Myoglobin Scavenges Peroxynitrite without Being Significantly Nitrated

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ABSTRACT: We have analyzed in detail hemoglobin (Hb) and myoglobin (Mb) after treatment of different forms of these proteins with variable amounts of peroxynitrite. HPLC analyses of the peroxynitrite-treated proteins subjected either to acid hydrolysis or Pronase digestion showed that only very low quantities of 3-nitrotyrosine are formed when equivalent amounts of peroxynitrite are allowed to react with the oxy form of these proteins. Comparable amounts of nitrated amino acids are formed when metMb and metHb are treated with peroxynitrite under analogous conditions, but significantly larger yields are observed with apoMb and metMbCN. Interestingly, in addition we found that also the tryptophan residues of Mb and Hb are nitrated to a low but detectable extent. Taken together, our data suggest that the heme center of Mb may act as an efficient scavenger of peroxynitrite, protecting the globin from nitration. As peroxynitrite can irreversibly inhibit cytochrome *c* oxidase, oxyMb may utilize an additional important pathway to maintain mitochondrial respiration, that is, rapidly react with peroxynitrite and thus prevent nitration of other cellular components.

Despite the fact that myoglobin (Mb[1]) is probably one of the most thoroughly studied protein, it has recently been proposed that it may display additional biological functions that remain a matter of continuing investigations. The original role attributed to Mb, that is, storage of dioxygen and facilitation of its transport through muscle tissue to mitochondria (*1*), has recently been challenged. The discovery that a mutant mouse devoid of Mb is capable of apparently normal muscle function raised questions about the real significance of this protein (*2*, *3*). In particular, it has been proposed that Mb may be involved in the regulation of intracellular nitrogen monoxide concentration.

Unlike other ligands such as carbon monoxide and dioxygen, nitrogen monoxide reacts with both the iron(II) and the iron(III) oxidation states of Mb (*4*). Reaction of NO$^\bullet$ with reduced deoxymyoglobin (deoxyMb, $MbFe^{II}$) yields the nitrosyl complex $MbFe^{II}NO$, whereas metmyoglobin (metMb, $MbFe^{III}$) binds NO$^\bullet$ to generate the corresponding oxidized nitrosyl complex $MbFe^{III}NO$, which then rapidly undergoes reductive nitrosylation and forms the same nitrosyl iron(II) complex $MbFe^{II}NO$ (*5*, *6*). In addition, oxymyoglobin (oxyMb, $MbFeO_2$) is readily oxidized by NO$^\bullet$ to metmyoglobin and nitrate (*7*−*9*). This reaction has recently been demonstrated to be relevant in vivo, as intracoronary perfusion of NO$^\bullet$ solutions has been shown to lead to a dose-dependent decrease in the total concentration of oxyMb and to a parallel increase in the formation of metMb (*10*).

Nitrogen monoxide can also bind reversibly to the reduced heme of cytochrome *c* oxidase to generate an iron(II) nitrosyl complex (*11*), which represents an inhibited form of the enzyme. As cytochrome *c* oxidase is essential for mitochondrial respiration, it has been proposed that an additional physiological function of Mb may be to scavenge NO$^\bullet$ and thus to contribute to preserve respiration in the skeletal muscle and in the heart by preventing cytochrome *c* oxidase inhibition (*10*, *12*, *13*).

Overproduction of NO$^\bullet$ and superoxide, both generated in large quantities as a response of the immune system or in inflammatory processes, favors the production of the powerful oxidizing and nitrating agent peroxynitrite[2] (*14*). The peroxynitrite anion ($ONOO^-$) is stable, but the protonated form, peroxynitrous acid [HOONO, pK_a 6.8 (*15*)], isomerizes to nitrate with a rate constant of 1.2 s^{-1} at 25 °C (*15*, *16*). However, under physiological conditions peroxynitrite reacts with a number of biological targets, in particular with thiols [the second-order rate constant for the reaction of peroxynitrite with cysteine is 5.9×10^3 M^{-1} s^{-1}, at pH 7.4 and 37 °C, (*17*)], with carbon dioxide [the pH-independent second-order rate constant is 5.8×10^4 M^{-1} s^{-1} at 37 °C, (*18*)], and with metalloproteins (*19*). Because of the relatively high concentration of CO_2 in both intra- and extracellular compartments (1−2 mM), the reaction of peroxynitrite with CO_2

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[1] Abbreviations: CNBr, cyanogen bromide; DTPA, diethylenetriaminepentaacetic acid; Hb, hemoglobin; $HbFeO_2$, oxyhemoglobin (oxyHb); metHb, iron(III)hemoglobin; $HbFe^{IV}$═O, oxoiron(IV), ferryl hemoglobin; HPLC, high-pressure liquid chromatography; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; Mb, myoglobin; $MbFe^{II}$, deoxymyoglobin (deoxyMb); $MbFeO_2$, oxymyoglobin (oxyMb); metMb, iron(III)myoglobin; metMbCN, cyanoiron(III)myoglobin; $MbFe^{IV}$═O, oxoiron(IV), ferryl myoglobin; NO_2-Tyr, 3-nitrotyrosine; NO_2-Trp, nitrotryptophan; TFA, trifluoroacetic acid; Trp, tryptophan; Tyr, tyrosine.

[2] The recommended IUPAC nomenclature for the peroxynitrite anion is oxoperoxonitrate(1-) and for peroxynitrous acid, hydrogen oxoperoxonitrate. The term peroxynitrite is used in the text to refer generically to both oxoperoxonitrate(1-) ($ONOO^-$) and its conjugate acid, hydrogen oxoperoxonitrate (ONOOH).

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is particularly relevant in biological systems. Carbon dioxide (*20*, *21*), metal complexes (*22*−*24*), and metal-containing proteins (*22*, *25*) have all been shown to catalyze the peroxynitrite-mediated nitration of aromatic compounds. Because of the instability of peroxynitrite under physiological conditions (*19*), the detection of 3-nitrotyrosine (NO_2-Tyr) has become a biochemical marker for the presence of peroxynitrite in pathophysiological processes (*26*). Extensive evidence supports the formation of NO_2-Tyr in vivo in a variety of different pathological conditions [for review, see ref *27*].

We have recently shown that the peroxynitrite-mediated oxidation of oxyMb and oxyHb proceeds via an intermediate ferryl complex, which, in a second step, reacts further with peroxynitrite to yield metMb and metHb, respectively (*28*). The rate constants for the two steps of the reaction of peroxynitrite with oxyMb, at pH 7.3 and 20 °C, were determined as $(5.4 \pm 0.2) \times 10^4$ and $(2.2 \pm 0.1) \times 10^4$ M^{-1} s^{-1}, respectively (*28*). The corresponding rates for the reaction with oxyHb, at pH 7.0 and 20 °C, are $(8.4 \pm 0.4) \times 10^4$ and $(9.4 \pm 0.7) \times 10^4$ M^{-1} s^{-1}, respectively (*28*). In addition, it has been shown that peroxynitrite is able to diffuse across the red blood cell membrane (*29*) and to oxidize oxyHb to metHb also within the erythrocytes. From the comparison of the values of the second-order rate constants, one can conclude that, if present in analogously high concentrations, these two proteins can efficiently compete with CO_2 and react with peroxynitrite. Thus, Hb and Mb are two of the main targets for peroxynitrite in the blood vessels and in the cardiac and skeletal muscles, where the concentration of these two proteins are high.

Here we present detailed analyses of Hb and Mb after treatment of different forms of these proteins with variable amounts of peroxynitrite. Our results show that only very low quantities of NO_2-Tyr are formed when equivalent amounts of peroxynitrite are allowed to react with the oxy form of these proteins. Comparable amounts of nitrated amino acids are formed when metMb and metHb are treated with peroxynitrite under analogous conditions, but significantly larger yields are observed with apoMb and metMbCN. Interestingly, in addition we found that also the tryptophan residues of Mb and Hb are nitrated to a low but detectable extent. Taken together, our data suggest that the heme center of Mb may act as an efficient scavenger of peroxynitrite, protecting the globin from nitration. As peroxynitrite can irreversibly inhibit cytochrome *c* oxidase (*30*), oxyMb may utilize an additional important pathway to maintain mitochondrial respiration, that is, rapidly react with peroxynitrite and thus prevent nitration of other cellular components.

EXPERIMENTAL PROCEDURES

Reagents. Buffer solutions were prepared from K_2HPO_4/KH_2PO_4 (Fluka) with deionized Milli-Q water. Sodium nitrite, sodium nitrate, sodium dithionite, potassium superoxide, potassium hexacyanoferrate(III), 3-nitro-L-tyrosine (NO_2-Tyr), 2-butanone, sulfanilamide, *N*-(1-naphthyl)-ethylenediaminedihydrochloride, ammonium sulfamate, and cyanogen bromide were obtained from Fluka. Sodium bicarbonate was obtained from Merck. L-Tyrosine and L-tryptophan were purchased from Sigma. Pronase was obtained from Roche Molecular Biochemicals. Nitrogen monoxide was obtained from Linde and passed through a NaOH solution as well as a column of NaOH pellets to remove higher nitrogen oxides before use.

Peroxynitrite and Protein Solutions. Peroxynitrite was prepared according to ref *31* from solid KO_2 and gaseous nitrogen monoxide and stored in a polyethylene bottle or in small aliquots at −20 or −80 °C. The concentration of the solutions was determined by measuring the absorbance at 302 nm [$\epsilon_{302} = 1705$ M^{-1} cm^{-1}, *32*]. The peroxynitrite solutions contained variable amounts of nitrite (maximally 50% relative to the peroxynitrite concentration) and no hydrogen peroxide. The stock solution was diluted to the required concentration immediately before use with 0.01 M NaOH. Peroxynitrite solutions were always kept on ice.

Horse heart myoglobin was purchased from Sigma and purified, after addition of a small amount of potassium hexacyanoferrate(III), over a Sephadex G25 column by using a 0.1 M phosphate buffer solution (pH 7.0) as the eluent. The concentration of the resulting metMb solutions was determined by measuring the absorbances at 408, 502, and/or 630 nm ($\epsilon_{408} = 188$ mM^{-1} cm^{-1}, $\epsilon_{502} = 10.2$ mM^{-1} cm^{-1}, and $\epsilon_{630} = 3.9$ mM^{-1} cm^{-1}) between pH 6.0 and 7.5 (*4*).

Oxymyoglobin was prepared by reducing the purchased Mb with a slight excess of sodium dithionite. The solution was purified chromatographically on a Sephadex G25 column by using a 0.1 M phosphate buffer solution (pH 7.0) as the eluent. In some cases, the protein was purified analogously a second time to ensure complete removal of sodium dithionite. However, this procedure proved not to be necessary, as it did not influence the results of the following experiments. The concentration of the oxyMb solutions was determined by measuring the absorbances at 417, 542, and/or 580 nm [$\epsilon_{417} = 128$ mM^{-1} cm^{-1}, $\epsilon_{542} = 13.9$ mM^{-1} cm^{-1}, and $\epsilon_{580} = 14.4$ mM^{-1} cm^{-1}, (*4*)].

ApoMb was prepared according to the method of Teale (*33*). In brief, a concentrated HCl solution was added dropwise to a Mb solution (ca. 0.2 g in 5 mL water) until the pH reached a value of 2. Ice cooled 2-butanone was then added, and the biphasic mixture was immediately vortexed. The two phases were separated by short centrifugation, and the upper porphyrin-containing organic layer was removed with a pipet. The extraction was repeated three times until the lower protein-containing aqueous phase was completely colorless. The apoprotein solution was dialyzed against water, 2, 10, 20, and finally 50 mM phosphate buffer, pH 7.0. During the last dialysis step a precipitate formed, which was removed by centrifugation. The concentration of the supernatant apoMb solution was determined by measuring the absorbance at 280 nm [$\epsilon_{280} = 13.5$ mM^{-1} cm^{-1}, (*34*)]. Contamination of the apoprotein with unreacted Mb, assessed spectrophotometrically, was routinely below 1% (ca. 0.3%).

The cyanide-bound form of metMb (metMbCN) was prepared by adding a slight excess of KCN to metMb and was purified over a Sephadex G25 column by using a 0.1 M phosphate buffer solution (pH 7.0) as the eluent. The concentration of the metMbCN solutions was determined by measuring the absorbances at 422 and/or 540 nm [$\epsilon_{422} = 116$ mM^{-1} cm^{-1} and $\epsilon_{540} = 11.3$ mM^{-1} cm^{-1}, (*4*)].

Purified human oxyHb stock solution (57 mg/mL solution of HbA_0 with approximately 1.1% metHb) was a kind gift from APEX Bioscience, Inc. (NC). The obtained solution was frozen in small aliquots (0.5−1 mL) and stored at −80

°C. Oxyhemoglobin solutions were prepared by diluting the stock solution with buffer, and concentrations (always expressed per heme) were determined by measuring the absorbance at 415, 541, and/or 577 nm [$\epsilon_{415} = 125$ mM^{-1} cm^{-1}, $\epsilon_{541} = 13.8$ mM^{-1} cm^{-1}, and $\epsilon_{577} = 14.6$ mM^{-1} cm^{-1}, (*4*)].

MetHb solutions were prepared by oxidizing oxyHb with a slight excess of potassium hexacyanoferrate(III). The solution was purified chromatographically on a Sephadex G25 column by using a 0.1 M phosphate buffer solution (pH 7.0) as the eluent. The concentration of the metHb solutions (always expressed per heme) was determined by measuring the absorbances at 405, 500, and/or 631 nm ($\epsilon_{405} = 179$ mM^{-1} cm^{-1}, $\epsilon_{500} = 10.0$ mM^{-1} cm^{-1}, and $\epsilon_{631} = 4.4$ mM^{-1} cm^{-1}) between pH 6.0 and 7.5 (*4*).

Absorption spectra were collected on a UVIKON 820, on an Analytik Jena Specord 200, or on an Analytik Jena Specord 100 spectrophotometer.

Preparation of the Solutions Containing Carbon Dioxide. Experiments in the presence of 1.1 mM CO_2 were carried out by adding to the protein solutions the amount of sodium bicarbonate required (from a freshly prepared solution, 500 mM $NaHCO_3$ in H_2O) (*35*). The values for the constant of the hydration−dehydration equilibrium $CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^-$ at pH 7.0 are 5.15×10^{-7}, 8.41×10^{-7}, and 1.00×10^{-6} M at 0, 20, and 37 °C, respectively (*36*). Thus, to obtain a constant CO_2 concentration of 1.1 mM, the $NaHCO_3$ concentrations present during the reactions were 6.7, 9.9, and 12.4 mM, at 0, 20, and 37 °C, respectively. After addition of $NaHCO_3$ to the protein solutions, 3−10 min elapsed (longer times when the experiments were carried out at 0 °C) before the peroxynitrite solution was added, to allow the equilibration of the various carbonated species (*35*).

HPLC Analysis. HPLC analysis was carried out with a Hewlett-Packard Series 1050 apparatus with a Series 1100 UV−vis detector, equipped with a VYDAC 218TP54 Protein&Peptide C18 Column (250 × 4.6 mm). Solvent A was 0.07% trifluoroacetic acid (TFA) in H_2O and solvent B 0.07% TFA in acetonitrile. Nitrotyrosine (NO_2-Tyr) was eluted (ca. 7.5 min after injection) by keeping the amount of B constant (5%) in the first 2 min and then by using an increasing linear gradient of B from 5% to 10% between 2 and 10 min and from 10% to 80% between 10 and 15 min. NO_2-Tyr was detected contemporaneously at 220, 280, 350, and 400 nm. Nitrotyrosine was quantified by measuring a calibration curve of 5−10 NO_2-Tyr standard solutions.

The nitrotryptophan products were eluted between 15 and 18 min, by following the same procedure. The different isomers were assigned by comparing the UV−vis spectra of the single peaks with those reported in the literature for 4-, 5-, 6-, and 7-nitrotryptophan (*37*, *38*).

Analysis of the 3-Nitrotyrosine Content in oxyHb, metHb, oxyMb, metMb, and apoMb after Reaction with Variable Amounts of Peroxynitrite. The reaction of peroxynitrite with oxyHb, metHb, oxyMb, metMb, or apoMb was carried out as follows: to 180 μL of a protein solution (112 μM in 0.1 M phosphate buffer, pH 7.0) kept at the required temperature (ice-cooled, room temperature, or in a thermostat at 37 °C) was added 20 μL of an iced-cooled peroxynitrite solution (different concentrations in 0.01 M NaOH) as a bolus and vortexed immediately. The final pH measured after mixing was always between 7.0 and 7.1. For each protein, an additional experiment was carried out with decomposed peroxynitrite as a control. For this purpose, the same peroxynitrite solution was acidified with HCl and then neutralized again with 0.1 M NaOH. The decomposition of peroxynitrite was confirmed by measuring an absorbance spectrum between 300 and 350 nm. Finally, this decomposed peroxynitrite solution was added, also in a volume ratio of 1:9, to the protein solutions. The reactions in the presence of 1.1 mM CO_2 were carried out analogously by adding the required amount of peroxynitrite to a solution containing $NaHCO_3$ (see above for the concentrations used at the different temperatures) and the protein (a total of 180 μL of a 112 μM protein solution) in a volume ratio of 1:9. After ca. 30 min, nitrite was removed by washing the solutions with a 25 mM ammonium hydrogencarbonate buffer, pH 7.8, through a 10000 MW cutoff filter (Centriplus YM-10, Amicon, Switzerland) at 3000 g until the ultrafiltrate did not show any qualitative reaction (pink coloring) with the Griess reagent (*39*). Alternatively ca. 200 μL of an ammonium sulfamate solution (100 mM in 0.5 M HCl) was added to convert nitrite to dinitrogen. This procedure proved to be efficient at removing nitrite, as confirmed by using the Griess reagent (see above). The NO_2-Tyr yields obtained with these two nitrite-removing procedures were equivalent.

The resulting proteins were hydrolyzed by treating the solutions (200−400 μL) with an equivalent volume of 12 M HCl and heating them for 18 h at 110 °C in a closed vial. The hydrolyzed mixtures were allowed to dry by maintaining the temperature at 110 °C and by opening the vials. The residuals were redissolved in 40 μL of 0.1% trifluoroacetic acid and 20 μL was analyzed by HPLC as described above.

Alternatively the proteins were digested with Pronase as follows. After the treatment with peroxynitrite, the proteins were first dialyzed against a 25 mM ammonium hydrogencarbonate buffer, pH 7.8, to remove nitrite (see above). Lyophilized Pronase was dissolved in water (20 mg/mL) and kept on ice before use. Protein samples (100 μM) were digested by adding Pronase and $CaCl_2$ to final concentrations of 1 mg/mL and 10 mM, respectively. The samples were incubated with gentle agitation at 40 °C for 24 h. Digestion was stopped by concentrating the samples to dryness in a speed-vac apparatus. Finally, the samples were redissolved in 0.1% trifluoroacetic acid and analyzed by HPLC as described above.

Western Blot Analysis. The protein samples were prepared in a way analogous to that described above for the HPLC analysis, by treating the protein solutions with one bolus of peroxynitrite (10% v/v). The unwashed samples were boiled for 3 min at 95 °C in an electrophoresis buffer (62.5 mM Tris-HCl, pH 6.8, 1% sodium dodecyl sulfate (SDS), 5% glycerol, 0.5 mM EDTA, and bromophenol blue) which did not contain any reducing thiols. This precaution was taken to avoid reduction of NO_2-Tyr, which has been reported to take place in the presence of hemoproteins and thiol-containing reductants (*40*). Samples containing 10 μg of protein per lane were separated by 11% SDS polyacrylamide gel electrophoresis (SDS−PAGE) (8 × 8 cm, and 1 mm thick) at 20 mA in a Novex X cell II apparatus (Invitrogen, Groningen, Netherlands). The resolved proteins were transferred onto nitrocellulose membranes (BA 83, Schleicher& Schüll, Dassel, Germany) by the application of 25 V for 90 min in a wet blot apparatus (transfer buffer: 12 mM Tris,

96 mM glycine, and 20% methanol). Transfer of proteins was checked by reversible Ponceau-S staining (0.1% Ponceau-S in 5% acetic acid). The same protein aliquots where run in parallel on a separate gel and were stained with Coomassie blue. The membranes were blocked by incubation with 5% (w/v) nonfat dry milk powder in phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4) for 1 h. After extensive washing with PBST (phosphate buffered saline with 0.05% Tween 20), the blots were incubated overnight at 4 °C either with polyclonal antibodies at 1:2000 dilution (*26*) or with the monoclonal 1A6 nitrotyrosine antibodies (Upstate Biotechnology, Lake Placid, USA) at 1:1000 dilution (in PBST with 0.05% (w/v) nonfat milk powder). The membranes were then washed five times for 5 min in PBST and incubated for ca. 45 min at room temperature with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (Jackson Immunoresearch, West Grove, PA) at 1:5000 dilution (in PBST with 0.05% (w/v) nonfat milk powder). The blots were again washed 5 times and the immunoreactive bands were detected by enhanced chemiluminescence (ECL) (Apbiotech, Dübendorf, Switzerland) according to the suppliers instructions (incubation with ca. 0.125 mL/cm^2 for 1 min). Labeled proteins were visualized by exposing medical X-ray films (Fuji RX) to the blot for 1−5 min.

Cyanogen Bromide Digestion. The nitrite-free protein samples, dialyzed against 25 mM ammonium hydrogencarbonate buffer at pH 7.8 as described above for the HPLC analysis, were dried in a speed-vac apparatus and then redissolved in 0.15 M HCl. Cyanogen bromide, dissolved immediately before use in acetonitrile (1 g/mL), was added in 200-fold excess relative to the methionine content of the protein, which is a 400-fold excess relative to horse heart Mb. The samples were degassed with N_2 and then incubated with gentle agitation for 24 h in the dark at room temperature. After addition of a 10-fold volume of water, the samples were incubated for 1 h at 65 °C. Finally, after concentration to dryness in a speed-vac apparatus, the samples were redissolved in 0.1% trifluoroacetic acid to a final concentration of either 100 or 200 μM.

Mass Spectrometry. Mass determination was performed by using a Perseptive Biosystem Voyager Elite matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF-MS) equipped with a standard nitrogen laser. 2,5-Dihydroxybenzoic acid or sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) dissolved in 0.1% trifluoroacetic acid (TFA)−acetonitrile (1/2, v/v) were used as sample matrixes. In each case, 1 μL of matrix was deposited on a stainless steel or gold covered MALDI target and air-dried. Then, 1 μL of the protein solution was applied on the matrix and air-dried. Finally, the sample/matrix mixture was recrystallized with 1 μL of 0.1% TFA−acetonitrile (1/2, v/v). Data were acquired under positive ionization conditions with external calibration. Proteins were detected with the linear time-of-flight modus and peptides after reflection. For data acquisition and extraction, the manufacturer's program Data explorer 5.0 was used.

Statistics. Most experiments reported in this article were carried out by different people at least in triplicate on independent days. The results are given as mean values of at least three experiments plus or minus the corresponding standard error.

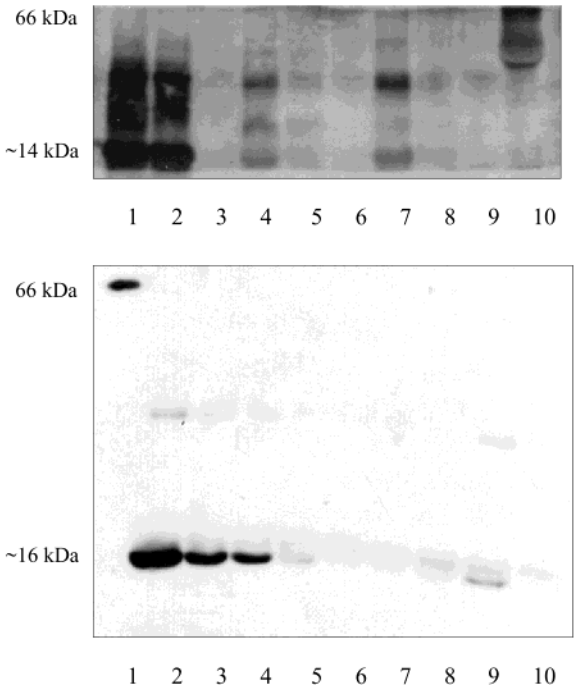

FIGURE 1: Anti-nitrotyrosine immunoblotting of Mb (upper gel) and hemoglobin (lower gel). Samples were separated on a 11% SDS polyacrylamide gel and examined by Western blot analysis with a polyclonal antibody against NO_2-Tyr as described in the Experimental Section. (Top) The proteins (100 μM) were incubated in 0.1 M phosphate buffer pH 7.0 at 0 °C with decomposed peroxynitrite (negative control) and with increasing amounts of peroxynitrite in the absence (lanes 2, 3, 5, 6, 8, 9, and 10) and presence of 1.1 mM CO_2 (lanes 1, 4, and 7). Lane 1, apoMb mixed with 25 equiv of peroxynitrite in the presence of CO_2; lanes 2 and 3, apoMb mixed with 25 equiv of peroxynitrite and with decomposed peroxynitrite; lane 4, metMb mixed with 25 equiv of peroxynitrite in the presence of CO_2; lanes 5 and 6, metMb mixed with 25 equiv of peroxynitrite and with decomposed peroxynitrite; lane 7, oxyMb mixed with 25 equiv of peroxynitrite in the presence of CO_2; lanes 8 and 9, oxyMb mixed with 25 equiv of peroxynitrite and with decomposed peroxynitrite; lane 10, BSA treated with 25 equiv of peroxynitrite. (bottom) The proteins (100 μM) were incubated in 0.1 M phosphate buffer pH 7.0 at 0 °C with decomposed peroxynitrite (negative control) and with increasing amounts of peroxynitrite in the absence (lanes 1−8 and 10) and presence of CO_2 (lane 9). Lane 1, bovine serum albumine (BSA) treated with 10 equiv of peroxynitrite; lanes 2−8, oxyHb mixed with 25, 10, 7.5, 5, 2, 1, and 0.5 equiv of peroxynitrite, respectively; lane 9, oxyHb mixed with 2 equiv of peroxynitrite in the presence of 1.1 mM CO_2; lane 10, oxyHb treated with decomposed peroxynitrite.

RESULTS

Immunohistochemical Analysis of the Globin after Reaction of oxyhemoglobin, oxy-, met-, and apomyoglobin with Peroxynitrite. Western blot analysis with anti-3-nitrotyrosine antibody showed that exposure of oxyMb to an excess of peroxynitrite (25 equiv) at pH 7.0 and 0 °C did not yield to significant nitration (lane 8 in Figure 1, top). In contrast, under our experimental conditions, that is, when an about 100 μM protein solution was treated with one bolus of peroxynitrite (10% v/v) at pH 7.0 and 0 °C, nitration of oxyHb was detectable when at least 5 equiv of peroxynitrite was added (lane 5 in Figure 1, bottom), in agreement to previous reports (*29*, *41*−*43*). As expected (*41*), in the presence of 1.1 mM CO_2, Hb was already nitrated by only 2 equiv of peroxynitrite (lane 9 in Figure 1, bottom), and

nitration of oxyMb could also be detected (lane 7 in Figure 1, top).

To identify whether the oxidation state of the heme influences significantly the yield of nitration, we treated metMb with peroxynitrite under analogous conditions. As depicted in Figure 1 top, similar amounts of nitrated protein were detected when metMb was mixed with 25 equiv of peroxynitrite both in the absence and presence of CO_2 (lanes 5 and 4, respectively). Finally, to elucidate the role of the heme center in the reaction of Mb with peroxynitrite, we determined the degree of nitration obtained by mixing peroxynitrite with apoMb. In contrast to oxy- and metMb, when apoMb was mixed with 25 equiv of peroxynitrite under the same conditions, the protein was nitrated to a significant degree (lane 2 in Figure 1, top). The amount of nitration was even more pronounced in the presence of 1.1 mM CO_2 (lane 1 in Figure 1, top).

From the Western blot of Mb (Figure 1, top), it appears that peroxynitrite treatment of oxy- and metMb causes the formation of dimers which are also nitrated. Visualization of the proteins by staining with Coomassie blue (Figure S1) showed that the amount of dimerized protein was very small, thus suggesting that the dimer is preferentially nitrated. Work is in progress to characterize this dimeric product and to understand the mechanism of its formation.

Quantification of the Amount of 3-Nitrotyrosine Generated. To quantify the yields of nitration, we hydrolyzed the proteins treated with peroxynitrite with hydrochloric acid and then analyzed them by HPLC. To avoid artifacts due to tyrosine nitration by nitrite (always present as a contaminant in our peroxynitrite solutions, up to 50% relative to the peroxynitrite concentration) under acidic conditions, we thoroughly washed the samples by using size exclusion membranes. Alternatively, nitrite was removed by addition of a concentrated acidic ammonium sulfamate solution. Control experiments showed that NO_2-Tyr does not react with ammonium sulfamate and is stable under our hydrolysis conditions (data not shown).

Exposure of oxy-, met-, and apoMb to a range of peroxynitrite concentrations (at pH 7.0 and 0 °C) induced a dose-dependent increase in the nitration of the proteins. Indeed, NO_2-Tyr could clearly be identified in the HPLC chromatogram of the hydrolyzed samples measured at 350 nm (Figure S2). The data, shown in Figure 2 and summarized in Table S1, confirm the results of the immunohistochemical analyses presented above. In the absence of CO_2, less than 5% of the two available tyrosine residues of oxyMb and about 7% of those in metMb are nitrated by a 20-fold excess of peroxynitrite, whereas apoMb is nitrated up to about 20%. Interestingly, addition of 5 or 10 equiv of peroxynitrite led to very similar nitration yields of the available tyrosine residues in the two different forms of the holoproteins, with the NO_2-Tyr yields in oxyMb slightly larger than those in metMb. In the presence of 1.1 mM CO_2 (Figure 2B), the same trend was observed at all peroxynitrite concentrations studied, and the absolute NO_2-Tyr yields were significantly higher. Addition of 20 equiv of peroxynitrite led to 30.5%, 14.7%, and 8.3% nitration of the available tyrosine residues in apoMb, oxyMb, and metMb, respectively. Addition of 1 equiv of peroxynitrite, still a large excess compared to the relative concentrations likely to be found in vivo, resulted only in the nitration of 2.7%, 1.3%, and 0.5% of the available tyrosine residues in apoMb, oxyMb, and metMb, respectively.

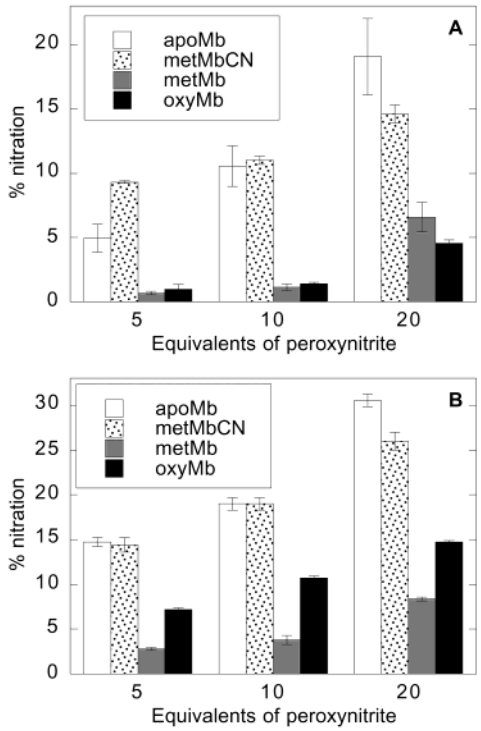

FIGURE 2: NO_2-Tyr yields (% relative to the total content of the protein, that is, 2 tyrosine residues per heme), determined by HPLC after acid hydrolysis, from the reaction of apo-, met-, oxyMb, and metMbCN (100 μM) with different amounts of peroxynitrite (5, 10, and 20 equiv) at 0 °C and pH 7.0 (A) in the absence and (B) in the presence of 1.1 mM CO_2. Note the different scale of the *y*-axis in the two plots.

To confirm our hypothesis that in oxy- and metMb the heme center scavenges peroxynitrite and thus prevents nitration of the two tyrosine residues of the globin, we determined the degree of NO_2-Tyr formation in metMbCN treated with peroxynitrite under analogous conditions. The cyanide-poisoned Mb is extremely stable and its UV−vis spectrum is only slightly modified even after treatment with 20 equiv of peroxynitrite (data not shown).[3] As depicted in Figure 2A (and summarized in Table S1), metMbCN was nitrated to an extent comparable to that of apoMb. In the presence of 1.1 mM CO_2 (Figure 2B) the same pattern was observed: nitration yields of peroxynitrite-treated metMbCN were comparable to those found for apoMb and significantly larger than those determined for oxy- and metMb. Peroxynitrite cannot react directly with the iron center of metMbCN, as the iron is coordinatively saturated by strong ligands which are not displaced during the reaction. As a consequence, peroxynitrite cannot be scavenged and thus leads to significant nitration of the tyrosine residues of the globin.

Analogous studies were carried out with oxyHb and metHb. As shown in Figure 3 and summarized in Table S1, treatment of these two forms of Hb (100 μM) with increasing concentrations of peroxynitrite (100−2000 μM), at pH 7.0

[3] Preliminary results showed that when oxyMb is treated with a large excess of peroxynitrite (≥20 equiv), a species is partly formed in which the heme is cross-linked to the globin, possibly via Tyr103. This species has a characteristic broad absorbance maximum around 590 nm and seems to be partly generated also by treatment of metMbCN with 20 equiv of peroxynitrite.

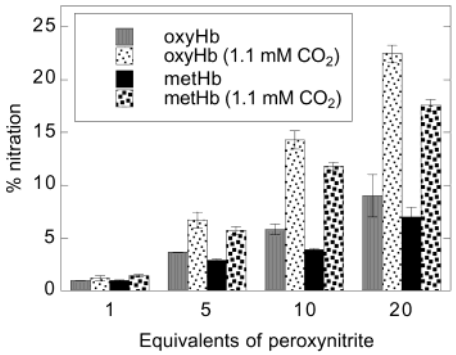

FIGURE 3: NO_2-Tyr yields (% relative to the total content of the protein, that is, 3 tyrosine residues per subunit), determined by HPLC after acid hydrolysis, from the reaction of met- and oxyHb (100 μM) with different amounts of peroxynitrite (1, 5, 10, and 20 equiv) at 0 °C and pH 7.0 in the absence and presence of 1.1 mM CO_2.

and 0 °C, both in the absence and presence of CO_2 (1.1 mM), induced a dose-dependent nitration of the tyrosine residues of the globins. As suggested by the immunohistochemical analyses presented above, the nitration yields were significantly higher than those obtained for the corresponding experiments with Mb. For instance, addition of 20 equiv of peroxynitrite in the presence of 1.1 mM CO_2 led to 22% and 17.6% nitration of the available tyrosine residues in oxyHb and metHb, respectively. Both in the absence and presence of 1.1 mM CO_2, under comparable conditions, the nitration yields were always higher for oxyHb than for metHb. Our data with 1 equiv of peroxynitrite are comparable to those reported recently for the nitration, at pH 7.4, of 1 mM oxyHb and metHb with 1 mM peroxynitrite (*41*, *44*).

As during the course of these studies Groves and coworkers reported significantly higher nitration yields of metMb by peroxynitrite (*45*), to validate our results we determined the yields of nitration by hydrolyzing the proteins with Pronase, a milder method. In an independent set of experiments, we treated the proteins with different amounts of peroxynitrite at pH 7.0 and 0 °C as described above. Each peroxynitrite-treated protein solution was then divided in two samples, one of which was hydrolyzed with HCl and the other one digested with Pronase. Nitrotyrosine yields in both samples were then determined by HPLC analysis. Comparison of the results obtained with the two different methods indicates that the nitration yields measured are reproducible (within 10% error) and are only slightly larger when determined after digestion with Pronase (Figure S3).

As mentioned above, the peroxynitrite solutions used for the experiments described in this paper contained variable amounts of nitrite (maximally 50%, expressed relative to the peroxynitrite concentration). Thus, we studied the influence of nitrite on the yield of the peroxynitrite-mediated nitration of the tyrosine residues in apo-, met-, and oxyMb, both in the absence and presence of 1.1 mM CO_2. For this purpose, we treated the proteins (at 0 °C and pH 7.0) with 20 equiv of peroxynitrite (final concentration 2 mM, contaminated with approximately 1 mM nitrite) premixed with different amounts of NO_2^-. The total final concentrations of NO_2^- were 1, 2, 3, and 5 mM (considering also the amount derived from the contamination of the peroxynitrite solution). Analysis of the globin indicated that the NO_2-Tyr yields apparently slightly decreased with increasing concentration of added nitrite (Table S2). However, the changes were higher than 10% only in the case of the reaction of peroxynitrite with apoMb in the presence of CO_2 (NO_2-Tyr yields: 30.5% ± 0.7%, 25.5% ± 0.2%, 24% ± 1%, and 20.8% ± 0.8% for 1, 2, 3, and 5 mM total NO_2^-, respectively). Taken together, these results suggest that variable amounts of contaminating nitrite in the peroxynitrite solutions may be one of the factors that cause slight discrepancies in the nitration yields obtained with different peroxynitrite preparations. However, as already stated above, our data were always reproducible within an error margin of ~10%.

Finally, we carried out one further experiment in the presence of the metal chelator diethylenetriaminepentaacetic acid (DTPA), to rule out any involvement of traces of free transition metal ions in the nitration reactions studied. For this purpose, we added 0.1 mM DTPA to our 0.1 M phosphate buffer pH 7.0 and determined the yield of nitration of oxyMb treated with 20 equiv of peroxynitrite, both in the absence and presence of 1.1 mM CO_2 (at 0 °C). Interestingly, under these conditions the NO_2-Tyr yields were slightly higher than those obtained in the absence of DTPA (no added CO_2, 4.6% ± 0.3% and 8.9% ± 0.7%; 1.1 mM CO_2, 14.7% ± 0.2% and 16.0% ± 0.5%, in the absence and presence of 0.1 mM DTPA, respectively). This result suggests that in our experiments free metal ions are not involved in the nitration of the proteins. In contrast, as already reported by Goldstein et al. (*46*), DTPA may interact with peroxynitrite and thus addition of this metal chelator may influence the reactivity of peroxynitrite.

Last, we studied the influence of the temperature on the yields of nitration, both in the absence and presence of 1.1 mM CO_2. When apo-, met-, oxyMb, met-, and oxyHb were treated with peroxynitrite at room temperature (at pH 7.0), the yields of nitration were significantly larger for the three forms of Mb, but were almost equivalent for the two Hb forms, compared to the results obtained at 0 °C (Table S1). For instance, addition of 20 equiv of peroxynitrite in the presence of 1.1 mM CO_2 led to 43.4%, 19%, and 15% nitration of the available tyrosine residues in apoMb, oxyMb, and metMb, respectively. In contrast, addition of 20 equiv of peroxynitrite in the presence of 1.1 mM CO_2 nitrated 23.6% and 17.8% of the available tyrosine residues in oxyHb and metHb, respectively.

Identification of Nitrated Tryptophan Residues. HPLC analyses of pronase-digested peroxynitrite-treated oxy-, met-, and apoMb suggested that, in addition to NO_2-Tyr, other nitrated species were formed, as additional peaks were present in the chromatograms measured at 350 nm. As shown for the reaction of apoMb with 10 equiv of peroxynitrite (Figure 4), four main peaks with elution times between 15 and 18 min displayed spectra characteristic for nitrotryptophan derivatives (insets of Figure 4) (*37*, *38*). As nitrotryptophan derivatives are not commercially available, we first reinvestigated (*47*, *48*) the products derived from the reaction of 100 μM tryptophan with different amounts of peroxynitrite (at pH 7.4 and room temperature), to be able to directly compare our results. As shown in Figure 5, the chromatographic traces monitored at 350 nm indicated the parallel formation of mainly four nitrated products. In addition, other products that absorb at 350 nm were eluted in the first 3−7 min of the chromatogram (data not shown) and probably represented nitrated forms of decomposed

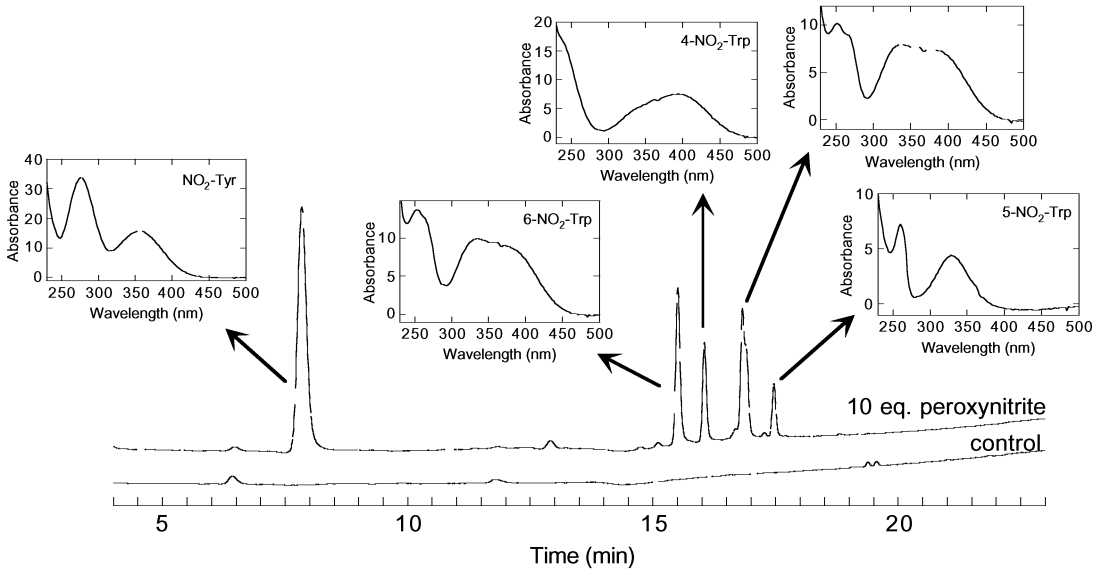

FIGURE 4: HPLC chromatograms (measured at 350 nm) of the products of the reactions of apoMb (~100 μM) with decomposed peroxynitrite (bottom, negative control) and with 10 equiv of peroxynitrite (top), in 0.1 M phosphate buffer pH 7.0 at 0 °C, after complete digestion with Pronase as described in the Experimental Section. In the insets, the UV−vis spectra of the peaks corresponding NO_2-Tyr (ca. 7.5 min), 6-NO_2-Trp (ca. 15.5 min), 4-NO_2-Trp (ca. 16 min), 5-NO_2-Trp (ca. 17.5 min), and that of the peak with an elution time of ca. 16.8 min.

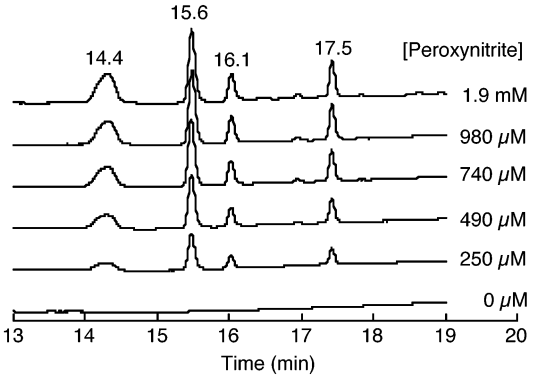

FIGURE 5: HPLC chromatograms (measured at 350 nm) of the products of the reaction of tryptophan (ca. 100 μM) with different amounts of peroxynitrite in 0.05 M phosphate buffer pH 7.4 at room temperature.

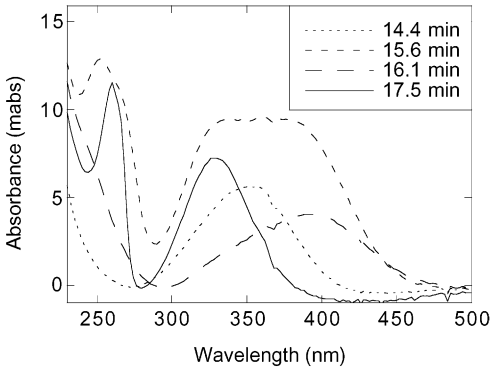

FIGURE 6: Absorbance spectra of the peaks of the four nitrated tryptophan products, which eluted after 14.4, 15.6, 16.1, and 17.5 min, respectively. The last three products were assigned as follows: 15.6 min, 6-NO_2-Trp; 16.1 min, 4-NO_2-Trp; 17.5 min, 5-NO_2-Trp.

tryptophan-derived species. In the presence of 1.1 mM CO_2, the total yields of nitrated products were higher but the same four species were generated at similar relative yields (data not shown).

Attempts were made to identify the nature of the four nitrated products. By direct comparison with the known absorbance spectra of the different nitrotryptophan derivatives (*37*, *38*), we could unambiguously assign the peaks at 15.6, 16.1, and 17.5 min to the 6-, 4-, and 5-nitrotryptophan, respectively. The absorbance spectra of the single peaks of the chromatogram are given in Figure 6. The peak at 14.4 min showed a spectrum with an absorbance maximum at 353 nm, whereas the last possible nitrotryptophan product (7-NO_2-Trp) shows a maximum at 371 nm (*37*). Thus, this peak could not be assigned with certainty. In agreement with previous studies (*47*), no hydroxylated products could be identified, but their formation cannot be completely excluded (*48*).

Comparison of the spectra of the four peaks of the chromatogram obtained from the pronase-digested peroxynitrite-treated apoMb (Figure 4 insets) with those depicted in Figure 6 allowed for the assignment of the peaks at ca. 15.5, 16, and 17.5 min to 6-, 4- and 5-NO_2-Trp, respectively. The peak with an elution time around 16.8 min has a spectrum nearly identical to that of the first peak and may thus represent a derivative of 6-NO_2-Trp, possibly an oxidized or a hydroxylated form. Interestingly, the additional nitrotryprotphan derivative, formed from the reaction of an excess peroxynitrite with tryptophan (14.5 min, Figure 6), which could not be identified, was not detected in the chromatograms of the peroxynitrite-treated proteins.

HPLC analyses of the pronase-digested peroxynitrite-treated metHb and oxyHb samples also indicated the formation of nitrated tryptophan residues. As shown in Figure 7, the two peaks of the chromatogram eluting at 15.5 and 16 min could be assigned to 6- and 4-nitrotryptophan, respectively. However, additional nitrated species and/or decomposition products also elute at similar retention times and are very likely to be responsible for the slightly modified absorption spectra obtained for these two nitrotryptophan products (Figure 7 insets). Preliminary results suggested that the yields of nitrated tryptophan residues of the two forms of Hb were lower than those of the corresponding Mb species.

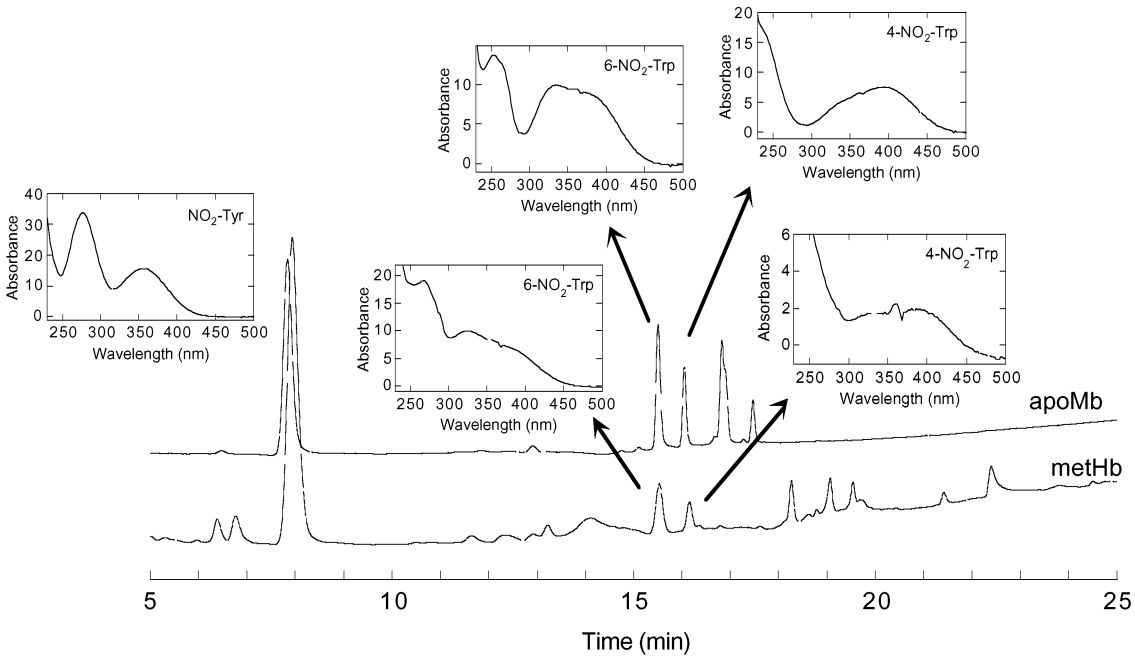

FIGURE 7: HPLC chromatograms (measured at 350 nm) of the products of the reactions of ~100 μM apoMb (top) and metHb (bottom) with 10 equiv of peroxynitrite, in 0.1 M phosphate buffer pH 7.0 at 0 °C, after complete digestion with Pronase as described in the Experimental Section. In the insets, the UV−vis spectra of the peaks corresponding NO_2-Tyr (ca. 7.5 min), 6-NO_2-Trp (ca. 15.5 min), and 4-NO_2-Trp (ca. 16 min).

Identification of Nitrated Tyrosine Residues in Myoglobin. To identify whether the two tyrosine residues of Mb, Tyr103, and Tyr146, were nitrated to a similar extent, we first cleaved the peroxynitrite-treated protein with cyanogen bromide (CNBr) and then analyzed them by immunoblotting techniques and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). CNBr is known to cleave methionine residues. As determined from the horse heart Mb sequence (Figure S4), the three resulting CNBr fragments (with the approximate molecular weight given in parentheses) are 1−55 (ca. 6 kDa), 56−131 (ca. 8 kDa), and 132−153 (ca. 2.5 kDa). The two tyrosine residues are localized in fragment 2 (Tyr103) and fragment 3 (Tyr146), whereas the two tryptophan residues are both in fragment 1 (Trp 7 and Trp 14).

As described above for the uncleaved protein, Western blot analysis of the CNBr fragments (Figure 8) indicated the presence of nitrated tyrosine residues only for peroxynitrite-treated apoMb. No staining was observed when oxyMb was mixed with peroxynitrite. As assessed from the blot in Figure 8, NO_2-Tyr was found in the uncleaved protein (upper band in lane 4) and in the fragment with the lowest molecular weight (lowest band in lane 4), which corresponds to that containing the tyrosine residue Tyr146 (fragment 3).

To confirm these results, we carried out MALDI-TOF mass spectrometric analyses of the CNBr digested proteins. The MALDI-TOF spectrum of apoMb treated with 10 or 20 equiv of peroxynitrite revealed a number of ions that could readily be assigned to the CNBr fragments. Several changes were evident when the spectrum of the nitrated fragments was compared with that of the negative control (apoMb treated with decomposed peroxynitrite and digested with CNBr). In the spectrum of fragment 3 (Figure 9C), which contains Tyr146, two new peaks appeared which have a mass of about 30 and 45 Da higher than that of the parent peak, respectively. In addition, the peak with a mass 16 Da higher

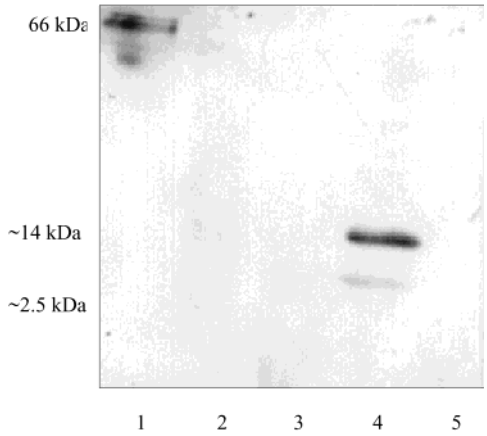

FIGURE 8: Anti-nitrotyrosine immunoblotting of Mb after digestion with CNBr. Samples were separated on a 20% SDS polyacrylamide gel and examined by Western blot analysis with a polyclonal antibody against NO_2-Tyr as described in the Experimental Section. The proteins (100 μM) were incubated in 0.1 M phosphate buffer pH 7.0 at 0 °C with decomposed peroxynitrite (negative control) and with 25 equiv of peroxynitrite and then were digested with CNBr. Lane 1, BSA treated with 10 equiv of peroxynitrite; lanes 2 and 3, oxyMb mixed with 25 equiv of peroxynitrite and with decomposed peroxynitrite; lanes 4 and 5, apoMb mixed with 25 equiv of peroxynitrite and with decomposed peroxynitrite.

than that of fragment 3, already present in the control spectrum, increased with increasing amounts of peroxynitrite. For fragment 2, which contains Tyr103, no significant differences were observed between the spectrum of the sample mixed with decomposed peroxynitrite and that of the sample treated with 10 equiv of peroxynitrite (Figure 9B). The spectrum of the sample treated with a larger excess of peroxynitrite (20 equiv) showed an increase in the intensity of the peak with a mass 16 Da higher than the parent peak and a new very weak peak with a mass 45 Da higher. Finally, for fragment 1 (Figure 9A), which does not contain tyrosine residues but Trp7 and Trp14, two new peaks, with a mass

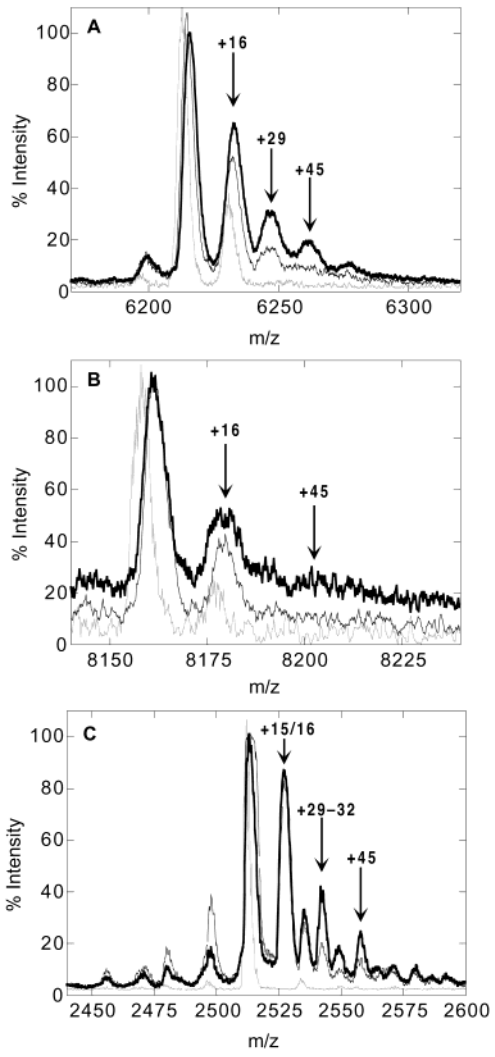

FIGURE 9: MALDI-TOF spectra of apoMb treated with decomposed peroxynitrite (negative control, gray line), 10 (thin line), and 20 (bold line) equiv of peroxynitrite and digested with CNBr. (A) Fragment 1 (1−55, 6213.2 Da), (B) fragment 2 (56−131, 8158.5 Da), and (C) fragment 3 (132−153, 2512.4 Da).

of about 29 and 45 Da higher than that of the parent peak, appeared in the spectrum of the sample treated with peroxynitrite. In addition, the peak with a mass 16 Da higher than that of fragment 1, already present in the control spectrum, increased with increasing amounts of peroxynitrite. As in the Western blots, nitration could only be identified when apoMb was allowed to react with peroxynitrite. Analogous studies with oxyMb and metMb did not lead to the unambiguous identification of any additional peak in the spectrum of the sample treated with 20 equiv of peroxynitrite, compared to that mixed with decomposed peroxynitrite (data not shown).

It has recently been shown that NO_2-Tyr can undergo a series of photodecomposition reactions in the mass spectrometer, which involve the loss of one or two oxygen atoms and can also be accompanied by further reductive reactions (*49*, *50*). The products of these decomposition processes have a mass of 15 Da (NH_2-Tyr), 29 Da (NO-Tyr), or 31 Da (NHOH-Tyr) higher than the parent peak. All these decomposition products seem to be generated during the MALDI-TOF analysis of nitrated apoMb. In addition, the peaks with a mass 16 or 32 Da higher than the parent peak could also represent oxidized derivatives of Tyr and/or Trp. Taken together, the mass spectrometric analyses confirm that when apoMb is treated with peroxynitrite Tyr146 is more readily nitrated than Tyr103. In addition, Trp7 and/or Trp14 are nitrated to an extent comparable to that of Tyr146.

DISCUSSION

The kinetic studies reported in our previous work (*28*) suggest that oxyMb may play an important role in vivo for the detoxification of peroxynitrite. Indeed, the second-order rate constant for the reaction of peroxynitrite with oxyMb is $(5.4\pm0.2) \times 10^4$ M^{-1} s^{-1} (at pH 7.3 and 20 °C), a value slightly larger than that for the reaction of peroxynitrite with CO_2 [2.3×10^4 M^{-1} s^{-1}, at pH 7.3 and 24 °C, (*51*)]. To be an efficient scavenger, Mb should not be damaged in the course of its reaction with peroxynitrite. In this work, we have analyzed extensively the globins after the reaction of different forms of Mb and Hb with variable amounts of peroxynitrite.

Nitration of Tyrosine Residues. Several qualitative studies with immunohistochemical analyses have shown that oxy- and metHb are nitrated by peroxynitrite to a dose-dependent extent (*29*, *41*−*44*). In agreement with previous studies (*41*, *44*), treatment of oxyHb and metHb with 1 equiv of peroxynitrite yields about 1% of NO_2-Tyr (expressed relative to the total amount of tyrosine in the protein, that is, 3 Tyr residues per heme). Furthermore, addition of 1 equiv of peroxynitrite to oxy- and metMb also led to approximately 1% nitration. In contrast, when the two proteins are treated with a larger excess of peroxynitrite our results show that oxy- and metMb are nitrated to a significantly lower extent than the corresponding Hb forms. Clear differences can already be detected with 5 equiv of peroxynitrite. As the rate constant for the reaction of peroxynitrite with oxyHb is larger than that for its reaction with oxyMb [$(8.4\pm0.4) \times 10^4$ M^{-1} s^{-1} and $(5.4\pm0.2) \times 10^4$ M^{-1} s^{-1}, respectively (*28*)], these data suggest that other factors, in addition to the rate of reaction with peroxynitrite, must influence the yields of nitration. Horse heart Mb has two Tyr residues (Tyr103 and Tyr146), whereas the two chains of human Hb have each three Tyr residues (Tyr24, Tyr42, and Tyr140 in the α- and Tyr35, Tyr130, and Tyr145 in the β-chain, respectively). The observation that Hb and Mb are nitrated to a different extent suggests that the exact position of the tyrosine residue within the protein structure strongly influences the nitration yields.

In most of our experiments, the nitration yields of the oxy forms of the proteins were slightly larger than those of the corresponding met-forms. The exact mechanism of the reaction of metMb and metHb with peroxynitrite is not known. However, in analogy to the mechanism of the reaction between His94-mutated metMb and peroxynitrite (*52*), it is conceivable that metMb and metHb simply catalyze the isomerization of peroxynitrite to nitrate. In contrast, we have previously shown that the reaction of oxyMb and oxyHb with peroxynitrite proceeds via the ferryl form of these proteins (*28*). Thus, the higher nitration yields obtained for the oxy forms of the proteins may derive from reactions with additional oxidizing and nitrating species generated from the interaction of peroxynitrite with oxyMb or oxyHb.

In the presence of 1.1 mM CO_2 the absolute yields of nitration are all significantly larger, in particular for the oxy forms of the proteins. In the presence of CO_2, the reaction

between oxyMb and peroxynitrite proceeds at a faster rate [$(4.6\pm0.3) \times 10^5$ M^{-1} s^{-1}, at pH 7.5, 20 °C, and 1.2 mM CO_2], but the yield of the reaction is significantly lower (M. Exner, and S. Herold, unpublished results). Thus, only a small amount of peroxynitrite is scavenged by the protein. The nitration yields of Mb are always lower than those of the corresponding forms of Hb. This result suggests that Mb can scavenge peroxynitrite better than Hb also in the presence of CO_2.

As we chose to keep the amount of CO_2 added constant throughout our experiments (1.1 mM), at the highest peroxynitrite concentrations used (20 equiv, 2 mM), CO_2 was present at a substoichiometric level relative to peroxynitrite. However, as the reaction of CO_2 with peroxynitrite is catalytic (*53*), a large amount of CO_2 is rapidly regenerated from this reaction. Nevertheless, the relative yields of nitration of all Mb and Hb forms studied are almost linearly dependent on the amount of added peroxynitrite in the absence of CO_2 but show a saturation effect in the presence of 1.1 mM CO_2. This result suggests that at higher peroxynitrite concentrations the direct, that is, not CO_2-mediated, oxidation of the protein by peroxynitrite may represent a significant reaction pathway. As the NO_2-Tyr yields are lower in the absence of added CO_2, this may lead to the reduced NO_2-Tyr yields obtained in the experiments with 20 equiv peroxynitrite.

To confirm our hypothesis that the heme center of Mb protects the protein from peroxynitrite-mediated modifications, we have also analyzed the NO_2-Tyr yields obtained from the reaction of apoMb and metMbCN with different amounts of peroxynitrite under the same conditions. Interestingly, our results show that apoMb and metMbCN are nitrated to a significantly larger extent than metMb and oxyMb, both in the absence and presence of 1.1 mM CO_2. ApoMb has been shown to retain the main features of the secondary and tertiary structure of holoMb (*54*, *55*). However, because of the observed loss of about 15−20% of its α-helicity (*54*, *55*), it cannot be excluded that small conformational changes in apoMb cause larger exposition of the Tyr residues to the surface and thus leads to higher nitration yields. Nevertheless, the observation that metMbCN is nitrated to an degree comparable to that of apoMb rather suggests that the main factor that determines the extent of nitration of the globin is the availability of the heme center to react directly with peroxynitrite or with species derived from the decay of HOONO and/or $ONOOCO_2^-$. Thus, our results demonstrate that the heme centers of oxy- and metMb efficiently scavenge peroxynitrite, which would otherwise nitrate the tyrosine residues of the globin.

It has recently been reported that reaction of metMb (100 μM) with 5, 10, and 20 equiv of peroxynitrite, carried out in the absence of added CO_2, yields 20, 36, and 50% nitration of selectively one of the two tyrosine residues (Tyr103) in the protein (*45*). The large discrepancies between our and these yields of nitration are difficult to rationalize (note that our results are expressed as percentage nitration of both tyrosine residues). However, as the experimental procedure used by Groves and co-workers (*45*) has not been described in detail, one can speculate that the reaction has been carried out at a different temperature and/or with different volumetric ratios of the two reagents. Our results show that the NO_2-Tyr yields were significantly larger at higher temperatures (20 vs 0 °C). However, preliminary results of the reactions carried out at 37 °C suggest that nitration yields are not significantly larger than those at 20 °C (data not shown). Different reactions that can take place in the presence of peroxynitrite: isomerization to nitrate, decomposition to dioxygen and nitrite via an adduct between the peroxynitrite anion and its conjugate acid (*56*, *57*), and finally reaction with the protein. Evidently, which of these reactions takes place depends on experimental conditions such as local peroxynitrite concentration, pH, and temperature. Thus, the technique used to carry out the experiments may greatly influence the results. In all our experiments, care was taken to rapidly mix the peroxynitrite solution with that containing the proteins and to always use the same volumetric ratio of 1:9.

In contrast to the preferential nitration of Tyr103 in metMb (*45*), we found that when apoMb is mixed with an excess of peroxynitrite, Tyr146 is nitrated first. Tyr103 is (possibly) nitrated only when Mb is treated with a very large molar excess of peroxynitrite (more than 20 equiv). The same preferential nitration of Tyr146 over Tyr103 is obtained when apoMb is treated with tetranitromethane (*58*), a reagent known to nitrate tyrosine residues (*59*). However, it has also been reported that electrooxidation of apo- and metMb at alkaline pH in the presence of sodium nitrite yields selectively nitrated Tyr103, together with small amounts of nitrated Phe106 and His97 (*60*). Analysis of the three-dimensional structure of Mb (*61*) suggests that nitration of Tyr103 and not Tyr146 would rather be expected. Indeed, Tyr103 is on the outside of the protein with the phenolic hydroxyl group facing the solvent and the closest ring carbon atom 3.3 Å from the heme iron center. In contrast, Tyr146 is significantly less accessible and its closest tyrosine ring carbon atom is 9.7 Å from the heme iron atom.

As mentioned above, despite the fact that apoMb retains the main features of the secondary and tertiary structure of holoMb (*54*, *55*), it has been shown that the accessibility of protein groups for chemical reagents changes significantly, thus pointing to an altered conformation and increased dynamics of the molecule (*62*). Consequently, the difference in the sites and yields of nitration between apo- and metMb may be due to these structural changes. The observation that electronitration of apoMb leads to a different selectivity may be explained by the significantly different conditions under which the nitration reaction was carried out, in particular the high pH (9.4) and the large nitrite concentration (50 mM) (*60*). Indeed, under these conditions conformational changes may have taken place and different kinds of reactive intermediates may be responsible for nitration.

The modifications of Hb by peroxynitrite have been investigated thoroughly by Minetti and co-workers (*41*, *44*). Treatment of oxyHb with 1 equiv of peroxynitrite in the presence of 1.3 mM CO_2 leads to the following approximate nitration yields (relative to the unmodified subunits): Tyr42 4.5%, Tyr140 1%, and Tyr24 0.05% in the α-chain plus Tyr130 3% in the β-chain (*41*). Interestingly, analogous studies with metHb lead to a somewhat different nitration pattern with the following approximate nitration yields (relative to the unmodified subunits): Tyr42 2.5%, Tyr140 2.5%, and Tyr24 0.1% in the α-chain and Tyr130 3% in the β-chain (*44*). For both forms of Hb, in the absence of CO_2 the total yield of nitration was about 30% lower without

significantly affecting the nitration pattern (*41*, *44*). These data also support our hypothesis that removal of the heme from metMb may lead to a different nitration pattern of the globin.

Nitration of Tryptophan Residues. Tryptophan is another amino acid that is known to be nitrated by peroxynitrite. In contrast to the reaction with tyrosine, the reaction between peroxynitrite and tryptophan is bimolecular and proceeds with a rate of 38 ± 3 M^{-1} s^{-1} (at pH 7.1 and 25 °C) (*47*). Previous reports agree that 6-NO_2-Trp is the main reaction product (*47*, *48*), but the formations of 5-NO_2-Trp (*48*) and oxidized products (*63*) have also been observed. Under our experimental conditions, that is, when tryptophan is allowed to react with an excess of peroxynitrite, we could also unambiguously identify 4-nitrotryptophan as an additional nitrated product.

Most mammalian myoglobins contain two tryptophan residues at invariant positions 7 and 14 of the amino acid sequence (*4*). Fluorescence data show that in holoMb both tryptophan residues are located in a hydrophobic environment and are equally exposed to the solvent (about 50%) (*64*). In contrast, in apoMb Trp7 is in a more polar environment and fully accessible to solvent while surroundings of Trp14 are hydrophobic, and it is inaccessible to external quenchers (*65*). This observation points to an altered conformation and increased dynamics of the N-terminal region in apoMb (*65*). Human Hb contains three tryptophan residues each $\alpha\beta$-dimer: one in the α-chain (Trpα14) and two in the β-chain (Trpβ15 and Trpβ37) (*4*).

Our studies with the different forms of Mb and Hb clearly show that in all species investigated, that is, apo-, met-, oxyMb, and met- and oxyHb, some of the tryptophan residues are nitrated by addition of peroxynitrite. All three derivatives, 6-, 5-, and 4-nitrotryptophan were definitely identified when apo-, oxy-, and metMb were treated with an excess of peroxynitrite, whereas only 6- and 4-nitrotryptophan were detected in peroxynitrite-treated oxy- and metHb. However, for all the proteins studied, 6-nitrotryptophan seems to be the isomer generated to the largest extent. It has recently been shown that when sperm whale Mb is treated with hydrogen peroxide, the ferryl form of the protein and, among other transient radical species, a peroxyl radical centered on tryptophan are formed (*66*). Site-directed mutations of all three tyrosine residues or Trp7 did not prevent formation of this radical, which in contrast was not formed when Trp14 was mutated, implicating Trp14 as the specific site of the peroxidation (*66*). Analogously, the two tryptophan residues of the Hb β-chain (Trp15 and Trp37) are also oxidized in the course of the reaction between hydrogen peroxide and oxyHb (*67*). With the results obtained up to now, we cannot assert whether Trp7 and/or Trp14 is modified by reaction of peroxynitrite with different forms of Mb. However, digestion of Mb with chymotrypsin should allow for the distinction of eventual modifications of each single tryptophan residues. These experiments are in progress in our laboratory.

Interestingly, we found only one other report, that appeared while this work was in progress (*68*), in which nitrated tryptophan residues have unambiguously been identified in a protein treated with peroxynitrite. Yakamura and coworkers showed that treatment of human recombinant Cu,Zn-superoxide dismutase with an excess of peroxynitrite leads to a dose-dependent decrease in the tryptophan fluorescence, which arises from the only tryptophan residue (Trp32) of the protein (human Cu,Zn-superoxide dismutase does not have any tyrosine residues) (*68*). The decrease in fluorescence was enhanced 20−25% in the presence of 1.3 mM CO_2 relative to the CO_2-free reaction. The difference absorption spectrum between the modified and the control enzymes has been mentioned to resemble that of 6-NO_2-Trp, even though the data have not been shown (*68*). Other authors have reported reduction of fluorescence in peroxynitrite-treated bovine serum albumine (BSA), attributed to oxidation and/or nitration of tryptophan residues (*69*), oxidation of Trp residues in BSA (*70*), oxidation of Trp residues in BSA and collagen IV (*63*), and formation of protein radicals centered on tryptophan residues in blood plasma (*71*).

In conclusion, our results suggest that tryptophan is an additional amino acid that can easily be nitrated by peroxynitrite in proteins, possibly even to a larger extent than tyrosine residues. Thus, the different nitrotryptophan derivatives may represent additional, not yet fully recognized, footprints for peroxynitrite and may also be used as biomarkers for oxidative stress-related diseases in vivo.

Summary and Possible Biological Implications. Peroxynitrite has been proposed to be formed when ischemic tissues are reperfused and to be one of the species responsible for oxidative lesions found in these tissues (*72*). However, our previous work showed that deoxyMb, which is also present in significant concentrations in ischemic tissues, is among the species which react at the highest rate with peroxynitrite [$k = \sim 10^6$ M^{-1} s^{-1}, at pH 7.4 and 20 °C (*28*)]. This reaction, which is thus likely to take place in vivo, yields nitrite and another oxidizing species, $MbFe^{IV}$=O. Ascorbate, present in tissues in mM concentration, can reduce $MbFe^{IV}$=O, though at a rather slow rate [2.7 ± 0.8 M^{-1} s^{-1} at pH 7.0 and 25 °C, (*73*)]. In addition, we have shown that $MbFe^{IV}$=O is rapidly reduced among others by peroxynitrite (*28*) and nitrogen monoxide (*9*). Taken together, these data suggest that the overall reaction of deoxy- or oxyMb with peroxynitrite may represent a detoxification pathway for this strong oxidant. Our current results indicate that the heme center of Mb protects against peroxynitrite-mediated oxidation and/or nitration of the globin. An excess of peroxynitrite nitrates Tyr146 and Trp7 and/or Trp14 in apoMb, but significantly lower yields, are obtained for oxyMb and metMb. Under physiological conditions, with Mb concentrations around 200 μM and considerably lower amounts of peroxynitrite, its half-life is reduced from about 4 s (in the absence of any other substrate) to less than 100 ms, and nitration yields are irrelevant. In addition, as the rate of the reaction between peroxynitrite and oxyMb (*28*) is in the same order of magnitude than that for the formation of $ONOOCO_2^-$ from peroxynitrite and CO_2 (*18*, *51*), Mb can partly scavenge peroxynitrite also in the presence of CO_2. This is supported also by the observation that the second-order rate constant for the reaction of oxyMb and peroxynitrite is larger in the presence of CO_2. Nitration yields in the presence of CO_2 are higher, but are still insignificant when physiological relevant concentrations of peroxynitrite are allowed to react with Mb.

It has recently been suggested that Mb may play an important role in the regulation of cellular respiration, by

scavenging $NO^{\bullet}$, an inhibitor of cytochrome *c* oxidase (*10*, *12*, *13*). Peroxynitrite has also been shown to irreversibly inhibit this enzyme (*30*). Thus, our results show that scavenging this strong oxidizing and nitrating agent may represent an additional important physiological function of Mb. In this contest, preliminary results showed that oxyMb can prevent peroxynitrite-mediated nitration of free tyrosine (K. Shivashankar, M. Exner and S. Herold, unpublished results). Under physiological conditions, this reaction may be relevant as the Mb concentration is mostly larger than that of free tyrosine, normally present in the range 20−80 μM (*74*). In addition, it is likely that Mb can protect also other proteins from oxidative modification by peroxynitrite.

Finally, we have identified two of the first examples of tryptophan nitration by peroxynitrite within proteins. Even though we have not quantified the results, 6-NO_2-Trp seems to be the preferred isomer, but 5- and 4-NO_2-Trp were also unambiguously identified in Mb and 4-NO_2-Trp in Hb. As we have found nitrated tryptophan residues when both apo- and holoMb were treated with peroxynitrite, our results suggest that this modification may not be found only in hemoproteins and may thus represent, in addition to NO_2-Tyr, a biomarker for peroxynitrite in vivo.

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SUPPORTING INFORMATION AVAILABLE

Figures of the Coomassie blue staining of peroxynitrite-treated myoglobin (S1), of the HPLC chromatogram of apoMb treated with peroxynitrite (showing the NO_2-Tyr and Tyr peaks, S2), of the relative NO_2-Tyr yields from the reaction of apo-, met-, and oxyMb with different amounts of peroxynitrite after acid hydrolysis and Pronase digestion of the proteins (S3), and of the horse heart Mb sequence (S4). Two Tables: (S1) summary of all the NO_2-Tyr yields obtained by mixing, at pH 7.0, 1, 5, 10, and 20 equiv of peroxynitrite with apo-, oxy-, metMb, metMbCN, oxy-, and metHb at 0 and 20 °C; (S2) summary of the NO_2-Tyr yields obtained by mixing, at pH 7.0 and 0 °C, 20 equiv of peroxynitrite previously mixed with different amounts of nitrite, with apo-, oxy-, and metMb. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Wittenberg, J. B. (1970) *Phys. Rev. 50*, 559−636.
2. Garry, D. J., Ordway, G. A., Lorenz, J. N., Radford, N. B., Chin, E. R., Grange, R. W., Bassel-Duby, R., and Williams, R. S. (1998) *Nature 395*, 905−908.
3. Merx, M. W., Flögel, U., Stmpe, T., Gödecke, A., Decking, U. K. M., and Schrader, J. (2001) *FASEB J. 15*, 1077−1079.
4. Antonini, E., and Brunori, M. (1971) *Hemoglobin and Myoglobin in Their Reactions with Ligands*, North-Holland, Amsterdam.
5. Hoshino, M., Ozawa, K., Seki, H., and Ford, P. C. (1993) *J. Am. Chem. Soc. 115*, 9568−9575.
6. Jourd'heuil, D., Mills, L., Miles, A. M., and Grisham, M. B. (1998) *Nitric Oxide: Biol. Chem. 2*, 37−44.
7. Doyle, M. P., and Hoekstra, J. W. (1981) *J. Inorg. Biochem. 14*, 351−358.
8. Eich, R. F., Li, T., Lemon, D. D., Doherty, D. H., Curry, S. R., Aitken, J. F., Mathews, A. J., Johnson, K. A., Smith, R. D., Phillips, G. N., Jr., and Olson, J. S. (1996) *Biochemistry 35*, 6976−6983.
9. Herold, S., Exner, M., and Nauser, T. (2001) *Biochemistry 40*, 3385−3395.
10. Flögel, U., Merx, M. W., Gödecke, A., Decking, U. K. M., and Schrader, J. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 735−740.
11. Brown, G. C. (2001) *Biochim. Biophys. Acta 1504*, 46−57.
12. Brunori, M. (2001) *Trends Biochem. Sci. 26*, 21−23.
13. Brunori, M. (2001) *Trends Biochem. Sci. 26*, 209−210.
14. Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., and Freeman, B. A. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1620−1624.
15. Koppenol, W. H., Moreno, J. J., Pryor, W. A., Ischiropoulos, H., and Beckman, J. S. (1992) *Chem. Res. Toxicol. 5*, 834−842.
16. Koppenol, W. H., and Kissner, R. (1998) *Chem. Res. Toxicol. 11*, 87−90.
17. Radi, R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991) *Arch. Biochem. Biophys. 288*, 481−487.
18. Denicola, A., Freeman, B. A., Trujillo, M., and Radi, R. (1996) *Arch. Biochem. Biophys. 333*, 49−58.
19. Koppenol, W. H. (1999) in *Metals in Biology* (Siegel, H., Ed.) pp 597−619, Marcel Dekker, New York.
20. Gow, A., Duran, D., Thom, S. R., and Ischiropoulos, H. (1996) *Arch. Biochem. Biophys. 333*, 42−48.
21. Lymar, S. V., Jiang, Q., and Hurst, J. K. (1996) *Biochemistry 35*, 7855−7861.
22. Beckman, J. S., Ischiropoulos, H., Zhu, L., van der Woerd, M., Smith, C., Chen, J., Harrison, J., Martin, J. C., and Tsai, M. (1992) *Arch. Biochem. Biophys. 298*, 438−445.
23. Balavoine, G. G. A., Geletii, Y. V., and Bejan, D. (1997) *Nitric Oxide: Biol. Chem. 1*, 507−521.
24. Crow, J. P. (1999) *Arch. Biochem. Biophys. 371*, 41−52.
25. Daiber, A., Herold, S., Schöneich, C., Namgaladze, D., Peterson, J. A., and Ullrich, V. (2000) *Eur. J. Biochem. 267*, 6729−6739.
26. Beckman, J. S., Ye, Y. Z., Anderson, P. G., Chen, J., Accavitti, M. A., Tarpey, M. M., and White, C. R. (1994) *Biol. Chem. Hoppe-Seyler 375*, 81−88.
27. Ischiropoulos, H. (1998) *Arch. Biochem. Biophys. 356*, 1−11.
28. Exner, M., and Herold, S. (2000) *Chem. Res. Toxicol. 13*, 287−293.
29. Denicola, A., Souza, J. M., and Radi, R. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 3566−3571.
30. Sharpe, M. A., and Cooper, C. E. (1998) *J. Biol. Chem. 273*, 30961−30972.
31. Koppenol, W. H., Kissner, R., and Beckman, J. S. (1996) *Methods Enzymol. 269*, 296−302.
32. Bohle, D. S., Glassbrenner, P. A., and Hansert, B. (1996) *Methods Enzymol. 269*, 302−311.
33. Teale, F. W. J. (1959) *Biochim. Biophys. Acta 35*, 543−550.
34. Bismuto, E., Sirangelo, I., Irace, G., and Gratton, E. (1996) *Biochemistry 35*, 1173−1178.
35. Radi, R., Denicola, A., and Freeman, B. A. (1999) *Methods Enzymol. 301*, 353−367.
36. Harned, H. S., and Bonner, F. T. (1945) *J. Am. Chem. Soc. 67*, 1026−1031.
37. Da Settimo, A. (1962) *Ann. Chim. (Rome) 52*, 17−24.
38. De Fazi, R., Berti, G., and Da Settimo, A. (1959) *Gazz. Chim. It. 89*, 2238−2257.
39. Schmidt, H. H. H. W., and Kelm, M. (1996) in *Methods in Nitric Oxide Research* (Feelisch, M., and Stamler, J. S., Eds.) pp 491−497, John Wiley & Sons, New York.
40. Balabanli, B., Kamisaki, Y., Martin, E., and Murad, F. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 13136−13141.
41. Pietraforte, D., Salzano, A. M., Scorza, G., Marino, G., and Minetti, M. (2001) *Biochemistry 40*, 15300−15309.
42. Alayash, A. I., Brockner Ryan, B. A., and Cashon, R. E. (1998) *Arch. Biochem. Biophys. 349*, 65−73.
43. Kikugawa, K., Nakauchi, K., Beppu, M., Hiramoto, K., Ando, K., and Hayakawa, M. (2000) *Biol. Pharm. Bull. 23*, 379−385.
44. Minetti, M., Pietraforte, D., Carbone, V., Salzano, A. M., Scorza, G., and Marino, G. (2000) *Biochemistry 39*, 6689−6697.
45. Bourassa, J. L., Ives, E. P., Marqueling, A. L., Shimanovich, R., and Groves, J. T. (2001) *J. Am. Chem. Soc. 123*, 5142−5143.
46. Goldstein, S., and Czapski, G. (1998) *J. Am. Chem. Soc. 120*, 3458−3463.
47. Padmaja, S., Ramezanian, M. S., Bounds, P. L., and Koppenol, W. H. (1996) *Redox Rep. 2*, 173−177.
48. Alvarez, B., Rubbo, H., Kirk, M., Barnes, S., Freeman, B. A., and Radi, R. (1996) *Chem. Res. Toxicol. 9*, 390−396.
49. Sarver, A., Scheffler, N. K., Shetlar, M. D., and Gibson, B. W. (2001) *J. Am. Soc. Mass. Spectrom. 12*, 439−448.

50. Petersson, A.-S., Steen, H., Kalume, D. E., Caidahl, K., and Roepstorff, P. (2001) *J. Mass. Spectrom. 36*, 616−625.
51. Lymar, S. V., and Hurst, J. K. (1995) *J. Am. Chem. Soc. 117*, 8867−8868.
52. Herold, S., Matsui, T., and Watanabe, Y. (2001) *J. Am. Chem. Soc. 123*, 4085−4086.
53. Pryor, W. A., Lemercier, J.-N., Zhang, H., Uppu, R. M., and Squadrito, G. L. (1997) *Free Radical Biol. Med. 23*, 331−338; *24*, 508.
54. Herskovits, T. T., and Solli, N. J. (1975) *Biopolymers 14*, 319−334.
55. Postnikova, G. B. (1999) *Biochemistry (Moscow) 64*, 267−286.
56. Kissner, R., Nauser, T., Bugnon, P., Lye, P. G., and Koppenol, W. H. (1997) *Chem. Res. Toxicol. 10*, 1285−1292.
57. Kissner, R., and Koppenol, W. H. (2002) *J. Am. Chem. Soc. 124*, 234−239.
58. Rischel, C., and Poulsen, F. M. (1995) *FEBS Lett. 374*, 105−109.
59. Solokovsky, M., Riordan, J. F., and L., V. B. (1966) *Biochemistry 5*, 3582−3589.
60. Kendall, G., Cooper, H. J., Heptinstall, J., Derrick, P. J., Walton, D. J., and Peterson, I. R. (2001) *Arch. Biochem. Biophys. 392*, 169−179.
61. Evans, S. V., and Brayer, G. D. (1990) *J. Mol. Biol. 213*, 885−897.
62. Griko, Y. V., Privalov, P. L., Venyaminov, S. Y., and Kutyshenko, V. P. (1988) *J. Mol. Biol. 202*, 127−138.
63. Kato, Y., Kawakishi, S., Aoki, T., Itakura, K., and Osawa, T. (1997) *Biochem. Biophys. Res. Commun. 234*, 82−84.
64. Takano, T. (1977) *J. Mol. Biol. 110*, 537−568.
65. Postnikova, G. B., and Yumakova, E. M. (1991) *Eur. J. Biochem. 198*, 241−246.
66. DeGray, J. A., Gunther, M. R., Tschirret-Guth, R., Ortiz de Montellano, P. R., and Mason, R. P. (1997) *J. Biol. Chem. 272*, 2359−2362.
67. Steffek, R. P., and Thomas, M. J. (1991) *Free Radical Res. Commun. 12−13*, 489−497.
68. Yamakura, F., Matsumoto, T., Fujimura, T., Taka, H., Murayama, K., Imai, T., and Uchida, K. (2001) *Biochim. Biophys. Acta 1548*, 38−46.
69. Ischiropoulos, H., and Al-Mehdi, A. B. (1995) *FEBS Lett. 364*, 279−282.
70. Pollet, E., Martinez, J. A., Metha, B., Watts, B. P., Jr., and Turrens, J. F. (1998) *Arch. Biochem. Biophys. 349*, 74−80.
71. Pietraforte, D., and Minetti, M. (1997) *Biochem. J. 321*, 743−750.
72. Grisham, M. B., Granger, D. N., and Lefer, D. J. (1998) *Free Radical Biol. Med. 25*, 404−433.
73. Kröger-Ohlsen, M., and Skibsted, L. H. (1997) *J. Agric. Food. Chem. 45*, 668−676.
74. Kettle, A. J., and Candaeis, L. P. (2000) *Redox Rep. 5*, 179−184.

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