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Electron Transfer between a Tyrosyl Radical and a Cysteine Residue in Hemoproteins: Spin Trapping Analysis

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Abstract: We investigated electron transfer between a tyrosyl radical and cysteine residue in two systems, oxyhemoglobin (oxyHb)/peroxynitrite/5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) and myoglobin (Mb)/hydrogen peroxide/DMPO, using a combination of techniques including ESR, immuno-spin trapping (IST), and ESI/MS. These techniques show that the nitrone spin trap DMPO covalently binds to one or more amino acid radicals in the protein. Treating oxyHb with peroxynitrite and Mb with H_2O_2 in the presence of a low DMPO concentration yielded secondary Cys-DMPO radical adduct exclusively, whereas in the presence of high DMPO, more of the primary Tyr-DMPO radical adduct was detected. In both systems studied, we found that, at high DMPO concentrations, mainly tyrosyl radicals (Hb-Tyr^{42}/Tyr^{24} and Mb-Tyr^{103}) are trapped and the secondary electron-transfer reaction does not compete, whereas in the presence of low concentrations of DMPO, the secondary reaction predominates over tyrosyl trapping, and a thiyl radical is formed and then trapped (Hb-Cys^{93} or Mb-Cys^{110}). With increasing concentrations of DMPO in the reaction medium, primary radicals have an increasing probability of being trapped. MS/MS was used to identify the specific Tyr and Cys residues forming radicals in the myoglobin system. All data obtained from this combination of approaches support the conclusion that the initial site of radical formation is a Tyr, which then abstracts an electron from a cysteine residue to produce a cysteinyl radical. This complex phenomenon of electron transfer from one radical to another has been investigated in proteins by IST, ESR, and MS.

Introduction

Protein-centered radicals are implicated in the pathogenesis of various diseases such as atherosclerosis, heart disease, and possibly aging.[1−3] Considerable attention has been focused on the formation, stability, and reactions of hemoprotein radicals formed from hemoglobin and myoglobin. A number of such globin-centered free radicals have been detected in biological systems.[4,5] In particular, biochemical studies have been focused on hemoprotein-derived radicals[6−8] such as the prostaglandin H synthase tyrosyl radical,[9] the mitochondrial cytochrome *c* oxidase thiyl radical,[10] the cytochrome *c* peroxidase tryptophanyl radical,[11] the cytochrome *c* tyrosyl radical,[12] the horse metmyoglobin tyrosyl radical,[13] and the hemoglobin thiyl[14,15] and tyrosyl radicals.[16]

Some of the potentially toxic effects of protein radicals, as exemplified by protein-mediated initiation of lipid peroxidation,[17−20] involve both intermolecular[21,22] and intramolecular[22,23] translocation of radical centers from one amino acid to another.[24] It has also been proposed that free radical damage in proteins may well occur through a chain process similar to that of lipid peroxidation.[25] One particularly well-studied example of radical translocation is provided by globin radicals formed by myo-

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globin upon reaction with hydrogen peroxide.[26−28] It has recently been reported that electron transfer from the Cys residue to the tyrosyl radical inhibits tyrosine nitration in model peptides.[29]

The technique presently most widely used to study protein-centered radicals is electron spin resonance (ESR), either direct or in combination with the spin-trapping technique. The spin-trapping technique involves the addition of the reactive free radical across the double bond of a diamagnetic spin trap to form a much more stable radical adduct that is examined with ESR. The spin-trapped adduct of the radical exists in three redox forms: (1) the nitroxide radical adduct, (2) the corresponding hydroxylamine formed by one-electron reduction of the radical adduct, and (3) the corresponding nitrone formed by one-electron oxidation of the radical adduct.[30] ESR is useful in detecting the nitroxide radical adduct, which is almost always very short-lived. The radical adduct then decays over time to the ESR-silent, stable nitrone adduct and, potentially, other product(s). Of the three redox forms of trapped radicals, the nitrone adduct is the most stable end product and preserves the chemical bond formed during spin trapping.[30] The residues where the spin trap is attached, as well as the molecular weight and sequence of the protein, can be determined with mass spectrometry, thus providing unambiguous identification.

Recently, a novel rabbit polyclonal antiserum against the nitrone spin trap 5,5-dimethyl-1-pyroline *N*-oxide (DMPO) was developed and used in the horse heart metmyoglobin/H_2O_2 system, where DMPO is known to trap the tyrosyl-Tyr[103] radical.[31] This antibody was used to develop immuno-spin trapping (IST), a technique that combines the specificity of spin trapping with the sensitivity of an antigen antibody-based assay and is a simple and reliable method for detecting protein radicals in the stable nitrone form.[30,32] The antibody has been extensively used in detecting several DMPO nitrone adducts in a number of biochemical systems[33−36] and has been successfully used to detect nitrones derived from thiyl radical adducts on small peptides as well.[37]

In hemoprotein oxidation chemistry, the precise location of the radical on the protein likely depends, to some extent, on both the mechanism by which it is formed and the stability of the radical species formed at the particular amino acid residue.[23] Globin radicals have generally been found on the tyrosine[38,39] and/or tryptophan residues[40] where the unpaired electron can be stabilized through extensive resonance delocalization;[16] such radicals may then undergo subsequent chemistry[40,41] to oxidize other biological molecules.[42]

Both ESR and immuno-spin trapping have been used to examine the reaction of human ferrous oxyhemoglobin (oxyHb) with peroxynitrite.[43] The term peroxynitrite is used to refer to the sum of the peroxynitrite anion ($ONOO^-$, oxoperoxonitrate (-1)) and peroxynitrous acid (ONOOH, hydrogen oxoperoxonitrate). Curiously, while ESR analysis of this system showed the trapped DMPO thiyl radical adduct, after blocking the cysteine site by reaction with *N*-ethylmaleimide (NEM), IST led to more detected nitrone adduct, consistent with trapping radicals at sites other than cysteine. Since the tyrosyl radical was known to react with DMPO, its nitrone was proposed as the moiety reacting with the antibody. It was further proposed that the anti-DMPO antibody may not recognize the DMPO thiyl radical adduct in this case. In this work, our aim was to explore the phenomenon of residue−residue radical transfer within proteins to see if this could give insight into the earlier result. To that end, we have studied two model systems, human oxyHb with peroxynitrite and human heart myoglobin (Mb) with H_2O_2,[26,27] to elucidate electron transfer between Tyr and Cys residues and its effect on DMPO adduct detection by ESR, MS, and IST. In both systems studied, formation of the globin-centered radical is well established.

We have now confirmed that, in both systems, the initial site of radical formation is at the tyrosine residue, followed by an electron transfer from tyrosine to the cysteine residue to form a thiyl radical. In the presence of high DMPO concentrations, tyrosyl radicals were trapped and the secondary reactions could not compete, whereas in the presence of low DMPO concentrations, the secondary reaction was able to proceed and a thiyl radical was formed and subsequently trapped. This type of result has been reported in the human heart myoglobin system with ESR,[26,27] but has been investigated here for the first time through the combined use of IST, ESR, and MS for both Mb and Hb systems. We further conclude that caution must be exercised by judiciously adjusting the DMPO concentration when an electron-transfer process is suspected.

Experimental Procedures

Reagents. Human oxyferrous Hb ($\epsilon_{541} = 13.8\ mM^{-1}\ cm^{-1}$) was a kind gift of Apex Biosciences Inc. Highly purified human cardiac myoglobin ($\epsilon_{408} = 188\,000\ M^{-1}\ cm^{-1}$)[44] was obtained from Life Diagnostics. ThioGlo-1 was purchased from Calbiochem. Diethylenetriamine pentaacetic acid (DTPA), 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), NEM, and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich. The spin trap DMPO was purchased from Alexis Biochemicals and purified twice by vacuum distillation at room temperature and stored under argon atmosphere at −80 °C. The DMPO concentration was measured at 228 nm assuming a molar absorption coefficient of 7800 $M^{-1}\ cm^{-1}$. Hydrogen peroxide was obtained from Fisher Scientific Company. The hydrogen peroxide concentration was verified using UV absorption at 240 nm ($\epsilon_{240} = 43.6\ M^{-1}\ cm^{-1}$).

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Peroxynitrite was synthesized and stored as described.[45−47] Contaminating H_2O_2 was eliminated with MnO_2, and peroxynitrite concentration was determined at 302 nm ($\epsilon_{302} = 1670\ M^{-1}\ cm^{-1}$). All buffers used were treated with Chelex 100 ion-exchange resin (Bio-Rad Laboratories) to avoid transition-metal-catalyzed reactions.

Chemical Modifications of the Hemoproteins. Modification of Cysteine Residue of the Heme Protein. Free Cys residues on human oxyHb and Mb were chemically modified by mixing native heme proteins with NEM at a molar ratio of 1:10. The mixture was incubated at room temperature for 1 h in the dark. The mixtures were then dialyzed against 10 mM sodium phosphate buffer, pH 7.4. A second aliquot of the hemoproteins was mixed with a 30 mM DTNB solution in dimethylsulfoxide at a molar ratio (heme/DTNB) equal to 1:10. The reaction mixtures were incubated in the dark for 1 h at 24 °C and, after three changes of buffer, were dialyzed overnight against sodium phosphate buffer, pH 7.4.

Iodination of Tyrosine Residues of the Heme Protein. The iodination reaction mixture contained 50 mM hemoprotein (as heme) in 50 mM phosphate buffer and NaI. The iodination of Tyr residues was initiated by the addition of two *N*-chlorobenzenesulfonamide-immobilized beads (IODO-Beads, Pierce Chemical Co.). The reaction was allowed to proceed with shaking at 25 °C for 10 min and then dialyzed against 10 mM phosphate buffer. Protein concentration was determined using the BCA protein assay kit using bovine serum albumin as the standard.

Total protein-SH in iodinated samples was measured by two different methods:

(1) Fluorescence assay. The concentration of protein-SH was determined using ThioGlo-1, which is a maleimide reagent that produces a highly fluorescent product upon its reaction with SH groups.[48] Protein-SH was determined from the fluorescence response after addition of 6 mM SDS to 10 mM of each sample. All reactions were carried out in 0.1 M phosphate buffer, pH 7.4, and ThioGlo concentration used was 10 mM. A fluorescence plate reader (Tecan) was used to detect fluorescence at excitation and emission wavelengths of 360 and 535 nm, respectively. Protein-SH concentration was determined to be 83%. This method used SDS to make available all the SH in the proteins. Thus fluorescence evoked by the addition of this detergent was due to all of the protein-SH groups and may be more accurate than the DTNB assay.

(2) DTNB assay. Samples were diluted 5 times in phosphate buffer containing 5 mM DTNB. After 15 min of incubations at room temperature, samples were transferred to Millipore 5 kDa mass cut-off filters and made to spin for 15 min to separate the protein (heme-containing fraction) from the low molecular weight (TNB-containing fraction). This was necessary to avoid interference since TNB and heme absorb strongly in the 400−420-nm region. After filtration, TNB was further diluted 10 times in phosphate buffer, pH 7.9, and absorbance at 412 nm ($\epsilon_{412} = 13.6 \times 10^3\ M^{-1}\ s^{-1}$) was measured. In the case of heme quantitation, the samples were diluted directly in phosphate buffer (100 times), and absorbance at 406 nm ($\epsilon_{406} = 154 \times 10^3\ M^{-1}\ cm^{-1}$) was recorded. The thiol content was normalized to the heme concentration and was determined to be 60%.

Chemical Reactions. Typically, the reaction mixture contained 0.5 mM native or modified oxyHb and 0.25 mM peroxynitrite (i.e., a molar ratio equal to 2:1, heme/$ONOO^-$) in the presence of varying amounts of DMPO. After 1 h of incubation at 37 °C, the reaction mixture was diluted by a factor of 50 in phosphate buffer and then analyzed by enzyme-linked immuno-sorbent assay (ELISA). In the myoglobin experiments, 10 μM human Mb (as heme) was mixed with 50 μM hydrogen peroxide (molar ratio 1:5, heme/H_2O_2) in the presence of varying amounts of DMPO for 1 h at 37 °C. Reactions were stored at −80 °C until analysis.

Immuno-Spin Trapping Analysis. A rabbit anti-DMPO polyclonal serum was obtained and applied in our laboratory[31] for the development of immuno-spin trapping.[30] In the present study, we used the antiserum to immunochemically evaluate the respective radical-derived DMPO nitrone adducts in two systems involving electron-transfer processes. Two heterogeneous immunoassays were used, ELISA and Western blot.

(1) ELISA. A standard ELISA procedure in 96 well plates (Greiner Labortechnik) was used. Ten microliters of the adduct solution in 190 μL of the coating buffer (100 mM sodium bicarbonate, pH 9.6) was incubated for 60 min at 37 °C. The plates were washed once with washing buffer (1 × Tris-buffered saline containing 0.05% Tween 20 and 0.05% casein) and blocked with coating buffer (0.1 M bicarbonate buffer, pH 9.6, containing 2.5% casein) for 60 min. Thereafter, the rabbit anti-DMPO serum, diluted 1:5000 in wash buffer, was added and incubated at 37 °C for 60 min. After three more washes, the secondary antibody (alkaline phosphatase (AP)-conjugated anti-rabbit $IgG_{(FC)}$, Pierce), diluted 1:5000 in wash buffer, was added and incubated for 60 min. After three washes, the antigen−antibody complexes were developed using a chemiluminescence system (CDP-Star, Roche Applied Bioscience), and the light emitted was recorded as arbitrary light units using X-Fluor software (Tecan).

(2) Western blot. Proteins (1 μg/lane) were separated on a reducing 4−12% BisTris NuPage system (Invitrogen) and electroblotted onto a nitrocellulose membrane. The membrane was blocked with a 4% cold water fish gelatin solution in 100 mM bicarbonate buffer, pH 9.6, for 90 min at room temperature. The membrane was washed once with washing buffer (0.2% cold water fish skin gelatin in Tris-buffered saline, pH 7.4), then exposed to the anti-DMPO serum (1:5000 in washing buffer) for 1 h, followed by three washing steps. The secondary antiserum anti-rabbit IgG (conjugated to AP) was added at a dilution of 1:5000 in washing buffer. After exposure for 1 h, the membrane was washed three times with washing buffer and one time with Tris-buffered saline, pH 9.6. The antigen−antibody complexes were detected using enhanced chemiluminescence (Nitroblock II and CDP-Star), and then the nitrocellulose membranes were exposed to an X-ray film.

ESR Spin-Trapping Experiments. Typically, 3 mM OxyHb was mixed with 1.5 mM peroxynitrite in the presence of varying concentrations of DMPO. The reaction was carried out in phosphate buffer containing 100 μM DTPA to minimize the possibility of transition-metal-mediated decomposition of peroxide by Fenton chemistry. The reaction mixture (250 μL, final volume) was transferred to a flat cell immediately after peroxynitrite addition, and the spectra were recorded within 1 min after starting the reaction. ESR spectra were obtained with a Bruker EMX ESR spectrometer equipped with an ER4122SHQ cavity operating at 9.76 GHz and at room temperature. The ESR spectrometer settings were as follows: scan range, 100 G; modulation frequency, 100 kHz; modulation amplitude, 3.0 G; microwave power, 20 mW; receiver gain, 1×10^5; time constant, 164 ms; and conversion time, 164 ms. The spectra shown are an average of three scans.

Mass Spectrometry Analyses. Typically, the reaction mixture consisted of 30 μM human Mb and 150 μM H_2O_2 in the presence of 100 mM or 6 mM DMPO. Samples of the reaction mixture were first dialyzed exhaustively against ammonium bicarbonate buffer and water to remove traces of inorganic contaminants. A Waters Q-Tof Global or a Waters Q-Tof Premier hybrid mass spectrometer was used for the acquisition of the electrospray ionization mass spectrometry (ESI/MS) and tandem mass spectra. These instruments were equipped with a nanoflow electrospray source and consisted 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 analysis. Samples for flow injection analyses were diluted 1:1

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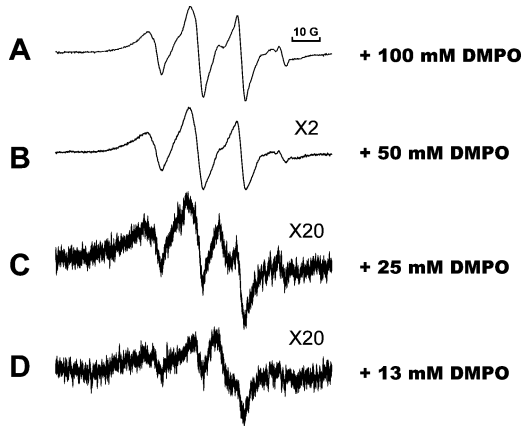

Figure 1. Formation of the hemoglobin radical-derived DMPO adducts from the reaction of native oxyHb with peroxynitrite in the presence of DMPO−DMPO spin trapping of the thiyl radicals formed from the reaction of oxyHb with peroxynitrite as a function of DMPO concentrations. Typically 3 mM oxyHb was mixed with 1.5 mM peroxynitrite in 10 mM phosphate buffer containing 0.1 mM DTPA, in the presence of various concentrations of DMPO. Instrumental conditions: modulation amplitude, 3.0 G; time constant, 164 ms; receiver gain, 1×10^5; and microwave power, 20 mW.

with a solution of 50:50 acetonitrile/water (0.1% formic acid) and infused into a Waters Ultima Global mass spectrometer at ~300 nL/min using a pressure injection vessel.

For the LC/MS/MS analyses, a Waters Q-Tof Premier spectrometer and automated data-dependent acquisition software were employed. For these acquisitions, the instrument can switch from the MS mode to the MS/MS mode and then return 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. A Waters NanoAquity 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 chromatographic 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, and a ProteinLynx software program supplied by the manufacturer.

Results

ESR Spin Trapping of DMPO Oxyhemoglobin Radical Adducts. When oxyHb (3 mM) was reacted with peroxynitrite (1.5 mM) and trapped with 100 mM DMPO, it produced a thiyl radical whose ESR spectrum (Figure 1A) showed a four-line pattern with a β-hydrogen hyperfine splitting constant of 15.4 G, a value consistent with previously assigned DMPO/protein thiyl radical adducts.[26,49] At lower DMPO concentrations (13−25 mM), the four-line spectrum of the DMPO thiyl radical adduct was superimposed on a broad single line, probably from untrapped protein tyrosyl radical (Figure 1B−D), which would indicate a low trapping efficiency of DMPO for this tyrosyl radical. These data demonstrate the formation of the thiyl radical adduct, but no other detectable radical adduct. The DMPO tyrosyl radical adduct, if present, is too short-lived to accumulate to a detectable level.

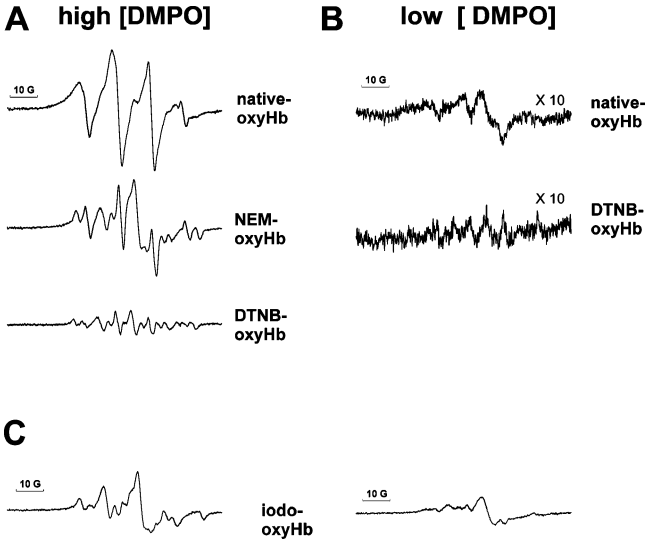

Figure 2. Effect of cysteine and tyrosine blocking on the nature of the ESR radicals trapped from the reaction of oxyHb with peroxynitrite in the presence of high and low concentrations of DMPO (A, B). Changes in the ESR spectral features obtained from the reaction of oxyHb and modified oxyHb. (A) Reactions carried out with 100 mM DMPO of native and NEM and DTNB chemically modified oxyHb and (B) reaction of native and DTNB-blocked oxyHb with 13 mM DMPO. Note that no thiyl radical is detected in the cysteine-blocked oxyHb. (C) The reaction of tyrosine-modified (iodinated) oxyHb with peroxynitrite fails to yield a significant amount of thiyl radical adduct over the entire DMPO concentration range. Experimental conditions used are as described earlier.

When the protein was chemically modified with NEM or DTNB to block Cys[93] and reacted under identical conditions, no DMPO protein thiyl radical adduct was produced (Figure 2A,B; DMPO concentration 100 and 13 mM, respectively). Instead, we primarily observed some unidentified, low molecular weight radical adducts, which have narrow lines. In an analogous experiment, when the oxyHb modified at the Tyr residue by iodination was reacted with peroxynitrite and 100 mM DMPO, no significant thiyl radical adduct was detected (Figure 2C). This experiment suggests tyrosyl as the initial site of radical formation, followed by an electron transfer from the Cys residue to the tyrosyl radical. With 13 mM DMPO, new, stronger unknown radical adducts were formed. It is known that iodotyrosines form radicals with peroxidases.[50,51] However, the radical is very unstable and decays extremely rapidly as is evident from the absence of any EPR characteristic data of iodotyrosyl radical as opposed to that of chloro- and fluorotyrosyl phenoxyl radical.[52] We used commercially available mono and diiodotyrosines (from Sigma-Aldrich) and used spin traps DMPO and 3,5-dibromo-4-nitrosobenzene sulphonate (DBNBS) to see if any radical is formed. No radical detection was observed with any of the spin traps (data not shown), which bind at different positions of the phenol ring. With earlier studies too, no iodotyrosyl radical was detected by either direct ESR[53] or by DMPO spin trapping.[54] The iodotyrosyl radical, if formed,

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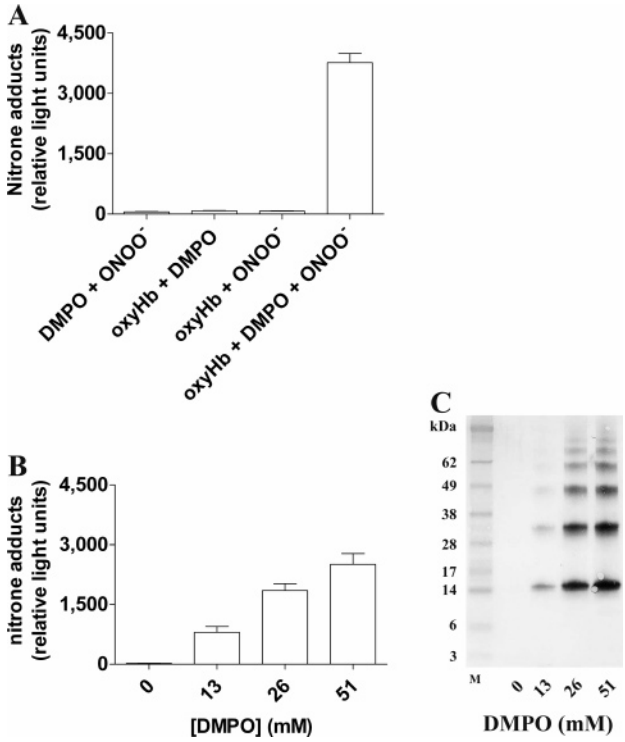

Figure 3. Immunological detection of the hemoglobin radical-derived DMPO adducts from the reaction of native oxyHb with peroxynitrite in the presence of DMPO. Reaction mixtures were incubated at 37 °C for 1 h and then analyzed as described in Experimental Procedures with IST detection. (A) In the absence of any component of the reaction mixture, significant luminescence is not produced. (B) IST of the nitrone DMPO adducts with increasing concentration of DMPO. (C) Corresponding Western blot experiment, under conditions described in Experimental Procedures.

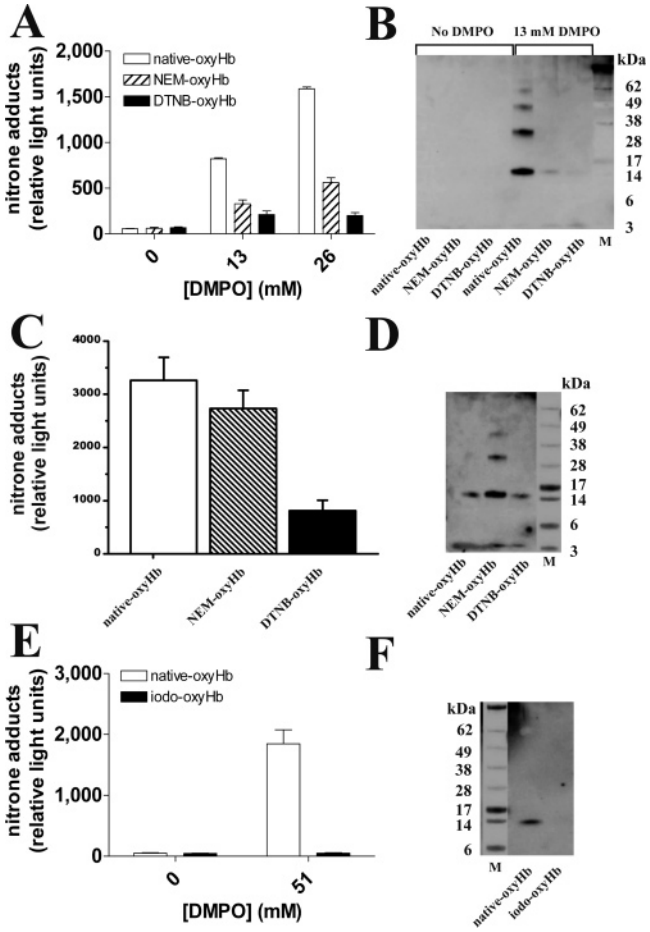

Figure 4. Immunological detection of the effect of cysteine blocking and tyrosine blocking on the reaction of oxyHb with peroxynitrite in the presence of varying concentrations of DMPO. IST analysis of oxyHb and biochemically modified oxyHb. The reaction mixture contained 0.5 mM oxyHb and 0.25 mM peroxynitrite in the presence of varying concentrations of DMPO. The reaction mixture was incubated at 37 °C for 1 h and diluted by a factor of 50 for immunological analysis. (A) Comparison between the nitrone adducts detected in native oxyHb, NEM oxyHb, and DTNB oxyHb with varying concentrations of DMPO. (B) Western blot detection of nitrone adducts with the same system as that in (A) for 0 and 13 mM DMPO. (C) Similar data with 100 mM DMPO; note that less inhibition occurs with NEM-blocked oxyHb as compared to lower doses of DMPO. (D) Corresponding Western blot experiment, under conditions described in the text. (E) Reaction of tyrosine-modified (iodinated) oxyHb with peroxynitrite fails to yield a significant amount of any radical adduct at any DMPO concentration. Reaction conditions used were as described in the text. (F) Complementary Western blot data with native and iodinated oxyHb using 51 mM DMPO; note the diminution of the nitrone adduct in the iodinated oxyHb.

decays by a mechanism other than radical transfer to the cysteine residue.

Immuno-Spin Trapping Analysis of DMPO Globin Nitrone Adducts. We next examined the above oxyHb thiyl protein nitrone adduct and its human heart myoglobin analogue with immuno-spin trapping analyzed with ELISA. To thoroughly investigate immuno-spin trapping with this type of system, we complemented our ELISA data for oxyHb by subjecting all our reaction mixtures to the corresponding Western blot experiments as well.

For the oxyHb system, oxyHb was reacted with peroxynitrite for 1 h in the presence of DMPO at 37 °C, and the globin nitrone adducts thus generated were measured. Production of nitrone adducts, as assessed by ELISA, required all the components of the reaction system (oxyHb, DMPO, and peroxynitrite) (Figure 3A). The nitrone adduct formation increased with increased DMPO concentration up to ~50 mM (Figure 3B,C). As has been reported, in addition to the Hb monomer, Hb dimer and higher molecular weight aggregates were detected.[32] When the ELISA and Western blot experiments were carried out with thiol-blocked oxyHb (NEM or DTNB), lower concentrations of nitrone adduct were observed as compared to the native oxyHb (Figure 4A,B). The decrease in production of protein nitrone adducts was more notable for DTNB oxyHb than NEM oxyHb; DTNB is apparently the better thiol-blocking agent under our experimental conditions. This trend was consistent with increasing concentration of DMPO up to ~50 mM (data not shown).

However, at higher DMPO concentrations, the difference in the signal intensity between native oxyHb and NEM oxyHb decreased as expected in ELISA (Figure 4C) and unexpectedly increased in Western blot experiments (Figure 4D and ref 43). This effect is presumably due to more effective trapping of other nonthiyl radicals, presumably tyrosyl, that may be produced during the reaction even though only DMPO thiyl radical adducts were detected by ESR (this work and ref 43).

Interestingly, when ELISA analysis was carried out with Tyr blocked in iodo oxyHb, no nitrone adduct formation was observed over the entire range of DMPO concentrations studied (Figure 4E,F). These results are consistent with the hypothesis

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that the primary site of radical formation is the tyrosyl radical, followed by an electron-transfer reaction from cysteine to the tyrosyl phenoxyl radical to form the cysteinyl radical. As a result, when the primary radical site was blocked, no significant radical adduct of any kind could be detected.

Human heart Mb is similar in sequence to other mammalian myoglobins. However, one of the most notable differences is the presence of a Cys residue at position 110 (Cys^{110}). Only a few mammalian myoglobins possess a Cys residue in their primary sequences. The reaction of human Mb and H_2O_2 is proposed to produce the thiyl radical via the initial formation of the tyrosyl phenoxyl radical[26,27] as does oxyHb with peroxynitrite. To further test the validity of the immuno-spin trapping technique in studying protein thiyl radicals and electron-transfer processes, we characterized the Mb/H_2O_2/DMPO system with varying DMPO concentrations using ELISA. If any of the components were omitted from the reaction mixture, no nitrone adducts were observed (Figure 5A).

When ELISA experiments were performed over a wide range of DMPO concentrations for the native, NEM, or DTNB Mb/H_2O_2 systems, some very interesting observations were made (Figure 5B). At a low DMPO concentration, thiol modification decreased nitrone adduct formation. As the concentration of DMPO increased (above 26 mM), the differences in the signal intensities for the nitrone adducts between the native, NEM, and DTNB Mb/H_2O_2 systems were clearly diminished. This observation is consistent with an earlier ESR finding[27] that, at low concentrations of DMPO, Mb thiyl radical is trapped almost exclusively, whereas at higher DMPO concentrations, either both tyrosyl and thiyl radicals are trapped or only the tyrosyl radical is. At low concentrations of DMPO, the trapping efficiency is very low; thus, the tyrosyl radical can participate in electron-transfer reactions with Cys^{110}. However, at high DMPO concentration, the trapping efficiency is high enough to trap most of the tyrosine phenoxyl radical, which is then unavailable to participate in electron-transfer reactions with Cys^{110}. The ELISA signal observed here is the sum of the nitrone form of the tyrosyl radical adduct and the nitrone form of the Cys^{110} radical adduct, if any. When similar experiments were carried out with iodinated Mb (Figure 5C), the signal intensity was decreased to near baseline, indicating an absence of radical formation when the Tyr residue is chemically modified.

Mass Spectrometric Analysis of DMPO Mb Adducts. The location of the DMPO binding was verified in the human Mb/H_2O_2/DMPO system by mass spectrometric analysis. The reaction mixtures were first analyzed by flow injection electrospray ionization mass spectrometry. In the deconvoluted ESI/MS spectrum of native human Mb alone (Figure 6A), a protonated molecule with an average molecular weight (M_r) of 17053 was observed (M_r calcd = 17 052.65). When H_2O_2 was added in the absence of DMPO (Figure 6B), both native human Mb (M_r = 17 052.3) and oxidized forms of the protein were observed, the latter representing incorporation of oxygen into the protein presumably by the reaction of protein radicals with molecular oxygen. The most abundant ion observed (M_r = 17 100.3) corresponds in mass to the addition of three oxygens to human Mb. In the absence of H_2O_2 (Figure 6C), the deconvoluted mass spectrum was similar to that of human Mb alone (Figure 6A). The deconvoluted ESI mass spectra of the human myoglobin/H_2O_2/6 mM DMPO reaction mixture (Figure 6D) and the human myoglobin/H_2O_2/100 mM DMPO reaction mixture (Figure 6E) showed an ion of M_r = 17 163 Da corresponding in mass to the addition of one DMPO (113Da-2H) molecule to human myoglobin as well as an ion due to protonated myoglobin, M_r = 17 053 Da. The incorporation of oxygen into Mb caused by H_2O_2 was inhibited by DMPO as is consistent with this oxidation being radical-mediated. In the reaction mixture containing 100 mM DMPO, an ion of M_r = 17 274.4 Da was also observed corresponding in mass to the addition of two DMPO molecules to the human Mb. This ion may also be present in the 6 mM DMPO reaction mixture (Figure 6D), but is of low relative abundance (<3%). These data indicate that the formation of the DMPO adduct on human Mb is dependent on the presence of both H_2O_2 and the DMPO

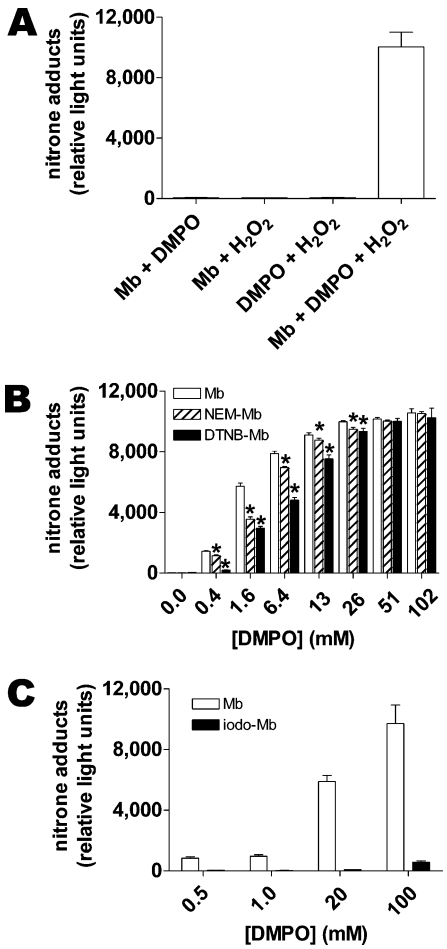

Figure 5. IST detection of the DMPO nitrone adduct from the reaction of human Mb with hydrogen peroxide in the presence of DMPO. Typically, 10 μM human Mb was mixed with 50 μM hydrogen peroxide in the presence of DMPO and incubated at 37 °C for 1 h. (A) No significant adduct formation in the absence of any of the components. (B, C) IST of the DMPO nitrone adducts derived from the reaction of human Mb and modified human Mb with hydrogen peroxide in the presence of varying amounts of DMPO. The reaction mixture was incubated at 37 °C for 1 h and subjected to ELISA analysis as described in Experimental Procedures. (B) Comparison of the native Mb, NEM-blocked, and DTNB-blocked Mb. At low DMPO concentrations, thiyl radicals are apparently trapped nearly exclusively, whereas at high DMPO concentrations, the secondary reactions are outcompeted by the trapping reaction for the primary radical. Thus, the effect of thiol blocking with both the blockers is significant at low DMPO concentration as opposed to high DMPO. (C) Native Mb compared to iodinated Mb. When tyrosine was blocked, no adduct formation was observed, indicating tyrosine as the primary radical site; neither the tyrosyl nor the cysteinyl radical was formed.

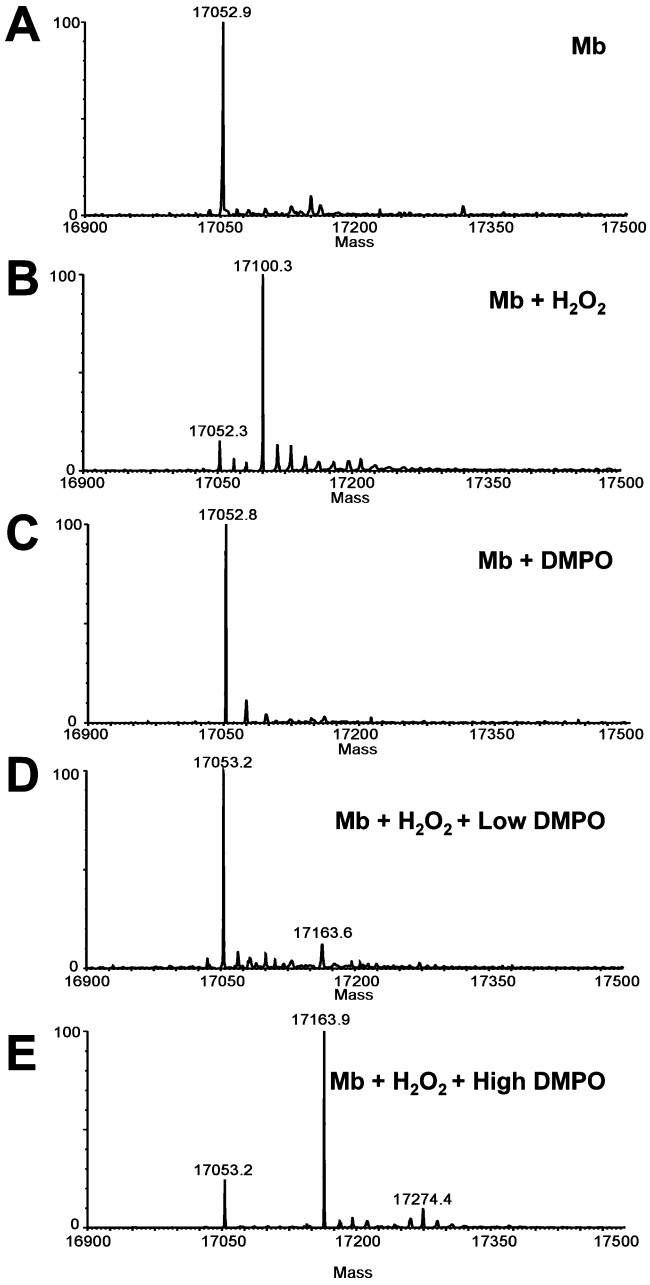

Figure 6. Deconvoluted electrospray mass spectra obtained from the reaction of human myoglobin (30 μM) with hydrogen peroxide (150 μM) in the presence of (D) 6 mM DMPO and (E) 100 mM DMPO. Experimental details were as given in the text. Note that in the ESI spectra of the human Mb/H_2O_2/DMPO systems, ions observed correspond to the addition of one (D) and one and two (E) DMPO molecules to the human Mb. In the absence of hydrogen peroxide (C), the spectrum is similar to the spectrum of Mb alone (A).

spin trap in the reaction mixture. It also indicates the presence of mainly one site of radical formation under the 6 mM DMPO reaction condition, whereas two sites of radical formation were observed from the reaction containing 100 mM DMPO.

To determine the location of the DMPO molecules on the human Mb, the reaction mixtures of Mb/H_2O_2/DMPO were subjected to tryptic digestion and analysis by LC/MS/MS. The spectra acquired from data-dependent MS/MS acquisitions were searched using ProteinLynx software. A user-defined modification of DMPO to all amino acid residues was included in the search. From the analysis of the myoglobin/H_2O_2/6 mM DMPO reaction, an $(M + 2H)^{2+}$ ion of *m*/*z* 1012.6 was observed that corresponds in mass to tryptic peptide 17 (amino acid residues 103−118) and 111 Da (one DMPO nitrone adduct). The resulting MS/MS spectrum of this ion (after transformation of all ions to the single-charge state) shows a series of y ions (y_2 through y_8) as well as the loss of 145 from the y_9 through y_{12} ions (Figure 7A).[55,56] The loss of 145 has been observed previously[16] and corresponds in mass to a DMPO modification on the sulfhydryl group of the cysteine side chain followed by cleavage between the CH_2 and the sulfhydryl with hydrogen transfer to the leaving moiety. In addition, several b ions (b_2 through b_4) are also observed. These structurally informative fragment ions not only allow the assignment of the DMPO molecule to the Cys^{110} residue of human Mb in this reaction, but also prove that DMPO reacted at the sulfur atom as expected when trapping the thiyl radical. An $(M + 3H)^{3+}$ ion of *m*/*z* 712.45, which corresponds in mass to T17 plus two DMPO moieties, was also observed in the mass spectra from this reaction, but because of the low relative abundance of this ion, an interpretable MS/MS spectrum was not obtained.

The reaction mixture of human myoglobin, H_2O_2, and 100 mM DMPO was then analyzed by LC/MS/MS. In these analyses, ions were observed that correspond in mass to predicted tryptic peptide 17 of human Mb with one and with two DMPO molecules. For the addition of one DMPO modification, the deconvoluted MS/MS spectra of both the $(M + 2H)^{2+}$ ion and the $(M + 3H)^{3+}$ ion (data not shown) resulted in a fragmentation spectrum similar to that shown in Figure 7A, thereby allowing assignment of the singly bound DMPO T17 peptide to be located at Cys^{110}. The MS/MS spectrum of the $(M + 3H)^{3+}$ ion of *m*/*z* 712.36 was deconvoluted; this ion corresponds in mass to tryptic peptide 17 and the trapping of two DMPO molecules, and the resulting MS/MS spectrum transformed to the single-charge state is shown in Figure 7B. An abundant ion of *m*/*z* 1990.02 (labeled as −145) corresponds in mass to the loss of S-DMPO and 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) was observed that corresponds to cleavages along the peptide backbone, providing unequivocal data to assign the DMPO modifications to Cys^{110} and Tyr^{103} in this peptide. In addition, an ion of *m*/*z* 247.15 was observed that corresponds in mass to an immonium ion of tyrosine and DMPO (labeled as I_{Y*}). These data demonstrate that DMPO does, in fact, trap the tyrosyl radical even though the adduct is not observed with ESR. Under the reaction conditions using 100 mM DMPO, these MS/MS data allow the assignment of DMPO to two amino acids in tryptic peptide 17 of human Mb, Cys^{110}, and Tyr^{103}.

Discussion

In both reaction systems studied, the oxyHb/peroxynitrite/DMPO system and the human Mb/H_2O_2/DMPO system, the formation of the ferryl heme and globin-centered radicals is well established. In the studies reported here, we have confirmed by immuno-spin trapping that at low DMPO concentration, thiyl radicals are trapped, and at high DMPO concentration, tyrosine radicals are trapped. Similar data were obtained earlier by ESR for human myoglobin.[26,27] The nature of the radicals trapped has been confirmed with the combined use of ESR and MS/

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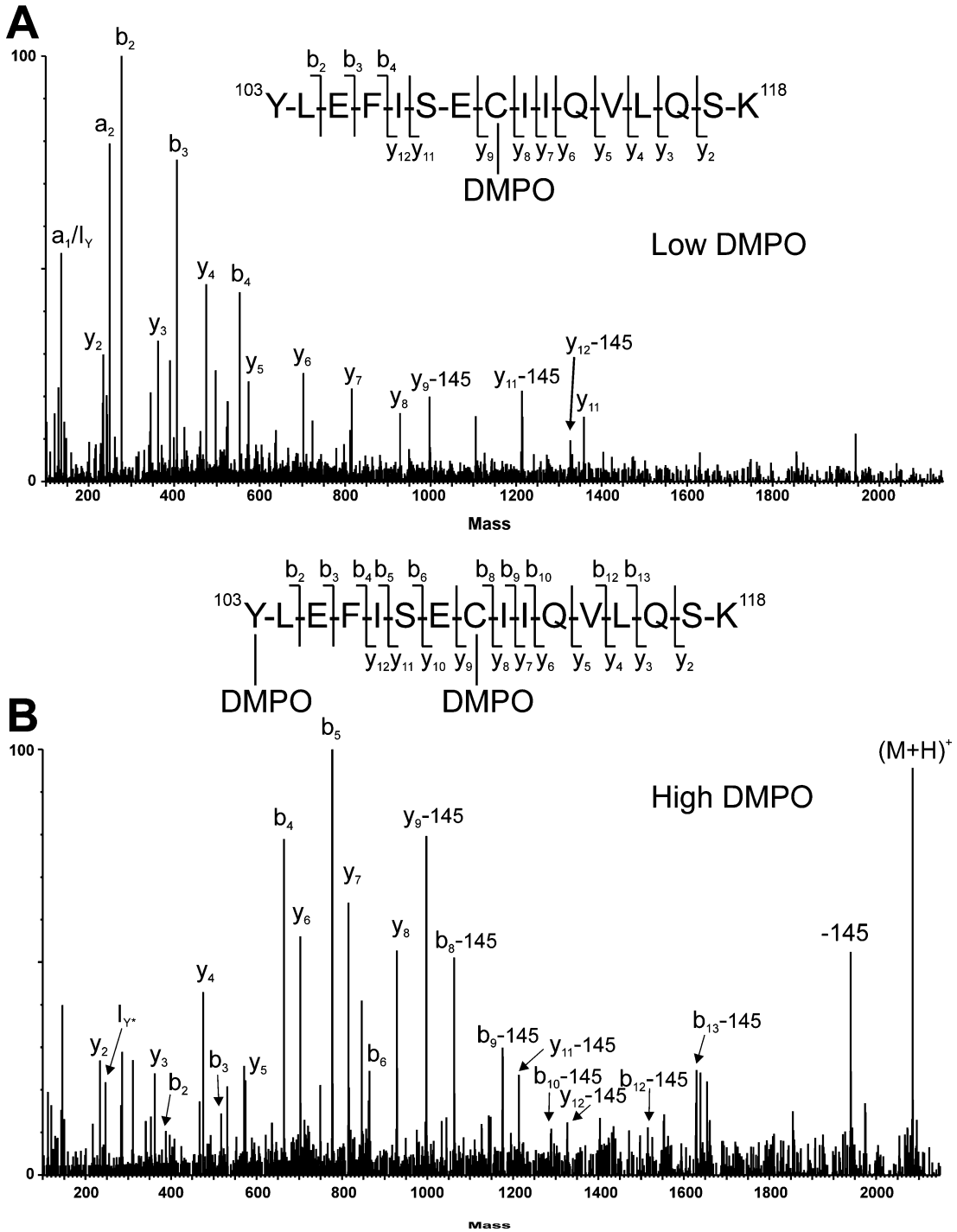

Figure 7. Deconvoluted ESI/MS/MS of the (A) $(M + 2H)^{2+}$ ion of 1012.60 which corresponds in mass to tryptic peptide T17 + DMPO of human Mb and (B) $(M + 3H)^{3+}$ ion of 712.36, which corresponds in mass to tryptic peptide T17 + 2 DMPO of human Mb. The reaction mixtures were digested with trypsin and analyzed by LC/MS/MS.

MS. A simplified mechanism can be outlined for the oxyHb/peroxynitrite system [peroxynitrite reacts with heme moieties ($k_{Hb} > 10^4$ M^{-1} s^{-1}, $k_{Mb} \approx 4 \times 10^4$ M^{-1} s^{-1},[57])], where amino acid-derived radicals form a minor pathway.[43]

$$\text{oxyHb} + \text{peroxynitrite} \rightarrow [\text{Hb tyrosyl radical } (Fe^{IV}{=}O)]^{\bullet+} + NO_2^-$$

$$\text{Hb tyrosyl radical} + \text{oxyHb Cys} \rightarrow \text{Hb Tyr} + \text{Hb Cys thiyl radical}$$

Spin-trapping experiments support the conclusion that a thiyl radical is formed. However, the trapping of the thiyl radical may be rationalized by either of two pathways: (i) the Cys^{93} of oxyHb or Cys^{110} of human Mb may be oxidized directly by the ferryl heme porphyrin cation radical or (ii) electron transfer between tyrosyl radical and the Cys residue results in the formation of the thiyl radical. Most of the peroxynitrite that reacts with heme is isomerized to produce NO_3^-;[43] however, a portion of the reaction leads to the production of ferryl heme hypervalent states, which can propagate radical transfer reactions (ref 43 and this work) to produce a thiyl radical product. In

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addition, our experiments with iodinated globin, where the tyrosine is chemically modified but the Cys is not, produced no significant nitrone adducts detected by ESR or by immuno-spin trapping. Thus, direct oxidation of cysteine to a thiyl radical is of very low efficiency if not ruled out altogether. If electron transfer occurred by either an intra- or intermolecular mechanism between the edge of the ferryl heme and Cys, the treatment of iodinated globin with the oxidants would be expected to yield significant amounts of DMPO Cys radical adducts in both our systems, which was not the case.

Our data reported here show the effect of DMPO concentration on trapping the different radicals that are formed. We used immuno-spin trapping to study the phenomenon where Tyr is a primary radical site followed by a secondary electron-transfer process producing a Cys radical. At low DMPO concentrations, a Cys thiyl radical is trapped exclusively, suggesting that this radical is thermodynamically favored under the experimental conditions and that cysteine acts as a radical sink. However, at high DMPO concentrations (i.e., high DMPO-trapping efficiency), DMPO will react with the tyrosyl radical, thus blocking the subsequent reactions of the tyrosyl radical.

The MS/MS data of the Mb/H_2O_2/6 mM DMPO system clearly indicates that a Cys thiyl radical adduct is trapped at this low DMPO concentration, while for the Mb/H_2O_2/100 mM DMPO system, both the tyrosyl and the Cys thiyl radicals are trapped. Iodinated oxyHb and iodinated Mb produced near-baseline nitrone adducts when reacted with peroxynitrite and H_2O_2, respectively, as assessed by ELISA and Western blot. This result indicates that the primary radical site is at Tyr and that this radical is essential in producing the subsequent thiyl radical. Conversely, as analyzed by ELISA and Western blot, Cys-blocked oxyHb and human Mb produced significantly lower nitrone adducts than the native hemoproteins at low concentrations of DMPO. However, the signal intensity was not reduced to baseline as in the case of iodinated heme proteins, indicating that the tyrosyl radicals are formed in the cysteine-modified heme proteins. These results indicate that the tyrosyl radical is formed in the unmodified proteins and is the initial site of radical formation. A recent ESR spin trapping study by Zhang et al.[29] showed that DBNBS thiyl radical adduct formation in model peptides treated with myeloperoxidase, H_2O_2, and NO_2^- was completely inhibited when free thiol groups were modified. However, tyrosyl radical formation was enhanced in these thiol-modified peptides as detected by the spin trap DBNBS. Thus, the precise position of the equilibrium between thiyl and tyrosyl radical may be variable.[58]

Our observation of the concentration dependence of DMPO spin trapping and the absence of significant DMPO adducts from iodinated human oxyHb and Mb treated with peroxynitrite or H_2O_2, respectively, indicates that a tyrosyl radical is formed before the formation of the thiyl radical in both systems studied. Also, from a structural standpoint, the formation of either the Hb (Tyr^{42} and Tyr^{24}) or Mb (Tyr^{103}) radicals before the formation of Hb (Cys^{93}) or Mb (Cys^{110}) radicals is consistent with the proximity of Tyr to the heme group in both hemoproteins. These results are consistent with the presence of a Cys residue adjacent to the Tyr group facilitating radical transfer between the tyrosyl radical and a Cys residue.[29,41] This process, in which Cys acts as a radical sink, will also impede the formation of nitrotyrosines.[29]

The MS/MS results show clearly that DMPO does trap the cysteinyl radical in large proteins; thus, the immunological results at high DMPO concentration in the oxyHb/peroxynitrite system cannot be due to the failure of DMPO to trap the thiyl radical in a large protein as proposed.[43] It is possible that the stability of the DMPO thiyl adduct, the very factor that allows it to be easily seen by ESR, prevents it from being detected in quantity by the anti-DMPO antibody, which recognizes only the nitrone oxidation product. In addition, the DMPO thiyl radical adduct is known to decompose, at least in part, unimolecularly to DMPO and the parent thiyl radical.[59] In this case, the bond formed during spin trapping is lost and nitrone adduct formation is impossible. Similarly, the DMPO tyrosyl adduct, which is not seen with ESR, is apparently oxidized so readily to the nitrone that it is easily detected by immuno-spin trapping.

In summary, immuno-spin trapping, ESR spin trapping, and mass spectrometry have been used in combination to elucidate a radical transfer mechanism by varying the DMPO concentration to trap different radicals. To our knowledge, the present work represents the first application of IST and MS to understanding the complex mechanism of electron-transfer processes in proteins. Because in electron-transfer reactions the identity of the radical trapped depends on the DMPO concentration, extreme caution must be exercised in interpreting spin trapping information. At high DMPO concentrations, adduct formation will occur exclusively with any primary radical that is sterically accessible, and data thus obtained can be misleading with respect to the subsequent radical chemistry, which may be inhibited. Although the type of free radical trapped can sometimes be determined by ESR alone, only MS can unambiguously determine the type of amino acid radical trapped and its location. The immuno-spin trapping technique which requires only simple and inexpensive equipment has promise as a simple, sensitive, and reliable tool for the investigation of radical species formed by electron transfer reactions, but the complexity of free radical chemistry makes it prudent that the combination of ESR, IST, ESI/MS, and MS/MS be used before mechanistic conclusions are drawn.

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