Mechanism of Peroxynitrite Interaction with Ferric Hemoglobin and Identification of Nitrated Tyrosine Residues. CO_2 Inhibits Heme-Catalyzed Scavenging and Isomerization†

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ABSTRACT: Hemoproteins are one of the major targets of peroxynitrite in vivo. It has been proposed that the bimolecular heme/peroxynitrite interaction results in both peroxynitrite inactivation (scavenging) and catalysis of tyrosine nitration. In this study, we used spectroscopic techniques to analyze the reaction of peroxynitrite with human methemoglobin (metHb). Although conventional differential spectroscopy did not reveal heme changes, our results suggest that, in the absence of bicarbonate, the heme in metHb reacts bimolecularly with peroxynitrite but is quickly back-reduced by the reaction products. This hypothesis is based on two indirect observations. First, metHb prevents the peroxynitrite-mediated nitration of a target dipeptide, Ala-Tyr, and second, it promotes the isomerization of peroxynitrite to nitrate. Both the scavenging and the isomerization activities of metHb were heme-dependent and inhibited by CO_2. Ferrous cytochrome *c* was an efficient scavenger of peroxynitrite, but in the ferric form did not show either scavenging or isomerization activities. We found no evidence of an increase in Ala-Tyr nitration with these hemoproteins. Peroxynitrite-treated metHb induced the formation of a long-lived radical assigned to tyrosine by spin-trapping studies. This radical, however, did not allow us to predict an interaction of peroxynitrite with heme. Hb was nitrated by peroxynitrite/CO_2 mainly in tyrosines β 130, α 42, and α 140 and, to a lesser extent, α 24. The nitration of α chain tyrosines more exposed to the solvent (α 140 and α 24) was higher in CO-Hb and metHb, while nitration of α 42, the tyrosine nearest to the heme, was higher in oxyHb. We deduce that the heme/peroxynitrite interaction, which is inhibited in CO-Hb and metHb, affects α tyrosine nitration in two opposite ways, i.e., by protecting exposed residues and by promoting nitration of the residue nearest to the heme. Conversely, nitration of β Tyr 130 was comparable in oxyHb, metHb, and CO-Hb, suggesting a mechanism involving only nitrating species formed during peroxynitrite decay.

Since the discovery of the unexpected biological activities of nitric oxide (•NO, nitrogen monoxide), it has become evident that hemoproteins play a major role in cellular events mediated by this radical (*1*−*5*). These studies demonstrated that the interaction of •NO with heme plays a crucial role in cell signaling as well as in limiting the radical activity (*6*, *7*). However, overproduction of •NO and the simultaneous generation of reactive oxygen species can prevail over the detoxification mechanisms, leading to •NO-dependent toxic reactions and tissue injury. Recent data clearly show that •NO has mainly antioxidant properties (*8*), whereas its toxic effects are largely dependent on reactions with molecular oxygen or with the superoxide radical anion ($O_2^{\bullet-}$). This latter reaction is faster than the dismutation catalyzed by superoxide dismutase and leads to the formation of peroxynitrite[1] (*9*), an oxidant species that is considerably more reactive than the parent radicals.

Although peroxynitrite has been shown to react and oxidize several biomolecules in vitro, its reaction with CO_2, which is ubiquitously present in tissues at 1−1.5 mM, suggests that relatively few targets can be modified directly by this oxidant (*10*, *11*). Peroxynitrite reacts with CO_2 ($k = 5.8 \times 10^4$ M^{-1} s^{-1} at pH 7.4 and 37 °C) (*12*) to form a postulated $ONOOCO_2^-$ adduct, which rapidly isomerizes to nitrate, reducing the lifetime of peroxynitrite to ∼20 ms. The reaction with CO_2 does not, however, detoxify peroxynitrite since the isomerization reaction produces about 30−35% of two reactive radical species, $CO_3^{\bullet-}$ and $^{\bullet}NO_2$, through homolysis of the peroxo bond (*13*, *14*). The CO_2-independent reactivity of peroxynitrite is restricted to acidic biological environments, where the CO_2 does not seem to affect the proton-catalyzed chemistry of peroxynitrite (*15*). This proton-catalyzed chemistry, however, also involves the formation of radicals (•OH and $^{\bullet}NO_2$) through homolysis of the peroxo

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[1] This term refers to both the anion oxoperoxynitrate(1−), $ONOO^-$, and its conjugate acid hydrogen oxoperoxynitrate, ONOOH.

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bond, a pathway that was initially rejected by thermodynamic calculations (*16*) but is now supported by experimental evidence (*15*) and thermodynamic and kinetic data (*17*).

Cellular targets that can react bimolecularly with peroxynitrite at neutral pH and physiological CO_2 are thiols and compounds containing metal centers, including, in particular, the hemoproteins. In fact, these targets react sufficiently fast with peroxynitrite and/or are sufficiently concentrated in tissues to compete with CO_2. In the case of oxyHb,[2] its high intracellular concentration (20 mM) and its second-order rate constant with peroxynitrite (8.8×10^4 M^{-1} s^{-1} at 20 °C and pH 7.0) (*18*) suggest that any peroxynitrite crossing the red blood cell will react preferentially with this target. It has been demonstrated that in blood at 45% hematocrit and at physiological concentrations of CO_2, about 60% of peroxynitrite generated extracellularly may react with oxyHb, making this protein a major sink of peroxynitrite (*12*, *19*).

We also previously showed that the peroxynitrite/oxyHb reaction proceeds through the formation of ferryl heme and globin tyrosyl radical intermediates (*20*), and the process of oxidation to ferryl heme has been further supported by stopped-flow spectroscopy (*18*). Thus, peroxynitrite reactivity with this hemoprotein resembles that of other peroxides that are well-known generators of ferryl heme and globin tyrosyl radicals (*21*, *22*). In the case of peroxynitrite, moreover, tyrosyl radical(s) can react with $^{\bullet}NO_2$ formed in the process, leading to the formation of 3-nitrotyrosine (*23*). Besides Hb, it has been shown that peroxynitrite reacts with some thiolate-based ferric hemoproteins and catalyzes nitration of phenolic groups in target molecules and/or the nitration of its tyrosine residues (*24*−*26*). According to these authors, the final effect of a bimolecular reaction between ferric heme and peroxynitrite is the amplification of peroxynitrite-dependent nitration, a phenomenon also observed with Mn-SOD (*27*), Fe-EDTA, and some Mn- and Fe-porphyrins (*28*−*30*). All these studies questioned the hypothesis that porphyrins (*31*) and hemoproteins (*32*) could be considered catalysts for peroxynitrite decomposition and suggest the possibility that metal centers may indeed increase tissue injury by increasing tyrosine nitration.

Clarification of the oxidation mechanism of hemoproteins is further complicated by the ability of peroxynitrite to be a one-electron oxidant of the protein moiety and a tyrosine nitrating agent independently of its reaction with heme.

Crucial to the understanding of peroxynitrite/hemoprotein chemistry is the elucidation of its interaction with the ferric forms. In general, peroxides—such as hydrogen peroxide—react more rapidly with ferric than with ferrous hemoproteins and lead to the formation of a species referred to as Compound I or perferryl heme, which is a complex between porphyrin π-cation radical and ferryl heme (or oxo-iron heme), $Por^{\bullet+}-Fe^{IV}=O-$globin. In some hemoproteins, such as Hb and myoglobin, the porphyrin π-cation radical is quickly reduced by globin to produce the more stable ferryl heme and a globin-centered radical, $Fe^{IV}=O-$globin$^{\bullet}$, but recently, transient formation of perferryl heme in H_2O_2-treated ferric myoglobin (metMb) has been demonstrated by stopped-flow visible spectroscopy (*33*). In the presence of a reducing substrate, Compound I is reduced, giving only ferryl heme, $Fe^{IV}=O-$globin, also referred to as Compound II.

It is generally accepted that peroxynitrite reacts bimolecularly with the ferrous heme of Hb and myoglobin (*18*, *20*, *34*), but it is not equally clear whether it can react with the ferric form of these hemoproteins. Some groups, using conventional or stopped-flow visible spectroscopy (*18*, *20*, *34*−*36*), were unable to demonstrate in metHb a peroxynitrite-dependent heme modification indicative of heme oxidation to ferryl or perferryl species. One exception was the report of Cooper et al. (*37*), who showed by conventional differential visible spectroscopy that peroxynitrite was indeed able to form ferryl heme from metHb. In the hypothesis that peroxynitrite interacts with ferric heme, it is reasonable to assume that the reaction may proceed through homolytic or heterolytic cleavage of the peroxynitrite peroxo bond, leading to one- or two-electron heme oxidation (reactions 1 and 2, respectively). Because of the instability of the porphyrin π-cation radical in Hb (reaction 3), both pathways can form a ferryl heme.

one oxidizing equivalent:

$$Fe^{III}\ \text{globin} + ONOO^- \rightleftarrows \underset{\text{ferrylHb}}{Fe^{IV}=O-\text{globin}} + {}^{\bullet}NO_2 \quad (1)$$

two oxidizing equivalents:

$$Fe^{III}\ \text{globin} + ONOO^- \rightleftarrows \underset{\text{perferrylHb}}{Por^{\bullet+}-Fe^{IV}=O\text{globin}} + NO_2^- \quad (2)$$

$$\underset{\text{perferrylHB}}{Por^{\bullet+}-Fe^{IV}=O-\text{globin}} \rightarrow Por-Fe^{IV}=O-\text{globin}^{\bullet} \quad (3)$$

In this study we investigated the reaction of peroxynitrite with human metHb using visible, EPR, and MS spectroscopic techniques. In the presence of CO_2, we found no evidence of a bimolecular reaction between metHb and peroxynitrite, and the relative amount of tyrosine nitration of some residues of the α chain was peculiarly different from that of oxyHb, suggesting that the heme/peroxynitrite reaction may affect the nitration pattern. On the contrary, indirect evidence for a reaction selectively directed toward the ferric iron center was obtained in the absence of CO_2, although ferryl intermediates were not detected by conventional spectroscopy. However, even when peroxynitrite interacts bimolecularly with Hb heme, the nitration of tyrosine in target molecules is inhibited, suggesting that peroxynitrite is scavenged and precluding the possibility that Hb may have a catalytic role in tyrosine nitration.

MATERIALS AND METHODS

Materials. Acetonitrile (highest grade purity) and formic acid were from J. T. Baker (Milan, Italy). Trifluoroacetic acid was from Carlo Erba (Milan, Italy) and tetramethylammonium salt of peroxynitrite from Alexis (San Diego, CA). All other reagents were from Sigma (St. Louis, MO).

Peroxynitrite Synthesis. Peroxynitrite was synthesized from nitrite and H_2O_2 as described by Radi et al. (*38*) and treated with MnO_2 to eliminate excess H_2O_2 (6 mg/mL at 4 °C for

[2] Abbreviations: oxyHb, oxyhemoglobin; metHb, ferric hemoglobin; CO-Hb, carbon monoxide hemoglobin; CN-metHb, cyanide-treated methemoglobin; metMb, ferric myoglobin; Cyt c^{+2}, ferrous cytochrome *c*; Cyt c^{+3}, ferric cytochrome *c*; DTPA, diethylenetriaminepentaacetic acid; MS, mass spectrometry; LC/ES liquid chromatography/electrospray; MNP, 2-methyl-2-nitrosopropane.

30 min). The mixture was then filtered 3 times to remove MnO_2. When freeze-fractionated (−80 °C), peroxynitrite solution forms a yellow top layer, which was retained for further studies. The peroxynitrite concentration was determined at 302 nm ($\epsilon = 1700\ M^{-1}\ cm^{-1}$). The top layer typically contained 100 mM peroxynitrite. The presence of NO_2^- and H_2O_2 as contaminants of peroxynitrite preparations interferes with the chemistry of both peroxynitrite and hemoproteins; to lower the NO_2^- contamination, we therefore synthesized peroxynitrite from a slight excess of H_2O_2 (NO_2^-:H_2O_2 = 1:1.5) (*39*). Under these conditions, a typical stock solution of peroxynitrite contained ≤2% residual H_2O_2, and the concentrations of contaminating nitrite and nitrate, as determined by peroxynitrite decomposition at pH 4.0 (*17*), were 49.4% and 185%, respectively. Peroxynitrite was added as a bolus to samples submitted to vigorous vortexing and buffered with 150 mM phosphate, 0.1 mM DTPA, pH 7.4 (phosphate/DTPA). Bicarbonate was added to phosphate/DTPA from a 1 M stock solution, and dissolved CO_2 was measured by a blood gas analyzer (ABL 330 Radiometer, Copenhagen, Denmark) and was 1.3 and 5.0 mM at 25 and 100 mM bicarbonate, respectively

Preparation of Hemoproteins. Heparinized fresh human blood was obtained from normal donors following informed consent. A concentrated hemolysate and oxyHb purification by an ion exchange column were performed as previously described (*20*). MetHb was prepared from oxyHb by the addition of potassium ferricyanide in a 1:1.2 molar ratio. Excess ferricyanide was removed by extensive dialysis and a further gel-filtration step through a Sephadex G-25 column. The concentrations of oxyHb and metHb expressed per heme group were determined spectrophotometrically (*40*). CO-Hb was obtained from oxyHb by bubbling with CO gas for 5 min. Commercial preparations of ferric cytochrome *c* (Cyt c^{+3}) contain about 4−5% of Cyt c^{+2}; to obtain a fully oxidized preparation, 5 mM Cyt c was treated with 0.24 mM potassium ferricyanide. Fully reduced Cyt c^{+2} was achieved by treatment of Cyt c^{+3} with dithionite in a 1:10 molar ratio (30 min, room temperature). Excess ferricyanide and dithionite were removed by extensive dialysis and gel filtration through a Sephadex G-25 column. The concentrations of Cyt c preparations were determined spectrophotometrically by the absorbance at 550 nm with $\epsilon_{ox} = 8.9$ and $\epsilon_{red} = 21.0\ mM^{-1}\ cm^{-1}$ (*41*). Hemoproteins were concentrated by centrifugation on Centriprep (cutoff 3000, Amicon, Beverly, MA). To avoid metal-catalyzed oxidations (*42*), buffers were treated extensively with Chelex 100 (Bio-Rad, Richmond, CA), and all samples contained 0.1 mM diethylenetriaminepentaacetic acid (DTPA).

Spectrophotometric Analysis of Hemoprotein Oxidation. The reactions of hemoproteins with peroxynitrite in phosphate/DPTA were followed by conventional visible differential spectroscopy (Lambda 14 P UV/VIS, Perkin-Elmer, Norwalk, CT). The difference spectrum (oxidized versus control) was recorded 30 s after the addition of the oxidant.

Nitrite and Nitrate Determination. Nitrite and nitrate were measured spectrophotometrically using a Cayman Nitrite/Nitrate Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI) with the following modifications. Nitrite determination: 20 μL of sample was diluted to 200 μL in phosphate, pH 7.4, and treated with 800 μL of Griess reagent. Nitrate determination: 20 μL of sample was treated with 20 μL of nitrate reductase/enzyme cofactors and diluted to 100 μL in phosphate, pH 7.4. After 2 h incubation at room temperature, samples were further diluted to 200 μL in phosphate, pH 7.4, and treated with the Griess reagent. To separate hemoproteins, samples were ultrafiltrated by Microcon (cutoff 3000, Amicon, Beverly, MA), and nitrite/nitrate was determined in the eluate.

Spectrophotometric Determination of 3-Nitrotyrosine. Treatment of Ala-Tyr with peroxynitrite in the presence of different concentrations of the hemoproteins was performed in phosphate/DTPA ± 25 mM bicarbonate, and 3-nitrotyrosine was detected in the eluate of samples after centrifugation at 4 °C on Microcon (cutoff 3000, Amicon, Beverly, MA). 3-Nitrotyrosine was measured using differential absorbance at 430 nm under acidic and alkaline conditions (*43*).

Liquid Chromatography/Electrospray Mass Spectrometry (*LC/ES/MS*). Peroxynitrite-treated hemoglobin samples were analyzed using an LCQ ion trap instrument (Finnigan Corp., San Jose, CA) equipped with an HPLC HP 1100 apparatus (Agilent Technologies, Palo Alto, CA). Individual globins were fractionated using a Phenomenex Jupiter C4 column (250 × 2.1 mm, 300 Å) (Torrance, CA) in the experimental conditions already reported (*23*). The eluate was directly injected into the electrospray ion source, and the spectra were acquired and elaborated using the software provided by the manufacturer. Modified globin chains were purified as described (*23*). Relative yields of nitrated globins were calculated as percentages of the area value associated with extracted ions for modified α and β chains compared with the corresponding unmodified globins. Extracted ion values were *m*/*z* 1221.6 (MH_{13}^{+13}), *m*/*z* 1323.5 (MH_{12}^{+12}), *m*/*z* 1443.5 (MH_{11}^{+11}), *m*/*z* 1587.7 (MH_{10}^{+10}) for the unmodified β chain, and *m*/*z* 1224.7, *m*/*z* 1327.1, *m*/*z* 1447.5, *m*/*z* 1592.2, respectively, for the same charged ions of the corresponding nitrated globin. For unmodified α globin, extracted ions used were *m*/*z* 1164.7 (MH_{13}^{+13}), *m*/*z* 1261.6 (MH_{12}^{+12}), *m*/*z* 1376.1 (MH_{11}^{+11}), and *m*/*z* 1513.6 (MH_{10}^{+10}), while *m*/*z* 1168.1, *m*/*z* 1265.3, *m*/*z* 1380.2, and *m*/*z* 1518.4 were used for the corresponding ions of nitrated α globin. All experiments were repeated at least 3 times to obtain statistically significant values for the percentages of nitration. Enzymatic hydrolysis by pepsin of nitrated globins was performed in 5% formic acid for 2 h at 37 °C using an enzyme-to-substrate ratio of 1:100 (w/w). The resulting peptide mixtures were fractionated on a narrowbore C18 column using a linear gradient from 5 to 60% of 0.07% trifluoroacetic acid in 95% acetonitrile over 60 min at a flow rate of 0.2 mL/min, and the absorbance was monitored at both 220 and 360 nm (characteristic of nitrotyrosine at acidic pH). Proteolytic mixtures were also directly analyzed by LC/ES/MS using the same chromatographic conditions and the LCQ instrument described above; effluent from the HPLC column was directly inserted into the ion source of the spectrometer, and ES/MS spectra were acquired throughout the entire analysis using the software provided by the manufacturer. Nitration of Tyr 140 and Tyr 24 was calculated through LC/ES/MS analysis of the hydrolysate of α nitrated globin by calculating the percentage ratios between the area values of the nitrated peptides and those of the corresponding unmodified species. For Tyr 140, area values were measured by extracting the following ions: *m*/*z* 699.7 and *m*/*z* 654.6, corresponding to singly charged peptides 137−141 nitrated

and unmodified, respectively. For Tyr 24, extracted ions for the nitrated peptide 1−29 were *m*/*z* 1478.8 (MH_2^{+2}) and *m*/*z* 986.6 (MH_3^{+3}), while *m*/*z* 1456.2 (MH_2^{+2}) and *m*/*z* 971.5 (MH_3^{+3}) were used for the corresponding unmodified species.

EPR (or ESR) Spectroscopy. Spectra were measured on a Bruker ECS 106 spectrometer (Bruker, Rheinstetten, Germany) equipped with a variable-temperature unit (ER4111VT). Samples were drawn up into a gas-permeable Teflon tube with 0.81 mm internal diameter and 0.05 mm wall thickness (Zeuss Industrial Products, Raritan, NJ). The Teflon tube was folded 4 times, inserted into a quartz tube, and fixed to the EPR cavity (4108 TMH). Samples were exposed to air at 37 °C. The dead time of sample preparation and EPR analysis was exactly 2 min after the last addition. All EPR spectra were corrected for baseline drift by a linear function and elaborated by Bruker WinEPR software. The 2-methyl-2-nitrosopropane (MNP) spin trap was suspended in deoxygenated 50 mM phosphate, pH 7.1, and stirred overnight in the dark at 25 °C. Spectrometer conditions common to all spectra were the following: modulation frequency, 100 kHz; microwave frequency, 9.5 GHz; microwave power, 20 mW; time constant, 81.92 ms; center field, 340.5 mT.

Fitting of Data. The Prism 2.0 software (GraphPad, San Diego, CA) was used to draw a curve from the experimental data, and differences were analyzed statistically by the Student's *t* test.

RESULTS

Direct EPR at 37 °C and Spin Trapping Studies. We previously reported that peroxynitrite oxidizes oxyHb, inducing the formation of a long-lived globin tyrosine-centered radical(s) similar to that induced by H_2O_2 to metHb and metMb (*20*, *44*). In this study, we investigated whether peroxynitrite can induce a long-lived radical(s) in metHb. As shown in Figure 1, a long-lived radical(s) at $g = 2.004$, with spectral features similar to those of peroxynitrite/oxyHb, was indeed formed in the peroxynitrite/metHb reaction, although the intensity was 52% lower (2 min after peroxynitrite addition, radical concentration was estimated as 0.5 and 0.2 μM in oxyHb and metHb, respectively). The radical(s) increased by 35% in samples containing bicarbonate and was not detectable if peroxynitrite decomposed before the addition of Hb, ruling out oxidative processes mediated by peroxynitrite decomposition products or contaminants (spectra not shown).

To identify the long-lived radical(s) generated by peroxynitrite/metHb, we performed spin trapping experiments with MNP followed by Pronase digestion of the adduct. Taking advantage of the high stability of the $g = 2.004$ radical(s) and to avoid trapping of radicals with a shorter lifetime, MNP was added 30 s after the addition of peroxynitrite. Figure 1C shows the immobilized nitroxide adduct ($2A_{zz} = 5.65$ mT) obtained after treatment of metHb in phosphate/DTPA/bicarbonate with tetramethylammonium peroxynitrite (this preparation of peroxynitrite was used because it is devoid of trace amounts of hydrogen peroxide). Proteolytic digestion with Pronase of the MNP/metHb adduct resulted in a spectrum showing mainly a three-line adduct ($a_N = 1.55 \pm 0.02$ mT) (Figure 1D), consistent with the trapping of a tyrosyl-centered radical(s) (*45*) and similar to the spectra

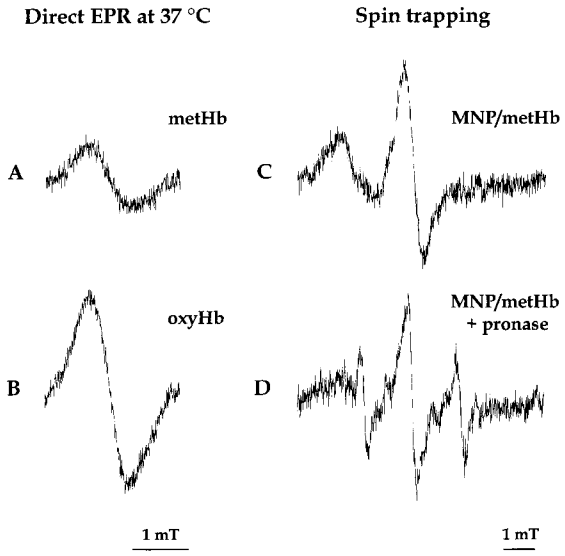


FIGURE 1: Direct EPR spectra at 37 °C and spin trapping with MNP of peroxynitrite-treated Hb. (A) MetHb (1 mM) treated with peroxynitrite (1 mM) in phosphate/DTPA, pH 7.4. (B) Sample as in panel A, but with oxyHb. (C) MetHb (1 mM) treated with peroxynitrite (5 mM) in phosphate/DTPA/100 mM bicarbonate, pH 7.4, and with the addition of 10 mM MNP after 30 s. (D) Sample as in panel C, but with the addition of 0.5 mg/mL Pronase. Spectrometer conditions of spectra A and B: modulation amplitude, 0.5 mT; conversion time, 40.96 ms; sweep time, 41.9 s; gain, 3.6×10^4; number of scans, 10. Spectrometer conditions of spectra C and D: modulation amplitude, 0.25 mT; conversion time, 163.84 ms; sweep time, 167.8 s; gain, 5×10^5; number of scans, 1.

previously detected in Pronase-treated MNP adducts of oxyHb/peroxynitrite and metHb/H_2O_2 (*20*). Notably, long-lived tyrosyl radicals were observed in peroxynitrite-treated metHb without bicarbonate as well as in CO-Hb and CN-Hb, although the spectra were of lower intensity.

These studies showed that when oxyHb and metHb are treated with peroxynitrite, tyrosyl radical(s) was (were) formed regardless of the interaction with the heme center. Since we previously demonstrated (*46*) that peroxynitrite can also induce the formation of tyrosyl radical(s) in non-heme proteins, we conclude that the formation of these long-lived radical(s) is not proof of the reaction with heme.

Differential Visible Spectroscopy. There is no consensus regarding the ability of peroxynitrite to induce ferryl species in the reaction with metHb (see the introduction). We reinvestigated the ability of peroxynitrite to modify visible spectra of metHb by conventional differential spectroscopy in the 500−700 and 300−500 nm (Soret) regions. However, the addition of peroxynitrite did not induce changes in the spectra of metHb (results not shown). Similarly, Cyt c^{+3} did not result in the formation of differential spectra, demonstrating that ferryl/perferryl species of these hemoproteins are not formed or, alternatively, are formed but rapidly back-reduced to the ferric forms. One reason to explain conflicting results (*18*, *20*, *34*−*37*) may be the presence of contaminating products in peroxynitrite stock solution (*47*). Our peroxynitrite preparation contained about 49% NO_2^- and ≤2% of residual H_2O_2 as contaminating products, but identical results were obtained with peroxynitrite prepared from isoamyl nitrite (*47*) or with a commercial preparation of peroxynitrite (tetramethylammonium salt of peroxynitrite). Cassina et al. (*48*) reported that peroxynitrite was able to induce spectral

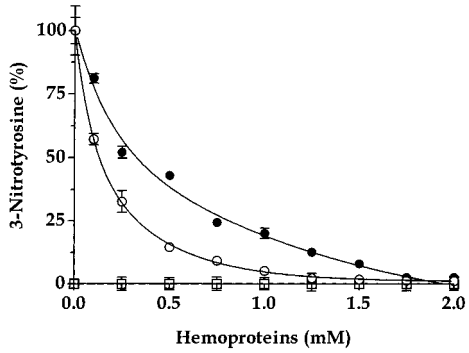


FIGURE 2: Effects of ferrous Hb and Cyt c on the peroxynitrite-mediated nitration of Ala-Tyr in the presence of 25 mM bicarbonate. Ala-Tyr (1 mM) treated with peroxynitrite (1 mM) in phosphate/DTPA/bicarbonate, pH 7.4, in the presence of different concentrations of oxyHb (○) and Cyt c^{+2} (●). Decomposed peroxynitrite (□) was obtained by mixing peroxynitrite with phosphate/DTPA/bicarbonate, pH 7.4, before the addition of metHb. The 3-nitrotyrosine yield detected in the absence of the hemoproteins was 154.3 ± 8.5 μM. Points represent % of control value ± SD, $n = 4$.

changes in Cyt c^{+3}. Indeed, the first change in the spectrum was reported with a 15 molar excess of peroxynitrite, while we did not observe a differential spectrum at 1:1 stoichiometry. We were interested in preferential reactions of peroxynitrite with the heme center that could be studied without using a large excess of peroxynitrite. The interpretation given by Cassina et al. (*48*) is that the spectral change is not due to a preferential reaction of peroxynitrite with the iron center, but reflects changes due to the oxidation of residues surrounding the heme.

Inhibition of Peroxynitrite-Induced Tyrosine Nitration. One indirect way to investigate whether a bimolecular peroxynitrite/heme interaction occurs is by looking at the effects of the protein on peroxynitrite-mediated oxidation of a suitable target molecule. In fact, if the interaction of the protein with peroxynitrite is faster than its proton-catalyzed isomerization to nitrate, indirect reactions of peroxynitrite should be inhibited. These reactions are due to radical species formed in the isomerization process (i.e., $^{\bullet}NO_2/^{\bullet}OH$ and, in the presence of bicarbonate, $^{\bullet}NO_2/CO_3^{\bullet-}$). Phenolic compounds such as tyrosine are one example of a biological target modified by peroxynitrite only indirectly, being oxidized to 3-nitrotyrosine by peroxynitrite-derived radical species (*10*, *11*). We previously reported (*23*) that oxyHb was an efficient scavenger of peroxynitrite as demonstrated by the complete protection from peroxynitrite-mediated and CO_2-enhanced nitration of a target dipeptide, Ala-Tyr. Figure 2 extends these results to Cyt c^{+2}, confirming that the ferrous center of these hemoproteins can successfully compete with CO_2 for peroxynitrite and inhibits tyrosine nitration. However, when similar experiments were performed with the hemoproteins in the ferric forms, we observed a poor and incomplete protection of Ala-Tyr (Figure 3 A), suggesting a less efficient scavenging of peroxynitrite. Moreover, the heme of metHb was not a preferential target of peroxynitrite/CO_2, as demonstrated by the comparable inhibition afforded by CN-metHb. The poor protection observed probably reflects reactions with globin amino acids. The cyanide ion is a high-affinity ligand for the ferric state of Hb and inhibits heme oxidation by peroxides. Notably, the omission of bicarbonate significantly improved the scavenging ability of metHb, without affecting

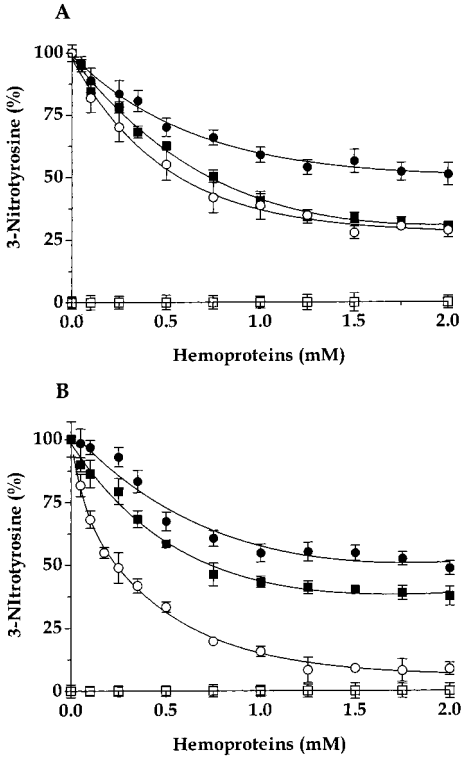


FIGURE 3: Protection by metHb and Cyt c^{+3} of peroxynitrite-mediated nitration of Ala-Tyr with (A) or without bicarbonate (B). Ala-Tyr (1 mM) treated with peroxynitrite (1 mM) in phosphate/DTPA/± bicarbonate, pH 7.4, in the presence of metHb (○), CN-metHb (■), and Cyt c^{+3} (●). Decomposed peroxynitrite (□) was obtained by mixing peroxynitrite with phosphate/DTPA, pH 7.4, before the addition of metHb. Without hemoproteins, the 3-nitrotyrosine yields were 154.3 ± 8.5 and 94.85 ± 6.5 μM with or without bicarbonate, respectively. Points represent % of control value ± SD, $n = 4$.

the scavenging by Cyt c^{+3}. As shown in Figure 3B, the concentration of metHb affording 50% protection of Ala-Tyr (IC_{50}) was about 2.5 times lower in the absence of bicarbonate ($IC_{50} = 0.60$ and 0.24 μM with and without bicarbonate, respectively), while the IC_{50} of Cyt c^{+3} was similar in the presence and in the absence of bicarbonate (IC_{50} about 2 mM).

The stronger inhibition of peroxynitrite-mediated nitration of Ala-Tyr by metHb without bicarbonate suggests the possibility that peroxynitrite may react preferentially with ferric heme, while the inefficient scavenging in the presence of bicarbonate suggests that ferric heme cannot compete successfully with CO_2 for peroxynitrite. In any case, even at the lowest concentration tested (50 μM) our data did not provide evidence of a catalytic role of these hemoproteins in the peroxynitrite-mediated tyrosine nitration of Ala-Tyr. However, it should be noted that the protection of Ala-Tyr from nitration in excess of metHb and without intentionally added bicarbonate was almost complete but did not reach the expected 100%. In excess metHb, a small amount of nitrotyrosine (about 7−8 μM) was a reproducible finding, and it is likely due to the unavoidable contamination of samples by atmospheric CO_2. This hypothesis was supported by the observation that this residual amount of nitrotyrosine was halved by thoroughly degassing of samples and doubled by addition of 0.1 mM bicarbonate. When peroxynitrite was decomposed before the addition of the biological targets (Ala-

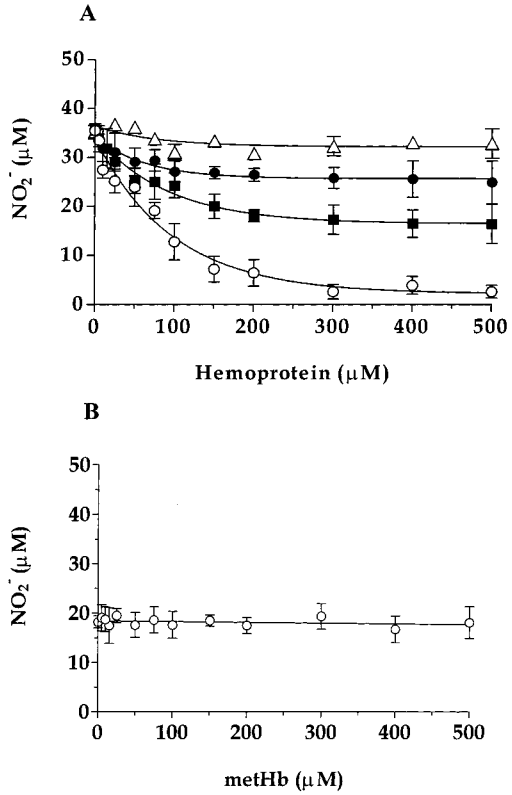


FIGURE 4: Effects of metHb on the nitrite yields derived from peroxynitrite decay. (A) Peroxynitrite (100 μM) added to phosphate/DTPA, pH 7.4, in the presence of different concentrations of metHb (○), CN-metHb (■), or CO-Hb (△). Decomposed peroxynitrite was obtained by mixing peroxynitrite with phosphate/DTPA, pH 7.4, before the addition of metHb (●). (B) Peroxynitrite added to phosphate/DTPA/25 mM bicarbonate, pH 7.4, in the presence of metHb (○). Points represent mean value ± SD, $n = 3$. The concentration of nitrite was obtained after subtraction of nitrite contaminating the peroxynitrite preparation. The mean value of total nitrogen in these experiments was 334 μM (49 and 185 μM derived from contaminating nitrite and nitrate, respectively, and 100 μM from peroxynitrite; see Materials and Methods for details).

Tyr with or without the hemoprotein), it did not induce the formation of 3-nitrotyrosine, excluding the participation of peroxynitrite decomposition or contaminating products (Figure 3).

To test the involvement of heme in the scavenging ability of metHb, the hemoprotein was treated with KCN. As shown in Figure 3B, without bicarbonate cyanide treatment significantly decreased the peroxynitrite scavenging ability of metHb. Compared with metHb, the IC_{50} of CN-metHb increased 2.8 times. It could thus be concluded that metHb possesses a CO_2-inhibitable peroxynitrite scavenging ability that depends on a heme/peroxynitrite interaction.

Effect of MetHb on Stable Peroxynitrite End Products. Previous studies have shown that without bicarbonate peroxynitrite decomposes at physiological pH by two distinct pathways: proton-catalyzed isomerization to nitrate and decomposition to NO_2^- and O_2 (*49*). Figure 4 shows nitrite yields from 100 μM peroxynitrite at pH 7.4 as a function of metHb concentration. Nitrite obtained from 100 μM peroxynitrite decomposed at pH 7.4 in phosphate/DTPA without bicarbonate was 84.3 ± 2.5 μM and is due to the sum of nitrite contaminating the peroxynitrite solution (unreacted nitrite) plus nitrite formed by the decomposition of peroxynitrite through the NO_2^-/O_2 pathway. The concentration of nitrite contaminating our peroxynitrite preparation was 49.4 ± 0.7%, as estimated by peroxynitrite isomerization at pH 4.0 when the NO_2^-/O_2 pathway is inhibited (*17*); thus, 100 μM peroxynitrite produced 84.3 − 49.4 = 34.9 μM nitrite derived from the NO_2^-/O_2 pathway and 65.1 μM nitrate derived from proton-catalyzed isomerization. The amount of contaminating nitrite was subtracted in Figure 4, which shows only the nitrite yield derived from peroxynitrite decomposition. The addition of metHb almost completely inhibited the formation of nitrite and, concomitantly, increased the nitrate yield (94−97 μM in excess of metHb). Figure 4A shows, in addition, that the decrease in nitrite yield was only minimally affected by the binding of nitrite to metHb as demonstrated by experiments with decomposed peroxynitrite. Moreover, CN-metHb and CO-Hb showed a significantly reduced inhibition of the nitrite yield (Figure 4A), indicating heme participation in the transformation of peroxynitrite to nitrate.

In samples containing 25 mM bicarbonate (Figure 4B), the yield of nitrite from 100 μM peroxynitrite was, as expected, decreased by the isomerase activity of CO_2 (about 18.2 μM nitrite[3] and 81.8 μM nitrate), but metHb was unable to modify the nitrite yield.

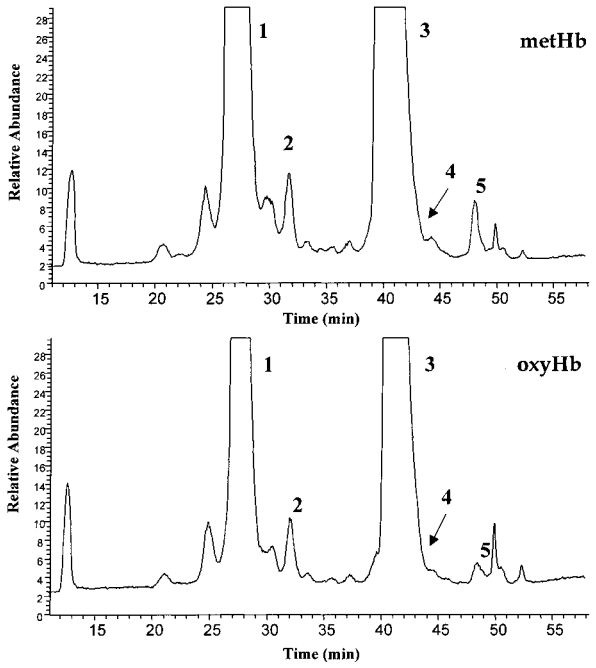
Cyt c^{+3} did not modify the NO_2^-/NO_3^- ratio either with or without bicarbonate (data not shown), supporting the hypothesis that peroxynitrite does not interact preferentially with Cyt c ferric heme.

The shift of peroxynitrite end products from nitrite to nitrate suggests that in the absence of bicarbonate metHb may act as a "peroxynitrite isomerase". The isomerase activity of metHb further supports the hypothesis of a bimolecular interaction between ferric heme and peroxynitrite, but does not prove whether reactive intermediates are involved.

Characterization by MS of Globin Residues Modified by Peroxynitrite. We analyzed the modifications induced by peroxynitrite (1 mM) to Hb (1 mM) in the presence of 25 mM bicarbonate using MS in combination with enzymatic hydrolysis. In all the experiments, we added 500 μM ascorbic acid to the reaction mixture 60 s after the addition of peroxynitrite and before LC/ES/MS analysis. It has previously been shown (*23*) that the addition of ascorbate does not affect the intensity of nitrated globin species, but avoids the post-addition modifications that occur under the denaturating conditions employed for MS. Figure 5 shows the total ion current profiles obtained for metHb and oxyHb after treatment with peroxynitrite. The yields of total nitration in $\alpha+\beta$ globin, estimated as percentages of the respective unmodified subunits, were 7.9% and 7.6% for oxyHb and metHb, respectively. To identify modified amino acids, nitrated species were purified by HPLC and then subjected to hydrolysis with pepsin. Peptide mixtures were then separated by HPLC and nitrated peptides easily identified by the characteristic absorption at 360 nm. Modified tyrosine residues were localized by ES/MS/MS analysis of the nitrated peptides.

Structural characterization showed a similar but not identical nitration pattern for oxyHb and metHb. Nitration was observed in Tyr 42, Tyr 140, and Tyr 24 of the α chain and in Tyr 130 of the β chain. Fraction 5 in Figure 5 contained both nitrated αTyr 140 and αTyr 24 and was also

[3] NO_2^- is due to $^{\bullet}NO_2$ hydrolysis formed through the different pathways discussed by Coddington et al. (*17*).



Fraction	Measured MW (Da) Hemoglobin	Interpretation	Target Residues
1	15866.8±0.5	β	-
2	15911.4±0.5	Mononitrated β chain	Tyr130
3	15126.6±0.4	α	-
4	15170.9±0.6	Mononitrated α chain	Tyr42
5	15171.2±0.7	Mononitrated α chain	Tyr140 + Tyr24

FIGURE 5: Total ion current profiles of peroxynitrite/CO_2-treated metHb and oxyHb. LC/ES/MS analysis of reaction products of 1 mM peroxynitrite with equimolar amounts of metHb (upper panel) or oxyHb (lower panel) in phosphate/DTPA/25 mM bicarbonate, pH 7.4. See Results for details.

the most evident difference between oxyHb and metHb. Previously, this fraction was assigned to nitrated αTyr 24 (*23*), but the use of on-line LC/ES/MS/MS in this study, at difference with the previous off-line one, revealed that αTyr 140 was indeed nitrated to an extent remarkably higher than αTyr 24 (note the different ordinate value in Figure 6). Interestingly, Figure 6 shows that nitration of αTyr 140 was low in oxyHb and about 3.5 times higher in metHb. This result may be correlated with the ability of peroxynitrite/CO_2 to interact with the ferrous but not with the ferric heme of Hb. To test this hypothesis, we investigated the nitration pattern of CO-Hb. Poisoning with CO would be expected to inhibit the interaction of peroxides with heme and to affect nitration if heme is somehow involved. This was indeed the case, and CO-Hb showed an increase in the nitration of αTyr 140 to a level comparable to that of metHb (Figure 6), supporting the hypothesis that oxyHb heme may have a protective role. The oxyHb heme also protected αTyr 24 from nitration, which was significantly lower in oxyHb than in metHb and CO-Hb (Figure 6).

The fraction containing nitrated αTyr 42 is almost superimposed on the unmodified α chain (see the arrow in Figure 5), making quantitative analysis of this residue more critical. However, careful comparison of this fraction in oxyHb and metHb revealed a difference worthy of note. In contrast to the α 140 and α 24 tyrosines, the αTyr 42 was

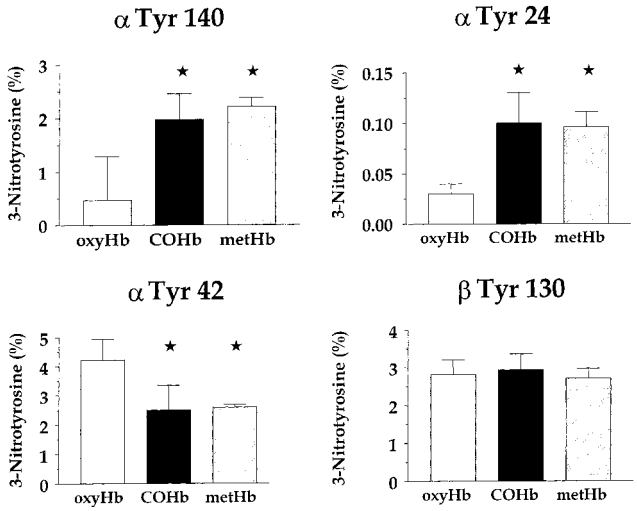


FIGURE 6: Comparison of nitrotyrosine in oxyHb, in CO-Hb, and in metHb after treatment with peroxynitrite/CO_2. Peroxynitrite (1 mM) was added to equimolar amounts of oxyHb (white bars), CO-Hb (black bars), or metHb (gray bars) in phosphate/DTPA/25 mM bicarbonate, pH 7.4. Relative yields of nitrated globins were calculated as percentages of the area value associated with extracted ions for modified α and *β* chains compared with the corresponding unmodified globins. * = $P \leq 0.05$ compared with oxyHb.

more nitrated (+40%) in oxyHb than in metHb or CO-Hb (Figure 6), suggesting a role of heme in the nitration of this residue.

The total nitration of the *β* chain was less than that of the α chain and localized in Tyr 130. The amount of *β*Tyr 130 nitration was similar in oxyHb, metHb, and CO-Hb (Figure 6), indicating that nitration of the *β* chain was apparently unaffected by the heme/peroxynitrite interaction. However, we cannot rule out that different mechanisms may be involved in the nitration of the *β* chain, although producing comparable nitration levels.

The omission of bicarbonate decreased the amount of tyrosine nitration in all tyrosine residues (mean decrease 29%, range 15−38%) without significantly affecting the nitration pattern. This result was also obtained with oxyHb (mean decrease 28%, range 6−33%). Moreover, it should be borne in mind that even if bicarbonate is not added intentionally, unavoidable contamination by atmospheric CO_2 may also contribute to tyrosine nitration.

DISCUSSION

Previous studies (*18, 20, 23*) have shown that oxyHb reacts bimolecularly with peroxynitrite, and forms the oxidant species ferrylHb, but protects cellular and extracellular targets from peroxynitrite-mediated nitration. We have now extended these studies to metHb.

Mechanism of Peroxynitrite/metHb Interaction. Although visible spectra of metHb were not appreciably affected by the addition of peroxynitrite, our data suggest that ferric heme can indeed react bimolecularly with this peroxide. This conclusion is based on two indirect observations. First, metHb was able to prevent the peroxynitrite-dependent nitration of a target dipeptide, Ala-Tyr, and, second, it acted as a peroxynitrite isomerase, favoring the transformation of peroxynitrite to nitrate. Both of these activities (scavenging and isomerization) were heme-dependent because they were strongly inhibited in CN-metHb. The inhibition afforded by

cyanide may be explained by its hindering the access of peroxynitrite to the catalytic iron center. After submission of this paper, Bourassa et al. (*50*) published that peroxynitrite reacts preferentially with the iron center of metMb and catalyzed peroxynitrite isomerization to nitrate; thus, present results obtained with metHb further support the hypothesis that these hemoproteins may act as peroxynitrite isomerases. From the residual 10% nitrite, Bourassa et al. (*50*) suggested that also a minor pathway exists that leads to $^{\bullet}NO_2$, accounting for 20% of peroxynitrite decay. We found a low level of residual nitrite when metHb was in molar excess to peroxynitrite (3−6%, see Figure 4), suggesting that the most relevant pathway of peroxynitrite decay is to nitrate, although a minor decay to $^{\bullet}NO_2$ cannot be completely ruled out. On the other hand, a somehow different reactivity of metMb is suggested by the faster second-order rate constants for peroxynitrite (1×10^4 and $1700 \times M^{-1}\ s^{-1}$ for metMb and metHb, respectively) (*50, 51*). Herold et al. (*52*) reported that mutants of distal His in metMb increase the reactivity for peroxynitrite, and, further, it has been suggested (*50*) that some double mutants of metMb not only decompose peroxynitrite at accelerated rates but are also extensively nitrated.

Although SH groups are known targets of peroxynitrite, protection of Ala-Tyr nitration by metHb was SH-independent, because cysteine alkylation did not modify metHb scavenging properties (D. Pietraforte and M. Minetti, unpublished results). Cyt c^{+3} did not provide efficient protection for Ala-Tyr and did not favor the transformation to nitrate, confirming the hypothesis (*48*) that the peroxynitrite reaction is not directed selectively toward its iron center.

The bimolecular peroxynitrite/metHb reaction we postulate and the lack of spectral changes imply that metHb is quickly regenerated without the requirement of exogenous reductants. The regeneration and isomerization of peroxynitrite to nitrate suggest that metHb can act as a peroxynitrite isomerase in a manner similar to that proposed for Fe porphyrins and metMb (*31, 50*). Although the mechanism of the peroxynitrite/porphyrin interaction is complex and a consensus has not yet been reached, it is generally accepted that the reaction proceeds through the formation of ferryl or perferryl species. In the case of Hb, perferryl heme is highly unstable and, further, may be rapidly bleached by the reaction products. When peroxynitrite acts bimolecularly, it is often a two-electron oxidant (*17*), and the reaction is expected to form nitrite besides perferryl heme (reaction 2). A fast bleaching of perferryl heme may be performed by nitrite, a known peroxidase substrate, leading to the formation of nitrate and metHb as end products.

However, other possibilities should be considered. Peroxynitrite itself may be the species responsible for bleaching ferrylHb (*24*). But this pathway, which would be expected to be more important when peroxynitrite is in excess, would yield a peroxynitrite radical, which would either decompose to $^{\bullet}NO$ and O_2 or react with itself. In either case, the final product would be NO_2^- and O_2, which is not compatible with our results.

A further possibility, that is compatible with our results, is the proposal (*24, 31, 50*) that peroxynitrite and ferric heme can form a complex that could be viewed as a radical pair between $^{\bullet}NO_2$ and Fe^{IV}═O−globin, leading to ferric iron and nitrate as end products (reaction 4).

$$Fe^{III}-\text{globin} + ONOO^- \rightarrow [^{\bullet}NO_2 \cdots Fe^{IV}{=}O-\text{globin}] \rightarrow Fe^{III}-\text{globin} + NO_2^- \quad (4)$$

The radical pair hypothesis is fairly common in peroxynitrite chemistry [protonation or addition of other Lewis acids, such as CO_2, or complexation by metal ions are all processes favoring the isomerization of peroxynitrite to nitrate through a caged radical pair mechanism (see, for example, references *16, 17*)] and can explain both the lack of spectral changes and isomerization to nitrate. Whatever the intermediate, the first event is the formation of a complex between peroxynitrite and metHb. Interestingly, such a complex has been hypothesized as a result of the reaction of oxyHb with $^{\bullet}NO$ (*53, 54*). The $^{\bullet}NO$/oxyHb reaction leads to the formation of an intermediate, detected by stopped-flow spectroscopy at alkaline but not neutral or acidic pH, and assigned to a peroxynitrito/metHb complex. The complex is short-lived and quickly decays to metHb and nitrate without significant formation of nitrotyrosine, thus suggesting that peroxynitrite does not leave the heme pocket (*54*). Therefore, the mechanism proposed for the oxyHb/$^{\bullet}NO$ reaction is fully compatible with the results obtained in this study for preformed peroxynitrite added to metHb.

The addition of bicarbonate strongly decreased both peroxynitrite scavenging and the isomerization activity of metHb, suggesting that CO_2 inhibits the postulated metHb/peroxynitrite bimolecular reaction by competing for peroxynitrite. The apparent second-order rate constant of the metHb/peroxynitrite reaction has been determined to be $1700\ M^{-1}\ s^{-1}$ at 37 °C and pH 7.4 (*51*), and this can explain the inhibition afforded by bicarbonate, since CO_2 reacts faster with peroxynitrite ($5.8 \times 10^4\ M^{-1}\ s^{-1}$) (*12*). Under these conditions, oxidation is not selectively directed toward the iron center while in the absence of bicarbonate a peroxynitrito/metHb complex can be formed. The rate of formation of this complex is, however, $\sim 5 \times 10^4$ times slower than that of $^{\bullet}NO$/oxyHb [1.7×10^3 and $8.9 \times 10^7\ M^{-1}\ s^{-1}$, respectively (*51, 54*)]. A slower build-up coupled with a fast decay of the complex presumably prevents the accumulation of the intermediate(s) in sufficient quantities to be observed by visible spectroscopy.

Tyrosyl Radical and Nitrotyrosine in the Peroxynitrite/metHb Interaction. Peroxynitrite was able to induce the formation of a long-lived tyrosyl radical(s) in both oxyHb and metHb. A similar long-lived radical, identified as a tyrosyl radical by MNP trapping, has been detected in metHb treated with H_2O_2 (*20, 44*) and is believed to be derived from perferryl or ferryl heme. However, peroxynitrite is a stronger one-electron oxidant than H_2O_2 and can induce the formation of tyrosyl radicals independently of the reaction with heme (*46*). It is, therefore, difficult to discriminate between radicals derived from the heme reaction and those derived from the one-electron oxidation pathway of peroxynitrite. In addition, it should be considered that tyrosine residues may function as a sink of other oxidizable residues (ferryl species, cysteines, and tryptophans). We conclude that the formation of long-lived tyrosyl radical(s) in the peroxynitrite/Hb interaction is not proof of a reaction with heme, and only assignment of the radical(s) to different globin residues will help to clarify the mechanisms involved in tyrosyl radical formation.

Collectively, MS results permit some general considerations. First, the heme/peroxynitrite bimolecular reaction in Hb modulates the nitration pattern without affecting the total amount of tyrosine nitration. Second, the amount of β chain nitration was (i) lower than that of the α chain, (ii) limited to tyrosine Tyr 130, and (iii) unaffected by the heme/peroxynitrite interaction. The reason for this lower reactivity of the β chain is currently unknown.

Despite the comparable nitration levels of oxyHb, metHb, and CO-Hb by peroxynitrite/CO_2, we observed that the heme affected the relative amount of nitration of all tyrosine residues of the α chain.

In the α chain, the tyrosine nearest to the heme is αTyr 42 (shortest distance ~4 Å, compatible with van der Waals contacts), and, for this reason, we previously hypothesized (*23*) that this residue could be coupled with the ferryl heme and oxidized to a tyrosyl radical through an electron-transfer process. Nitration may follow from the reaction of a tyrosyl radical with $^{\bullet}NO_2$. The hypothesis of a relationship between αTyr 42 nitration and ferryl heme formation seems to be confirmed by the present results. In fact, when the bimolecular interaction of heme with peroxynitrite was inhibited (CO-Hb and metHb), the amount of nitration of αTyr 42 was halved. For this tyrosine, it could be hypothesized that in oxyHb formation of a relatively stable ferryl heme, as demonstrated spectroscopically (*18, 23*), favors its self-nitration. Therefore, the nitration mechanism of αTyr 42 in oxyHb may involve a heme-dependent process similar to that of Tyr 334 in $P450_{BM-3}$ (*26*). The self-nitration of oxyHb was, however, compensated by a decreased nitration of the other two tyrosines of the α chain. In fact, the nitration of αTyr 140, the most exposed tyrosine residue of the α chain, and of αTyr 24, a buried residue with an external hydroxyl group (*55*), was low in oxyHb and higher in metHb and CO-Hb, balancing the effect on αTyr 42. In this case, the heme/peroxynitrite interaction protects tyrosine from nitration rather than promoting it. Compared to metHb and CO-Hb, the decrease in nitration of αTyr 24 and αTyr 42 in oxyHb was high (about 70−80%), as would be expected by heme-dependent scavenging of peroxynitrite. In summary, the interaction of heme with peroxynitrite protects the two tyrosines most exposed to the solvent, but promotes nitration of the tyrosine nearest to the heme, thus leading to comparable total nitration levels in oxyHb, metHb, and CO-Hb.

Without bicarbonate, the amount of nitration in metHb decreased in all tyrosines, and no relative changes of nitration attributable to the heme reaction were observed. The instability of the intermediate(s) formed in the metHb/peroxynitrite reaction may underlie the lack of effects on the tyrosine nitration pattern. The residual nitration observed in peroxynitrite-treated metHb without bicarbonate may be due to peroxynitrite escaping the heme reaction. We admit, however, that these considerations are largely speculative because both the initial site(s) of oxidation and the patterns of electron transfer in Hb are unknown. Comparison with other peroxides and further studies with different hemoproteins will be useful to test these hypotheses.

Biological Implications. Our results show that in the presence of physiological concentrations of bicarbonate, oxyHb and Cyt c^{+2} can protect biological targets against peroxynitrite-mediated nitration and act as *preventive antioxidants* because they eliminate the species involved in the initiation of free radical reactions. This confirms and extends the hypothesis that some hemoproteins are among the major physiological scavengers of peroxynitrite in tissues (*23, 32*). We found no evidence for catalysis of Ala-Tyr nitration with or without bicarbonate and, regardless, the heme valence for both Hb and Cyt c. It should be pointed out, however, that this conclusion cannot be extended to all hemoproteins, because it has been shown that some thiolate/ferric hemoproteins may indeed catalyze the nitration of tyrosine residues (*24, 25*). Because of the inhibitory role of bicarbonate in some bimolecular reactions of peroxynitrite, it is unclear if these ferric hemoproteins can also be nitration catalysts in the presence of physiological concentrations of CO_2. MetHb was an efficient scavenger of peroxynitrite in the absence of bicarbonate, while Cyt c^{+3} was not, but bearing in mind the high CO_2 concentration in tissues, it could be concluded that also metHb is not a significant peroxynitrite scavenger. Any peroxynitrite formed in vivo would be expected to be intercepted by other biological targets, including oxyHb and CO_2, before its interaction with metHb.

Nevertheless, the peroxynitrite/metHb chemistry is probably of biological relevance in $^{\bullet}NO$ detoxification mediated by oxyHb as discussed above. In agreement with results obtained with metMb (*50*), we have shown in this study that preformed peroxynitrite added to metHb can catalyze peroxynitrite isomerization to nitrate, thus preventing nitration of other targets. Our data bridge the gap between the mechanism proposed by Herold et al. (*53, 54*) and the inconsistency derived from the postulated unreactivity of ferric Hb with peroxynitrite (*18, 20, 34*−*36*).

Notably, a strong correlation between metHb and plasma nitrate was observed in human volunteers during $^{\bullet}NO$ inhalation (*56*), suggesting that in vivo the predominant pathway of $^{\bullet}NO$ inactivation is metabolic oxidation to nitrate. The metHb-mediated isomerization of peroxynitrite may therefore be an important $^{\bullet}NO$ detoxification mechanism, particularly under conditions of $^{\bullet}NO$ overproduction. Collectively this and other studies confirm that hemoproteins are among the major targets of $^{\bullet}NO$ and $^{\bullet}NO$-related oxidants. Although regulatory functions are of great importance, a secondary role of some hemoproteins seems to be the inactivation of the nitrogen oxides $^{\bullet}NO$, NO_2^-, $^{\bullet}NO_2$, and $ONOO^-$.

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