosciences Inc. (Research Triangle Park, NC). Diethylenetriamine pentaacetic acid (DTPA) was purchased from Sigma. Beef liver catalase was purchased from Roche Applied Science and used as received. The spin trap DMPO was purchased from Alexis Biochemicals (San Diego, CA) and purified by double distillation at room temperature. The DMPO concentration was measured at 228 nm, assuming a molar absorption coefficient of 7800 M⁻¹ cm⁻¹ (25). Reagent grade 30% H₂O₂ was obtained from Fisher Scientific Co. The H₂O₂ concentration was verified using ε₄₃₅nm = 43.6 M⁻¹ cm⁻¹. All buffers were stored over Chelex 100® (Bio-Rad) at 4 °C for 24 h followed by the addition of 100 μM DTPA to avoid possible transition metal-catalyzed reactions.

Spin Trapping Reaction Conditions—Purified human oxyHb (oxyferrous form) was dissolved in 0.1 M sodium phosphate buffer (pH 7.4) treated with Chelex 100® and containing DTPA (100 μM). Before use, the oxyHb was passed through a prepacked Sephadex G-25 column (PD-10, Amersham Biosciences) equilibrated with 100 mM phosphate buffer. For the SDS-PAGE and Western analyses, the reactions of oxyHb with H₂O₂ (H₂O₂:heme molar ratio equal to 10) were carried out in the presence of 100 mM DMPO in 0.1 M sodium phosphate (pH 7.4) at 37 °C for 2 h. Reactions were stopped by consumption of any excess of H₂O₂ by 100 IU catalase. Because nonvolatile buffers cause mass spectrometer source contamination and the formation of salt adducts, the oxyHb samples for the MS analyses were prepared in 100 mM ammonium bicarbonate (pH 8) instead of the sodium phosphate buffer, and the reactions were stopped by freezing.

SDS-PAGE and Silver Staining—Reactions were mixed with 4× loading buffer with the addition of dithiothreitol and heated at 80 °C for 10 min. After cooling to room temperature, 75 pmol of heme (1.2 μg Hb) was loaded in each lane in precast 10 well 4%–15% Bis-Tris gels and separated by SDS-PAGE. 5 μl SeeBlue® Plus (Invitrogen) molecular weight markers were included on each gel. The gels were stained using Silver Stain Plus (BioRad) as described by the manufacturer.

Western Blotting—After SDS-PAGE, the separated proteins were blotted on a nitrocellulose membrane, blocked with 4% cold water fish gelatin solution in 100 mM carbonate/bicarbonate buffer (pH 9.6) for 90 min at room temperature, and then washed with a Tris buffer saline solution (pH 7.4) containing 0.2% cold water fish skin gelatin. Exposure of the membrane to anti-DMPO serum at a dilution of 1:5000 was performed for 1 h. Positive immuno-complexes were detected by exposure of the membrane to 1-Step NBT/BCIP reagent (Pierce) for 15 min.

Tryptic Digestion Conditions—The hemoglobin control sample and the hemoglobin + DMPO + H₂O₂ reaction sample in 100 mM ammonium bicarbonate buffer (pH 8) were subjected to tryptic digestion. Porcine trypsin (Promega Corp., Madison, WI) was added to a 175-μl aliquot of the samples at a protein:enzyme ratio of 20:1. The reactions were allowed to proceed overnight at 37 °C.

Electrospray Mass Spectrometry—A Micromass Q-Tof Ultima Global (Waters/Micromass, Milford, MA) hybrid tandem mass spectrometer (26) was used for the acquisition of the electrospray ionization (ESI) mass spectra and tandem mass spectra. This instrument is equipped with a nanoflow electrospray source and consists of a quadrupole mass filter and an orthogonal acceleration time-of-flight mass spectrometer. The needle voltage was ~3000 V, and the collision energy was 10 eV for the MS analyses. Samples for flow injection analyses were diluted 1:1 with a solution of 50:50 acetonitrile:0.1% trifluoroacetic acid. The samples were allowed to equilibrate with the electrospray source for 1 min before the start of each injection. The spray voltage was set at 1.5 kV, and the desolvation gas flow rate was 600 l/h. The analysis for each sample was performed at a resolution of 1000 at m/z 447.
formic acid and infused into the mass spectrometer at ∼300 nL/min using a pressure injection vessel.

For the LC/MS/MS analyses, a Waters CapLC HPLC system (Waters/Micromass, Milford, MA) consisting of binary pumps and a micro autosampler was used to deliver the gradients. Injections of 8 μL were made and a linear gradient of 5–75% acetonitrile (0.1% formic acid) over 35 min was used for the chromatographic separations. The column used was a 15 cm × 75 μm i.d. Hypersil C18 (“pepmap”) column (LC Packings, San Francisco, CA) at a flow rate of ∼300 nL/min.

During the LC/MS/MS experiments, automated data dependent acquisition software was employed. For these acquisitions, the instrument can switch from the MS mode to the MS/MS mode and then returns back to the MS mode based on predetermined or operator-entered parameters, such as abundance and time. The advantage of this software is that both MS and MS/MS data can be acquired from a single chromatographic separation of the mixture. The collision energy used for these experiments was set according to the charge state and the m/z of the precursor as determined from a charge state recognition algorithm. Data analysis was accomplished with a MassLynx data system, MaxEnt deconvolution software, and ProteinLynx software supplied by the manufacturer.

RESULTS AND DISCUSSION

Formation of Protein-centered Radicals on Hemoglobin—In the present study, the formation of protein-centered radicals on hemoglobin has been investigated. As demonstrated previously (16), when hemoglobin is reacted with hydrogen peroxide, radicals are formed which can be detected using immuno-spin trapping with an anti-DMPO antibody. Based upon these studies (16), tyrosine and possibly cysteine residues were thought to be involved in the formation of radicals in hemoglobin. In the ESR spin trapping investigation, the formation of DMPO radical adducts on both tyrosyl and cysteinyl amino acids on hemoglobin in the presence of peroxides have been proposed (16, 19).

To characterize the hemoglobin radicals, hemoglobin was reacted with hydrogen peroxide in the presence of DMPO spin trap. The SDS-PAGE separation and silver staining of the reaction mixtures is shown in Fig. 1, upper panel. The exposure of human oxyHb to hydrogen peroxide produces hemoglobin subunit dimers (∼30 kDa) and higher order aggregation (e.g. trimer (∼48 kDa), tetramer (∼62 kDa), etc . . . ) (Fig. 1). DMPO protects the Hb monomers from hydrogen peroxide-mediated destruction, presumably by trapping the protein-derived radicals thus preventing subsequent protein damage (Fig. 1, upper panel, lanes C and D).

Previously, antiserum that recognizes protein radical-derived nitrone adducts was developed and used to characterize myoglobin radical-derived nitrone adducts in the myoglobin/ H2O2 system and in the supernatant of rat heart (10). Recently,
this antiserum was shown to be useful in detecting hemoglobin radical-derived nitrone adducts by exposure of red blood cells to a bolus of H$_2$O$_2$ (16) and on Hb where H$_2$O$_2$ was generated by UVA irradiation of ketoprofen (27). The covalent binding of DMPO to the hemoglobin requires the presence of H$_2$O$_2$ showing that this process operates by a peroxidase-type mechanism (Scheme 1; Refs. 16, 28, and 29). Hemoglobin, hydrogen peroxide, and DMPO were all necessary to obtain hemoglobin-derived nitrone adducts (Fig. 1, lower panel).

**ESI/MS of DMPO Adducts on Hemoglobin**—To verify that DMPO had been trapped on the hemoglobin protein, the reaction mixtures were analyzed by flow injection ESI/MS. The resulting deconvoluted ESI mass spectra for the hemoglobin samples are shown in Fig. 2. In the deconvoluted ESI mass spectrum of native hemoglobin (Fig. 2A), molecular ions of the $\alpha$ chain and the $\beta$-chain of average molecular weight ($M_r$) of 15128.5 ($M_r$ calculated = 15126.4) and of 15869.0 ($M_r$ calculated = 15867.2), respectively, are observed as well as oxidized forms of these ions. In the absence of hydrogen peroxide (Fig. 2B), the deconvoluted mass spectrum is identical to that of hemoglobin alone (Fig. 2A). In the absence of DMPO spin trap in the reaction (Fig. 2C), oxidized forms of the protein are observed (e.g. ions labeled with + O and + 2O), which represents incorporation of oxygen into the protein presumably by the reaction of protein-derived free radicals with molecular oxygen. The deconvoluted electrospray mass spectrum of the reaction mixture containing hemoglobin, H$_2$O$_2$, and DMPO (Fig. 2D) shows four major protonated molecules of $M_r$ = 15128.8 Da, native $\alpha$ chain of hemoglobin, $M_r$ = 15239.8 Da, $\alpha$ chain plus one DMPO molecule, $M_r$ = 15869.1 Da, native $\beta$ chain of hemoglobin, and $M_r$ = 15980.6 Da, native $\beta$ chain plus one DMPO molecule. In addition, minor amounts of oxidized forms of these ions are observed. These data suggest that a single DMPO molecule is trapped on each of the hemoglobin chains. None of these ions were observed in any of the control experiments (Fig. 2, A–C). These data indicate that the formation of the DMPO adduct on hemoglobin is dependent on the presence of both the hydrogen peroxide and the DMPO spin trap in the reaction mixture.

In the control experiment where hemoglobin is reacted with hydrogen peroxide in the absence of DMPO as the spin trap (Fig. 2C), oxidized forms of the protein are observed. Presumably these species form from the reaction of protein-centered radicals with molecular oxygen. The hydrogen peroxide-initiated oxygen incorporation observed in Fig. 2C is protein free radical-dependent as demonstrated by its inhibition with DMPO (Fig. 2D). DMPO is known to be an anti-oxidant that blocks subsequent reactions of radicals with oxygen by trapping them. The partial protection by DMPO of Hb against H$_2$O$_2$-induced destruction is also apparent in the immuno-spin trapping experiments (Fig. 1, upper panel, lanes C and D). These data implicate competition between molecular oxygen and DMPO for the radical sites.

**LC/ESI/MS and MS/MS of Tryptic Digest of DMPO Adducts on Hemoglobin**—Identification of the specific location of the DMPO adducts on hemoglobin was determined by peptide mapping and MS sequencing analyses. To determine which amino acids in hemoglobin contained a trapped DMPO molecule, hemoglobin alone and the Hb/H$_2$O$_2$/DMPO reaction mixture were subjected to tryptic digestion and analysis by LC/ESI/MS/MS.
Because of the complexity of the digestion mixture, on-line LC was used in conjunction with the MS analyses. The data were acquired using the automated data dependent acquisition software, which enables the instrument to switch from the MS to the MS/MS mode and then return to the MS mode based on criteria set by the user. The resulting MS/MS data were then searched using ProteinLynx software. In the ProteinLynx system, DMPO was entered as a user-defined modifier agent on all the amino acids. The software will then automatically search for tryptic peptides modified by the addition of adducts of 111 Da. The corresponding MS/MS spectra will be interpreted on the basis of the presence of a DMPO adduct and indicate which amino acid modified by DMPO best fits the MS/MS data. With the ProteinLynx software, three amino acid residues were identified as containing a DMPO molecule in the Hb/H2O2/DMPO reaction mixture. None of these ions were observed in the LC/MS/MS of the hemoglobin alone control tryptic digest.

These data were confirmed manually by first transforming all ions in the MS/MS spectra to the single charge state using the MaxEnt software (Figs. 3–5). Fig. 3 shows the deconvoluted MS/MS spectrum of the tyrosine-containing tryptic peptide T6 of hemoglobin, α chain. A nearly complete series of both y and b ions (30, 31) are observed which correspond to cleavages along the peptide backbone. In addition, the loss of H2O from several b ions is observed (ions not labeled). The y series ions result from C-terminal peptide backbone cleavages and the b series ions result from N-terminal backbone cleavages. The mass of the b2 ion corresponds to amino acids threonine plus a DMPO-modified tyrosine. In addition, the observation of the y14 and y15 ions provides the necessary data to definitively assign the location of the DMPO adduct. The observed mass difference between the y14 and y15 ions corresponds to the mass of a tyrosine residue plus the mass of one DMPO molecule. These structurally informative fragment ions allow the assignment of the DMPO molecule to the Tyr-42 residue of tryptic peptide T6 of hemoglobin, α chain.

The deconvoluted MS/MS spectrum of tryptic peptide T10–11 (amino acids residues 83–104) of hemoglobin, α chain is shown in Fig. 4. An abundant ion of m/z 2495.3 is observed, which corresponds in mass to the loss of DMPO and H2S from the molecular ion. A series of y ions (y1 through y20) and b ions (b2 through b5) are observed as well as the loss of DMPO and H2S from the y12 through y19 ions (ions labeled with an asterisk). The mass difference between y11 and y12 corresponds to the mass of a DMPO-modified cysteine residue. These data allow the unequivocal assignment of a DMPO molecule to Cys-93 of tryptic peptide T10–11 of hemoglobin, α chain.

The MS/MS spectrum of the (M+2H)2+ ion at m/z 821.4 which corresponds in mass to tryptic peptide T4 of α chain plus a DMPO molecule was deconvoluted, and the resulting MS/MS...
spectrum in which all ions are transformed to the single charge-state is shown in Fig. 5. Based on the ProteinLynx software interpretation of these data, the tyrosine residue in this peptide was identified as the site of the DMPO adduct. The corresponding MS/MS spectrum and fragment ions are shown in Fig. 5A. A complete series of y ions are observed as well as the
Identification of Hemoglobin Radicals by Mass Spectrometry

Fig. 6. Amino acid residues trapped by DMPO as determined by mass spectrometry. The figure was generated from the crystal structure of the hemoglobin tetramer (32). The α chains are shown in blue, the β chains are shown in purple, and the hemes are shown in red. The Cys-93 of the β chains is shown in orange, the His-20, Tyr-24, and Tyr-42 of the α chains are shown in yellow, violet, and green, respectively.

After interpretation of the T4 MS/MS spectrum, however, many fragment ions are observed in the higher m/z range of the spectrum that cannot be accounted for by a tyrosine-DMPO adduct, e.g., ions at m/z 908.5, 1094.5, 1165.6 (labeled with an asterisk). In addition, some ions in the lower m/z range are observed which correspond to a fragment ion plus a DMPO molecule. For example, the ion of m/z 476.3 corresponds to a fragment ion plus a DMPO molecule and the ion of m/z 547.3 corresponds in mass to a b5 ion (m/z 436.2) plus DMPO. These data indicate the presence of a DMPO adduct on a second amino acid in the T4 tryptic peptide. Further interrogation of these data indicates that there is a DMPO adduct on the histidine residue (Fig. 5B). The mass difference between the ion of m/z 1165.6 (labeled as y2 in Fig. 5B) and the ion of m/z 1413.7 (labeled as y3 in Fig. 5B) corresponds to the mass of a histidine residue plus a DMPO. These data indicate the presence of a DMPO molecule on His-20 of T4 from hemoglobin, α chain. Other fragment ions observed which support the presence of DMPO on the histidine residue are labeled as y3 through y7, b4 through b9, a4, and His-DMPO (immonium ion for a histidine residue plus DMPO) in Fig. 5B. The parent ion of m/z 1640.8, therefore, is actually a mixture of two T4 tryptic peptides, one with a DMPO located at His-20 and one with a DMPO located at Tyr-24. The relative abundance of the corresponding fragment ions would lead one to speculate the His-20 DMPO adduct is present in a higher relative abundance than the Tyr-24 DMPO adduct. There is no evidence for the presence of a doubly adducted T4 peptide.

Although three sites of radical formation were identified on the α chain of hemoglobin (His-20, Tyr-24, and Tyr-42), there is currently no evidence observed that multiple sites are trapped by DMPO at the same time. In the deconvoluted electrospray mass spectrometry spectrum of the hemoglobin, H2O2, and DMPO reaction mixture (Fig. 2D) only ions that correspond in mass to the native α and β chains as well as ions that correspond to the addition of a single DMPO molecule to each of these chains is observed. For example, no ions are observed which would correspond to the α chain plus two DMPO molecules.

The amino acid residues that have been identified by mass spectrometry as a site for DMPO spin trapping in hemoglobin are shown in Fig. 6. The figure was derived from the x-ray crystal structure of the hemoglobin tetramer (32). The backbone of the α and β chains of hemoglobin are illustrated as ribbons, whereas the trapped amino acids and hemes are shown as space-filled models. Hemes are shown in red, His-20, Tyr-24, and Tyr-42 on the α chain are shown in yellow, violet, and green, respectively, whereas Cys-93 on the β chain is shown in orange. Based on previous studies, it has been determined that the initial product between the reaction of heme proteins and hydrogen peroxide is an oxidized heme consisting of a ferryl iron and a porphyrin cation radical usually referred to as compound I (33). An electron is then transferred from an amino acid residue to the oxidized heme. These electron transfers can occur either intermolecularly or intramolecularly (12). In the absence of DMPO, the hydrogen peroxide-dependent oxidation of hemoglobin leads to the incorporation of oxygen into hemoglobin.

In Fig. 6 it is observed that Tyr-24 and His-20 of the α chain of hemoglobin are nearly coplanar to one another. Because of the pi-pi interaction of Tyr-24 and His-20, the unpaired electron is apparently delocalized on both the tyrosine and histidine residue (pi-pi stacked radical pair) as in the chlorophyll dimer radical (34, 35). Therefore, only one radical is trapped at a time. To our knowledge, this is the first report of a histidinyl radical in a hemeprotein, although histidine is a common ligand of the redox active iron in such proteins and has been reported in the SOD/H2O2 system (36). The pi-pi stacking of histidine with tyrosine must lower the oxidation of histidine significantly because the oxidation potential of histidine (1.170 V) is normally well above that of tyrosine, cysteine, or even tyroptophan (1.015 V) (37).

In summary, using peptide mapping and MS sequencing methodologies, the DMPO radical adducts on hemoglobin were identified. Tandem mass spectrometry provided structural information as to the location of the DMPO molecules in the hemoglobin. It was determined that Cys-93 on hemoglobin, β chain, Tyr-42, His-20, and Tyr-24 on the α chain of hemoglobin all form a DMPO adduct. Thus, peptide mapping in combination with mass spectrometry is an extremely powerful technique for determining the location of radical adducts in proteins.

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b7, b8, a4, and b5 fragment ion. The mass difference between the y7 ion of m/z 745.4 and the y9 ion of m/z 1019.5 corresponds to the mass of a tyrosine residue plus a DMPO.

In summary, using peptide mapping and MS sequencing methodologies, the DMPO radical adducts on hemoglobin were identified. Tandem mass spectrometry provided structural information as to the location of the DMPO molecules in the hemoglobin. It was determined that Cys-93 on hemoglobin, β chain, Tyr-42, His-20, and Tyr-24 on the α chain of hemoglobin all form a DMPO adduct. Thus, peptide mapping in combination with mass spectrometry is an extremely powerful technique for determining the location of radical adducts in proteins.

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