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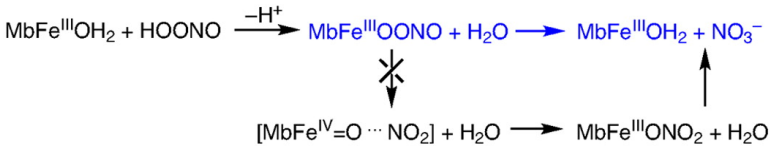

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Mechanistic Studies of the Isomerization of Peroxynitrite to Nitrate Catalyzed by Distal Histidine Metmyoglobin Mutants

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Abstract: Hemoproteins are known to react with the strong nitrating and oxidizing agent peroxynitrite according to different mechanisms. In this article, we show that the iron(III) forms of the sperm whale myoglobin (sw Mb) mutants H64A, H64D, H64L, F43W/H64L, and H64Y/H93G catalyze the isomerization of peroxynitrite to nitrate. The two most efficient catalysts are H64A ($k_{cat} = (5.8 \pm 0.1) \times 10^6$ M^{-1} s^{-1}, at pH 7.5 and 20 °C) and H64D metMb ($k_{cat} = (4.8 \pm 0.1) \times 10^6$ M^{-1} s^{-1}, at pH 7.5 and 20 °C). The pH dependence of the values of k_{cat} shows that HOONO is the species which reacts with the heme. In the presence of physiologically relevant concentrations of CO_2 (1.2 mM), the decay of peroxynitrite is accelerated by these metMb mutants via the concurring reaction of HOONO with their iron(III) centers. Studies in the presence of free added tyrosine show that the metMb mutants prevent peroxynitrite-mediated nitration. The efficiency of the different sw metMb mutants correlates with the value of k_{cat}. Finally, we show that sw WT-metMb is nitrated to a larger extent than horse heart metMb, a result that suggests that the additional Tyr151 is a site of preferential nitration. Again, the extent of nitration of the tyrosine residues of the metMb mutants correlates with the values of k_{cat}.

Introduction

Peroxynitrite,[1] a strong oxidizing and nitrating agent generated in vivo from the diffusion-controlled reaction between $NO^{\bullet}$ and $O_2^{\bullet -}$,[2] reacts with hemoproteins according to radically different pathways. In general, the rate constants for the reactions of peroxynitrite with hemoproteins are rather large. Thus, when present in large concentrations these proteins can conceivably compete with CO_2, the major target of peroxynitrite in vivo. The reaction between the peroxynitrite anion and CO_2 is quite fast (3×10^4 M^{-1} s^{-1} at 24 °C)[3,4] and yields the adduct 1-carboxylato-2-nitrosodioxidane ($ONOOCO_2^-$),[3,5] a nitrating agent stronger than peroxynitrous acid. The iron(III) forms of peroxidases such as horseradish peroxidase, lactoperoxidase, myeloperoxidase, and chloroperoxidase have been reported to catalyze the decay of peroxynitrite by a complex mechanism which involves the high valent oxoiron(IV) (ferryl) species.[6−8] In the presence of phenolic compounds, these peroxidases have been shown to significantly increase the yield of the peroxynitrite-mediated nitration.[7] A similar mechanism was observed for the reactions of peroxynitrite with the iron(III) forms of cytochrome P450 enzymes such as $P450_{BM-3}$ and $P450_{NOR}$.[9] Furthermore, oxyhemoglobin (oxyHb) and oxymyoglobin (oxyMb) are oxidized by an excess of peroxynitrite to their corresponding iron(III) forms (metHb and metMb, respectively) in a two-step mechanism: first the proteins are oxidized to their oxoiron(IV) forms, which in the second reaction step are reduced by peroxynitrite.[10] Cytochrome *c* oxidase reacts with peroxynitrite to generate $NO^{\bullet}$ (in its reduced iron(II) form) or nitrite (in its iron(III) form).[11,12] However, high peroxynitrite concentrations irreversibly inhibit this enzyme.[11,12]

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(1) The recommended IUPAC nomenclature for peroxynitrite is oxoperoxonitrate(1−); for peroxynitrous acid, hydrogen oxoperoxonitrate. The term peroxynitrite is used in the text to refer generically to both oxoperoxonitrate-(1−) ($ONOO^-$) and its conjugate acid, hydrogen oxoperoxonitrate (ONOOH). Abbreviations used: TMPyP, 5,10,15,20-tetrakis-(*N*-methyl-4'-pyridyl)-porphyrin; TPPS, 5,10,15,20-tetrakis-(4'-sulfonatophenyl)porphyrin; sw WT-Mb or WT-Mb, recombinant sperm whale myoglobin; Hb, hemoglobin; oxyHb, oxyhemoglobin ($HbFeO_2$); Mb, myoglobin; hh Mb, horse heart myoglobin; metMb, iron(III)myoglobin; oxyMb, oxymyoglobin ($MbFeO_2$); PDB, Protein Data Bank; NO_2-Tyr, 3-nitrotyrosine.

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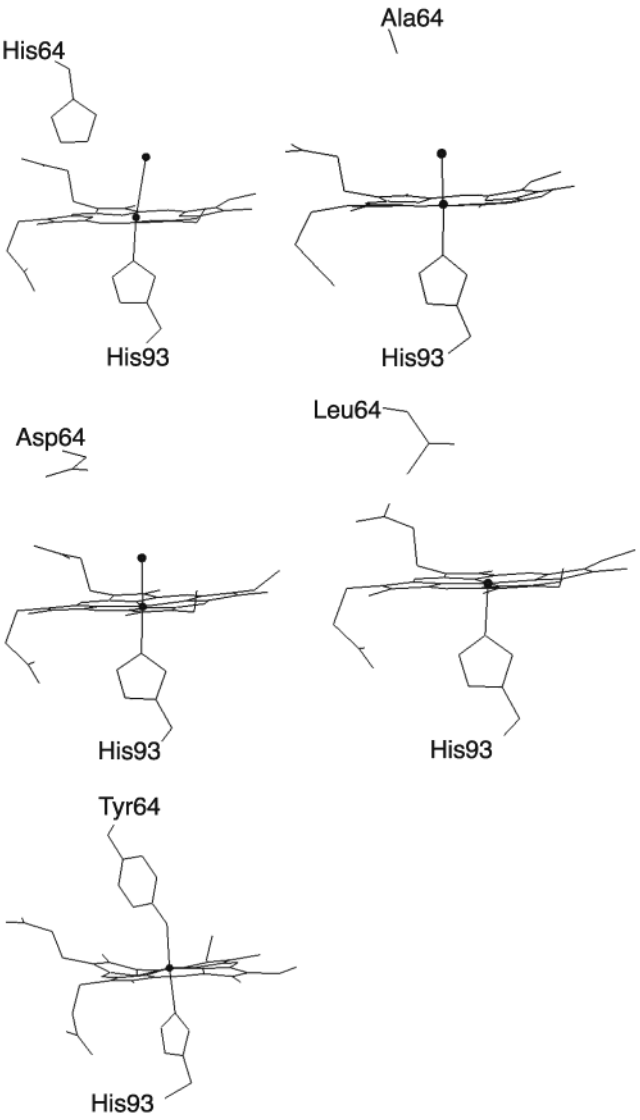

Figure 1. Heme ligation of wild-type metMb (His64, PDB no. 1YMA),[46] H64A-metMb (PDB no. 102M),[47] H64D-metMb (H64D/V68A, PDB no. 1LUE),[16] H64L-metMb (in the crystallized double mutant H64L/F43H, PDB no. 1OFK, a water molecule is bound to the heme),[48] and H64Y-metMb (PDB no. 1YMA).[49]

We have recently shown that human metHb and horse heart metMb (hh metMb) catalyze, albeit not very efficiently, the isomerization of peroxynitrite to nitrate.[13] The reduced reactivity of the iron(III) form of these proteins contrasts the intrinsic high reactivity of iron(III)−porphyrin complexes toward peroxynitrite and is due to the strong hydrogen bond present in metMb and metHb between the distal histidine (His64) and the water molecule coordinated to the iron(III) center (Figure 1).[13] Indeed, synthetic water-soluble iron(III)−porphyrin complexes efficiently catalyze the isomerization of peroxynitrite to nitrate, the best catalyst being $[Fe(III)(TMPyP)]^{5+}$ ($k_{cat} = 1.6 \times 10^6\ M^{-1}\ s^{-1}$, at pH 7.4 and 37 °C).[14]

In this article, we extended our preliminary studies[15] and investigated the reactivity toward peroxynitrite of the iron(III) forms of the sperm whale Mb mutants, H64A, H64D, H64L, F43W/H64L, and H64Y/H93G. In all these mutants, the distal histidine is replaced by an amino acid which cannot undergo a hydrogen bond with the water molecule bound to the heme (Figure 1). Asp64 could theoretically undergo a hydrogen bond, but it has been shown by X-ray crystallography that it does not point to the heme but toward Arg45 (Figure 1).[16] In H64D-metMb, the water molecule bound to the iron is stabilized by hydrogen bonds to other water molecules present in the distal pocket.[16] Moreover, the H64L Mb mutant was chosen because the iron(III) center is pentacoordinated, the coordinated water being absent.[17] We have recently shown that, upon mixing with different forms of hh Mb, peroxynitrite induces not only formation of nitrotyrosine but also of different nitrotryptophan isomers.[18] Thus, the double mutant F43W/H64L Mb was investigated to determine the extent of nitration of a tryptophan residue close to the heme. Finally, the last Mb mutant studied, H64Y/H93G, exhibits a completely different axial ligation pattern. In its iron(III) form, only the tyrosine residue Tyr64 is coordinated to the heme on the distal side (compare structure of H64Y-metMb in Figure 1). The originally coordinated proximal histidine (His93) is replaced by the noncoordinating glycine. Thus, reaction of peroxynitrite with H64Y/H93G metMb will necessarily take place on the proximal side of the heme, and peroxynitrite will have to follow a different pathway to reach the iron center. We have carried out kinetic studies of the decay of peroxynitrite catalyzed by sw metMb and by these five metMb mutants at different pH values and CO_2 concentrations. In addition, we have analyzed the nitrogen-containing products of these reactions, the efficiency of these proteins to protect peroxynitrite-mediated nitration of free tyrosine, and the extent of tyrosine (and tryptophan) nitration upon addition of an excess peroxynitrite.

Experimental Procedures

Chemicals. Buffers were prepared from KH_2PO_4/K_2HPO_4 (Fluka) with deionized Milli-Q water. Sodium nitrite, sodium nitrate, 3-nitrotyrosine, L-tyrosine, ammonium sulfamate, trifluoroacetic acid, hydrogen peroxide, calcium chloride, and acetonitrile (99.8%) were obtained from Fluka. Sodium bicarbonate was purchased from Merck. Pronase was obtained from Roche Molecular Biochemicals. The Enhanced Chemiluminescence kit and the nitrocellulose membrane were purchased from Amersham Biosciences and Schleicher & Shuell, respectively. 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 19 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 before each experiment by measuring the absorbance at 302 nm ($\epsilon_{302} = 1705\ M^{-1}\ cm^{-1}$).[20]

Experiments in the presence of CO_2 were carried out by adding the required amount of a freshly prepared 0.5 or 1 M sodium bicarbonate

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solution to the protein solutions as described in detail in ref 13. The values for the constant of the hydration−dehydration equilibrium $CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^-$ were derived from ref 21 by taking into consideration the ionic strength of the solutions. After addition of bicarbonate, the protein solutions were allowed to equilibrate for at least 5 min at 20 °C. For the experiments carried out in the absence of CO_2, the buffers and the 0.01 M NaOH solutions were thoroughly degassed.

Overexpressed and purified recombinant sperm whale Mb (WT-Mb) was purchased from Sigma. MetMb was prepared by adding a small amount of potassium hexacyanoferrate(III) and purified over a Sephadex G25 column by using a 0.1 M phosphate buffer solution (pH 7.0) as the eluent. H64A, H64L, and H64 D sperm whale Mb mutants were prepared according to refs 22 and 23. The Phe43 → Trp mutation was introduced by the polymerase chain reaction (PCR)-based method as reported before.[22,23] The His64 → Tyr mutation on H93G sperm whale Mb was introduced by the cassette mutagenesis (Roach, M. P.; Ozaki, S.-I.; Watanabe, Y. *Biochemistry* **2000**, *39*, 1446−1454). The concentrations of WT-metMb and the iron(III) form of the sperm whale Mb mutants were determined by measuring (at pH 7.0) the absorbance at 409 nm for WT-metMb ($\epsilon_{409} = 157\ mM^{-1}\ cm^{-1}$),[24] at 407 nm for H64A and H64D metMb ($\epsilon_{407} = 136\ mM^{-1}\ cm^{-1}$ and $\epsilon_{407} = 154\ mM^{-1}\ cm^{-1}$, respectively), at 393 nm for H64L metMb ($\epsilon_{393} = 103\ mM^{-1}\ cm^{-1}$), at 394 nm for F43W/H64L metMb ($\epsilon_{394} = 103\ mM^{-1}\ cm^{-1}$), and at 406 nm for H64Y/H93G metMb ($\epsilon_{406} = 115\ mM^{-1}\ cm^{-1}$). All the absorption spectra were collected on a UVIKON 820 spectrophotometer.

Stopped-Flow Experiments. The kinetics of peroxynitrite decay in the presence of WT-metMb and its mutants were studied with an Applied Photophysics SX18MV-R single-wavelength stopped-flow instrument. The length 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 Kaleidagraph, version 3.52. The results of the fits of the traces (averages of at least 5−8 single traces) from at least three experiments were averaged to obtain each observed rate constant, given the corresponding standard deviation. The protein solutions of the required concentrations were prepared under aerobic conditions in 0.1 M phosphate buffer. In general, the pH of the buffer was slightly lower than the desired final pH, since upon mixing with the alkaline peroxynitrite solution the pH always rose between 0.3 and 0.6 units. For the experiments carried out in the presence of CO_2, the pH of the buffer had to be even lower, because addition of sodium bicarbonate also raised the pH of the protein solutions. Obviously, the concentration of bicarbonate needed to obtain a final (after mixing) concentration of 1.2 mM CO_2 depended on the pH and varied from 2 to 65 mM as the pH was raised from 6.0 to 8.5. Thus, the required initial pH of the protein solution had to be determined separately for each experiment. Specifically, for the experiments at pH 6.0, the protein solutions had a pH of 5.7 (in the absence of CO_2) or of 5.5 (in the presence of CO_2); for the experiments at pH 6.5, the protein solutions were prepared in phosphate buffer pH 6.2 (in the absence of CO_2) or pH 6.0 (in the presence of CO_2); for the experiments at pH 7.0, the protein solutions had a pH of 6.7 (in the absence of CO_2) or of 6.5 (in the presence of CO_2); and for the experiments at pH 7.5, the protein solutions were prepared in phosphate buffer pH 7.1 (in the absence of CO_2) or pH 6.8 (in the presence of CO_2). Finally, for the experiments under alkaline conditions at pH 8.0, the protein solutions were prepared in phosphate buffer pH 7.4 (in the absence of CO_2) or pH 7.0 (in the presence of CO_2); for those at pH 8.5, the protein solutions had a pH of 7.8 (in the absence of CO_2) or of 7.5 (in the presence of CO_2). If not specified, all the measurements were carried out at 20 °C and the pH was always measured at the end of the reactions for control.

Rapid-scan UV−vis spectra were measured by using an OLIS RSM 1000 rapid scanning monochromator stopped-flow apparatus (On-Line Instrument System, Inc). The solutions were prepared as described above for the single-wavelength stopped-flow experiments.

Determination of Nitrite and Nitrate. Product analysis was carried out as described previously[25] 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 100 μL of WT-metMb and metMb mutants (1−25 μM in 0.1 M phosphate buffer pH 6.9 or in 0.1 M phosphate buffer pH 6.8 containing 22 mM sodium bicarbonate) at room temperature with 100 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. The contamination of nitrite and nitrate in peroxynitrite was determined as reported recently.[13] Usual nitrite and nitrate contaminations were in the range 20−50% and 0−10% of the peroxynitrite concentration, respectively.

Analysis of the Free 3-Nitrotyrosine Content. The reaction of peroxynitrite with sperm whale WT-metMb and metMb mutants in the presence of L-tyrosine was 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 protein concentrations (0−10 μM in 0.05 M phosphate buffer pH 7.4), in the absence and presence of 1.2 mM CO_2. All the samples were analyzed by reverse-phase HPLC on 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^2), and fluorescence detection (222 and 400 nm, excitation and emission wavelength, respectively) as described previously.[18] Nitrotyrosine was eluted at ca. 7.5 min after injection and was detected contemporaneously at 220, 280, 350, and 400 nm. NO_2-Tyr was quantified by measuring a calibration curve of 5−10 nitrotyrosine standard solutions.

Analysis of the Protein-Bound 3-Nitrotyrosine Content. The reaction of peroxynitrite with WT-metMb and its mutants was carried out at 20 °C as described previously.[18] 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 at room temperature. 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.[18] Finally, the samples were analyzed by HPLC as described above.

Western Blot Analysis. After treatment with peroxynitrite as described above for the HPLC samples, the proteins were boiled for 3 min at 95 °C in Laemmli sample buffer containing no reducing agents. Samples (5 μg of per lane) were subjected to an 11% SDS-polyacrylamide gel, and were electrophoretically transferred to a pure nitrocellulose membrane in 25 mM Tris, 192 mM glycine, and 20% methanol. Membranes were blocked overnight at 4 °C with 5% dry milk in phosphate-buffered saline containing 0.2% Tween 20. Then the membranes were probed with either monoclonal or a polyclonal antibody to 3-nitrotyrosine as described previously.[18] Complexes were detected by using peroxidase conjugated goat anti-mouse or goat anti-rabbit (1:5000) IgG and then visualized by using the enhanced chemiluminescence detection kit.

Pronase Digestion of Nitrated Proteins. Proteins for Pronase digestion were treated with 20 equiv of peroxynitrite at 20 °C and pH

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7.0, as described in detail above for the HPLC samples. Nitrated proteins (200 μL) were mixed with 200 μL of ammonium sulfamate (50 mM) to remove excess nitrite prior to digestion. After ca. 30 min, $CaCl_2$ was added to the samples (100 μM) to a final concentration of 10 mM. After addition of Pronase (1 mg/mL), the samples were incubated for 12 h at 40 °C, and then another 1 mg/mL Pronase was added and incubated again for 12 h at 40 °C. Before HPLC separation the samples were dried by speed-vac (Eppendorf concentrator 5301 connected to a vacuum pump) at 45 °C, dissolved in 0.1% TFA, and analyzed by HPLC as described above.

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.

Results and Discussion

Kinetic Studies at pH 7.5. Kinetic studies of the decay of peroxynitrite (100 μM) in the presence of the sperm whale WT-metMb, the distal histidine mutants (H64L, H64A, and H64D metMb), and the double mutants (F43W/H64L and H64Y/H93G metMb) were performed by single-wavelength stopped-flow spectroscopy at 20 °C. The traces were collected at 302 nm, the characteristic absorbance maximum for peroxynitrite. All the measured traces could be fitted well to a single-exponential expression. For all proteins studied, the observed peroxynitrite decay rates increased linearly with increasing protein concentration (Figure 2 and S1). The values of k_{cat} obtained from the linear fits are summarized in Table 1. In similar studies with horse heart metMb, we have previously shown that the acceleration of the rate of decay of peroxynitrite is due to its reaction with the heme center of the protein.[13] Indeed, we showed that the heme-free apoMb or the heme-blocked MbFe(III)CN do not catalyze the decay of peroxynitrite.[13]

The values of the rate constants of the reactions of peroxynitrite with H64A and H64D metMb are among the largest measured for reactions with peroxynitrite[26] and are comparable to those of the most efficient iron(III) porphyrin catalysts such as $[Fe(III)TPPS]^{3-}$ ($k_{cat} = 8.6 \times 10^5$ M^{-1} s^{-1}, at pH 7.4 and 37 °C) and $[Fe(III)TMPyP]^{5+}$ ($k_{cat} = 1.6 \times 10^6$ M^{-1} s^{-1}, at pH 7.4 and 37 °C).[14] Interestingly, the only protein containing a pentacoordinated heme (H64L metMb) was not the most efficient catalyst: the two most active mutants were H64A and H64D metMb, which both have a water molecule as the sixth ligand of the iron center. As leucine is larger than alanine and aspartic acid, our results indicate that, when the hydrogen bond that stabilizes the coordinated water is not present, the reactivity toward peroxynitrite is regulated mostly by steric factors. Indeed, substitution of Phe43, a residue close to the heme on its distal side, with the larger Trp residue also slightly reduced the reactivity of the heme toward peroxynitrite (compare the k_{cat} value of F43W/H64L metMb with that of H64L metMb). The H64Y/H93G metMb mutant was the least efficient catalyst (Table 1): its k_{cat} value was even lower than that of sw WT-metMb, which is somewhat lower than that recently reported for hh metMb under the same conditions ($k_{cat} = 2.9 \times 10^4$ M^{-1} s^{-1}).[13] This observation suggests that the proximal side of the heme is less accessible to peroxynitrite. Alternatively, the lower catalytic activity may be attributed to the reduced Lewis acidity of the iron center in H64Y/H93G metMb, a consequence of the tyrosinate ligand bound to the heme.

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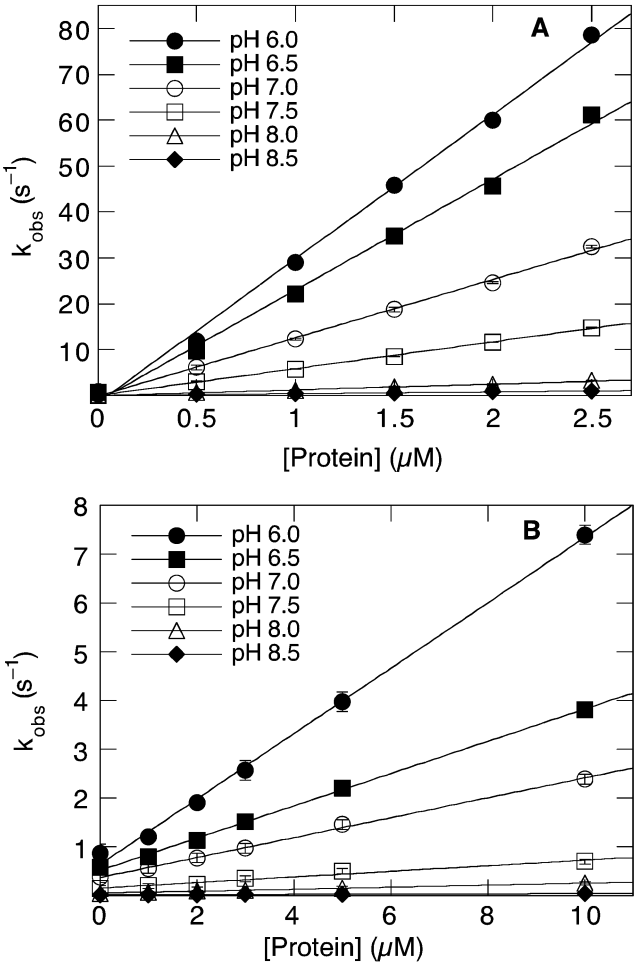


Figure 2. Plots of k_{obs} versus different concentrations of (A) H64A metMb and (B) H64L metMb for the protein-catalyzed decay of peroxynitrite (100 μM) in 0.05 M phosphate buffer in the pH range 6.0−8.5 at 20 °C.

Table 1. Summary of the Catalytic Rate Constants (in M^{-1} s^{-1}) for the Protein-Catalyzed Decay of Peroxynitrite (100 μM) Measured at pH 7.5 and 20 °C in the Presence of Different Amounts of CO_2

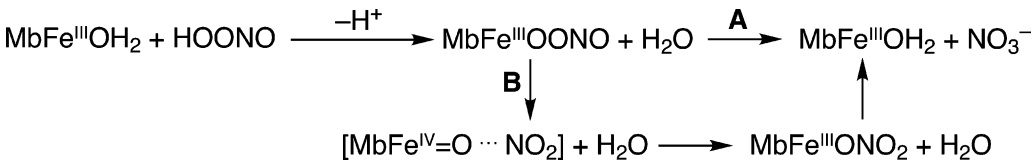
protein	no CO_2	0.6 mM CO_2	1.2 mM CO_2
H64A metMb	$(5.8 \pm 0.1) \times 10^6$	$(5.0 \pm 0.1) \times 10^6$	$(5.4 \pm 0.1) \times 10^6$
H64D metMb	$(4.8 \pm 0.1) \times 10^6$	$(3.8 \pm 0.1) \times 10^6$	$(4.9 \pm 0.1) \times 10^6$
H64L metMb	$(5.7 \pm 0.1) \times 10^4$	$(5.2 \pm 0.4) \times 10^4$	$(6.1 \pm 0.9) \times 10^4$
F43W/H64L metMb	$(5.2 \pm 0.2) \times 10^4$	n.d.[a]	$(7.2 \pm 0.3) \times 10^4$
H64Y/H93G metMb	$(0.9 \pm 0.1) \times 10^4$	n.d.[a]	$(3.7 \pm 0.1) \times 10^4$
sw WT-metMb	$(1.6 \pm 0.1) \times 10^4$	n.d.[a]	n.d.[a]
hh metMb[b]	$(1.2 \pm 0.1) \times 10^4$	n.d.[a]	$(3.9 \pm 0.2) \times 10^4$

[a] Not determined. [b] Reference 13.

Interestingly, a correlation seems to be present between the second-order rate constants of azide binding to H64A-, H64L-, and WT-metMb and the catalytic rate constant for the protein-catalyzed decay of peroxynitrite. At pH 7.0 and 20−25 °C, the values of the azide binding constants also follow the order H64A-metMb ≫ H64L-metMb > WT-metMb (2000, 34, and 2.9 mM^{-1} s^{-1}, respectively).[17] Indeed, it has been shown that azide binding is also strongly influenced by the size of the distal ligands.[17]

Analogous kinetic studies were carried out in the presence of different amounts of CO_2. For all proteins studied, the

Scheme 1. Possible Mechanisms for the MetMb-Catalyzed Isomerization of Peroxynitrite



measured traces could be fitted well to a single-exponential expression. As expected, the values of the observed rate constants were significantly larger than those measured in the absence of CO_2 (Figure S1) because of the rapid reaction of peroxynitrite and CO_2.[4] However, as summarized in Table 1, no significant changes were found between the catalytic rate constants (obtained from the linear fit of the plot of the observed rate constants versus protein concentrations) determined in the absence or in the presence of either 0.6 or 1.2 mM CO_2.[27] As recently observed for the reaction of peroxynitrite with metMb and metHb,[13] the results obtained here suggest that also in the presence of CO_2 the metMb mutants accelerate the decay of peroxynitrite via the fast concurring reaction between peroxynitrite and the iron(III) center of the proteins.

For all Mb mutants studied, we followed their reactions with peroxynitrite, both in the absence and presence of CO_2, also by rapid-scan spectroscopy in the range 480−650 nm (each millisecond, up to 50 s). In all cases, no changes were observed in the UV−vis spectra of the proteins upon addition of an excess of peroxynitrite (data not shown), in analogy to the parallel reaction with hh metMb and human metHb.[13] We have previously reported that the Fe(III)OONO complex, generated as an intermediate during the reaction of $MbFeO_2$ or $HbFeO_2$ with NO•, can be detected only under alkaline conditions.[28,29] Moreover, at pH 9.5, the metMb and metHb−peroxynitrite complexes decay to nitrate and the corresponding iron(III) form of the proteins without formation of observable high valent intermediates.[28,29] At pH 7.0, the NO•-mediated oxidation of oxyMb and oxyHb yields metMb and metHb, respectively, without formation of any detectable intermediates.[28,29] Taken together, these previous results are in excellent agreement with the data presented here. Binding of peroxynitrite to the iron(III) center of the metMb mutants is likely to be the rate-determining step, followed by isomerization and dissociation of nitrate. Thus, no intermediates can be observed during these reactions.

The formation of nitrate and metMb from the intermediate MbFe(III)OONO can proceed according to two possible mechanisms: the coordinated peroxynitrite either isomerizes to free nitrate in a concerted mechanism (pathway A in Scheme 1) or generates an oxoiron(IV)/nitrogen dioxide intermediate which rapidly recombines to the metMb-nitrato complex from which nitrate dissociates (pathway B in Scheme 1). We have previously shown that, under alkaline conditions the formation of metMb and nitrate from MbFe(III)OONO (generated from the reaction between oxyMb and NO•) must proceed in a concerted way according to pathway A (in Scheme 1).[29] Indeed, the rate of dissociation of nitrate from $MbFe(III)NO_3$ is significantly slower than that of MbFe(III)OONO decay[29] and would thus have to be the rate-determining step of the reaction between oxyMb and NO•. In pathway B, the oxoiron(IV) intermediate can be detected only if its reaction with NO_2• is the rate-determining step. Interestingly, Groves and co-workers recently reported that the reaction of peroxynitrite with the H64D/V68L sperm whale metMb mutant leads to the formation of detectable amounts of the high valent oxoiron(IV) form.[30]

The intermediate produced from the reaction of CO_2 with $ONOO^-$, $ONOOCO_2^-$, has been shown to partly generate $CO_3^{\bullet-}$ upon its decomposition.[5,31] The yield of radicals formed from this decay reaction is still a matter of discussion. Indeed, it has been proposed that 30%[32,33] or less than 5%[34] of $ONOOCO_2^-$ decays to form the radicals NO_2• and $CO_3^{\bullet-}$. Our data suggest that the heme center of the iron(III) form of the Mb mutants does not react with these radicals. Indeed, preliminary pulse radiolysis studies of the reaction between hh metMb and $CO_3^{\bullet-}$ show that the iron center is not oxidized and that $CO_3^{\bullet-}$ reacts only with amino acids of the globin.[35]

pH Dependence of the Catalytic Rate Constants. Kinetic studies of the decay of peroxynitrite in the presence of different concentrations of H64A and H64L metMb were carried out in the pH range 6.0−8.5 (at 20 °C). As shown in Figure 2 and summarized in Table 2, for both proteins the values of k_{cat} increased with decreasing pH. At pH 6.0 the values of the catalytic rate constants for H64A and H64L metMb were nearly 2 orders of magnitude larger than those obtained at pH 8.5. A possible explanation for this observation is that peroxynitrous acid (ONOOH) is the species that reacts with the iron center of these proteins. If one assumes that the pH dependence originates from a single ionizable group, the relationship given in eq 1 exists between k_{cat} and the proton concentration.

$$k_{cat} = (k_{cat'} \times [H^+])/(K + [H^+]) \quad (1)$$

In eq 1, $k_{cat'}$ stays for the pH-independent catalytic rate constant and K for the dissociation constant of the protonable group. Despite the narrow pH range studied, 6.0−8.5, the experimental data could be fitted rather well to eq 1. The best fits for the two reactions gave values of $pK = 6.7 \pm 0.5$ and $k_{cat'} = (3.7 \pm 0.1) \times 10^7\ M^{-1}\ s^{-1}$ for H64A metMb and values of $pK = 6 \pm 1$ and $k_{cat'} = (1.1 \pm 0.1) \times 10^5\ M^{-1}\ s^{-1}$ for H64L metMb. Although a large error was associated with the value of the pK obtained for the reaction with H64L metMb, these results support the hypothesis that HOONO ($pK = 6.8$)[36] is the species that reacts with the iron(III) center of these proteins.

(27) Since the reaction is strongly pH dependent (see below), the small differences observed in the second-order rate constants may be attributed to experimental errors in the preparation of solutions with an exact final pH value of 7.5 (see Experimental Procedures).

(28) Herold, S. *FEBS Lett.* **1999**, *443*, 81−84.
(29) Herold, S.; Exner, M.; Nauser, T. *Biochemistry* **2001**, *40*, 3385−3395.
(30) Groves, J. T. *Third International Conference on Peroxynitrite and Reactive Nitrogen Species in Biology and Medicine*; Pacific Grove, CA, 2001.
(31) Bonini, M. G.; Radi, R.; Ferrer-Sueta, G.; Ferreira, A. M. D. C.; Augusto, O. *J. Biol. Chem.* **1999**, *274*, 10802−10806.
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Table 2. Summary of the Catalytic Rate Constants for the Protein-Catalyzed Decay of Peroxynitrite (100 μM) Measured at Different pH (at 20 °C) in the Absence and Presence of CO_2

	H64A metMb (in 10^6 M^{-1} s^{-1})		H64L metMb (in 10^4 M^{-1} s^{-1})	
pH	no CO_2	1.2 mM CO_2	no CO_2	1.2 mM CO_2
6.0	31 ± 1	n.d.[a]	67 ± 1	n.d.[a]
6.5	24 ± 1	26 ± 1	33 ± 1	49 ± 1
7.0	13 ± 1	n.d.[a]	20 ± 1	n.d.[a]
7.5	5.8 ± 0.5	5.4 ± 0.5	5.7 ± 0.1	5.4 ± 0.2
8.0	1.2 ± 0.1	n.d.[a]	2.0 ± 0.1	n.d.[a]
8.5	0.40 ± 0.01	1.3 ± 0.2	0.24 ± 0.01	6 ± 2

[a] Not determined.

This result was unexpected, as it might rather have been anticipated that the positively charged $[(porphyrin)Fe(III)OH_2]^+$ complex reacts preferentially with the deprotonated form $ONOO^-$. Interestingly, previous studies of the reaction between oxyMb and peroxynitrite showed that HOONO is the reactive species also in this system.[10] Taken together, these results may suggest that the diffusion of HOONO toward the heme is facilitated compared to that of $ONOO^-$.

The identical pH dependence of k_{cat} for H64A and H64L metMb suggests that the water molecule bound to the heme in H64A metMb (and absent in H64L metMb) is not responsible for the decrease of the value of k_{cat} at higher pH (deprotonation of the coordinated H_2O can generate a hydroxide, which is likely to be more difficult to displace). Finally, a last explanation for the observed pH dependence of the k_{cat} values may be that conformational changes take place in the protein under acidic conditions and cause the observed acceleration.

Analogous kinetic studies at different pH values were carried out with H64A and H64L metMb also in the presence of 1.2 mM CO_2. As summarized in Table 2, the catalytic rate constants increased with decreasing pH (Figure S2). Moreover, in most cases the values obtained in the presence of added CO_2 were nearly identical to those measured in its absence. This result supports the hypothesis that the acceleration observed is independent of CO_2 and always arises from the reaction of HOONO with the iron(III) center of the protein. To further confirm this hypothesis, we studied the reactions of H64A and H64L metMb with peroxynitrite under alkaline conditions (pH 8.0) and in the presence of a large excess of CO_2 (5 mM). Under these conditions, essentially no HOONO is left after the mixing time. As shown in Figure 3, the decay rate of peroxynitrite was not affected by addition of increasing amounts of either of the proteins. The large errors associated with the values of k_{obs} and the scattering of the data are due to the experimental problems linked to the preparation of different solution with exactly the same CO_2 concentration and pH. Taken together, our results show that the acceleration of the decay of peroxynitrite observed in the presence of physiologically relevant CO_2 concentrations (1.2 mM) arise from the concurring reaction of peroxynitrite with the iron(III) center of the proteins. However, with the results obtained from these kinetic studies it is not possible to find out whether the heme center of the Mb mutants reacts with $ONOOCO_2^-$, the adduct generated from the reaction of $ONOO^-$ with CO_2, or with the products of its decomposition. Nevertheless, since during the reaction of the metMb mutants with peroxynitrite no changes were observed in the UV−vis spectra,

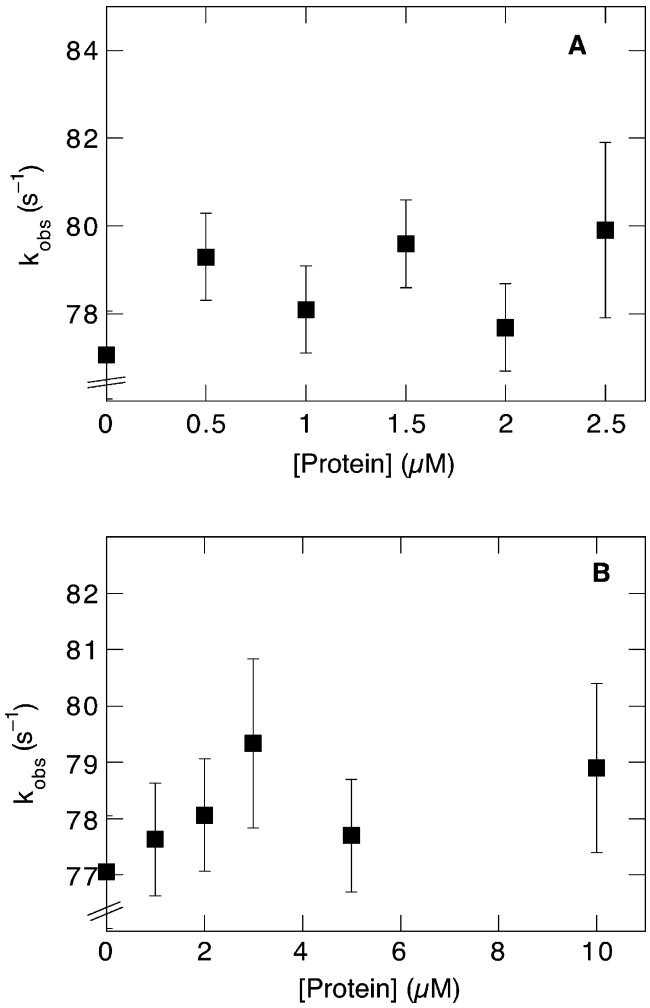

Figure 3. Plots of k_{obs} versus different concentrations of (A) H64A metMb and (B) H64L metMb for the protein-catalyzed decay of peroxynitrite (100 μM) in 0.05 M phosphate buffer pH 8.0 in the presence of 5 mM CO_2 (20 °C).

and since $CO_3^{\bullet-}$ does not oxidize metMb (see above), reactions may take place only with the amino acids residues of the globin.

Analysis of the Nitrogen-Containing Products. The products of the protein-catalyzed decay of peroxynitrite were analyzed by ion chromatography with conductivity detection. As expected,[37] in the absence of the proteins, the decomposition of peroxynitrite (100 μM) yielded 67 ± 3% nitrate and 32 ± 3% nitrite (at pH 7.0). As summarized in Table 3, addition of different amounts of the proteins led to a concentration-dependent increase of the amount of nitrate formed, accompanied by a corresponding decrease of the nitrite yields. The efficiency of the proteins to catalyze the isomerization of peroxynitrite to nitrate correlated with the value of k_{cat}. Thus, addition of 5 μM of either of the two best catalysts, H64A or H64D metMb, led to complete isomerization of peroxynitrite (100 μM) to nitrate, and no nitrite could be detected. In contrast, larger amounts of all other proteins were required for complete conversion of peroxynitrite to nitrate. These results are in agreement with our previous observation that addition of a large amount (750 μM) of hh metMb or human metHb also leads to the quantitative isomerization of peroxynitrite to nitrate.[13] Taken together, our data indicate that when the decomposition of

(36) Koppenol, W. H.; Moreno, J. J.; Pryor, W. A.; Ischiropoulos, H.; Beckman, J. S. *Chem. Res. Toxicol.* **1992**, *5*, 834−842.

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Table 3. Distribution of the Products of Peroxynitrite (100 μM) Decay in the Presence of Different Concentrations of WT-metMb and metMb Mutants (in 0.05 M Phosphate Buffer pH 7.0 and 20 °C), in the Absence and Presence of 1.2 mM CO_2[a]

	no added CO_2			1.2 mM CO_2		
proteins (μM)	NO_2^-	NO_3^-	total	NO_2^-	NO_3^-	total
no protein	32 ± 3	67 ± 3	99	15 ± 3	87 ± 2	102
H64A (1 μM)	3 ± 1	95 ± 1	98	2 ± 3	99 ± 4	101
H64A (5 μM)	0	100 ± 2	100	0	100 ± 3	99
H64D (1 μM)	5 ± 2	96 ± 3	101	3 ± 1	97 ± 2	100
H64D (5 μM)	0	100 ± 3	100	0	100 ± 2	100
H64L (1 μM)	29 ± 3	73 ± 4	102	20 ± 3	81 ± 3	101
H64L (5 μM)	21 ± 2	81 ± 2	102	18 ± 2	81 ± 2	99
H64L (25 μM)	10 ± 2	91 ± 3	101	8 ± 2	93 ± 2	101
F43W/H64L (25 μM)	15 ± 2	86 ± 2	101	13 ± 4	87 ± 3	100
H64Y/H93G (25 μM)	20 ± 2	80 ± 3	100	18 ± 3	84 ± 3	102
WT-metMb (25 μM)	24 ± 3	77 ± 2	101	20 ± 2	82 ± 3	102

[a] The data are expressed as percentage yields.

peroxynitrite occurs exclusively via the heme, either in the presence of a very efficient catalyst such as H64A metMb or by addition of very large amounts of the ineffective catalyst hh metMb, no nitrite is generated. This conclusion is in contrast to the results of Groves and co-workers[38] who observed that a constant level of approximately 10% nitrite was produced from the decay of peroxynitrite even in the presence of large concentrations of metMb. According to the mechanism proposed by these authors (pathway B in Scheme 1),[38] nitrite is generated from $NO_2^{\bullet}$, which partly eludes the reaction with ferrylMb and undergoes hydrolysis to nitrite and nitrate.

As expected, in the presence of 1.2 mM CO_2 the yield of nitrate generated from the decay of 100 μM peroxynitrite (in the absence of the proteins, at pH 7.0 and 20 °C) was significantly larger: we obtained 15 ± 3% nitrite and 87 ± 2% nitrate. As summarized in Table 3, also under these conditions addition of small amounts (1−5 μM) of the active catalysts H64A and H64D metMb led to the complete conversion of peroxynitrite to nitrate. In contrast, in the presence of 25 μM of the less effective catalysts (H64L, H64L/F43W, H64Y/H63G, and WT-metMb), the products distribution remained approximately unchanged. These results are in agreement with the hypothesis that the best Mb catalysts can efficiently isomerize the peroxynitrite that eludes reaction with CO_2.

Protection against Peroxynitrite-Mediated Nitration of Free Tyrosine. To find out whether the metMb mutants investigated protect against peroxynitrite-mediated nitration or catalyze this reaction, we determined the yield of 3-nitrotyrosine (NO_2-Tyr) formed from the reaction of peroxynitrite with free tyrosine in the presence of different concentrations of the proteins. For this purpose, we mixed 200 μM peroxynitrite with 100 μM tyrosine in the presence of increasing amounts (0−10 μM) of H64A, H64D, H64L, F43W/H64L, H64Y/H93G, and WT-metMb (in 0.05 M phosphate buffer, pH 7.4 and 20 °C). As shown in Figure 4A (and summarized in Table S1), H64A and H64D metMb are very efficient scavengers of peroxynitrite: in the presence of 5 μM of these proteins no NO_2-Tyr was generated. As expected from the values of k_{cat}, the other proteins studied were significantly less efficient. However, the NO_2-Tyr yields always decreased with increasing amounts of proteins added (Figure 4A).

The different extent of protection exhibited by the metMb mutants can be explained by comparing the rate of the protein-

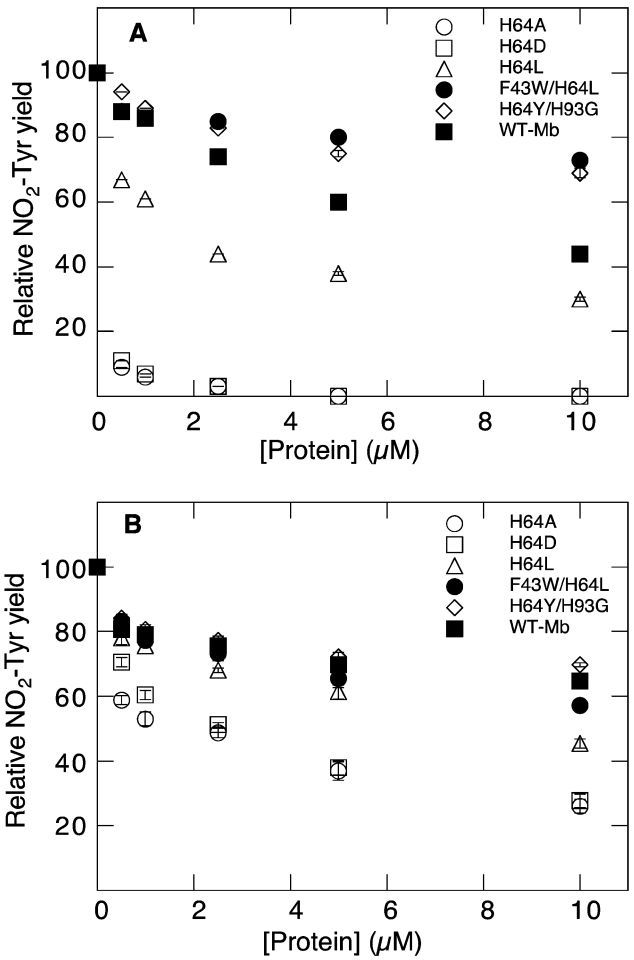

Figure 4. Relative yield of NO_2-Tyr formed from the reaction of peroxynitrite (200 μM) and tyrosine (100 μM), in the presence of WT-metMb and metMb mutants in 0.05 M phosphate buffer pH 7.4, 20 °C. Experiments (A) in the absence and (B) in the presence of 1.2 mM CO_2. Relative yield = (yield with added protein/yield with no protein) × 100%. The data are given in Table S1.

catalyzed isomerization of peroxynitrite with that of the proton-mediated process. For instance, under the conditions of our experiment the rate of the reaction between 5 μM H64A metMb and 200 μM peroxynitrite is 29 s^{-1} (k_{obs} = (5.8 × 10^6 M^{-1} s^{-1}) × (5 × 10^{-6} M) = 29 s^{-1}) and the decay of peroxynitrite is 0.3 s^{-1} at pH 7.4. Thus, in agreement with our experimental results, in the presence of 5 μM of H64A metMb less than 1% of peroxynitrite is expected to react with tyrosine and over 99% will be isomerized through its reaction with H64A metMb. In contrast, the reaction of peroxynitrite (200 μM) with 10 μM of less efficient mutant H64L proceeds at a rate of 0.57 s^{-1} (k_{obs} = (5.7 × 10^4 M^{-1} s^{-1}) × (10 × 10^{-6} M) = 0.57 s^{-1}). Thus, under these conditions only approximately 34% of peroxynitrite is expected to be available for reaction with free tyrosine and should lead to a relative NO_2-Tyr yield of 34% (a relative yield of 100% corresponds to the amount obtained in the absence of the protein, with 100% peroxynitrite), in excellent agreement with our experimental value of 30 ± 1%.

Analogous studies were carried out in the presence of 1.2 mM CO_2. As expected,[4] addition of CO_2 led to an increase of the absolute NO_2-Tyr yields generated from the reaction of

(38) Bourassa, J. L.; Ives, E. P.; Marqueling, A. L.; Shimanovich, R.; Groves, J. T. *J. Am. Chem. Soc.* **2001**, *123*, 5142−5143.

peroxynitrite with tyrosine. As observed in the absence of CO_2, addition of increasing amounts of all metMb mutants studied induced a concentration-dependent decrease of the NO_2-Tyr yields (Figure 4B and Table S1). The order of efficiency of the different mutants was not influenced by addition of CO_2, and once more mirrored the values of k_{cat}. However, in the presence of 1.2 mM CO_2, the metMb mutants were all less efficient to prevent the peroxynitrite-mediated nitration of added tyrosine. For instance, in the presence of 5 μM H64A metMb the relative NO_2-Tyr yield was 37 $\pm$ 3%. This result can be explained by the significant increase of the rate of decay of peroxynitrite in the presence of 1.2 mM CO_2, the process that leads to NO_2-Tyr formation. Indeed, under the conditions of our experiments the decay rate of peroxynitrite is approximately 24.5 s^{-1}. In the presence of 5 μM H64A metMb peroxynitrite reacts with the protein at a rate of 27 s^{-1}, and thus a relative nitration yield of 47% would be expected. The observation that the expected yield is larger than that measured experimentally suggests that the protein reacts with $ONOOCO_2^-$ and/or the products derived from its decomposition and thus partly prevents the formation of free NO_2-Tyr. This hypothesis is supported by the yields obtained in the presence of the less efficient catalysts which were all significantly lower than the calculated yields. For instance, the expected NO_2-Tyr yield in the presence of 10 μM H64L metMb is 96%, whereas only 45 $\pm$ 1% NO_2-Tyr was obtained. As discussed above, the heme is not involved in this scavenging process, which must thus take place through reaction with the amino acid residues of the globin.

To further support the hypothesis that the nitrating agent derived from the reaction of peroxynitrite with CO_2 can be scavenged by the globin, we carried out a similar experiment at high pH (8.0) and in the presence of a very large CO_2 concentration (5 mM). As already mentioned above, under these conditions essentially no HOONO is left after the mixing time. Under these conditions, addition of 200 μM peroxynitrite to 100 μM Tyr in the presence of 2.5−10 μM H64A or H64L reduced the amount of NO_2-Tyr formed up to 20%. Taken together, our data show that in the presence of physiological concentrations of CO_2 the peroxynitrite that eludes reaction with CO_2 is scavenged by the iron(III) center, whereas the amino acid residues of the globin scavenge, albeit not very efficiently, $ONOOCO_2^-$ and/or the products derived from its decomposition.

Nitration of the Globins After Treatment of the metMb Mutants with Excess Peroxynitrite. Sperm whale Mb contains three tyrosine residues (Tyr103, Tyr146, and Tyr151), one more (Tyr151) than hh Mb. The extent of nitration of these residues after treatment of the mutants with an excess of peroxynitrite was first assessed by Western blot analysis with anti-NO_2-Tyr antibody. As shown in Figure 5, exposure of H64A and H64D metMb to 10 or 20 equiv of peroxynitrite induced nitration in a dose-dependent way. In comparison, the less efficient scavenger H64L metMb was nitrated to a larger extent. Moreover, the amount of nitration of F43W/H64L, H64Y/H93G metMb was comparable to that of sw WT-metMb, but was significantly higher than that of hh metMb.[18] Taken together, these blots suggest that the metMb mutants were nitrated to an extent inversely correlated to their efficiency as peroxynitrite scavengers, that is, H64A $\approx$ H64D $\ll$ H64L $\approx$ F43W/H64L $<$ H64Y/H93G $\approx$ WT-metMb. Interestingly, after treatment with 20 equiv of peroxynitrite a second nitrated band of approximately 29 kDa was present in the blots, in particular H64L, H64Y/H93G, and F43W/H64L metMb. A much smaller amount of nitrated dimer was formed from the reaction with hh metMb.[18] Thus, our results suggest that Tyr151, which is not present in hh metMb, is responsible for the formation of the dimer. A similar observation was made upon reaction of hydrogen peroxide with sw metMb and hh metMb.[39] Indeed, Ortiz de Montellano and co-workers demonstrated that sw metMb (but not hh metMb) forms a dityrosine cross-link between Tyr151 on one molecule and Tyr103 on the other.[39] Moreover, site-directed mutagenesis of Tyr103 and/or Tyr146 to a phenyalanine still led to the formation of dimers, whereas mutation of Tyr151 completely blocked dimerization.[40] Thus, Tyr151−Tyr151 dimers must also be formed.

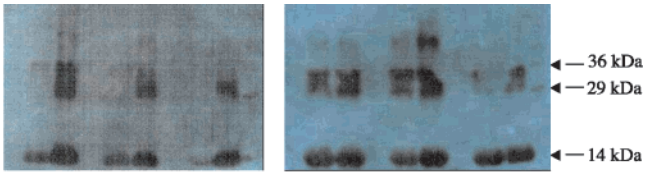

Figure 5. Anti-nitrotyrosine immunoblotting of WT-metMb and metMb mutants. Samples were separated on an 11% SDS polyacrylamide gel and examined by Western blot analysis with a polyclonal antibody against nitrotyrosine as described in the Experimental Procedures. The proteins (100 μM) were incubated in 0.1 M phosphate buffer pH 7.0 at 0 °C with decomposed peroxynitrite (negative control) and with increasing amounts of peroxynitrite in the absence of added CO_2. Lanes 1−3, H64L metMb mixed with decomposed peroxynitrite, 10, or 20 equiv of peroxynitrite; lanes 4−6, H64D metMb mixed with decomposed peroxynitrite, 10, or 20 equiv of peroxynitrite; lanes 7−9, H64A metMb mixed with decomposed peroxynitrite, 10, or 20 equiv of peroxynitrite; lane 10, molecular weight marker; lanes 11−13, H64Y/H93G metMb mixed with decomposed peroxynitrite, 10, or 20 equiv of peroxynitrite; lanes 14−16, F43W/H64L metMb mixed with decomposed peroxynitrite, 10, or 20 equiv of peroxynitrite; lanes 17−19, WT-metMb mixed with decomposed peroxynitrite, 10, or 20 equiv of peroxynitrite; lane 20, molecular weight marker.

As shown in Figure 6, in the presence of 1.2 mM CO_2, the blots of all the proteins treated analogously with peroxynitrite showed much more intense bands, suggesting a larger extent of nitration. Moreover, the amount of dimeric and trimeric nitrated proteins was considerable, in particular F43W/H64L, H64L, and H64D metMb. Interestingly, visualization of the proteins by staining with Coomassie Blue (Figure 4S) indicated that the largest fraction of the proteins was in the monomeric form, thus suggesting that the oligomers are preferentially nitrated. It has been reported that under neutral conditions Trp radicals generated within a protein can transfer their electron deficiency to tyrosine residues.[41] This process takes place at rates in the range 10^2-10^4 s^{-1} and leads to the formation of Tyr radicals.[41] Our results suggest that Trp43, located close to the heme, may be oxidized by peroxynitrite in the presence of CO_2, lead to the formation of additional Tyr radicals, and consequently generate larger amounts of nitrotyrosine as well as of dimeric and/or oligomeric species. Interestingly, recent reinvestigation of the reaction of sw metMb with H_2O_2 also showed the formation of trimeric as well as tetrameric products.[42]

(39) Tew, D.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **1988**, *263*, 17880−17886.
(40) Wilks, A.; Ortiz de Montellano, P. *J. Biol. Chem.* **1992**, *267*, 8827−8833.
(41) Butler, J.; Land, E. J.; Prütz, W. A.; Swallow, A. J. *Biochim. Biophys. Acta* **1982**, *705*, 150−162.

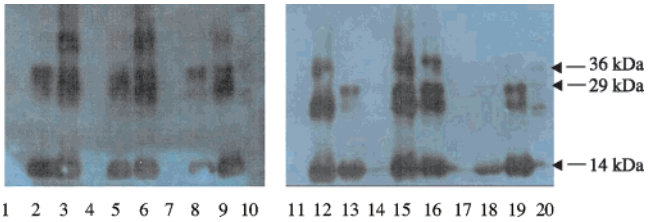

Figure 6. Anti-nitrotyrosine immunoblotting of WT-metMb and metMb mutants. Samples were separated on an 11% SDS polyacrylamide gel and examined by Western blot analysis with a polyclonal antibody against nitrotyrosine as described in the Experimental Procedures. The proteins (100 μM) were incubated in 0.1 M phosphate buffer pH 7.0 at 0 °C with decomposed peroxynitrite (negative control) and with increasing amounts of peroxynitrite in the presence of 1.2 mM CO_2. Lanes 1−3, H64L metMb mixed with decomposed peroxynitrite, 10, or 20 equiv of peroxynitrite; lanes 4−6, H64D metMb mixed with decomposed peroxynitrite, 10, or 20 equiv of peroxynitrite; lanes 7−9, H64A metMb mixed with decomposed peroxynitrite, 10, or 20 equiv of peroxynitrite; lane 10, molecular weight marker; lanes 11−13, H64Y/H93G metMb mixed with decomposed peroxynitrite, 10, or 20 equiv of peroxynitrite; lanes 14−16, F43W/H64L metMb mixed with decomposed peroxynitrite, 10, or 20 equiv of peroxynitrite; lanes 17−19, WT-metMb mixed with decomposed peroxynitrite, 10, or 20 equiv of peroxynitrite; lane 20, molecular weight marker.

To quantify the amount of nitration of the tyrosine residues in H64A, H64D, H64L, F43W/H64L, H64Y/H93G, and WT-metMb, we mixed these proteins with an excess of peroxynitrite (at 20 °C and pH 7.0), subjected them to acid hydrolysis, and analyzed the products by HPLC. To avoid artifactual nitration by nitrite present in the peroxynitrite solution (up to 50% relative to the peroxynitrite concentration), we treated the samples with a concentrated acidic ammonium sulfamate solution (before hydrolysis with HCl). The results, shown in Figure 7 (and summarized in Table S2), confirm the qualitative data of the immunohistochemical analyses discussed above. The three tyrosine residues of the two efficient scavengers, H64A and H64D metMb, were nitrated less than 1%, even upon addition of 20 equiv of peroxynitrite. Interestingly, despite the similar values of k_{cat}, F43W/H64L metMb was always nitrated to a significantly larger extent than H64L metMb. As discussed above, Trp43 may be responsible for the formation of larger amounts of NO_2-Tyr, upon reaction of F43W/H64L metMb with peroxynitrite. Indeed, Trp43 radicals, generated either from the direct reaction of Trp43 with peroxynitrite or by a mechanism mediated by the heme, may form additional Tyr radicals, which may recombine with $NO_2^{\bullet}$ to yield NO_2-Tyr.

As shown in Figure 7, HPLC analyses confirmed that sw metMb is nitrated to a significantly larger extent than hh metMb, confirming the hypothesis that Tyr151 is nitrated more readily than Tyr103 and Tyr146. Finally, the most nitrated Mb mutant was H64Y/H93G metMb. Because the UV−vis spectrum of the protein remained unchanged even after treatment with a large excess of peroxynitrite (data not shown), the increased extent of nitration does not arise from nitration of the additional Tyr64. Indeed, such a modification would be expected to cause discernible changes in the absorbance spectrum of the protein. Taken together, these results rather indicate that the higher degree of nitration is due to the poor ability of H64Y/H93G metMb to scavenge peroxynitrite.

In the presence of 1.2 mM CO_2, the yields of NO_2-Tyr produced after treatment of all proteins with an excess of peroxynitrite were generally higher but followed a trend analogous to that observed in the absence of CO_2 (Table S2 and Figure 7B; note the different scale of the *y*-axis in the two

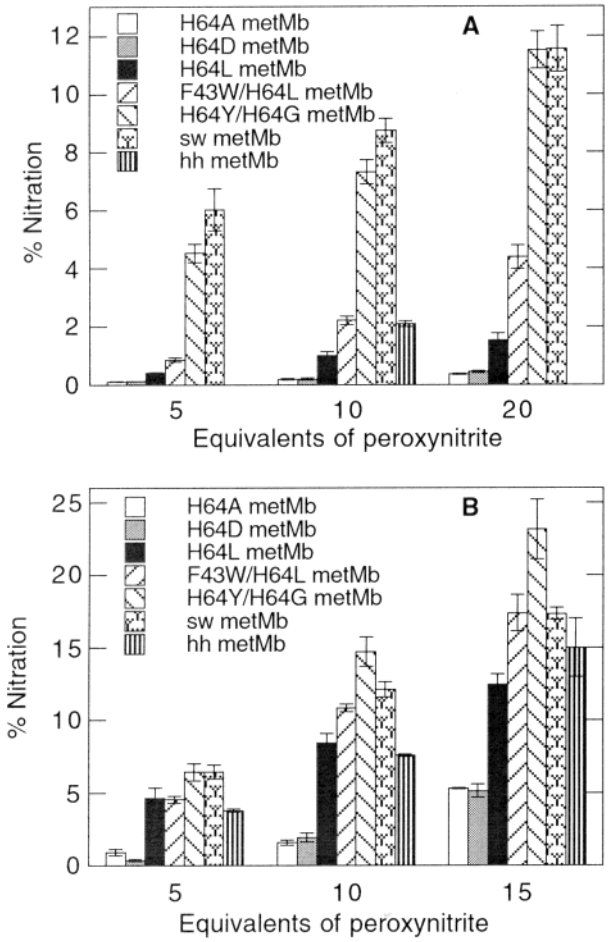

Figure 7. NO_2-Tyr yields (% relative to the total content of tyrosine residues available for nitration, that is, three residues per heme for sw Mb and two for hh Mb), determined by HPLC after acid hydrolysis of the proteins, from the reaction of WT-metMb and metMb mutants (100 μM) with 5, 10, and 20 equiv of peroxynitrite (A) in the absence and (B) in the presence of 1.2 mM CO_2 at 20 °C and pH 7.0. The data are given in Table S2.

plots of Figure 7). Because of the large values of k_{cat}, H64A and H64D metMb can still compete with CO_2 for peroxynitrite and significantly reduce the extent of nitration of the tyrosine residues in the protein. All other metMb mutants studied display a value of k_{cat} close to that of the second-order rate constant of the reaction between peroxynitrite and CO_2 (3×10^4 M^{-1} s^{-1} at 24 °C).[4] Thus, in the presence of 1.2 mM, only a small amount of peroxynitrite can be detoxified by the heme center of these mutants. It is important to note that in the experiments with 10−20 equiv of peroxynitrite (1−2 mM) the concentration of CO_2 is comparable to that of peroxynitrite. However, it has been shown that the reaction between CO_2 and $ONOO^-$ is catalytic and regenerates CO_2,[43] and thus these reaction conditions may simply lead to a slower reaction (since the pseudo-first-order conditions are not maintained). Finally, as clearly depicted in Figure 7B, the yields of nitration of the tyrosine residues of sw metMb were significantly larger than those of hh metMb, again indicating that Tyr151 is a preferential site of nitration also in the presence of CO_2.

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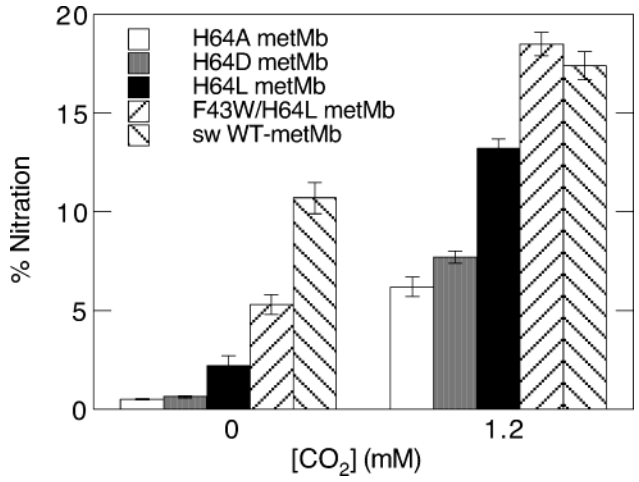

Figure 8. NO_2-Tyr yields (% relative to the total content of the proteins, that is, three tyrosine residues per heme), determined by HPLC after Pronase digestion after treatment of WT-Mb and Mb mutants (100 μM) with 20 equiv of peroxynitrite, in the presence of 0 or 1.2 mM added CO_2 (at 20 °C and pH 7.0).

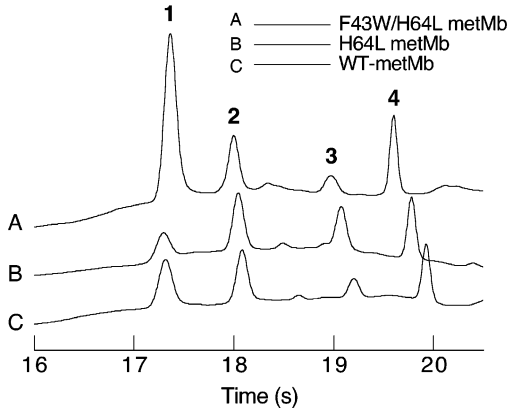

Figure 9. Sections corresponding to the nitrotryptophan species of the HPLC chromatograms (measured at 350 nm) of the products of the reactions of 100 μM WT-metMb, F43W/H64L metMb, and H64L metMb with 20 equiv of peroxynitrite, in 0.1 M phosphate buffer pH 7.0 at 0 °C, after complete digestion with Pronase as described in the Experimental Procedures.

To confirm the NO_2-Tyr yields obtained after acid hydrolysis of the treated proteins, we hydrolyzed the nitrated proteins also by a milder method, that is, with Pronase, and then analyzed the samples by HPLC. As shown in Figure 8 (and summarized in Table S3), both in the absence and in the presence of 1.2 mM CO_2, the yields of NO_2-Tyr were slightly larger (5−25%) than those quantified after acid hydrolysis. As recently reported,[18] this small difference may be due to partial decomposition of NO_2-Tyr during acid hydrolysis.

Interestingly, HPLC analyses of pronase-digested protein samples showed that, in addition to NO_2-Tyr, other nitrated species were formed, as additional peaks were present in the chromatograms measured at 350 nm (Figure 9). For H64L, F43W/H64L, and WT-metMb, four peaks of the chromatogram measured between 17 and 20 min displayed absorbance spectra characteristic for nitrotryptophan derivatives. The assignment of the peaks to the corresponding NO_2-Trp isomers was rather difficult because of the presence of an overlapping absorbance probably derived from heme impurities. However, comparison of the spectra with those known for the different NO_2-Trp isomers[18,44,45] allowed for the assignment of the peaks at 17.3 (Peak 1), 18.0 (Peak 2), and 19.8 min (Peak 4) to the 6-, 4-, and 5-nitrotryptophan, respectively. We have recently shown that 6-, 5-, and 4-NO_2-Trp are generated also when apo-, oxy- or metMb (horse heart) are treated with excess of peroxynitrite.[18] Additional studies are required to identify which of the two tryptophan residues, Trp7 and/or Trp14, is modified upon reaction with peroxynitrite. As shown in Figure 9, comparison of the intensity of the NO_2-Trp peaks suggests that the additional Trp residue present in F43W/H64L metMb may also be nitrated. Indeed, especially the peak at 17.3 min assigned to 6-NO_2-Trp was significantly larger for peroxynitrite-treated F43W/H64L metMb.

Conclusions

We have shown that the reactivity of peroxynitrite toward metMb is regulated by the distal amino acid residues. The strong hydrogen bond between the distal histidine and the water molecule bound to the iron(III) center in WT-metMb considerably reduces the efficiency of the protein to catalyze the isomerization of peroxynitrite to nitrate. In the absence of a residue which can undergo hydrogen bonds, the size of the distal ligands determines the effectiveness of the catalysis. In the presence of CO_2, only the fraction of peroxynitrite that eludes reaction with CO_2 reacts with the iron(III) center of the protein, and this reaction is responsible for the observed catalytic effect. The adduct $ONOOCO_2^-$ and/or the products derived from its decay are partly scavenged by the globin. However, this reaction is not catalytic and consequently significantly less effective.

In contrast to peroxidases, the iron(III) forms of WT-metMb and its mutants protect free tyrosine from peroxynitrite-mediated nitration. The extent of protection is directly correlated to the value of k_{cat}. In the absence of free tyrosine, sperm whale metMb is significantly more nitrated by an excess of peroxynitrite than hh metMb. This result suggests that, analogously to the reaction with H_2O_2, Tyr151 is more easily modified, and thus larger amounts of nitrotyrosine and oligomerization products are detected upon reaction with an excess of peroxynitrite. The double mutant F43W/H64L metMb, which contains an additional Trp residue close to the distal pocket of the heme, is the mutant nitrated to the largest extent. This result suggests that facilitated generation of Trp radicals followed by H-atom transfer produces Tyr radicals, which can then lead to nitrotyrosine or dityrosine modifications. In conclusion, our results show that myoglobin represents an ideal platform to investigate the influence of single amino acid residues on the reactivity of hemoproteins with peroxynitrite.

Supporting Information Available: Primary kinetic data for the H64Y/H93G and F43W/H64L metMb-catalyzed decay of 100 μM peroxynitrite at pH 7.5 and 20 °C in the absence and presence of 1.2 mM CO_2 (Figure S1). Primary kinetic data for

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the H64A and H64L metMb-catalyzed decay of 100 μM peroxynitrite at pH 6.5, 7.5, and 8.5 (at 20 °C) in the presence of 1.2 mM CO_2 (Figure S2). Primary kinetic data for the H64A and H64L metMb-catalyzed decay of 100 μM peroxynitrite at pH 7.5 and 20 °C in the presence of 0.6 mM CO_2 (Figure S3). Coomassie Blue staining of proteins treated with peroxynitrite in the presence of 1.2 mM CO_2 (Figure S4). All the relative NO_2-Tyr yields formed from the reaction of 200 μM peroxynitrite and 100 μM tyrosine in the presence of different concentrations of H64A, H64D, H64L, F43W/H64L, H64Y/H93G, and sw WT-metMb (Table S1). All the NO_2-Tyr yields determined by mixing H64A, H64D, H64L, F43W/H64L, H64Y/H93G, and sw WT-metMb with 5, 10, or 20 equiv of peroxynitrite at pH 7.0 and 20 °C (Table S2). All NO_2-Tyr yields determined by Pronase digestion after mixing H64A, H64D, H64L, F43W/H64L, and sw WT-metMb with 10 equiv of peroxynitrite at pH 7.0 and 20 °C (Table S3) (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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