Metmyoglobin and Methemoglobin Catalyze the Isomerization of Peroxynitrite to Nitrate†

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ABSTRACT: Hemoproteins, in particular, myoglobin and hemoglobin, are among the major targets of peroxynitrite in vivo. The oxygenated forms of these proteins are oxidized by peroxynitrite to their corresponding iron(III) forms (metMb and metHb). This reaction has previously been shown to proceed via the corresponding oxoiron(IV) forms of the proteins. In this paper, we have conclusively shown that metMb and metHb catalyze the isomerization of peroxynitrite to nitrate. The catalytic rate constants were determined by stopped-flow spectroscopy in the presence and absence of 1.2 mM CO_2 at 20 and 37 °C. The values obtained for metMb and metHb, with no added CO_2 at pH 7.0 and 20 °C, are $(7.7 \pm 0.1) \times 10^4$ and $(3.9 \pm 0.2) \times 10^4$ M^{-1} s^{-1}, respectively. The pH-dependence of the catalytic rate constants indicates that HOONO is the species that reacts with the iron(III) center of the proteins. In the presence of 1.2 mM CO_2, metMb and metHb also accelerate the decay of peroxynitrite in a concentration-dependent way. However, experiments carried out at pH 8.3 in the presence of 10 mM CO_2 suggest that $ONOOCO_2^-$, the species generated from the reaction of $ONOO^-$ with CO_2, does not react with the iron(III) center of Mb and Hb. Finally, we showed that different forms of Mb and Hb protect free tyrosine from peroxynitrite-mediated nitration. The order of efficiency is metMbCN < apoMb < metHb < metMb < ferrylMb < oxyHb < deoxyHb < oxyMb. Taken together, our data show that myoglobin is always a better scavenger than hemoglobin. Moreover, the globin offers very little protection, as the heme-free (apoMb) and heme-blocked (metMbCN) forms only partly prevent nitration of free tyrosine.

Peroxynitrite,[1] the strong oxidizing and nitrating agent generated from the diffusion-limited reaction of $NO^\bullet$ with $O_2^{\bullet-}$ (*1*), is a molecule of considerable biological interest (*2*). A variety of compounds present in biological systems have been shown to be modified by peroxynitrite (for a recent review, see ref *3*). Targets for peroxynitrite-mediated oxidation and/or nitration are amino acids such as tyrosine (*4*), tryptophan (*5*, *6*), cysteine (*7*), and methionine (*8*); nucleic acids (*9*); and membrane lipids (*10*). Moreover, the selenocysteine-containing glutathione peroxidase has been reported to catalytically reduce peroxynitrite to nitrite by using glutathione (*11*).

Metal-containing proteins, in particular, hemoproteins, can also be modified by peroxynitrite. Among others, peroxynitrite inhibits the cytosolic iron-thiolate protein aconitase (*12*, *13*), Mn-superoxide dismutase (*14*), and induces the release of zinc and the oxidation of the thiol moiety of zinc-finger-containing proteins (*15*). Amid hemoproteins that have been reported to react with peroxynitrite are peroxidases (*16*), catalase (*16*), cytochrome *c* (*17*), cytochrome P450 (*18*), nitric oxide synthase (*19*), and cytochrome *c* oxidase (*20*). Moreover, synthetic iron(III)- and manganese(III)-metalloporphyrins have been shown to catalyze the isomerization of peroxynitrite to nitrate in vitro (*21*, *22*) and to be cytoprotective against peroxynitrite in vivo (*23*).

Under physiological conditions, one of the main targets of peroxynitrite is CO_2, present in millimolar concentration in most tissues. Reaction of $ONOO^-$ with CO_2 leads to $ONOOCO_2^-$, a stronger nitrating agent than peroxynitrous acid.

Hemoglobin (Hb)[2] and myoglobin (Mb), by large the hemoproteins present in the highest amount in the human body, also react with peroxynitrite. We have shown that, in the presence of an excess of peroxynitrite, oxyMb and oxyHb are oxidized to their corresponding iron(III) forms (metMb and metHb) via the formation of ferrylMb and ferrylHb, respectively (*24*, *25*). In addition, we have recently proposed that, since the proteins are nitrated only to a very small extent by an excess of peroxynitrite, this reaction represents an efficient scavenging pathway for peroxynitrite (*26*). Here,

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[1] The recommended IUPAC nomenclature for the peroxynitrite anion is oxoperoxonitrate(1−) and for peroxynitrous acid, it is 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).

[2] Abbreviations: Hb, hemoglobin; oxyHb, $HbFeO_2$, oxyhemoglobin; metHb, iron(III)hemoglobin; ferrylHb, $HbFe^{IV}{=}O$, oxoiron(IV)hemoglobin; deoxyHb, $HbFe^{II}$, deoxyhemoglobin; HPLC, high-pressure liquid chromatography; Mb, myoglobin; deoxyMb, $MbFe^{II}$, deoxymyoglobin; oxyMb, $MbFeO_2$, oxymyoglobin; metMb, iron(III)myoglobin; ferrylMb, $MbFe^{IV}{=}O$, oxoiron(IV)myoglobin; metMbCN, cyanide bound metMb; NO_2-Tyr, 3-nitrotyrosine; TFA, trifluoroacetic acid; Tyr, tyrosine; BSA, bovine serum albumin.

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we report kinetic studies of the reaction of peroxynitrite with metMb and metHb in the presence and absence of added CO_2. Analysis of the nitrogen-containing products suggests that the iron(III) forms of these proteins catalyze the isomerization of peroxynitrite to nitrate. Comparison with the heme-free (apoMb) and the heme-blocked (metMbCN) forms of Mb indicate that the reaction is mediated by the heme center, despite no absorbance changes being detected in the UV−vis spectrum of metMb upon mixing with peroxynitrite. In the presence of physiological amounts of CO_2 (1.1−1.2 mM), metMb and metHb catalyze the decay of peroxynitrite by competing with CO_2 for peroxynitrite. No direct reaction seems to take place between $ONOOCO_2^-$ and the iron(III) center of the proteins.

EXPERIMENTAL PROCEDURES

Chemicals. Potassium phosphate buffers were prepared from KH_2PO_4/K_2HPO_4 (Fluka) with deionized Milli-Q water. Sodium nitrite, sodium nitrate, sodium dithionite, potassium hexacyanoferrate(III), 2-butanone, sulfanilamide, *N*-(1-naphthyl)-ethylenediaminedihydrochloride, ammonium sulfamate, trifluoroacetic acid, hydrogen peroxide, and acetonitrile (99.8%) were obtained from Fluka. Sodium bicarbonate was purchased from Merck. Bovine serum albumin (fraction V) and L-tyrosine were obtained from Fluka. Catalase (bovine liver, 17 000 units/mg of protein) was purchased from Sigma. Nitrogen monoxide was obtained from Linde and passed through a column of NaOH pellets to remove higher nitrogen oxides before use.

Peroxynitrite, Carbon Dioxide, and Protein Solutions. Peroxynitrite was synthesized from KO_2 and nitrogen monoxide according to ref *27* and stored in small aliquots at −80 °C. The peroxynitrite solutions contained variable amounts of nitrite (maximally 50% relative to the peroxynitrite concentration) and no hydrogen peroxide. The stock solution was diluted with 0.01 M NaOH, and the concentration of peroxynitrite was determined spectrophotometrically prior to each experiment by measuring the absorbance at 302 nm ($\epsilon_{302} = 1705\ M^{-1}\ cm^{-1}$, ref *28*).

Experiments in the presence of CO_2 were carried out by adding to the protein solutions the required amount of a freshly prepared 0.5 or 1 M sodium bicarbonate solution. The values for the constant of the hydration−dehydration equilibrium $CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^-$ were derived from ref *29* by taking into consideration the ionic strength of the solutions. After the addition of bicarbonate, the protein solutions were allowed to equilibrate for at least 5 or 3 min at 20 or 37 °C, respectively. For the experiments carried out in the absence of CO_2, the buffers and the 0.01 M NaOH solutions were thoroughly degassed.

Horse heart myoglobin was purchased from Sigma. Solutions of oxyMb, metMb, ferrylMb, and the cyanide-bound form of metMb (metMbCN) were prepared as described previously (*26*, *30*). ApoMb was prepared according to the method of Teale (*26*, *31*). 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. (Research Triangle Park, NC). MetHb and deoxyHb were prepared as reported earlier (*32*). The concentration of Hb was always expressed per heme. Absorption spectra were collected on a UVIKON 820 spectrophotometer.

Kinetic Studies. The decay rates of peroxynitrite in the presence of different forms of Mb and Hb were studied with an Applied Photophysics SX18MV-R single-wavelength stopped-flow instrument. The width of the cell in the spectrophotometer is 1 cm, and the mixing time of the instrument is about 2 ms. The kinetic traces were collected at 302 nm, and the data were analyzed with the SX18MV-R operating software or with Kaleidagraph, version 3.52. The results of the fits of the traces (averages of at least 10 single traces) from at least three experiments were averaged to obtain each observed rate constant, given with the corresponding standard deviation.

For the experiments at pH 7.0, the protein solutions of the required concentrations were prepared under aerobic conditions in 0.1 M phosphate buffer pH 6.8 (in the absence of CO_2) or pH 6.6 (in the presence of CO_2). Peroxynitrite solutions were prepared by diluting the stock solution immediately before use with 0.01 M NaOH to achieve the required concentration. The experiments were carried out either at 20 or at 37 °C. The pH was always measured at the end of the reactions. The protein solutions for the experiments at pH 6.5 and 8.0 were prepared analogously in 0.1 M phosphate buffer pH 6.3 and 7.5, respectively. The protein solutions for the experiments in the presence of 10 mM CO_2 at pH 7.3 and 8.3 were both prepared in 0.1 M phosphate buffer pH 6.5 containing 71.5 mM sodium bicarbonate. The two different pH values were obtained by varying the concentration of the NaOH solution with which the peroxynitrite stock solution was diluted. Specifically, 0.01 M NaOH was used to reach a final pH of 7.3 and 0.05 M NaOH to obtain a final pH of 8.3. In some cases, the protein/bicarbonate solution was prepared and kept in a gastight SampleLock Hamilton syringe to avoid loss of CO_2. This additional precaution proved not to be necessary.

Nitrite and Nitrate Analysis. Product analysis was carried out as described previously (*33*) by anion chromatography with conductivity detection with a Metrohm instrument (IC Separation Center 733, ICDetector 732 and IC pump 709) equipped with an Anion SUPER-SEP (6.1009.000) column and an Anion SUPER-SEP (6.1009.010) precolumn. Calibration curves were obtained by measuring 5−10 standard sodium nitrite and sodium nitrate solutions in 5 mM phosphate buffer. The samples were prepared by mixing 500 μL of the apoMb, metMb, and metHb solutions (50−1500 μM in 0.1 M phosphate buffer pH 6.8 or in 0.1 M phosphate buffer pH 6.6 containing 22 mM sodium bicarbonate) at room temperature with 500 μL of an ice-cooled peroxynitrite solution (200 μM in 0.01 M NaOH) while vortexing. The reaction mixture was diluted 1:10 with water and analyzed within ca. 5 min. At least two analyses of three separate experiments were carried out for each protein. Nitrite and nitrate contamination in peroxynitrite was determined by adding to 500 μL of an ice-cooled 50 mM phosphoric acid solution 500 μL of the same peroxynitrite solution used for the reactions. Under these conditions, peroxynitrite exclusively isomerizes to nitrate. Thus, ion chromatographic analysis after dilution of the samples with water (1:10) gives directly the amount of nitrite present in the peroxynitrite solutions. Nitrate contamination was determined by subtracting from the amount of nitrate found in this experiment the peroxynitrite concentration measured spectrophotometrically. Usual nitrite and nitrate contaminations were in the range

of 20−50 and 0−10% of the peroxynitrite concentration, respectively.

Reverse Phase 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% TFA in H_2O and solvent B was 0.07% TFA in acetonitrile. Nitrotyrosine 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. NO_2-Tyr was quantified by measuring a calibration curve of five to 10 nitrotyrosine standard solutions.

Analysis of the Free Nitrotyrosine Content Generated by the Reaction of Peroxynitrite with oxyMb, metMb, apoMb, metMbCN, ferrylMb, oxyHb, deoxyHb, and metHb in the Presence of Added Free Tyrosine. The reactions were carried out at room temperature by adding 100 μL of an ice-cooled peroxynitrite solution (2 mM in 0.01 M NaOH) to 900 μL of a solution containing tyrosine (112 μM) and different concentrations of oxyMb, metMb, apoMb, metMbCN, metHb, and oxyHb (0−55.5 μM in 0.05 M phosphate buffer pH 7.5), in the absence and presence of 1.1 mM CO_2. For the reactions with deoxyHb, 1.8 mL of an oxyHb solution (16.6, 27.8, or 55.5 μM in 0.05 M phosphate buffer pH 7.5) containing tyrosine (112 μM) was placed in a sealable cell for anaerobic applications and thoroughly degassed until the recorded UV−vis spectrum indicated the complete formation of deoxyHb. Then, 200 μL of an ice-cooled degassed peroxynitrite solution (2 mM in 0.01 M NaOH) was added directly in the cell by using a gastight SampleLock Hamilton syringe. For the reactions with ferrylMb, 1.765 mL of a metMb solution (5.6−56.6 μM in 0.05 M phosphate buffer pH 7.5) was allowed to react with 10 equiv of H_2O_2 (10 μL of a 1−10 mM solution) for 6 min. Then, excess H_2O_2 was destroyed by the addition of 5 μL of a solution of catalase in water (approximately 1 mg/mL). After 1 min, 20 μL of a 10 mM tyrosine solution (basic) was added, followed by the addition of 200 μL of an ice-cooled peroxynitrite solution (2 mM in 0.01 M NaOH). All the samples were analyzed by HPLC as described above.

Analysis of the Nitrotyrosine Content in BSA, Tyrosine, apoMb, oxyMb, metMb, oxyHb, and metHb after Reaction with Variable Amounts of Peroxynitrite. The reaction of peroxynitrite with BSA, apoMb, oxyMb, metMb, oxyHb, and metHb was carried out at 37 °C as described previously (*26*). In brief, 20 μL of an ice-cooled peroxynitrite solution (different concentrations in 0.01 M NaOH) was added as a bolus while vortexing to 180 μL of a protein solution (112 μM in 0.1 M phosphate buffer, pH 7.0) kept in a thermostat at 37 °C. After ca. 30 min, nitrite was removed by adding ca. 200 μL of an ammonium sulfamate solution (100 mM in 0.5 M HCl) and subjected to acid hydrolysis as described previously (*26*). Finally, the samples were analyzed by HPLC as described above. For comparison, an analogous experiment was carried out by adding a peroxynitrite solution to a tyrosine solution under identical experimental conditions.

Statistics. The experiments reported in this article were carried out 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 deviation.

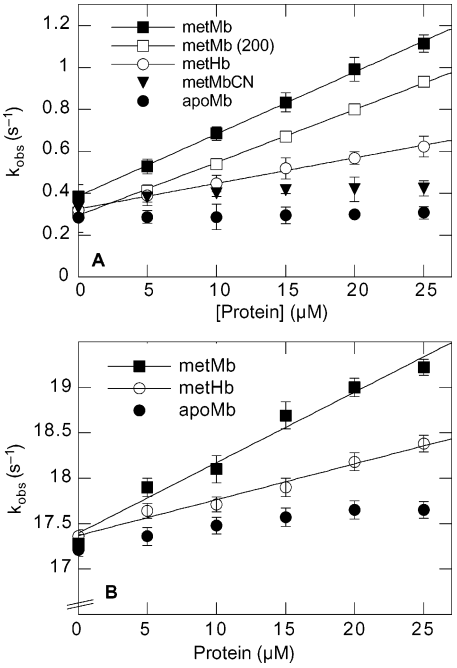


FIGURE 1: Plots of k_{obs} vs protein concentration (apoMb, metMb, CN, metMb, and metHb) for the protein-catalyzed decay of peroxynitrite (100 μM) in 0.05 M phosphate buffer pH 7.0 and 20 °C. metMb (200): metMb-catalyzed decay of 200 μM peroxynitrite. Data in the absence (A) and presence (B) of 1.2 mM CO_2. The second-order rate constants resulting from the linear fits depicted are given in Table 1.

Table 1: Summary of the Catalytic Rate Constants (in 10^4 M^{-1} s^{-1}) for the Protein-Mediated Decay of Peroxynitrite (100 μM) in 0.05 M Phosphate Buffer pH 7.0, at 20 and 37 °C

	no CO_2		1.2 mM CO_2	
protein	20 °C	37 °C	20 °C	37 °C
metMb	2.9 ± 0.1	8.8 ± 0.2	7.7 ± 0.1	26.5 ± 0.2
metHb[a]	1.2 ± 0.1	2.1 ± 0.1	3.9 ± 0.2	18.2 ± 0.1

[a] The rate constants for metHb are expressed per heme.

RESULTS

Kinetic Studies at 20 and 37 °C. The decay rate of peroxynitrite (100 μM) in the presence of the iron(III) forms of Mb and Hb was determined by stopped-flow spectroscopy at 20 °C and pH 7.0. The reactions were studied by following the absorbance changes at 302 nm, the characteristic absorbance maximum for peroxynitrite. All the measured traces could be fitted well to a single-exponential expression. As shown in Figure 1A, the observed peroxynitrite decay rates (k_{obs}) increased linearly with increasing metMb and metHb concentration. The values of k_{cat} (Table 1), obtained from the linear fits of the two plots, are in the order of magnitude of 10^4 M^{-1} s^{-1}, but metMb is a slightly more efficient catalyst than metHb.

For comparison, we determined the decay rates of peroxynitrite also in the presence of different amounts of the heme-free protein (apoMb) and a heme-blocked form (metMbCN). As depicted in Figure 1A, the values of the observed rate constants for the decay of peroxynitrite (100 μM) were nearly unchanged when the concentration of these two forms of Mb was varied in the range of 0−25 μM.

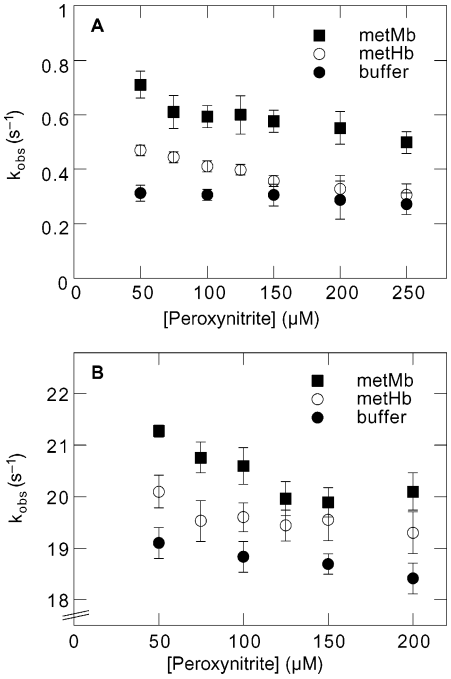


FIGURE 2: Plots of k_{obs} vs peroxynitrite concentration for the decay of peroxynitrite (at pH 7.0 and 20 °C) in the absence of added protein (buffer) and in the presence of 10 μM metMb or 10 μM metHb. Data in the absence (A) and presence (B) of 1.3 mM CO_2.

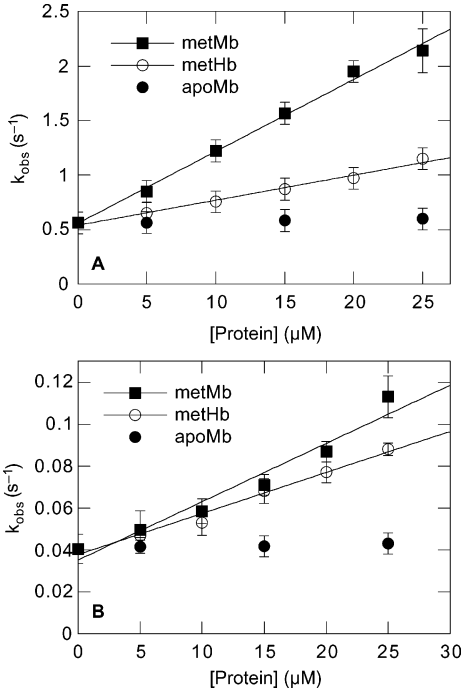


FIGURE 3: Plots of k_{obs} vs protein concentration (apoMb, metMb, and metHb) for the protein-catalyzed decay of peroxynitrite (100 μM) in 0.05 M phosphate buffer (A) pH 6.5 and (B) pH 8.0 (20 °C). The second-order rate constants resulting from the linear fits depicted are given in the text.

Because of the physiological relevance attributed to the reaction between carbon dioxide and peroxynitrite, we studied the protein-mediated decay of peroxynitrite also in the presence of 1.2 mM CO_2. As shown in Figure 1B and summarized in Table 1, metMb and metHb accelerated the decay of peroxynitrite (100 μM) in a concentration-dependent extent. In general, the values of k_{cat} were all approximately 2−3 times larger than those obtained in the absence of CO_2. Thus, also in the presence of CO_2, metMb was more efficient than metHb. The analogous experiment with apoMb showed that this protein form only slightly accelerated the decay of peroxynitrite.

The kinetics of the protein-catalyzed decay of peroxynitrite in the absence and presence of 1.2 mM CO_2 were studied also at 37 °C (Figure 1S), to better evaluate the physiological relevance of these reactions. As expected, at a higher temperature, the values for k_{cat} were all 2−4 times larger (Table 1), and metMb was still the most efficient catalyst, both in the absence and in the presence of CO_2.

To confirm the catalytic nature of the protein-mediated decay of peroxynitrite, we determined the observed rate constants for the disappearance of peroxynitrite in the presence of metMb and metHb over the peroxynitrite concentration range of 50−250 μM (at 20 °C and pH 7.0). The protein concentration was always kept constant (10 μM), and the reactions were carried out both in the absence and in the presence of CO_2. As shown in Figure 2, in both cases the values of the observed rate constants were nearly constant and only slightly decreased with increasing peroxynitrite concentration. In agreement with previous reports (*34*), a similar trend was observed also in the absence of the proteins. Indeed, at peroxynitrite concentrations higher than 50 μM and under neutral conditions, it has been proposed that an adduct is formed between the peroxynitrous acid and its deprotonated form (*34*, *35*). The decrease in the observed rate constant at higher peroxynitrite concentrations may suggest that either this adduct reacts at a lower rate with the heme or that it has to dissociate before it can react with the heme.

To find out whether the peroxynitrite concentration influences the value of k_{cat}, we measured the decay of 200 μM peroxynitrite in the presence of various amounts of metMb (0−50 μM) under similar conditions. As shown in Figure 1A (metMb 200), at each metMb concentration the observed rate constants (k_{obs}) were slightly lower than those obtained with 100 μM peroxynitrite (metMb). However, the values of k_{cat}, obtained from the linear fits of the two plots, were nearly identical: $(2.9 \pm 0.1) \times 10^4$ and $(2.5 \pm 0.1) \times 10^4$ M^{-1} s^{-1} for the reactions with 100 and 200 μM peroxynitrite, respectively.

pH-Dependence of the Catalytic Rate Constant in the Absence of CO_2. The decay rate of peroxynitrite (100 μM) in the presence of apoMb, metMb, and metHb was determined also at pH 6.5 and 8.0 (at 20 °C). As shown in Figure 3, in both cases apoMb did not influence the rate of peroxynitrite decay. At pH 6.5, the catalytic rate constants for the metMb- and metHb-mediated decay of peroxynitrite were significantly larger than those obtained at pH 7.0, that is $(6.6 \pm 0.3) \times 10^4$ and $(2.3 \pm 0.1) \times 10^4$ M^{-1} s^{-1}, respectively (Figure 3A). In contrast, at pH 8.0, metMb and metHb were significantly less effective (Figure 3B). Indeed, the values of the catalytic rate constants for the metMb- and metHb-mediated decay of peroxynitrite measured at pH 8.0 were $(0.27 \pm 0.03) \times 10^4$ and $(0.19 \pm 0.01) \times 10^4$ M^{-1} s^{-1}, respectively.

Peroxynitrite Decay in the Presence of a Large Excess of CO_2 (10 mM) and apoMb, metMb, or metHb. The influence of apoMb, metMb, and metHb on the decay rate of peroxynitrite (100 μM) was determined also in the presence

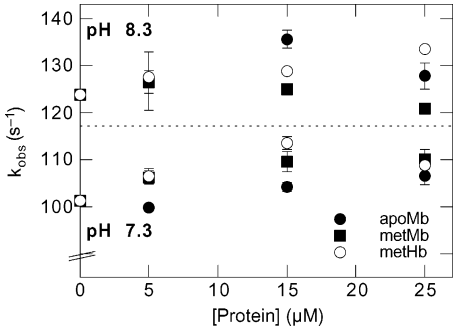


FIGURE 4: Plots of k_{obs} vs protein concentration (apoMb, metMb, and metHb) for the decay of peroxynitrite (100 μM) in 0.05 M phosphate buffer in the presence of 10 mM CO_2 (bottom) at pH 7.3 or (top) at pH 8.3 (20 °C). The second-order rate constants resulting from the linear fits depicted are given in the text.

Table 2: Product Distribution of Peroxynitrite (100 μM) Decay in the Presence of Different Concentrations of apoMb, metMb, and metHb (in 0.05 M Phosphate Buffer pH 7.0 and 20 °C), in the Absence and Presence of 1.1 mM CO_2[a]

[proteins] (μM)	no added CO_2			1.1 mM CO_2		
	NO_2^-	NO_3^-	total	NO_2^-	NO_3^-	total
no protein	34 ± 3	67 ± 2	101	17 ± 2	85 ± 3	102
apoMb 25	29 ± 3	65 ± 1	94	17 ± 3	81 ± 4	99
apoMb 50	26 ± 5	66 ± 2	92	16 ± 3	83 ± 4	99
apoMb 100	24 ± 3	68 ± 4	92	18 ± 1	83 ± 2	101
apoMb 200	20 ± 1	66 ± 3	86	12 ± 2	84 ± 2	96
metMb 25	20 ± 3	78 ± 4	98	17 ± 3	84 ± 3	101
metMb 50	16 ± 4	81 ± 2	97	14 ± 2	85 ± 2	99
metMb 100	13 ± 5	85 ± 3	98	9 ± 2	85 ± 2	94
metMb 200	8 ± 3	86 ± 2	94	7 ± 4	91 ± 3	98
metMb 500	4 ± 2	96 ± 3	100			
metMb 750	<1	99 ± 2	99			
metHb 25	24 ± 4	76 ± 4	100	16 ± 4	81 ± 4	97
metHb 50	19 ± 5	81 ± 4	100	12 ± 3	85 ± 2	97
metHb 100	15 ± 4	87 ± 4	103	6 ± 2	91 ± 3	97
metHb 200	8 ± 3	85 ± 4	93	3 ± 2	95 ± 2	98
metHb 500	5 ± 2	94 ± 2	99			
metHb 750	<1	97 ± 2	97			

[a] The data are expressed as percentage yields.

of a large excess of CO_2. Interestingly, with 10 mM CO_2, both at pH 7.3 and at pH 8.3, the rate of decay of peroxynitrite was not affected by the addition of increasing amounts of apoMb, metMb, or metHb (Figure 4).

Analysis of the Nitrogen Containing Products. The products of the protein-catalyzed decay of peroxynitrite were analyzed by ion chromatography. In agreement with previous reports (*35*), spontaneous decay of 100 μM peroxynitrite at pH 7.0 and 20 °C yielded 67 ± 2% nitrate and 34 ± 3% nitrite. In the presence of the iron(III) forms of Mb and Hb, the nitrite yield continuously decreased with increasing protein concentration (25−750 μM), and the nitrate yields increased to a comparable extent (Table 2). In the presence of 750 μM metMb or metHb, peroxynitrite isomerized nearly quantitatively to nitrate, and the amount of nitrite found corresponded to that already present in the peroxynitrite solution. Increased nitrate yields have recently been reported for the metHb-catalyzed decay of peroxynitrite (100 μM) at pH 7.4 (*36*) and for the metMb-mediated isomerization of peroxynitrite (500 μM) at pH 7.6 (*37*). ApoMb did not influence significantly the nitrate yields, but lowered the nitrite yields; thus, the total amount of nitrogen-containing products decreased with increasing protein concentration (Table 2). As previously shown (*26*), this observation

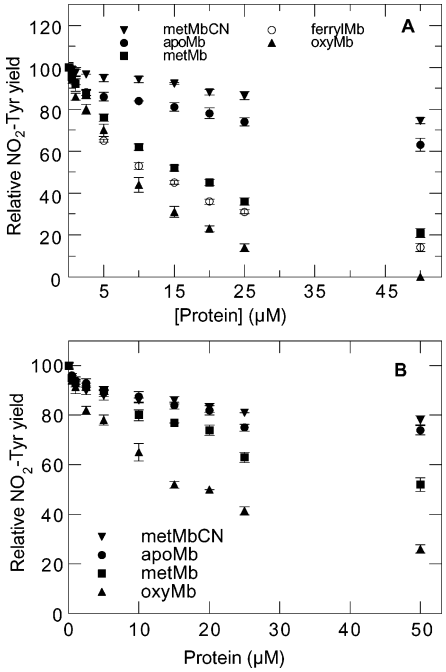


FIGURE 5: Relative yield of NO_2-Tyr formed from the reaction of peroxynitrite (200 μM) and tyrosine (100 μM), in the presence of different concentrations of oxyMb, metMb, apoMb, ferrylMb, and metMbCN in 0.05 M phosphate buffer pH 7.4, 20 °C. Experiments in the absence (A) and presence (B) of 1.1 mM CO_2. Relative yield = (yield with added protein/yield with no protein) × 100%.

suggests that under these conditions the protein is nitrated to a significant extent.

As expected, in the presence of 1.1 mM CO_2, the yield of nitrate generated from the decay of 100 μM peroxynitrite (at pH 7.0 and 20 °C) was significantly larger. In agreement with previous reports (*35*), we obtained 17 ± 2% nitrite and 85 ± 3% nitrate. Also in the presence of 1.1 mM CO_2, the addition of metMb and metHb led to a concentration-dependent increase of the nitrate yields (Table 2). In contrast, apoMb caused a slight decrease of the nitrite yields without influencing significantly the nitrate yields (Table 2). As pointed out above, this result is in agreement with our previous observation that apoMb is significantly nitrated under these conditions.

Protection Against Peroxynitrite-Mediated Nitration of Free Tyrosine. To investigate whether different forms of myoglobin can protect against peroxynitrite-mediated nitration, we determined the yield of 3-nitrotyrosine (NO_2-Tyr) formed from the reaction of peroxynitrite with free tyrosine in the presence of increasing quantities of the protein. For this purpose, we mixed 200 μM peroxynitrite with 100 μM tyrosine in the presence of increasing amounts of apoMb, oxyMb, metMb, ferrylMb, and metMbCN (in 0.05 M phosphate buffer, pH 7.4 and 20 °C). As shown in Figure 5A and summarized in Table 1S, oxyMb, ferrylMb, and metMb are rather efficient scavengers of peroxynitrite. In the presence of 50 μM of these proteins, the relative NO_2-Tyr yields (relative to the amount found in the absence of the proteins) were 0, 14 ± 2, and 21 ± 2%, respectively. In contrast, the addition of 50 μM heme-free apoMb and heme-blocked metMbCN lowered the relative NO_2-Tyr yields only to 63 ± 3 and 74 ± 1%, respectively.

To study the efficiency of the reduced form of the proteins under the same conditions, we chose to use deoxyHb as it

can be prepared in a much simpler way than deoxyMb, by thoroughly degassing an oxyHb solution. DeoxyHb was slightly more effective than oxyHb (Table S1). For instance, the addition of 50 μM deoxyHb or oxyHb led to relative NO_2-Tyr yields of 0 and 8 ± 3%, respectively. In agreement with our kinetic studies, oxyHb and metHb were found to be less efficient scavengers than the corresponding Mb forms. The addition of 50 μM metHb led to the formation of 27 ± 1% NO_2-Tyr, whereas only 21 ± 2% was formed in the presence of 50 μM metMb.

Analogous studies were carried out in the presence of CO_2. As previously reported (*38*), the addition of CO_2 leads to an increase of the absolute NO_2-Tyr yields produced from the reaction of peroxynitrite with tyrosine. As observed in the absence of CO_2, the addition of increasing amounts of oxyMb and metMb led to a significant, concentration-dependent decrease of the NO_2-Tyr yields. However, in the presence of 1.1 mM CO_2, these two forms of Mb were less efficient to prevent the peroxynitrite-mediated nitration of added tyrosine (Figure 5B and Table 1S). The order of efficiency of the four Mb forms studied was identical to that obtained in the absence of CO_2. In the presence of 50 μM oxyMb, the best scavenger, the NO_2-Tyr yield was 26 ± 2% of the amount obtained in the absence of the protein. Interestingly, small quantities of metMbCN reduce the nitration of free tyrosine to a larger extent in the presence than in the absence of CO_2.

Nitration of the Globin After Treatment of Mb and Hb with Peroxynitrite at 37 °C. We have previously determined the extent of nitration of the tyrosine residues upon reaction of different forms of Mb and Hb with an excess of peroxynitrite at 0 and 20 °C (*26*). Our preliminary results showed that the NO_2-Tyr yields were only slightly larger when the reactions were carried out at 37 °C instead of 20 °C (*26*). Thus, we completed these studies by exposing at 37 °C apoMb, oxyMb, metMb, oxyHb, and metHb (100 μM) to an excess of peroxynitrite (1000 μM) in phosphate buffer pH 7.0. For comparison, we treated also tyrosine and BSA with peroxynitrite under identical conditions.

As shown in Figure 6A, at 37 °C the percentages of nitration of the two available tyrosine residues of all forms of Mb and of the three tyrosine residues of oxyHb and metHb were 2−3 times larger than those obtained at 20 °C (Table 2S). In contrast, in the presence of 1.1 mM CO_2, the yield of nitrotyrosine produced when the oxygenated forms of Mb and Hb were allowed to react with peroxynitrite at 37 °C were nearly identical to those obtained at 20 °C (Figure 6B and Table 2S). The percentages of nitration of apoMb, metMb, and metHb were larger at 37 °C also in the presence of CO_2. As observed previously at lower temperatures (*26*), also at 37 °C apoMb was always nitrated to the largest extent, and the tyrosine residues of the iron(III) forms of Mb and Hb were slightly less nitrated than those of the corresponding oxygenated forms, both in the absence and in the presence of CO_2. As anticipated, the addition of CO_2 always led to higher relative NO_2-Tyr yields.

As expected, the exposure of L-tyrosine and BSA to a range of peroxynitrite concentrations under similar conditions (at 20 and 37 °C) induced a dose-dependent increase in the nitration yields (Figures 7 and 2S). For both tyrosine and BSA, the nitration yields were mostly larger at higher temperatures (Table 3S). Interestingly, in the absence of CO_2,

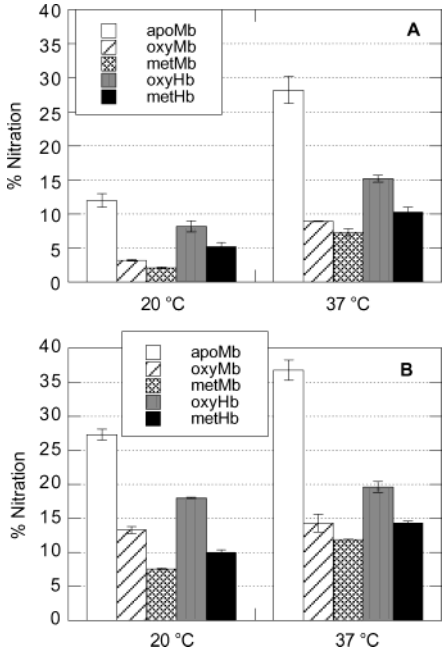


FIGURE 6: NO_2-Tyr yields, determined by HPLC after acid hydrolysis of the proteins, from the reaction of apoMb, oxyMb, metMb, oxyHb, and metHb (100 μM) with 10 equiv of peroxynitrite in the absence (A) and presence (B) of 1.1 mM CO_2 at 20 and 37 °C, pH 7.0.

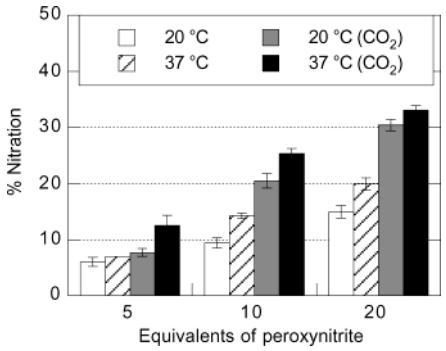


FIGURE 7: NO_2-Tyr yields, determined by HPLC, from the reaction of tyrosine with different amounts of peroxynitrite in the absence and presence of 1.1 mM CO_2, at 20 and 37 °C, pH 7.0.

this temperature-mediated increase in the nitration yields was more pronounced at higher concentrations of peroxynitrite, in particular for tyrosine. In contrast, in the presence of CO_2, larger differences between the NO_2-Tyr yields obtained at 20 and 37 °C were observed when smaller amounts of peroxynitrite were used.

DISCUSSION

The reaction of peroxynitrite with oxyMb and oxyHb has been reported to yield the corresponding iron(III) forms (*39*−*41*). We have shown that these two reactions proceed in two steps via the formation of the corresponding ferryl species (*24*). The second-order rate constants for the two steps of the reaction between peroxynitrite and oxyHb are (3.3 ± 0.1) × 10^4 and (3.3 ± 0.4) × 10^4 M^{-1} s^{-1} (at pH 7.4 and 20 °C), respectively (F. Boccini and S. Herold, unpublished results). The values for the rate constants of the reaction with oxyMb are slightly larger, (5.4 ± 0.2) × 10^4 and (2.2 ± 0.1) × 10^4 M^{-1} s^{-1} (at pH 7.3 and 20 °C), respectively. It is conceivable that the reaction between peroxynitrite and

oxyHb takes place in vivo, as peroxynitrite has been shown to be able to diffuse across the erythrocyte membrane and oxidize oxyHb within the red blood cells (*39*, *42*).

In contrast, we and others have shown that upon mixing peroxynitrite with the iron(III) forms of Hb and Mb, no changes are detected in the UV−vis absorbance spectra of the proteins (ref *43* and references therein). This observation may suggest that no reaction takes place between peroxynitrite and the heme centers of metMb or metHb. However, despite the lack of absorbance changes in the UV−vis spectra of the proteins, the iron(III) forms of sperm whale myoglobin mutants, in which the distal histidine residue had been replaced with either an aspartic acid or an alanine, have been shown to be efficient catalysts for the isomerization of peroxynitrite to nitrate (*44*). In addition, we have recently reported that the tyrosine residues of metMb and metHb are nitrated by peroxynitrite to a significantly smaller extent than those of apoMb (*26*). Taken together, these results suggest that a reaction must take place between peroxynitrite and heme centers of metMb and metHb.

Kinetic Studies of the Reaction of Peroxynitrite with the Iron(III) Forms of Mb and Hb. In this paper, we have shown that there is a linear dependence between the observed rate of decay of an excess peroxynitrite and the concentration of both metMb and metHb. This result suggests that the iron(III) forms of these proteins catalyze the decay of peroxynitrite. This assumption is supported by the further observation that a variation of the peroxynitrite concentration does not affect significantly the value of the observed rate of peroxynitrite decay. Finally, analysis of the nitrogen-containing products showed that metMb and metHb increase the production of nitrate from the decay of peroxynitrite in a concentration-dependent way. Taken together, our results indicate that metMb and metHb catalyze the isomerization of peroxynitrite. The measured values of the catalytic rate constants are $(2.9 \pm 0.1) \times 10^4$ and $(1.2 \pm 0.1) \times 10^4$ M^{-1} s^{-1} (at pH 7.0 and 20 °C), respectively. Thus, as for the reaction with the oxygenated forms of these proteins, the rate of the reaction between peroxynitrite and myoglobin is larger than that of its reaction with hemoglobin. The value for the reaction with metMb is consistent with those recently reported: $(1.4 \pm 0.1) \times 10^4$ M^{-1} s^{-1} (at pH 7.4 and 20 °C) (*44*) and 1.03×10^4 M^{-1} s^{-1} (at pH 7.6) (*37*). For comparison, the sperm whale metMb mutant H64A has a k_{cat} value of $(6.0 \pm 0.1) \times 10^6$ M^{-1} s^{-1} (at pH 7.4 and 20 °C) (*44*). This significantly larger rate is reflected in the extremely small quantities (1.6 μM) of this mutant needed to completely convert 100 μM peroxynitrite to nitrate under conditions analogous to those used in the present work (*44*).

The pH-dependence of the catalytic rate constant for metMb and metHb suggests that HOONO is the species that reacts with the iron(III) center of these proteins. Alternatively, the decrease of the value of k_{cat} with increasing pH may be a consequence of the deprotonation of the water coordinated to the iron(III) center, which leads to a stronger Fe^{III}−O bond. However, as the pK_a value for the acid-alkaline transition of metMb is 8.05 and that for metHb is 8.93 (*45*), no significant difference would be expected between the rate constants at pH 6.5 and 7.0. In contrast, at pH 6.5, the values of k_{cat} are approximately twice as large as those at pH 7.0. The acid-alkaline transition could be at least in part responsible for the significant decrease of k_{cat} at pH 8.0. A further possible explanation for the observed pH-dependence is that protonation of the distal histidine at low pH and its consequent shift away from the active site pocket (*46*) facilitate the entrance of peroxynitrite and its binding to the iron. Nevertheless, if this was true, no significant difference would be expected among the k_{cat} values at pH 7.0 and 8.0, as the pK_a of the distal histidine is lower than 6. In contrast, at pH 8.0, the values of k_{cat} are approximately 10 times smaller than those at pH 7.0. Taken together, these results strongly indicate than HOONO is the species that reacts at a larger rate with metMb and metHb. This conclusion is in agreement with the results obtained with the H64A-metMb mutant. Also for this protein, the values of k_{cat} increase with decreasing pH (*44*).

To further probe the role of the iron center for the catalysis of the decay of peroxynitrite, we also investigated the reaction of peroxynitrite with increasing amounts of apoMb and metMbCN. As expected, the heme-free apoMb and the heme-blocked metMbCN do not affect the decay rate of peroxynitrite. Moreover, analysis of the nitrogen-containing products showed that apoMb does not induce an increase of the nitrate yield produced by the decay of peroxynitrite. Taken together, these results confirm that the iron(III) centers of metMb and metHb play a critical role in the catalysis of the isomerization of peroxynitrite.

The rapid reaction between the peroxynitrite anion and the ubiquitous CO_2 has been shown to be one of the principal pathways for peroxynitrite consumption in biological systems (*38*). Despite the high CO_2 concentration present in the plasma (ca. 1.3 mM), it has been established that significant amounts of peroxynitrite can diffuse inside the red blood cells, via the anion channels or by simple diffusion and oxidize oxyHb (*42*). We have previously shown that the peroxynitrite-mediated oxidation of oxyMb proceeds via the ferrylMb also in the presence of added CO_2 (*25*). The second-order rate constants for the two reaction steps are $(41 \pm 7) \times 10^4$ and $(3.2 \pm 0.2) \times 10^4$ M^{-1} s^{-1} (at pH 7.5, 20 °C, and in the presence of 1.2 mM CO_2), respectively (*25*). The corresponding values of the rate constants for the reaction with oxyHb are $(35 \pm 3) \times 10^4$ and $(10.6 \pm 0.6) \times 10^4$ M^{-1} s^{-1} (at pH 7.4, 20 °C, and in the presence of 1.2 mM CO_2) (F. Boccini and S. Herold, unpublished results). Thus, for both proteins the first reaction step, the formation of the ferryl form from the oxy form, is significantly accelerated by the addition of CO_2, and once more Mb reacts faster than Hb.

Also in the presence of 1.1 mM CO_2, the reaction of peroxynitrite with metMb and metHb does not induce any changes in the UV−vis spectra of the proteins (data not shown). However, our stopped-flow and ion chromatography studies show that, also in the presence of physiological concentrations of CO_2 (1.1−1.2 mM), metMb and metHb catalyze the isomerization of peroxynitrite to nitrate, whereas apoMb has no effect on its decay rate. The values of the catalytic rate constants are approximately 3 times larger than those determined in the absence of CO_2 (Table 1).

Interestingly, in the presence of 10 mM CO_2 both at pH 7.3 and 8.3, metMb and metHb did not influence the decay rate of peroxynitrite. After the 4 ms approximately needed for mixing, under the conditions chosen for the experiment at pH 8.3, a large fraction of peroxynitrite is already converted to $ONOOCO_2^-$. Because of the alkaline pH, most of the remaining peroxynitrite is in its deprotonated form.

Thus, nearly no HOONO will be available for reaction with metMb or metHb. As no effect is observed by the addition of increasing amounts of metMb or metHb, our data indicate that no fast reaction takes place between $ONOOCO_2^-$ and the iron(III) center of these two proteins. Taken together, these results suggest that the catalytic effect observed in the presence of 1.2 mM CO_2 is due to the competing reaction between HOONO, still present in significant amounts after mixing the reagents at pH 7.0, and the iron(III) center of the proteins. However, according to this mechanism, the value of k_{cat} should not be influenced by the addition of CO_2. The larger values of k_{cat} obtained in the presence of 1.2 mM CO_2 could reflect the increased rate observed with lower amounts of peroxynitrite (Figure 2). Indeed, despite the fact that 100 μM peroxynitrite is mixed with the proteins, a large amount of peroxynitrite is rapidly consumed by its reaction with CO_2; thus, lower amounts of peroxynitrite are available for reaction with the protein. Alternatively, large concentrations of HCO_3^-/CO_2 could cause conformational changes of the proteins, which could lead to a faster reaction with HOONO.

The hypothesis that $ONOOCO_2^-$ does not react with metMb or metHb also explains why in the presence of 1.2 mM CO_2 larger amounts of metMb and metHb were needed to get a significant increase in the nitrate yields. Under the conditions of our experiment, the very fast reaction between peroxynitrite and CO_2 has a half-life of ca. 40 ms, whereas in the presence of 50 μM metMb, the half-life of the protein-catalyzed process is 180 ms. Thus, only about 20% of peroxynitrite will react with metMb, and the nitrate yields will be only slightly larger than those obtained in the absence of the protein.

Protection of metMb and metHb Against Peroxynitrite-Mediated Nitration. The ability of metMb and metHb to protect against peroxynitrite-mediated nitration of free tyrosine is another feature that confirms that these proteins are active catalysts for the isomerization of peroxynitrite. Pietraforte et al. (*36*) have shown that metHb prevents nitration of the dipeptide Ala-Tyr (1 mM) by 1 mM peroxynitrite in a concentration-dependent way. We carried out a similar experiment with different forms of Mb and showed that oxyMb is the species that most efficiently inhibits the nitration of free tyrosine by peroxynitrite. Higher concentrations of metMb were needed to achieve a similar extent of protection, and significant nitration of free tyrosine was obtained also in the presence of large amounts of apoMb and metMbCN. These data further confirm that the heme center of Mb plays a key role to scavenge peroxynitrite. The protection provided by high concentrations of apoMb and metMbCN is likely to be due to the concurrent reaction of peroxynitrite with the two tyrosine, the two tryptophan, and/or the three methionine residues of the globin. The observation that metMbCN is slightly more efficient than apoMb in protecting free tyrosine from peroxynitrite-mediated nitration suggests that in apoMb, because of partial unfolding of the globin (*47*, *48*), some of these residues may be less accessible.

The protection efficiency of metMb can be rationalized by comparing the rate of the protein-catalyzed isomerization of peroxynitrite with that of its spontaneous decay. For instance, under the conditions of our experiment, the rate of the reaction between 25 μM metMb and 200 μM peroxynitrite is 0.72 s^{-1} ($k_{obs} = (2.9 \times 10^4\ M^{-1}\ s^{-1}) \times (25 \times 10^{-6}\ M) = 0.72\ s^{-1}$) and that of the decay of peroxynitrite is 0.3 s^{-1} (*34*). Thus, in the presence of 25 μM metMb, approximately 70% of peroxynitrite will be converted to nitrate by its reaction with metMb, and 30% will be available for reaction with free tyrosine. In this experiment, we have expressed the amount of nitrotyrosine produced in relative yields, that is, we have arbitrarily set the nitration yield obtained from the reaction with 200 μM peroxynitrite (in the absence of protein) equal to 100%. Thus, if 30% of peroxynitrite is available for reaction with tyrosine, we expect a relative nitration yield of 30%. This calculated value is in good agreement with the experimental yield obtained, that is, 36 $\pm$ 2%.

As the reaction between oxyMb and peroxynitrite is not catalytic, the higher protection provided by this form of Mb in the presence of a large excess of peroxynitrite over the protein is probably due to a combination of the reactions of peroxynitrite with oxyMb and with metMb. Indeed, the half-life of the reaction of 200 μM peroxynitrite with 2.5 μM oxyMb is approximately 64 ms (*25*), whereas that of the spontaneous peroxynitrite decay is ca. 2.3 s. Thus, the rapid oxidation of oxyMb generates metMb, which catalyzes the decay of excess peroxynitrite, still present in large quantities when all oxyMb is oxidized.

FerrylMb is slightly less efficient than oxyMb as fewer equivalents of peroxynitrite are consumed to generate metMb (*25*), which also in this case catalyzes the isomerization of the rest of peroxynitrite present after complete reduction of ferrylMb. As expected by comparing the kinetic parameters, metHb and oxyHb were a little less effective than the corresponding Mb forms. The observation that deoxyHb is more efficient than oxyHb is probably also due to the larger value of the rate constant for the reaction between peroxynitrite and deoxyHb (F. Boccini and S. Herold, unpublished results).

In the presence of CO_2, higher concentrations of the proteins, in particular of metMb and oxyMb, are required to achieve similar protection. This observation can also be explained by comparing the rates of all the reactions in the system. In the presence of 1.1 mM CO_2, the observed rate of peroxynitrite decay is 17.4 s^{-1}, whereas the isomerization catalyzed by 25 μM metMb proceeds at a rate of 1.9 s^{-1}. Thus, in the presence of 25 μM metMb, most peroxynitrite (90%) will be available for reaction with free tyrosine, and only 10% will be converted to nitrate by its reaction with the iron center of metMb. This calculated NO_2-Tyr yield (90%) is significantly larger than that obtained experimentally (63 $\pm$ 2%). This difference is due to the protection imparted by the globin. Indeed, the addition of 25 μM apoMb or metMbCN reduces the amount of nitration of free tyrosine by 20−25%. Moreover, amino acid analysis of metMb after treatment with an excess of peroxynitrite showed significant nitration of the tyrosine residues (*26*). In summary, by considering the protection provided both by the globin (20−25%) and by the iron(III) center (10%), the expected NO_2-Tyr yield is 65−70%, a value close to the experimentally obtained value of 63 $\pm$ 2%. As discussed above for the reaction in the absence of CO_2, the higher protection imparted by oxyMb probably derives from the combination of the reactions of peroxynitrite/CO_2 with oxyMb and with metMb.

Under physiological conditions, the protein concentration will always be significantly larger than that of peroxynitrite.

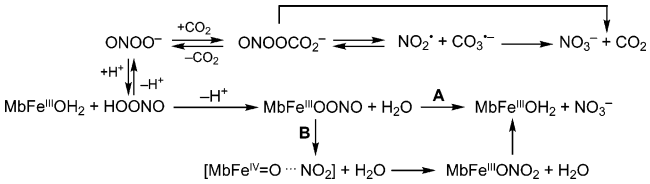
Mb and Hb are mostly found in the oxygenated form, with only a small percentage present in the oxidized iron(III) form. Consequently, since the second-order rate constants of the reactions of peroxynitrite with oxyMb and oxyHb are in the same order of magnitude as the catalytic rate constants for its metMb- and metHb-mediated isomerization, oxyHb and oxyMb will be the species primarily involved in the scavenging of peroxynitrite in vivo. The deoxygenated forms of Mb and Hb have been shown to react with peroxynitrite at a rate 1−2 orders of magnitude larger than that for the reaction with the oxygenated proteins (*24*). Thus, the reaction with deoxyHb may play a role in venous blood, whereas deoxyMb will scavenge peroxynitrite only under hypoxic conditions, for instance, such as found in ischemia.

Comparison of the Extent of Nitration at 20 and 37 °C. We have previously shown that the extent of nitration of the tyrosine residues upon mixing, at 0 or 20 °C, different forms of Mb and Hb with an excess of peroxynitrite are larger when the reactions are carried out at 20 °C (*26*). The results described in this paper show that, in the absence of added CO_2, the NO_2-Tyr yields are even larger at 37 °C. As already observed at lower temperatures, metMb is always nitrated to a significantly smaller extent than metHb. The catalytic rate constants determined in this work for the metMb- and metHb-mediated isomerization of peroxynitrite at 20 and 37 °C provide a key to interpret this result. Indeed, both at 20 and at 37 °C the values of k_{cat} for metMb are 2.5−4 larger than those for metHb. Thus, in the presence of metMb, a larger amount of peroxynitrite is isomerized to NO_3^- and cannot nitrate the tyrosine residues of the protein.

At higher temperatures, all the proteins are nitrated to a larger extent despite the fact that the catalytic rate constants are 2−3 times larger. This result is due to the accelerated decay of peroxynitrite, the process that leads to the reactive species responsible for nitration and that proceeds significantly faster at higher temperatures. For instance, at 20 °C, the rate of the reaction between 100 μM metMb and 1000 μM peroxynitrite is ca. 3 s^{-1} and that of the decay of peroxynitrite is 0.3 s^{-1}. At 37 °C , the rate of the protein-catalyzed (100 μM metMb) reaction is 3 times larger (8.8 s^{-1}), whereas that of the decay of peroxynitrite is 5 times larger (1.5 s^{-1}). Interestingly, nitration yields of tyrosine and BSA are only slightly larger at 37 °C. Thus, the significant increase in the nitration of metMb and metHb arises from a decrease in the potency of these proteins to scavenge peroxynitrite rather than from an intrinsic higher nitration efficiency of peroxynitrite at higher temperatures.

At both temperatures studied, oxyMb and oxyHb are nitrated to a larger extent than metMb and metHb, despite the fact that the oxy forms prevent more effectively the nitration of free tyrosine by peroxynitrite (Figure 5). This result suggests that, as has recently been shown by EPR spectroscopy (*41*, *49*), tyrosine radicals are generated in some extent from the reaction of peroxynitrite with oxyHb, and most likely, with oxyMb. A similar EPR spectrum has been observed upon mixing of metHb with peroxynitrite, but its intensity was 52% lower than that obtained with oxyHb (*36*). As the nitration yields of the two tyrosine residues of oxyHb and metHb are very low, the pathways leading to the tyrosine radicals must be secondary. Nevertheless, the higher radical yields obtained for oxyHb may be responsible for the larger nitration yields of oxyHb (and oxyMb).

Scheme 1: Possible Pathways for the Reaction between metMb and Peroxynitrite, in the Absence and Presence of CO_2



A further noteworthy result is the significant increase of nitration of apoMb at 37 °C (almost twice as much as at 20 °C). This result suggests that apoMb may be significantly unfolded at higher temperatures; thus, the tyrosine residues may be more exposed and nitrated more easily.

As expected, since it has been shown that carbon dioxide increases the peroxynitrite-mediated yields of NO_2-Tyr (*38*, *50*), the addition of carbon dioxide in all the systems discussed above led to increased nitration. Moreover, also in the presence of added CO_2, the yields of nitration were mostly larger at a higher temperature. Two exceptions are represented by metMb and metHb. Interestingly, treatment of the iron(III) forms of these proteins with peroxynitrite at 20 or 37 °C led to comparable yields of nitration of their tyrosine residues. This observation can be explained by comparing the rates of all the reactions in the system at the two temperatures. At 20 °C, in the presence of 1.1 mM CO_2, the rate of the reaction between 100 μM metMb and 1000 μM peroxynitrite is ca. 7.7 s^{-1} and that of the decay of peroxynitrite is 17.4 s^{-1}. At 37 °C, the rate of the protein-catalyzed (100 μM metMb) reaction is ca. 3.5 times larger (26.5 s^{-1}) and that of the decay of peroxynitrite is also ca. 3.5 times larger (60 s^{-1}). Thus, as both reactions are accelerated to the same extent, no significant changes are observed in the nitration yields.

Mechanistic Considerations and Conclusions. The work presented here shows conclusively that metMb and metHb catalyze the isomerization of peroxynitrite to nitrate, despite the fact that no changes are observed in the UV−vis spectrum of the proteins upon treatment with peroxynitrite. As shown in Scheme 1, two mechanisms may be formulated to interpret these results. It is conceivable that the first step of the reaction is represented by a ligand substitution reaction. The coordinated water is replaced by peroxynitrite, and the peroxynitrito-complex $MbFe^{III}OONO$ is generated. Unexpectedly, our results suggest that the protonated form of peroxynitrite, HOONO, is the species that reacts with the iron(III) center. Because of the strong hydrogen bond between the distal histidine and the aquo-ligand in metMb, this reaction is likely to proceed at a slow rate. Indeed, it has previously been established that ligand binding to metMb is significantly accelerated in mutants in which the coordinated water is absent or in which the distal histidine has been replaced by an amino acid residue that cannot form hydrogen bonds (*51*). The metMb peroxynitrito complex has been shown to be unstable at neutral pH and to rapidly decay to metMb and nitrate (*52*). Thus, if the binding of peroxynitrite is the rate-limiting step, one would expect that $MbFe^{III}OONO$ does not accumulate in concentrations large enough to be detected. Moreover, the UV−vis spectrum of $MbFe^{III}OONO$ is very similar to that of metMb under neutral or acidic

conditions (*52*), a feature that makes its detection even less likely.

The corresponding hemoglobin complex $HbFe^{III}OONO$ is more stable, and under alkaline conditions, its increased stability and UV−vis spectrum significantly different from that of the basic form of metHb ($HbFe^{III}OH$) may allow for its detection (*53*). Nevertheless, ligand substitution reactions of metHb and metMb are significantly slower under alkaline conditions, as the coordinated hydroxide ion is a stronger ligand for iron(III) than the water molecule (*45*). Thus, if both the rate of formation and the rate of decay are slowed, also in this case $HbFe^{III}OONO$ will not accumulate in concentrations large enough to be detected.

Our previous mechanistic studies of the decay of $MbFe^{III}OONO$ and $HbFe^{III}OONO$ to their corresponding iron(III) forms and nitrate suggest that this reaction proceeds in a concerted way (pathway A in Scheme 1) (*52*). However, Groves and co-workers proposed an alternative mechanism (*37*). According to their hypothesis, the reaction of the peroxynitrito complex to the final products proceeds in three steps: homolytic cleavage of the O−O bond of the peroxynitrito ligand with generation of nitrogen dioxide and the ferryl form of the protein, partial recombination to the nitrate complex, and finally, dissociation of nitrate (pathway B in Scheme 1). Groves and co-workers based their mechanism on the observation that in the presence of 100−200 μM metMb the nitrate yields generated from the decay of 500 μM peroxynitrite leveled off around 11%. Thus, they suggested that part of $NO_2^{\bullet}$ eludes the reaction with ferrylMb and undergoes hydrolysis to nitrite and nitrate. However, our data in the presence of a large excess of metMb or metHb (750 μM) show that, if the experimental conditions are chosen so that the decay of peroxynitrite proceeds exclusively via the protein-catalyzed pathway, no nitrite is generated. Thus, no $NO_2^{\bullet}$ is produced from the peroxynitrite complex, which yields quantitatively nitrate (*52*). This conclusion is supported by our previous observation that no nitrite is formed when peroxynitrite is allowed to react with 1.6 μM of the effective isomerization catalyst H64A-metMb. Moreover, Pietraforte et al. also obtained nearly quantitative nitrate yields (94−97%) from the decay of 100 μM peroxynitrite in the presence of 500 mM metHb (at pH 7.4) (*36*).

The values of the catalytic rate constants for the metMb- and metHb-mediated isomerization of peroxynitrite are in the same order of magnitude as that of the reaction between CO_2 and peroxynitrite. Thus, in the presence of physiological amounts of CO_2, metMb and metHb can compete with CO_2 for peroxynitrite, and at least in part, catalyze its isomerization to nitrate (Scheme 1). Our data at high pH in the presence of 10 mM CO_2 clearly indicate that no direct reaction takes place between $ONOOCO_2^-$ and the iron(III) center of the proteins. Interestingly, Pietraforte et al. arrived to the same conclusion, partly based on experiments that in our hands gave different results (*36*). Specifically, they showed that the product distribution of the decay of 100 μM peroxynitrite in the presence of 1.2 mM CO_2 is not influenced by the addition of increasing amounts of metHb (5−500 μM) (*36*). The only difference between these two experiments is the temperature: from the bicarbonate concentration used to reach a CO_2 concentration of 1.2 mM, it looks as if these experiments were carried out at 37 °C, whereas our data were obtained at 20 °C.

In conclusion, our results confirm and extend previous studies demonstrating that the oxy-, deoxy-, and met-forms of Mb and Hb are efficient scavengers for peroxynitrite (*24*−*26*, *36*, *54*). The catalytic rate constants obtained in this work for the metMb- and metHb-catalyzed isomerization of peroxynitrite are essential to appraise the role of the different protein forms in this scavenging process.

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SUPPORTING INFORMATION AVAILABLE

Primary kinetic data for the protein-mediated decay of peroxynitrite at 37 °C (Figure S1) and NO_2-Tyr yields from the reaction of BSA with different amounts of peroxynitrite in the absence and presence of 1.1 mM CO_2, at 20 and 37 °C (Figure S2). Table S1 with all the relative NO_2-Tyr yields formed from the reaction of peroxynitrite and tyrosine in the presence of different concentrations of oxyMb, metMb, apoMb, metMbCN, oxyHb, deoxyHb, and metHb. Table S2 with all the NO_2-Tyr yields determined by mixing apo-, oxy-, metMb, oxy-, and metHb with 10 equiv of peroxynitrite at 20 and 37 °C. Table S3 with all the NO_2-Tyr yields determined by mixing BSA and tyrosine with 5, 10, and 20 equiv of peroxynitrite at 20 and 37 °C. This material is available free of charge via the Internet at http://pubs.acs.org.

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