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Top-Down and Bottom-up Mass Spectrometric Characterization of Human Myoglobin-Centered Free Radicals Induced by Oxidative Damage

Leesa J. Deterding[1,*], **Suchandra Bhattacharjee**[2], **Dario C. Ramirez**[2], **Ronald P. Mason**[2], and **Kenneth B. Tomer**[1]

1 *Laboratory of Structural Biology, National Institute of Environmental Health Sciences, National Institutes of Health, PO Box 12233, MD F0-03, Research Triangle Park, NC 27709*

2 *Laboratory of Pharmacology and Chemistry, National Institute of Environmental Health Sciences, National Institutes of Health, PO Box 12233, MD F0-03, Research Triangle Park, NC 27709*

Abstract

In an effort to determine the utility of top down mass spectrometric methodologies for the characterization of protein radical adducts, top down approaches were investigated and compared to the traditional bottom up approaches. Specifically, the nature of the radicals on human myoglobin induced by the addition of hydrogen peroxide and captured by the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) was investigated. The most abundant ion observed in the electrospray mass spectrum of this reaction mixture corresponds in mass to the human myoglobin plus one DMPO molecule. In addition, a second ion of lower abundance is observed which corresponds to a second DMPO molecule being trapped on myoglobin. Top down analyses using Fourier transform ion cyclotron resonance (FTICR) mass spectrometry can be used to characterize proteins; and thus, were performed on several different charge state ions of both the native and the mono-DMPO nitrone adduct of human myoglobin. Data produced from the top down analyses are very complex yet information rich. In the case of DMPO-modified human myoglobin, the top down data localized the DMPO spin trap to residues 97–110 of the myoglobin. The observation of the y_{43}^{+5} fragment ion arising from C-terminal cleavage to the cysteine-110 residue in the MS/MS spectrum of DMPO-modified myoglobin and not in the unmodified myoglobin implicates a change to this residue, specifically, DMPO adduction. On the other hand, using the traditional bottom up approach of peptide mapping and MS sequencing methodologies, two DMPO radical adducts on human myoglobin were identified, Cys-110 and Tyr-103. The bottom-up approach is more proven and robust than the top down methodologies. Nonetheless, the bottom up and top down approaches to protein characterization are complementary rather than competitive approaches with each having its own utility.

INTRODUCTION

Reactive oxygen species play a key role in both normal biological function and in the pathogenesis of a variety of human diseases. As a result of their implications in diseases, much research effort has focused on understanding the function of these reactive species (1,2). When reactive oxygen species overwhelm the cell's antioxidant system, oxidation of biomolecules occurs, with the intermediacy of biomolecule-centered free radicals. Targets for reactive oxygen species oxidation include DNA/RNA, proteins, and unsaturated lipids (3–6). Proteins,

*To whom correspondence should be addressed. Leesa J. Deterding, Ph.D., NIEHS, PO Box 12233, MD F0-03, RTP, NC 27709, Phone: 919-541-3009, Fax: 919-541-0220, E-mail: deterdi2@niehs.nih.gov.

in particular hemoproteins, are exposed to reactive oxygen species (such as hydrogen peroxide) in sites of chronic inflammation. Upon exposure, these hemoproteins can form hemoprotein-centered free radicals which may contribute to tissue and/or organ damage (7–9). The nature and localization of the radical in hemoproteins may be important to understanding the mechanisms of these reactions and their contributions to human diseases. In fact, a recent study has reported evidence for the association of nitrotyrosine (which forms via a tyrosyl radical) and coronary artery disease (10).

Protein-centered radicals have traditionally been studied through either direct electron spin resonance (ESR) or by spin-trapping (11). Because most radicals are very reactive, and thus, short-lived (i.e., μsec to secs), the spin-trapping approach has been more widely used. With the spin-trapping approach, spin trap molecules, such as 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), can react with radical sites in proteins in situ and in real time. During this reaction, the spin trap forms a covalent bond at the radical site in the protein primary sequence forming a protein-spin trap radical adduct (e.g. protein-DMPO radical adduct). In doing so, the radical adduct is, essentially, footprinting where the radical was initially formed and consequently trapped in the protein. The protein radical adduct is more stable and, consequently, more long-lived (i.e. secs to minutes) than a protein radical, but decays to form an ESR-silent DMPO-protein nitrone adduct in which the DMPO is covalently-bound to the protein.

To take advantage of these more stable, trapped adducts, an antibody with specificity for the nitrone adduct, which is the only stable product of trapping radicals with DMPO, was developed (12). 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 (immuno-spin trapping), we have successfully detected DMPO adducts in several heme-containing proteins (13–15), as well as in DNA (16). In proteins, however, structural assignment of the specific amino acid residue or residues that form radical adducts with the spin trap molecule is difficult with immuno-spin trapping or ESR; thereby, requiring the use of additional biochemical techniques, such as site-directed mutagenesis and amino acid derivatization. These methods, however, suffer from potential drawbacks, such as perturbation of the protein's stability and conformation by mutations or derivatization. Hence, the formation of the amino acid radical(s) and/or transfer of the radical may be affected.

Our groups have been collaborating for a number of years on using mass spectrometry to study protein free radicals where the use of peptide mapping methodologies with mass spectrometry allows for the unequivocal assignment of the trapped radical on the protein (12–14,17,18). We have been using a combination of peptide mapping by mass spectrometry and the immuno-spin trapping technique to investigate the formation and structures of the protein radical adducts generated on heme-containing proteins. To identify the precise amino acid residues (i.e. radical site) trapped by DMPO, we have utilized mass spectrometry-based sequencing. This bottom up approach includes proteolysis of the protein-derived DMPO adducts by trypsin or other suitable proteases, followed by MS peptide mapping, and finally MS/MS analyses of the peptides. With these combined techniques, we have identified the specific location of the DMPO adducts on several heme-containing proteins (13,14).

With the bottom up approach to the characterization of any protein of interest, it is common that many peptides resulting from digestion of the protein are not observed in the subsequent MS analyses, thus making complete characterization of the protein difficult. Therefore, as an alternative to the bottom up approach, we are currently developing a top down based methodology (19,20) for the identification of protein-DMPO adducts. Top down protein characterization refers to gas phase ionization of the intact protein subsequently followed by fragmentation in the mass spectrometer and was recently demonstrated on a protein of >200 kilodalton molecular weight (21). Because of the complexity of the resulting MS/MS spectra,

these analyses are generally performed on Fourier transform mass spectrometers (FTMS) which, characteristically, have high mass resolution capabilities. This approach has the advantage that by performing an MS/MS experiment on the intact protein, in theory, the entire sequence of the protein is analyzed in the mass spectrometer. This should enable more complete characterization of the protein and any post-translational modifications than the bottom up approach.

Several techniques exist for fragmenting peptides and proteins in the mass spectrometer. Collisional activation decomposition (CAD) has been successfully used for many years to fragment peptides and determine their sequence. The CAD process of proteins, however, is, in general, not as efficient as for peptides. Typically, CAD of proteins produces a limited number of fragment ions, thereby, resulting in low sequence coverage of the protein. Consequently, additional fragmentation techniques, such as electron capture dissociation (ECD) (22), infrared multiphoton dissociation (IRMPD) (23), and sustained off-resonance irradiation (SORI) CAD (24–26), have been developed to increase the efficiency of gas-phase fragmentation of proteins and are the topics of recent review articles (27–30). Briefly, the IRMPD and SORI-CAD fragmentation processes involve a slow increase in the internal energy of the ion (activation time ~10^{-2} s) (31). Internal energy is deposited in small increments until the ion of interest dissociates via the weakest bonds. This can often result in the loss of labile post-translational modifications. These methods are referred to as slow heating methods. Conversely, in ECD, low-energy electrons are used to irradiate the precursor ion preferentially cleaving the N-Cα amine backbone bond, thereby, generating c and z• (or c• and z) fragment ions. This is in contrast to the b and y fragment ions which are formed as a result of cleavage of the amide bond and are usually observed in the low energy collisionally induced fragmentation processes. Because ECD generally produces backbone fragmentations, post-translational modifications on proteins are typically retained. This can be highly advantageous when one is interested in determining the sites of post-translational modification, e.g. sites of phosphorylation.

For this study, both the top down and bottom up approaches were used to characterize DMPO-modified human myoglobin. Top down analyses were performed using either IRMPD or a combination of IRMPD/ECD on a Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR MS) or CAD on a quadrupole time-of-flight (Q-Tof) mass spectrometer (32). The results obtained from these top down approaches are compared to those obtained using the more traditional bottom up LC/MS/MS approach.

EXPERIMENTAL

Spin Trapping Reaction Conditions

The formation of the heme-protein radicals was initiated by treating a solution of human cardiac myoglobin (Life Diagnostics, West Chester, PA) with hydrogen peroxide in the presence of the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) (Alexis Biochemicals, San Diego, CA). The reactions of 30 μM myoglobin with 150 μM H_2O_2 (H_2O_2:heme molar ratio being 5:1) were carried out in the presence of 100 mM DMPO at 37 °C for 1 hr. Control experiments of myoglobin plus DMPO and myoglobin plus H_2O_2 under identical reaction conditions were also performed. Reactions were dialyzed against 50 mM ammonium bicarbonate buffer (pH 8) overnight to remove excess DMPO and H_2O_2. The reactions were analyzed by both immuno-spin trapping (ELISA) and mass spectrometry.

Enzyme-linked Immunosorbent Assay (ELISA)

The radical-derived DMPO nitrone adducts were determined using a standard ELISA in a 96 well plate. Ten μL of the reaction solution in 190 μL of 0.1 M bicarbonate buffer (pH 9.6) was

incubated for 60 min at 37 °C. The plates were washed once with 1X TBS washing buffer (0.10% Tween 20, 0.05% casein and 0.05% BSA) and blocked with coating buffer (0.1M bicarbonate buffer, pH 9.6, containing 2.5% casein and 2.5% BSA) for 60 min. Thereafter, the rabbit anti-DMPO serum (1:5000) in washing buffer was added and incubated for 60 min. After three washes, the secondary antibody (anti-rabbit IgG-alkaline phosphatases 1:5000 in wash buffer) was added and incubated for 60 min. Following three additional washes, the antigen-antibody complexes were developed using a CDP Star chemiluminescence system and the light emitted was recorded in arbitrary units using Xfluor Software.

Tryptic Digestion Conditions

Ten microliters of the human myoglobin control sample and ten microliters of the protein +DMPO+H_2O_2 reaction sample were each diluted to 50 μL in 50 mM ammonium bicarbonate buffer (pH 8) were subjected to tryptic digestion. Porcine trypsin (Promega Corporation, Madison, WI) was added at a protein:enzyme ratio of 20:1. The reactions were allowed to proceed overnight at 37 °C.

Electrospray Mass Spectrometry

Q-Tof MS—A Waters Micromass Q-Tof Ultima Global or a Waters Micromass Q-Tof Premier (Beverly, MA) hybrid tandem mass spectrometer was used for the acquisition of the electrospray ionization mass spectra and tandem mass spectra. These instruments are equipped with a nanoflow electrospray source and consist of a quadrupole mass filter and an orthogonal acceleration time-of-flight mass spectrometer. The needle voltage was ~3500 V and the collision energy was 10 eV for the MS analyses. For the MS/MS analyses of intact protein ions, various charge state ions were isolated with the quadrupole and the collision energy was ramped manually from 20 eV to 50 eV. Samples for flow injection analyses were diluted (1:1 to 1:10) with a solution of 50:50 acetonitrile:water (0.1% formic acid) and infused into the Ultima Global mass spectrometer at ~300 nL/min using a pressure injection vessel (33).

For the LC/MS/MS analyses, the Waters Micromass Q-Tof Premier instrument and automated data dependent acquisition software were employed. A Waters NanoAcquity Ultra Performance LC system was used to deliver the gradients. Injections of 2 μL were made onto a Waters Symmetry C18 trapping column (20 mm × 180 μm id) and a linear gradient of 2–40% (0.1% formic acid) over 60 min was used for the hromatographic separations. The column used was a 100 mm × 100 μm id Waters Atlantis™ dC18 column at a flow rate of 300 nL/min. Data analysis was accomplished with a MassLynx data system, MaxEnt deconvolution software (34), and ProteinLynx software supplied by the manufacturer.

FT-ICR MS—FT-ICR MS and MS/MS data were acquired on an IonSpec (Lake Forest, CA) quadrupole Fourier Transform ICR mass spectrometer with a 9.4 Tesla actively shielded magnet, equipped with a Waters Z-spray electrospray interface. For the MS analyses, ions were accumulated for 3 seconds in the hexapole region and then pulsed into the ICR cell. The ICR transient was measured at 1 MHz with 512k data points (~0.5 sec transient). Each transient was processed with a Blackman window function, zero-filled once, and Fourier transformed to the frequency domain. For the MS/MS analyses, fragmentation was produced by using either infrared multiphoton dissociation (IRMPD) or IRMPD in combination with electron capture dissociation (ECD). IRMPD fragmentation was performed with a 25 W CO_2 laser set at 42–55% of maximum power for 0.5–1 second. For the combined IRMPD/ECD MS/MS analyses, electrons were generated with a dispenser cathode operated with a filament current setting of 6.5 volts. Dissociation was performed by using a 1s IRMPD pulse at 20% power followed by a 100 ms pulse of electrons from an initial voltage of +5 v to a voltage of −1.25 v. For all MS/MS analyses, the ICR transient was measured at 1 MHz with 512k data points (~0.5 sec transient) and 20–50 scans were accumulated.

RESULTS AND DISCUSSION

In the present study, the utility of top down mass spectrometry has been investigated for the characterization of protein-centered radicals. For this work, the nature of the radical in the heme-containing protein human myoglobin was examined. The amino acid sequence of human myoglobin is similar to horse heart and sperm whale myoglobin sequences, except for a unique cysteine at position 110. Human myoglobin was reacted with hydrogen peroxide in the presence of the DMPO spin trap. Detection of the resulting protein radicals was investigated using immuno-spin trapping, enzyme-linked immuno-sorbent assay (ELISA) with an anti-DMPO antibody (12), and mass spectrometry. With the ELISA methodology, an abundant signal for a DMPO-nitrone adduct was detected in the complete reaction mixture of human myoglobin plus hydrogen peroxide and DMPO (35; data not shown). The observation of this adduct was dependent upon all of the components of the reaction mixture (data not shown).

To further verify that DMPO adducts of the protein free-radicals had been formed, the reaction mixtures were analyzed by electrospray ionization mass spectrometry on the Global Q-Tof mass spectrometer and the FTICR mass spectrometer. The resulting ESI mass spectra for the human myoglobin samples acquired by FTMS are shown in Figure 1. The resolved isotopic distributions for the most abundant +15 charge state ions are shown in the insets (observed R_s = ~20k; theoretical R_s = ~25k). For the human myoglobin alone sample (Figure 1A), the monoisotopic mass (M_I) was determined to be 17041.9142 (theoretical M_I = 17041.9142) whereas the monoisotopic mass from the myoglobin/H_2O_2/DMPO reaction mixture (Figure 1B) was determined to be 17152.9220 (theoretical M_I = 17152.9827). The observed mass errors from these data (3 ppm or less) fall within the expected mass error (~5–10 ppm) for externally calibrated data on this instrument. The resulting mass difference (Δm_{obs} = 111.0078; Δm_{theor} = 111.0685) observed between the most abundant ions in these spectra corresponds in mass to the addition of a DMPO spin trap molecule (C6H11NO, 113.0841). Additional ions observed at a much lower abundance relative to the myoglobin plus one DMPO ions in the spectrum from the complete reaction mixture are ions corresponding in mass to native myoglobin as well as those corresponding in mass to myoglobin plus two DMPO molecules.

Similarly, the most abundant ions observed in the deconvoluted ESI data acquired on a Waters Q-tof instrument (35, data not shown) are an ion of average molecular weight (M_r) of 17053.0 (calc M_r = 17052.7) and an ion of M_r = 17163.9 Da (calc M_r = 17163.8) for the native human myoglobin alone sample and the human myoglobin/H_2O_2/DMPO sample, respectively. These ions correspond in mass to the predicted amino acid sequence of human myoglobin and the addition of one DMPO molecule to native human myoglobin sequence, respectively. Also observed in the spectrum from the complete reaction mixture is a protonated molecule of M_r = 17053.2 Da, which corresponds in mass to native human myoglobin, and a low abundance ion of M_r = 17274.4 Da, which corresponds in mass to the addition of two DMPO molecules on the human myoglobin. In the absence of the DMPO spin trap (data not shown) in the reaction, native human myoglobin is observed (M_r = 17052.3) as well as oxidized forms of the protein which represents incorporation of oxygen into the protein presumably by the reaction of protein-derived free radicals with molecular oxygen. In this spectrum, the most abundant ion observed (M_r = 17100.3) corresponds in mass to the addition of three oxygens to human myoglobin. In the absence of hydrogen peroxide (data not shown), the deconvoluted mass spectrum is similar to that of myoglobin alone. These data indicate that the formation of the DMPO adduct on the human myoglobin is dependent on the presence of both the hydrogen peroxide and the DMPO spin trap in the reaction mixture.

Top Down Characterization

The utility of using top down approaches to determine the nature of the radicals formed on human myoglobin was explored. The solutions of myoglobin alone and the myoglobin/H_2O_2/

DMPO reaction mixture were subjected to MS/MS analyses on an FTMS and on a Q-Tof instrument. Dissociation of the parent ion was performed by CAD on the Q-Tof and by IRMPD and a combination of IRMPD and ECD on the FTMS. Several of the most abundant multiply charged ions were randomly selected for MS/MS analyses on a Q-Tof mass spectrometer (i.e. +13, +14, +16, and +18 charge states). The Q-Tof/CAD MS/MS spectra of the ions of *m/z* 948.4 and 954.7 which correspond to the $(M+18H)^{18+}$ ions of myoglobin and myoglobin plus DMPO, respectively, are shown in Figure 2. The identity of several low *m/z* ions could be readily determined in these spectra; however, one of the limitations to top down approaches on the Q-Tof instruments is that many of the fragment ions are not resolved to a point that the charge state can be accurately determined. Because proteins typically undergo the loss of water and ammonia upon CAD fragmentation, however, these common losses can be used to help determine the charge state of large fragment ions (36). To illustrate this, the expanded mass range of *m/z* 985–1005 for the MS/MS spectra of the $(M+18H)^{18+}$ ions (Figure 2) are shown in the spectra in Figure 3. Assuming these ion clusters result from fragmentation of the intact protein and subsequent water and/or ammonia losses, the charge state of the fragment ion observed at *m/z* 994.2 (Figure 3A) and of *m/z* 1000.8 (Figure 3B) is determined to be +17. These ions can then be assigned as the y_{151} fragment ion (amino acid residues 3–153) of myoglobin (calculated average y_{151}^{+17} = *m/z* 994.1) and of myoglobin plus one DMPO modification (calculated average y_{151}^{+17} = *m/z* 1000.6), respectively. The shift in the observed average *m/z* ratio of these two ions at this charge state, therefore, corresponds to the average mass of one DMPO molecule. Assignment of large fragment ions that do not show the multiple losses of water and/or ammonia is extremely difficult from data acquired on lower mass resolving instruments. None the less, many fragment ions could be assigned for all the charge states of the DMPO-modified myoglobin and the native myoglobin analyzed by this MS/MS approach and are mapped on the amino acid sequence of human myoglobin (Figure 4, fragment ions shown in red). Based on a comparison of these data, the site of DMPO modification can be determined to exist somewhere between residues 22 and 111 of human myoglobin.

Alternatively, gas phase fragmentation of the intact proteins was investigated on a high mass resolving FT mass spectrometer. Parent ion selection was performed in the ICR cell using an arbitrary wave function or in the quadrupole of the instrument prior to ion insertion into the ICR cell. Dissociation of the parent ion was performed by IRMPD or a combination of IRMPD and ECD. Several different charge state ions (i.e. +14, +15, +16, and +18 charge states) were selected for IRMPD MS/MS analyses. For parent ion selection prior to fragmentation, data are acquired in the narrow-band mode which allows for increased resolution and sensitivity (37, 38). As the mass window for parent ion selection on this instrument is approximately ± 10 Da, the narrow-band spectrum for isolation of the multiply charged ions of the myoglobin containing a single covalently-bound DMPO also includes the corresponding multiply charged ion of unmodified myoglobin as well as the multiply charged ion which corresponds in mass to myoglobin plus two DMPO molecules. Consequently, both unmodified myoglobin as well as myoglobin plus one and two DMPO adducts were fragmented in the MS/MS analyses from the DMPO-modified myoglobin. The IRMPD MS/MS data of the $(M+15H)^{15+}$ ions of *m/z* 1137.14 and of *m/z* 1144.54 which correspond in mass to myoglobin alone and myoglobin plus one DMPO are shown in Figure 5. (Due to the low relative abundance of the di-DMPO modified human myoglobin, an interpretable MS/MS spectrum was not obtained.) Because the MS/MS spectrum of the modified myoglobin is the result of fragmentation of unmodified as well as modified myoglobin and the fact that there is mainly a single modification in the myoglobin plus DMPO ion, these spectra are extremely similar and no obvious differences are readily observed.

To assist in the comparison of these data, the MS/MS spectra were overlaid. Upon doing so, two major interpretable differences were observed between the two spectra. An ion of *m/z* 1282.8751 (+5 charge state) is observed in the myoglobin MS/MS spectrum (Figure 6A, black

line) and to a much lesser extent in the myoglobin+DMPO MS/MS spectrum (Figure 6A, red line). This ion corresponds in mass to a y_{57}^{+5} ion (residues 97–153). The relative abundances of the y_{57}^{+5} ions are consistent with the relative abundances of unmodified myoglobin vs modified myoglobin that was transmitted to the ICR cell. This indicates that the modified myoglobin does not produce a y_{57}^{+5} fragment ion nor was a y_{57}+DMPO^{+5} ion from DMPO-modified myoglobin observed. Because this ion is not observed in the MS/MS spectrum of myoglobin+DMPO, residues 97–153 are implicated as the location of the DMPO adduct. Additionally, a +5 charge state ion of *m/z* 945.4806 is observed in the myoglobin+DMPO spectrum (Figure 6B, red line) and not in the control spectrum (Figure 6B, black line). This ion corresponds in mass to a y_{43}^{+5} ion (residues 111–153) arising from cleavage at the C-terminus of the cysteine-110 residue. This ion is not observed in the MS/MS spectrum of the unmodified myoglobin. (Note: The low abundance ion observed in the control spectrum of Figure 6B is a +4 charge state ion, not a +5 charge state ion). Therefore, we hypothesis that the DMPO adduct may be directing the fragmentation of this cleavage. This implicates a structural change to the Cys-110 residue, i.e. DMPO modification. Because the y_{57}^{+5} ion (aa 97–153) is not observed in the DMPO-modified MS/MS spectrum and the y_{43}^{+5} ion (aa 111–153) is observed in the modified myoglobin MS/MS spectrum, these data indicate that the DMPO modification is located within residues 97–110 (HKIPVKYLEFISEC) of human myoglobin. Many internal cleavage ions as well as various backbone cleavages (y and b ions) are also observed. No fragment ions were observed which could be readily attributed to a second DMPO modification in the protein's sequence. The amino acid residues observed from these various fragment ions are mapped onto the sequence of human myoglobin (Figure 4, fragment ions are shown in black and residues from internal cleavages are underlined), thereby, illustrating that, collectively, 94% sequence coverage of the protein was achieved using this top down approach.

Using ECD MS/MS conditions alone, the yield of ECD produced fragment ions from the myoglobin proteins was relatively low. Therefore, IRMPD was used in combination with ECD to increase the efficiency of fragmentation. It has been reported previously (39–41) that increased ECD fragmentation can be achieved by gently activating the ions by combining CAD or IRMPD with ECD. The IRMPD/ECD MS/MS spectra of the $(M+15H)^{15+}$ ion of *m/z* 1137.85 of human myoglobin (Figure 7) and DMPO-human myoglobin nitrone adducts (data not shown) were acquired. In these spectra, mostly c ions (various c ions from c_6 to c_{43}) are observed which correspond to cleavages at the N-terminus of this protein. Very few z ions (z_8 to z_{17}) are observed under the experimental conditions used. Using the IRMPD/ECD approach, no ions were observed which would allow confirmation of a DMPO trapping at Cys-110.

Although the cleavage of only 49 interresidue bonds was observed using IRMPD and IRMPD/ECD FTICR top down approaches, the IRMPD data provide sufficient information to localize the major site of DMPO trapping. The IRMPD MS/MS data from unmodified myoglobin shows an abundant y_{57}^{+5} ion (aa 97–153) whereas this ion is not observed in the fragment ion spectrum of modified myoglobin, thereby, suggesting that the DMPO modification is within the C-terminal residues of 97–153. In addition, the observation of the +5 fragment ion of *m/z* 945.4806 (y_{43}^{+5} fragment ion corresponding to aa 111–153) in the modified myoglobin MS/MS spectrum and not the unmodified myoglobin MS/MS spectrum suggests that the site of DMPO spin trapping may be directing this fragmentation. This implicates the Cys-110 as the possible site of DMPO modification within residues 97–110 (HKIPVKYLEFISEC).

From these analyses, the IRMPD/ECD approach has a tendency to generate a series of ions which differ by single amino acid residues. Thus, these data provided backbone sequence information, primarily from the termini of the myoglobin protein. Conversely, the IRMPD spectra yielded information from throughout the entire sequence of the protein. In general,

these two fragmentation approaches provide complementary structural information about the protein.

Bottom Up Characterization

To confirm the results obtained from the top down approaches, the nature of the radicals formed on human myoglobin was also explored using the traditional bottom up approach, i.e. proteolysis techniques followed by liquid chromatography/mass spectrometry and tandem mass spectrometry analyses. The reaction mixtures of myoglobin/H_2O_2/DMPO were subjected to tryptic digestion and analysis by LC/ESI/MS/MS. The data acquired from data dependant acquisitions were searched using ProteinLynx software. A user defined modification of DMPO to all amino acid residues was included in the search. From these analyses, two ions were observed which correspond in mass to predicted tryptic peptides of human myoglobin plus the addition of one and two DMPO modifications. These ions are observed as an $(M+3H)^{3+}$ ion of *m/z* 675.35 and as an $(M+3H)^{3+}$ ion of *m/z* 712.36 and correspond in mass to tryptic peptide 17 containing amino acid residues 103–118 plus 111 Da and 222 Da, respectively. The resulting MS/MS spectrum, after transformation of all ions to the single charge-state for the $(M+3H)^{3+}$ ion of *m/z* 675.35, is shown in Figure 8A. This spectrum shows a series of y ions (y_2 through y_{14}) and b ions (b_1 through b_6) as well as the loss of 145 from the y_9 through y_{14} ions. The loss of 145 corresponds in mass to a DMPO binding on the S of the cysteine side chain and then cleavage between the CH_2 and S with hydrogen transfer to the leaving moiety. These structurally informative fragment ions confirm the assignment of the bound DMPO to the Cys-110 residue of human myoglobin.

The MS/MS spectrum of the $(M+3H)^{3+}$ ion of *m/z* 712.36 which corresponds in mass to tryptic peptide 17 plus two DMPO adducts was deconvoluted using MaxEnt deconvolution software. The resulting MS/MS spectrum in which all ions are transformed to the single-charge state is shown in Figure 8B. An abundant ion of *m/z* 1990.2 (labeled as -145) is observed which corresponds in mass to the loss of S-DMPO plus hydrogen (cleavage between the CH_2 and S of the cysteine side chain). A nearly complete series of both y and b ions (or y-145 and b-145) are observed which correspond to cleavages along the peptide backbone. These data allow the assignment of DMPO to two amino acids in tryptic peptide 17 of human myoglobin, Cys-110 and Tyr-103. The major DMPO reaction site appears to be located at Cys-110, whereas, the second, lower abundance, reaction site was determined to be at Tyr-103.

Human Myoglobin Radicals

Based on previous studies (42), it has been determined that the initial product of the reaction of heme proteins and hydrogen peroxide is an oxidized heme consisting of ferryl iron and a porphyrin cation radical usually referred to as compound I. An electron is then transferred from an amino acid residue to the oxidized heme. These electron transfers can occur either intermolecularly or intramolecularly. Witting *et al.* (43,44) have reported previously on the formation of a thiyl radical in the human myoglobin/H_2O_2/DMPO system when the protein:DMPO concentration was 1:5. At higher DMPO concentrations (protein:DMPO 1:100) a tyrosine radical was observed by EPR and this adduct was assigned to Tyr-103 based on comparison of the EPR signal to that reported on sperm whale and horse heart myoglobins. Additionally, Witting *et al.* (45) showed that a DMPO thiyl radical adduct (Cys-110) was detected by electron paramagnetic resonance spectroscopy when human myoglobin was treated with peroxynitrite in the presence of DMPO.

The only structure in the database for human myoglobin is an x-ray crystal structure of a double mutant, K45R and C110A (Figure 9) (46). Assuming the cysteine to alanine mutation does not greatly alter the overall structure, the 3-dimensional location of the cysteine can be identified using the structure. The amino acid locations that have been determined by mass spectrometry

as sites for DMPO spin trapping in human myoglobin are shown in Figure 9. The backbone of myoglobin is illustrated as a ribbon whereas the amino acids at position 103 and 110 (DMPO trapped residues) and the heme are shown as spacefilled models. The heme is shown in red, while Ala-110 (corresponding to Cys-110) and Tyr-103 are shown in yellow and purple, respectively.

At the protein:DMPO concentration ratios used for this study (> 1:100) the most abundant DMPO modification appears to be located at the Cys-110 based on the MS/MS data of the mono-DMPO modified T17 tryptic peptide. The MS/MS spectrum of the di-DMPO modified T17 tryptic peptide shows modifications at the Cys-110 and Tyr-103. From these data, either the Cys-110 is the preferred site of radical formation or the Tyr-103 residue is the initial radical site which then gets transferred intramolecularly to Cys-110. This type of intramolecular transfer between tyrosine and cysteine residues has been recently reported in model peptides (47). This intramolecular radical transfer has been investigated in human myoglobin in further detail (35). The results from this investigation suggest that the primary site of radical formation is the tyrosyl radical followed by electron transfer of the radical from the tyrosine to the cysteine. It should also be noted that, in electron transfer reactions, the nature of the radical trapped depends on the DMPO concentration used. At high DMPO concentrations, trapping will occur at the reactive radical that is formed first and, thus, these results can be misleading.

CONCLUSIONS

This study combined top down and bottom up mass spectrometric methods to study heme-protein free radicals. In summary, using top down methodologies, MS/MS data for native human myoglobin and DMPO-modified human myoglobin were obtained; however, these spectra are extremely complex. Manual interpretation of these spectra is very tedious and time consuming. None the less, manual interpretation of these spectra allows for the determination that the location of the most abundant DMPO adduct on human myoglobin is within residues 97–110, though without the rigor provided by the MS/MS data in the bottom up approach. In comparison, using the traditional bottom up approach of peptide mapping and MS sequencing methodologies, two DMPO radical adducts on human myoglobin were identified. Tandem mass spectrometry provided structural information as to the location of the DMPO adducts, Cys-110 and Tyr-103.

In conclusion, the bottom up and top down approaches to protein characterization are complementary rather than competitive approaches with each one having its own utility. Data produced from the top down analyses are very complex yet information rich. FTICR top down experiments can be used to characterize proteins, and, in the case of DMPO-modified human myoglobin, localize the DMPO modification to within 14 residues. On the other hand, bottom up approaches are more robust and proven, however, 100% complete sequence coverage of a protein can often be difficult to achieve. Perhaps the two approaches will ultimately prove most beneficial by meeting somewhere in between in which large domains or fragments of proteins are analyzed intact as a “middle down” approach (48). Ultimately, the incessant developments in the field of mass spectrometry allow for the continual determination of more complete protein primary structures and their posttranslational modifications.

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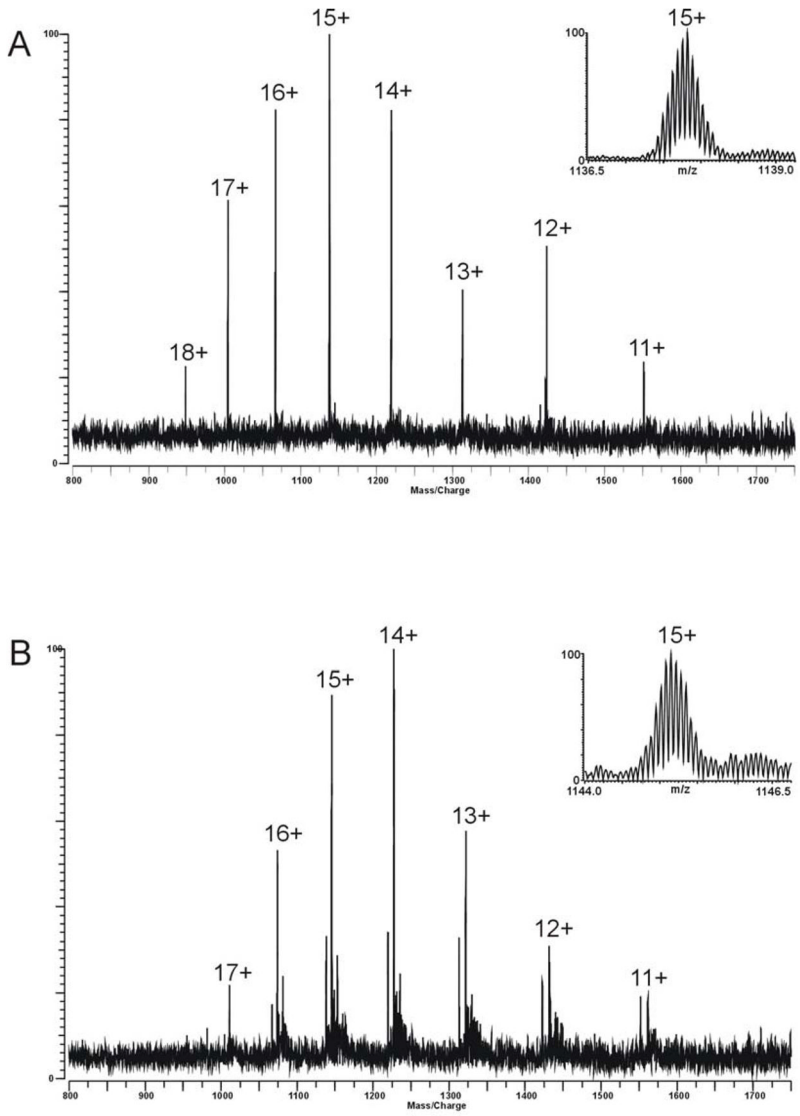

Figure 1.

FTICR MS data obtained from the reaction of human myoglobin with hydrogen peroxide in the presence of DMPO. A) Human myoglobin and B) Human myoglobin plus DMPO and H_2O_2. An expanded view of the +15 charge state ion is shown in the insets.

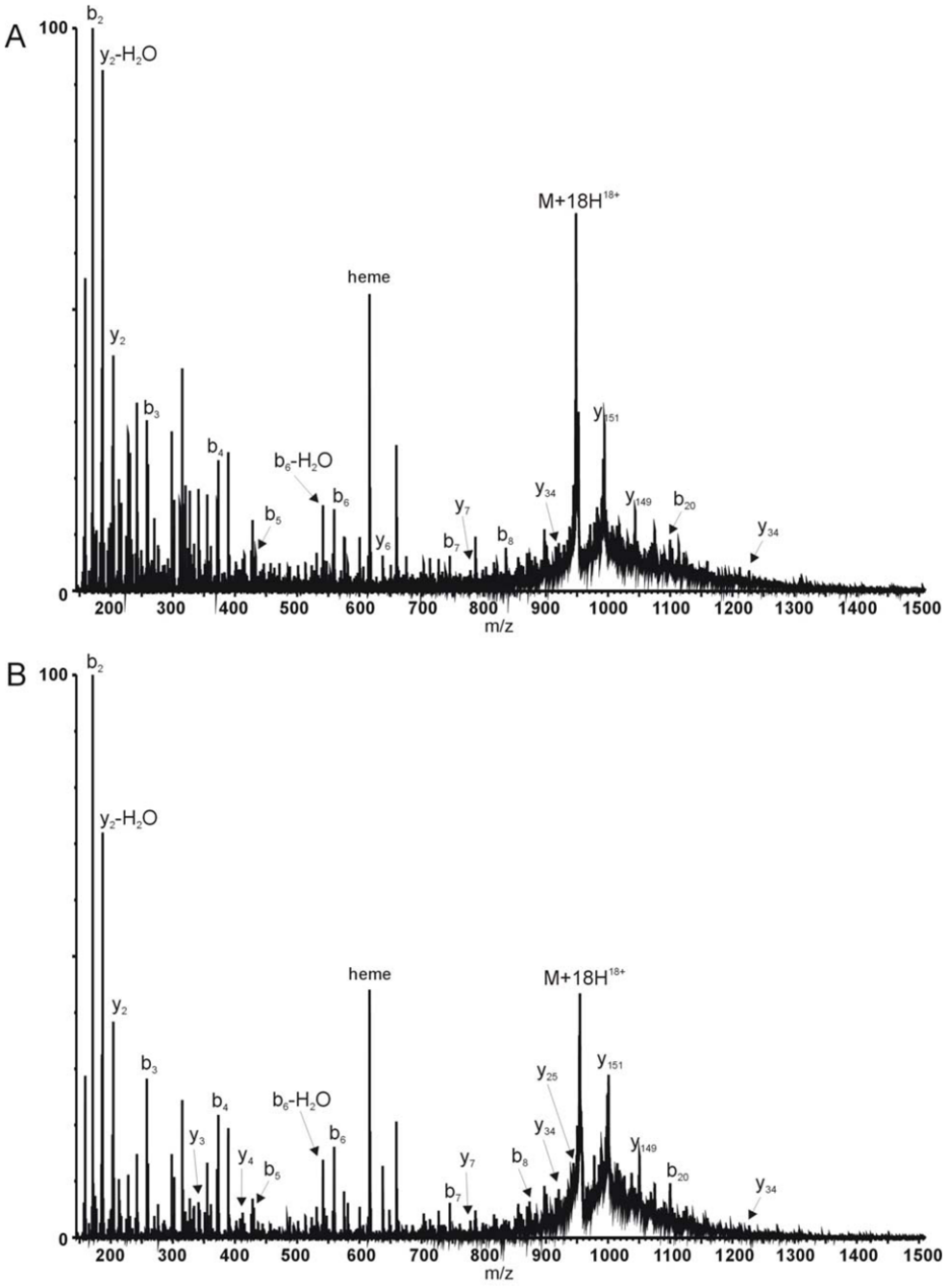

Figure 2.
Q-Tof/CAD MS/MS spectra of the $(M+18H)^{18+}$ ion of A) *m/z* 948.4 of human myoglobin and B) *m/z* 954.7 of human myoglobin-DMPO nitrone adduct.

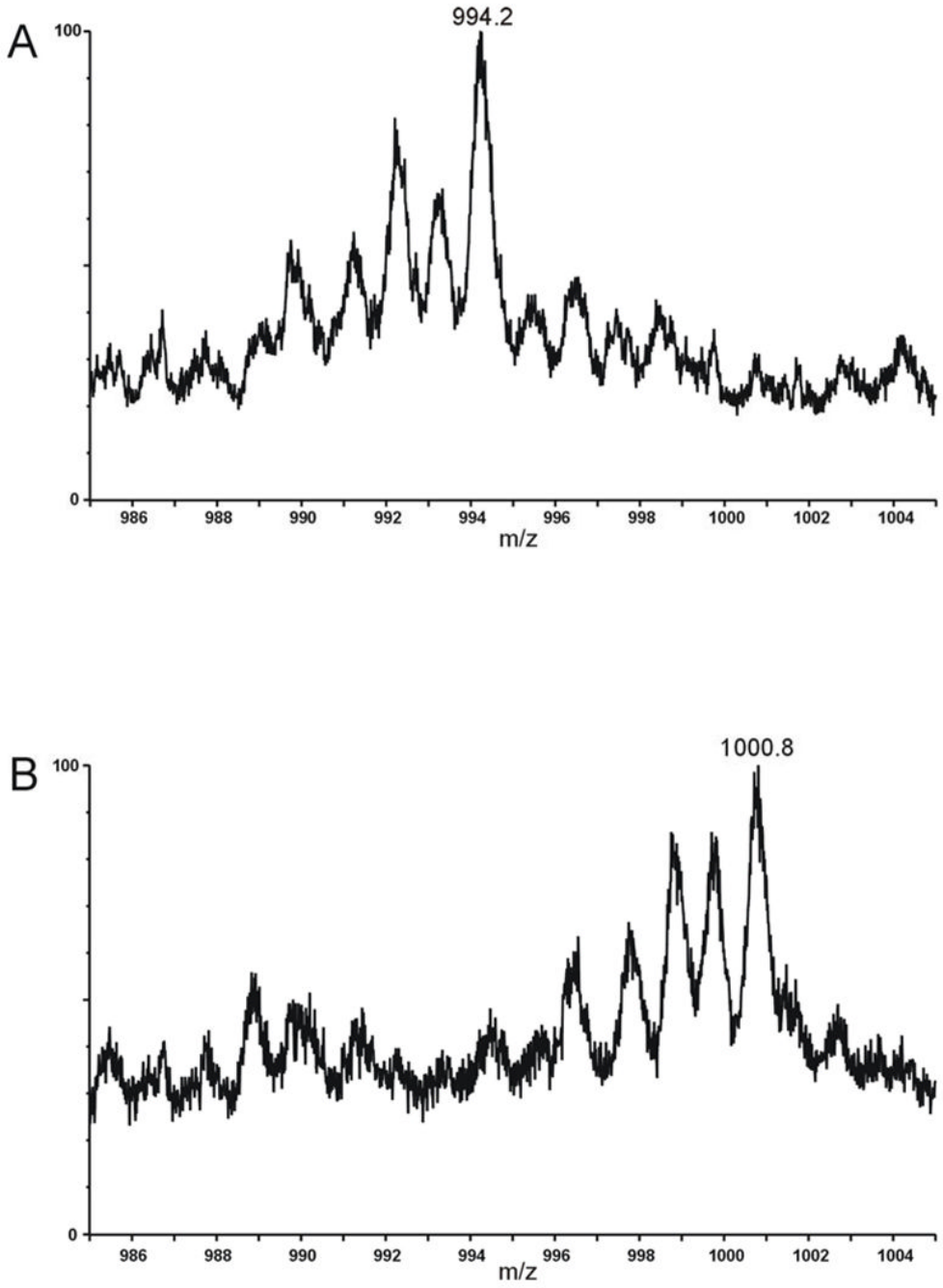

Figure 3.

Expanded mass range from *m/z* 985–1005 from the Q-Tof/CAD MS/MS spectra of the $(M+18H)^{18+}$ ion of A) *m/z* 948.4 of human myoglobin and B) *m/z* 954.7 of human myoglobin-DMPO nitrone adduct.

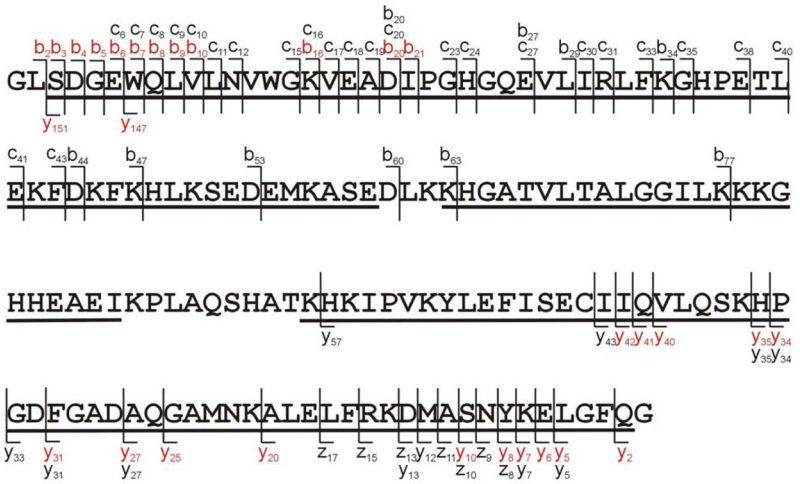

Figure 4.

Top down map of the cleavages observed in the tandem mass spectra of human myoglobin. Fragment ions in red were observed in the Q-Tof CAD MS/MS data. Fragment ions in black were observed in the FTICR IRMPD and/or IRMPD/ECD data. Underlined residues are observed as internal fragmentations in the IRMPD spectra.

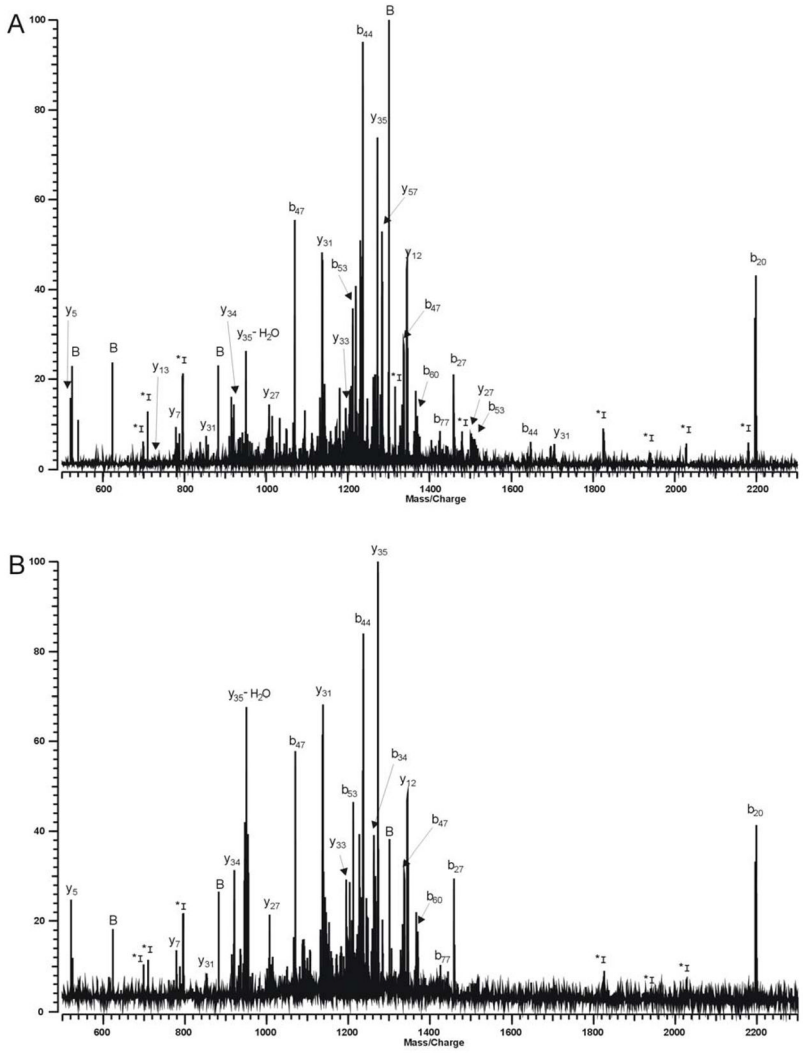

Figure 5.
FTICR IRMPD MS/MS spectra of the $(M+15H)^{15+}$ ions of A) human myoglobin and B) human myoglobin-DMPO nitrone adduct. Ions labeled as "*I" are internal fragment ions and ions labeled as "B" are background ions.

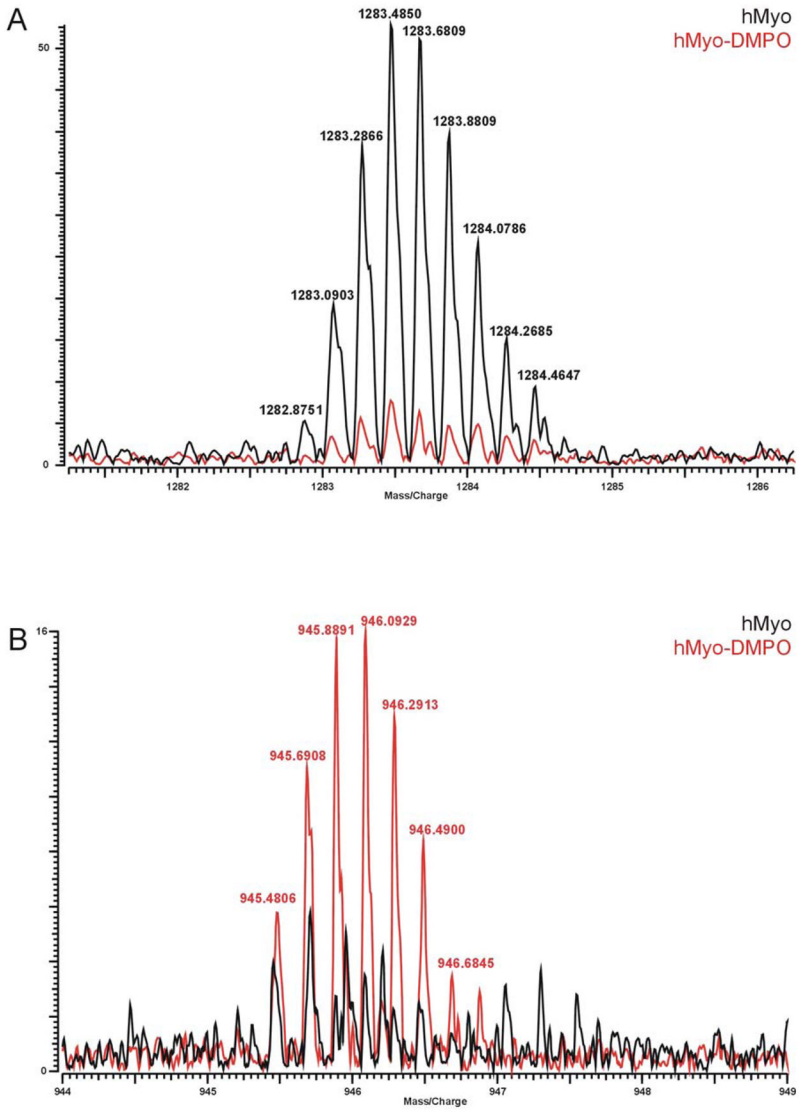

Figure 6.

Overlay of FTICR IRMPD MS/MS spectra of the $(M+15H)^{15+}$ ions of human myoglobin (black line) and human myoglobin-DMPO nitrone adduct (red line). Expanded mass range of A) *m/z* 1282.5 to 1285.5 and B) *m/z* 945.0 to 957.5.

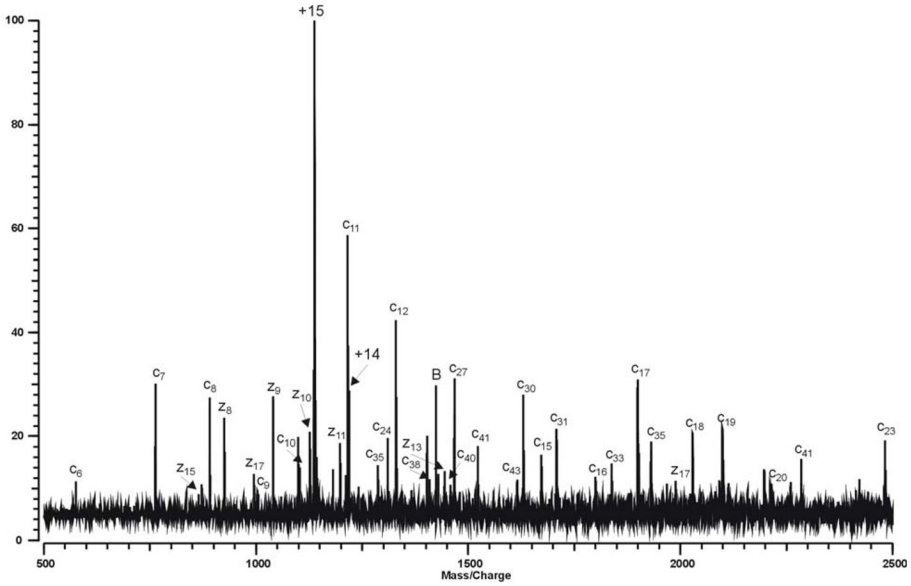

Figure 7.
FTICR IRMPD/ECD MS/MS spectrum of the $(M+15H)^{15+}$ ion of human myoglobin.

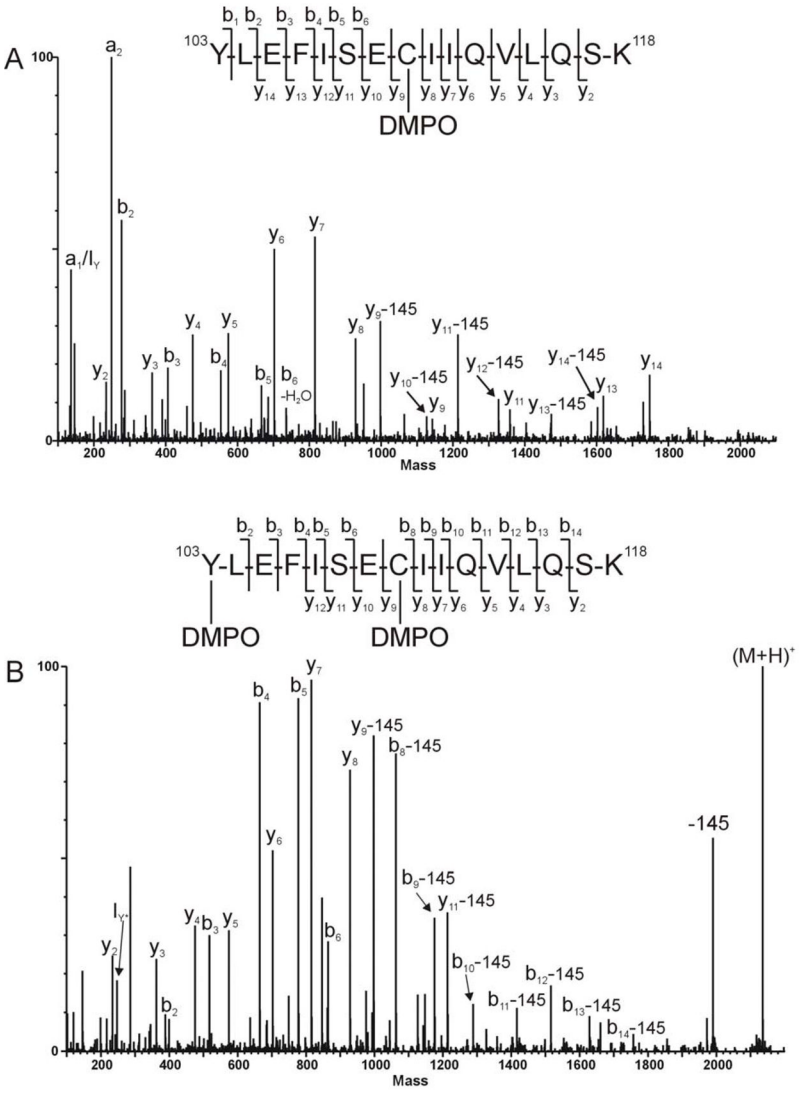

Figure 8.

A) Deconvoluted ESI/MS/MS spectrum of the $(M+3H)^{3+}$ ion of *m/z* 675.35 which corresponds in mass to tryptic peptide T17 plus one DMPO adduct of human myoglobin. B) Deconvoluted ESI/MS/MS spectrum of the $(M+3H)^{3+}$ ion of *m/z* 712.36 which corresponds in mass to tryptic peptide T17 plus two DMPO adducts of human myoglobin.

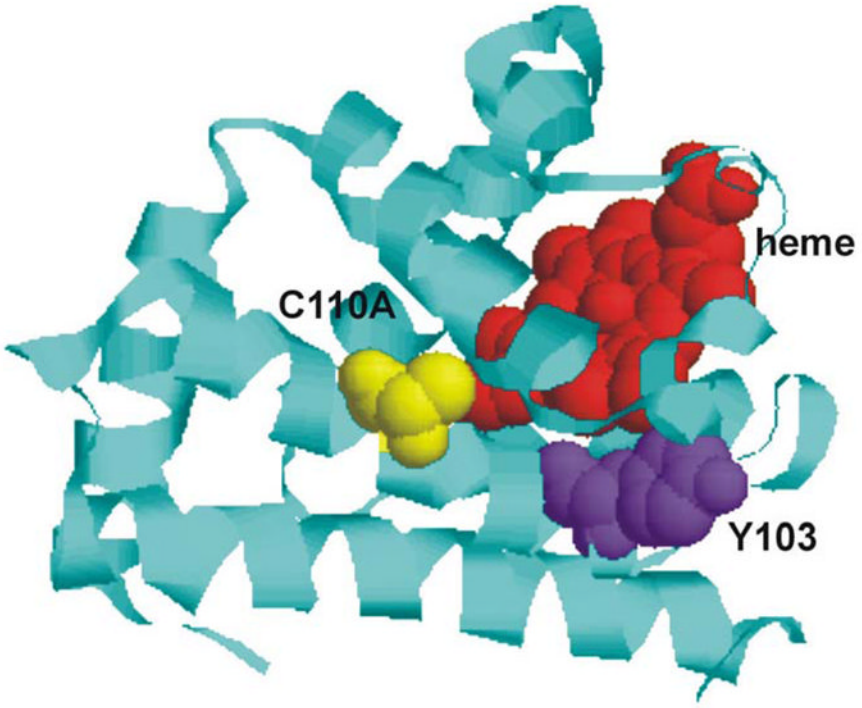

Figure 9.

Positions of the amino acid residues in human myoglobin treated with hydrogen peroxide and trapped by DMPO as determined by mass spectrometry. Figure was generated from the x-ray crystal structure of the human myoglobin double mutant, K45R and C110A (46). The backbone of myoglobin is shown in cyan. The heme is shown in red, Ala-110 (corresponding to Cys-110) is shown in yellow and Tyr-103 is shown in purple.