Kinetic and Mechanistic Studies of the NO•-Mediated Oxidation of Oxymyoglobin and Oxyhemoglobin†

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ABSTRACT: The second-order rate constants for the reactions between nitrogen monoxide and oxymyoglobin or oxyhemoglobin, determined by stopped-flow spectroscopy, increase with increasing pH. At pH 7.0 the rates are $(43.6 \pm 0.5) \times 10^6$ M^{-1} s^{-1} for oxymyoglobin and $(89 \pm 3) \times 10^6$ M^{-1} s^{-1} for oxyhemoglobin (per heme), whereas at pH 9.5 they are $(97 \pm 3) \times 10^6$ M^{-1} s^{-1} and $(144 \pm 3) \times 10^6$ M^{-1} s^{-1}, respectively. The rate constants for the reaction between oxyhemoglobin and NO• depend neither on the association grade of the protein (dimer/tetramer) nor on the concentration of the phosphate buffer (100−1 mM). The nitrogen monoxide-mediated oxidations of oxymyoglobin and oxyhemoglobin proceed via intermediate peroxynitrito complexes which were characterized by rapid scan UV/vis spectroscopy. The two complexes $MbFe^{III}OONO$ and $HbFe^{III}OONO$ display very similar spectra with absorption maxima around 500 and 635 nm. These species can be observed at alkaline pH but rapidly decay to the met-form of the proteins under neutral or acidic conditions. The rate of decay of $MbFe^{III}OONO$ increases with decreasing pH and is significantly larger than those of the analogous complexes of the two subunits of hemoglobin. No free peroxynitrite is formed during these reactions, and nitrate is formed quantitatively, at both pH 7.0 and 9.0. This result indicates that, as confirmed from protein analysis after reacting the proteins with NO• for 10 times, when peroxynitrite is coordinated to the heme of myoglobin or hemoglobin it rapidly isomerizes to nitrate without nitrating the globins in physiologically significant amounts.

Nitrogen monoxide (NO•) is currently a species of extreme biological interest because of the variety of physiological functions that are associated with this inorganic messenger molecule (*1*−*4*). Among other things, it is involved in the transmission of nerve impulses, in smooth muscle relaxation, in the inhibition of platelet aggregation, in regulation of blood pressure and of mitochondrial respiration, and in the immune response to bacterial infection. Overproduction of NO• triggers toxicological events such as toxic shock, tissue free radical injury, and DNA damage (*5*, *6*) and is responsible for pathological conditions such as inflammation, diabetes, stroke, neurodegeneration, and sepsis (*7*).

NO• undergoes two major processes in biological systems: nearly diffusion-controlled reaction with superoxide ($O_2^{\bullet-}$) to form peroxynitrite[1] (*8*, *9*) and reaction with different metal centers of proteins (*10*). Peroxynitrite, which is relatively stable in the anionic form ($ONOO^-$) but rapidly isomerizes to nitrate when protonated (HOONO, $pK_a = 6.8$) (*8*, *11*), has been shown to initiate lipid peroxidation (*12*), damage DNA (*13*), oxidize thiols (*14*, *15*) and methionine (*16*−*18*), and hydroxylate aromatic compounds (*19*−*21*). In addition, peroxynitrite can also nitrate aromatic compounds (*22*), a reaction that can be catalyzed by metal complexes (*23*−*25*) or metal-containing proteins (*23*, *26*−*28*). As many products resulting from all these reactions are indistinguishable from those seen with other oxidants such as hydroxyl radical and nitrogen dioxide (*29*), peroxynitrite is likely to be responsible for the cytotoxicity originally attributed to NO• or other oxygen-derived species.

Probably one of the most significant aspects of NO• chemistry is its ability to react in a unique way with the heme centers of different proteins such as nitric oxide synthase, guanylate cyclase, hemoglobin (Hb),[2] and myoglobin (Mb) (*30*). Of particular interest is the rapid reaction of NO• with oxyhemoglobin ($HbFeO_2$), which significantly reduces the half-life of NO• in vivo and is the cause for an increase in blood pressure observed when extracellular Hb-based blood substitutes are administered (*31*, *32*). As NO• can rapidly diffuse away from where it is produced (*33*, *34*) and can easily cross membranes − because of its solubility in hydrophobic phases (*35*) − it has been argued that the rapid reaction with $HbFeO_2$ would result in a NO• concentration too low to activate its target enzyme, guanylate cyclase (*36*, *37*). Recently, two theories have been presented to explain this paradox. First, it has been shown that the reaction of NO• with intact red blood cells is limited by the rate of

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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).

[2] Abbreviations: Hb, hemoglobin; Mb, myoglobin; $HbFeO_2$, oxyhemoglobin; $MbFeO_2$, oxymyoglobin; metHb, iron(III)hemoglobin; metMb, iron(III)myoglobin; $MbFe^{IV}$═O, ferrylmyoglobin; $HbFe^{IV}$═O, ferrylhemoglobin; TFA, trifluoroacetic acid; HPLC, high-pressure liquid chromatography.

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diffusion of NO• through the erythrocyte membrane and thus is about 3 orders of magnitude slower than that with an equivalent amount of free $HbFeO_2$ (*36*). Second, it has been demonstrated that the intravascular flow generates a red blood cell-free zone near the vessel wall, which contributes to the reduced consumption of NO• by the red blood cells under physiological conditions (*38*). It has also been proposed that the low concentration of NO• generated from the endothelial cells of the blood vessels is not scavenged by $HbFeO_2$ but instead reacts with the partially deoxygenated Hb, is stored as an iron(II)nitrosyl complex, and is transferred to β-Cys83 and liberated when needed (*39*).

It has often been proposed that, in analogy to the reaction between NO• and superoxide, the reaction between $HbFeO_2$ and NO• might generate peroxynitrite, free or coordinated to methemoglobin (*7*, *31*, *40*, *41*). It is of great importance to verify this hypothesis because peroxynitrite is a powerful oxidizing and nitrating agent which could possibly damage amino acid residues of the protein. To gain a better understanding of the mechanism of the NO•-mediated oxidations of oxymyoglobin ($MbFeO_2$) and $HbFeO_2$, we have studied the kinetics of these reactions by stopped-flow spectroscopy in the pH range 5−9.5. As partially reported in a short communication (*42*), intermediate peroxynitrito−metmyoglobin and −methemoglobin complexes can be spectroscopically characterized under alkaline conditions but rapidly decay under neutral or acidic conditions. Analysis by ion chromatography of the nitrogen-containing products show that nitrate is formed quantitatively, and analyses by HPLC of the globins after cycling the reaction − oxidation with NO• and reduction with ascorbate − 10 times indicated that only trace amounts (<0.1%) of nitrotyrosine are generated. We thus conclude that the coordinated peroxynitrite isomerizes too rapidly to be able to damage the proteins in physiologically significant amounts.

EXPERIMENTAL PROCEDURES

Reagents. Buffer solutions for kinetic measurements (0.1 M) were prepared from K_2HPO_4/KH_2PO_4 (Fluka) or from $Na_2B_4O_7{\cdot}10H_2O$/NaOH (Fluka) with deionized Milli-Q water. Buffer solutions for ion chromatography analysis (1 mM) were prepared from Na_2HPO_4/NaH_2PO_4 (Fluka), as these salts were found to be less contaminated with nitrite and nitrate ions. Sodium nitrite, sodium nitrate, sodium dithionite, sodium L-ascorbate and nitro-L-tyrosine were supplied from Fluka. NO• was obtained from Linde and passed through a degassed NaOH solution as well as a column of NaOH pellets to remove higher nitrogen oxides before use. Purified human $HbFeO_2$ stock solution HbA_0 (57 mg/mL solution with approximately 1.1% methemoglobin) was a kind gift from APEX Bioscience, Inc. (NC). Lyophilized horse heart Mb was purchased from Sigma.

NO• and Protein Solutions. NO• solutions were prepared by degassing water for at least 30 min with N_2 and then saturating it with NO•. The obtained stock solution (ca. 2 mM) was diluted with degassed buffer in gastight Sample-Lock Hamilton syringes. The final NO• concentrations were measured with an ANTEK Instruments nitrogen monoxide analyzer, with a chemiluminescent detector.

$MbFeO_2$ was prepared by reducing the Sigma metmyoglobin with a slight excess of sodium dithionite. The solution was purified chromatographically on a Sephadex G25 column using a 0.1 M phosphate buffer solution, pH 7.0, as the eluant. $MbFeO_2$ solutions were prepared by diluting this stock solution with buffer, and concentrations were determined by measuring the absorbance at 417, 542, and/or 580 nm (ϵ_{417} = 128 mM^{-1} cm^{-1}, ϵ_{542} = 13.9 mM^{-1} cm^{-1}, and ϵ_{580} = 14.4 mM^{-1} cm^{-1}) (*43*).

$HbFeO_2$ solutions were diluted analogously, and concentrations were determined by measuring the absorbance at 415, 541, and/or 577 nm (ϵ_{415} = 125 mM^{-1} cm^{-1}, ϵ_{541} = 13.8 mM^{-1} cm^{-1}, and ϵ_{577} = 14.6 mM^{-1} cm^{-1}) (*43*). Absorption spectra were collected on a UVIKON 820 spectrophotometer.

Stopped-Flow Kinetic Analysis. Kinetic studies were carried out with an Applied Photophysics SX17MV single-wavelength stopped-flow instrument and an On-Line Instrument Systems, Inc. stopped-flow instrument equipped with an OLIS RSM 1000 rapid scanning monochromator. The width of the cells in the two spectrophotometers was 1 and 2 cm, respectively. The mixing time of the instruments was 2−4 ms. With the Applied Photophysics apparatus, kinetic traces were taken at different wavelengths between 280 and 650 nm and the data were analyzed with the SX17MV operating software or with Kaleidagraph, version 3.0.5. Traces (averages of at least 10 single traces) from at least five experiments were averaged to obtain each observed rate constant. The pH was measured at the end of the reactions for control.

Nitrite and Nitrate Analysis. Product analysis was carried out by anion chromatography with conductivity detection with a Metrohm instrument (ICSeparation 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. A phthalic acid solution (2.5 mM phthalic acid, 5% acetonitrile, pH 4.6, TRIS) was used as eluant. Calibration curves were obtained by measuring 5−10 standard sodium nitrite and sodium nitrate solutions in 1 mM sodium phosphate buffer. All experiments were carried out at least in double, and all the solutions were measured at least three times.

The protein solutions to be analyzed were prepared by adding to 10 mL of an $MbFeO_2$ or $HbFeO_2$ solution (10−20 μM in 1 mM sodium phosphate buffer), placed in a 10-mL Schlenk flask sealed with a rubber septum, the required equimolar amount of NO• (50−100 μL of saturated 2 mM NO• solution) with a gastight microliter Hamilton syringe under constant stirring. A long needle was used in order to add the NO• solution at the bottom of the flask to avoid any contact of NO• with the oxygen present in the headspace of the flask. Flushing with argon the headspace for a few minutes prior to addition of the NO• solution was found not to influence the results of the experiments. After stirring for about 10 min the reaction mixture was, if necessary, diluted with 1 mM sodium phosphate buffer and analyzed.

The amount of nitrite present in the NO• solution was determined by injecting with a gastight syringe 1 mL of the saturated NO• solution into 9 mL of dioxygen-saturated water placed in a sealed 10-mL Schlenk flask. After stirring for about 10 min the reaction mixture was diluted with 1 mM sodium phosphate buffer and analyzed. With this procedure nitrite is quantitatively formed from the reaction of NO• with O_2 in water. Thus, the amount of nitrite found in excess

relative to the $NO^{\bullet}$ used for this experiment corresponded to the amount of nitrite already present in the $NO^{\bullet}$ solution.

Alternatively, to measure directly the amount of nitrite present in the $NO^{\bullet}$ solution, 1 mL of $NO^{\bullet}$-saturated solution was injected in an evacuated flask, degassed by a series of vacuum degassing and nitrogen purges (pump freeze and thaw), diluted with 1 mM sodium phosphate buffer, and then analyzed.

Analysis of the Nitrotyrosine Content in Mb after 10 Reaction Cycles. An $MbFeO_2$ solution in 0.1 M phosphate buffer, pH 7.4, was first mixed with a solution of $NO^{\bullet}$ in 0.1 M phosphate buffer, pH 7.4 (ca. 0.9 equiv). After the instantaneous generation of metmyoglobin and nitrate, the reaction mixture was concentrated and washed by spinning it through a 10 000 MW cutoff filter (Centriplus YM-10, Amicon, Switzerland) at 3000*g* to remove the nitrite present in the added $NO^{\bullet}$ solution. The solution was washed until the ultrafiltrate did not show any qualitative reaction (pink coloring) with the Griess reagent (*44*). The concentrated metmyoglobin solution was placed in a Schlenk flask and purged with N_2 for ca. 30 min. Then, an excess of sodium L-ascorbate, dissolved in degassed H_2O (ca. 1 M), was added using a gastight syringe. The solution was stirred until reduction was complete (determined by UV/vis spectrophotometry). The excess ascorbate was removed by spinning it through a 10 000 MW cutoff filter at 3000*g*. The solution was washed until the ultrafiltrate did not show any qualitative reaction (bleaching of the pink coloring) with a MnO_4^- solution. The entire procedure was repeated 9 more times and stopped after removing the nitrite.

The protein was hydrolyzed by treating it (0.5 mL of a 500 μM solution) for 24 h with 0.3 mL of 6 M HCl at 100 °C in a closed vial. The solution was allowed to dry on air by maintaining the temperature at 100 °C and by opening the vial. The residual was redissolved in 100 μL of H_2O and analyzed by HPLC 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. 15 min after injection) by an increasing linear gradient of B from 0 to 20% between 7 and 20 min and from 20 to 80% between 20 and 40 min, and detected contemporaneously at 250, 300, 350, and 400 nm. Nitrotyrosine was quantified by measuring a calibration curve of 5−10 nitrotyrosine standard solutions. As a control $MbFeO_2$ (500 μM) was allowed to react with peroxynitrite (500 μM) in 0.1 M phosphate buffer, pH 7.0 (*45*), and analyzed analogously.

Analysis of the Nitrotyrosine Content in Hb after 10 Reaction Cycles. An $HbFeO_2$ solution in 0.1 M phosphate buffer, pH 7.4, was first mixed with a solution of $NO^{\bullet}$ in 0.1 M phosphate buffer, pH 7.4 (ca. 0.9 equiv). After the instantaneous generation of methemoglobin and nitrate, the reaction mixture was concentrated and washed by a procedure analogous to that described above for the Mb reaction. Alternatively, the concentrated solution was purified by chromatography on a Sephadex G25 column. The concentrated methemoglobin solution was placed in a Schlenk flask and purged extensively with N_2 for ca. 60 min. Then, an excess of sodium L-ascorbate, dissolved in degassed H_2O (ca. 1 M), was added using a gastight syringe. The solution was stirred under anaerobic conditions until reduction was complete (determined by UV/vis spectrophotometry). The excess ascorbate was removed by rapidly purifying the solution by chromatography on a Sephadex G25 column to avoid the formation of a green precipitate, described also in ref *46*. Equilibration of the solution with air yielded $HbFeO_2$. The entire procedure was repeated 9 more times and stopped after removing the nitrite.

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Analysis of the Free Nitrotyrosine Content Generated by the $NO^{\bullet}$-Mediated Oxidation of $MbFeO_2$ in the Presence of Added Free Tyrosine. $MbFeO_2$ in 0.1 M phosphate buffer, pH 7.0, was mixed with tyrosine to give a concentration of 400 μM $MbFeO_2$ and 200 μM tyrosine. This solution was immediately (within 1 min) mixed with a 400 μM $NO^{\bullet}$ solution in 0.1 M phosphate buffer in the Applied Photophysics stopped-flow machine. The reaction products were collected at the outlet and divided in 16 different fractions. Eight of these fractions were analyzed by HPLC as described above for Hb. To four of the samples 1, 2, 5, and 10% of pure nitrotyrosine (relative to free tyrosine) was added, respectively, and analyzed analogously by HPLC.

Pulse Radiolysis. Solutions were irradiated with a 2-MeV electron accelerator (Febetron 705, Hewlett-Packard) as described before (*47*). A solution of 3.1 μM metmyoglobin in 0.1 M borate buffer, pH 9.5, was mixed with an excess of hydrogen peroxide to generate ferrylmyoglobin ($MbFe^{IV}$═O). The $MbFe^{IV}$═O solution, which proved to be stable for the length of the experiment, was purged with N_2O for ca. 30 min, mixed immediately prior to the experiment using a peristaltic pump with a N_2O-saturated (24.4 mM) 10 mM nitrite solution, and irradiated. Under these conditions $NO_2^{\bullet}$ is generated at a concentration of about 150 μM. Kinetic traces were taken at 410 or 550 nm using an Acton SP308 monochromator with 150 grooves/mm gratings, appropriate order sorting filters, and a Hamamatsu R928 photomultiplier detector.

RESULTS

Stopped-Flow Kinetic Studies of the $NO^{\bullet}$-Mediated Oxidation of $MbFeO_2$ and $HbFeO_2$. The $NO^{\bullet}$-induced oxidations of oxy- to metmyoglobin (horse heart) and oxy- to methemoglobin (human) were studied by single-wavelength stopped-flow spectroscopy in the pH range 5−9.5 at 20 °C. To avoid the difficulties linked with the accurate determination of the concentration of $NO^{\bullet}$ solutions during the measurements, the proteins were present in 10-fold excess to maintain pseudo-first-order conditions.

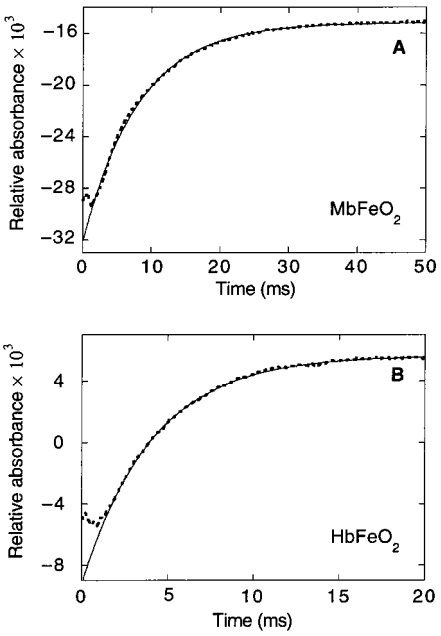

FIGURE 1: Time course (average of 10 traces) measured (A) at 409 nm for the reaction of 2.9 μM $MbFeO_2$ with ~0.2 μM $NO^\bullet$ and (B) at 406 nm for the reaction of 2.6 μM $HbFeO_2$ with ~0.2 μM $NO^\bullet$ at pH 7.0 and 20 °C. The dotted lines represent the experimental data, whereas the full lines correspond to the best fit to the data resulting in the observed rate constants (A) $k_{obs} = (121.7 \pm 0.5)\ s^{-1}$ and (B) $k_{obs} = (247.4 \pm 0.9)\ s^{-1}$.

The kinetic traces, measured mostly by following the absorbance increases at 409 and 406 nm for Mb and Hb, respectively, could all be fitted well to a single-exponential expression (Figure 1). This indicated that the α- and β-subunits of Hb reacted at the same rate. Identical observed rate constants were obtained at several other wavelengths on the Soret absorbance band. As the spectra of the resulting iron(III) proteins are pH-dependent, at pH 9.5 the reactions were studied by measuring the absorbance decreases near 420 nm, where the differences between the extinction coefficients of oxy- and Mb/Hb are the largest.

The second-order rate constants, obtained from the linear plots of the observed pseudo-first-order rate constants versus $MbFeO_2$ or $HbFeO_2$ concentration (Figure 2), were pH-dependent. At pH 7.0 we obtained $(43.6 \pm 0.5) \times 10^6\ M^{-1}\ s^{-1}$ for the $NO^\bullet$-mediated oxidation of $MbFeO_2$, a value slightly larger than what had previously been reported for recombinant sperm whale Mb at the same pH, $34 \times 10^6\ M^{-1}\ s^{-1}$ (*31*). The same rate constant was obtained when this reaction was carried out in the pH range 5−7, whereas under alkaline conditions the second-order rate constants continuously increased up to $(97 \pm 3) \times 10^6\ M^{-1}\ s^{-1}$ at pH 9.5 (Figure 3).

A parallel trend was observed for the $NO^\bullet$-mediated oxidation of $HbFeO_2$. Between pH 5 and 7 the values measured for the second-order rate constants did not vary significantly. At pH 7.0 we obtained $(89 \pm 3) \times 10^6\ M^{-1}\ s^{-1}$ (per heme), a value slightly larger than what had previously been reported (*31*, *48*). At alkaline pH the second-order rate constants continuously increased up to a value of $(144 \pm 3) \times 10^6\ M^{-1}\ s^{-1}$ (per heme) at pH 9.5 (Figure 3).

As the dissociation constant for the tetrameric Hb $(\alpha\beta)_2$ into its dimers $\alpha\beta$ is about 3 μM (*43*) and we used $HbFeO_2$ concentrations between 0.5 and 5 μM, under our experimental conditions the protein is largely in its dimeric form. To determine whether the rate constant for the reaction between $NO^\bullet$ and $HbFeO_2$ is identical for the tetrameric and dimeric forms of the protein, a stopped-flow experiment was carried out in which we mixed between 20 and 35 μM $HbFeO_2$ in a 250-μL syringe with a $NO^\bullet$ solution between 0.2 and 0.35 μM placed in a 2.5-mL syringe. As the tetramer−dimer dissociation rate is $(2.5 \pm 0.4) \times 10^{-5}\ s^{-1}$ (*49*), a value significantly lower than that for the rate of reaction of $HbFeO_2$ with $NO^\bullet$, this experimental setup allows for the determination of the rate of reaction of the tetrameric form of $HbFeO_2$ with $NO^\bullet$. The second-order rate constant (per heme) obtained at pH 7.0 from the linear fit (data not shown) was $(94 \pm 2) \times 10^6\ M^{-1}\ s^{-1}$, identical to that measured by a 1:1 mixing experiment ($(89 \pm 3) \times 10^6\ M^{-1}\ s^{-1}$).

As it has recently been argued that the phosphate concentration influences the nature of the reaction between $HbFeO_2$ and $NO^\bullet$ (*39*), we also studied the reaction between $HbFeO_2$ and $NO^\bullet$ in a 1 mM phosphate buffer, pH 7.0. The second-order rate constant (per heme) obtained from the

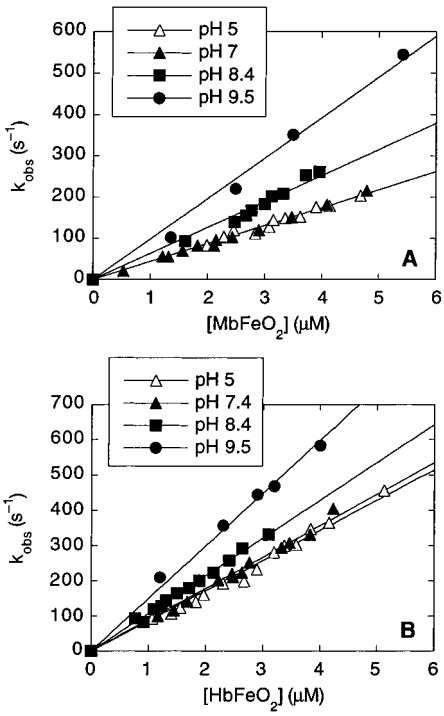

FIGURE 2: Plots of k_{obs} versus (A) $[MbFeO_2]$ and (B) $[HbFeO_2]$ for the $NO^\bullet$-mediated oxidation of $MbFeO_2$ (A) and $HbFeO_2$ (B) at 20 °C and different pH values.

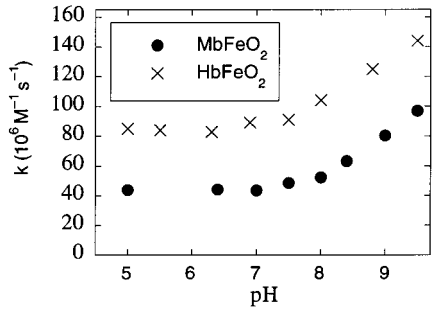

FIGURE 3: pH dependence of the second-order rate constants (per heme) of the $NO^\bullet$-mediated oxidations of $MbFeO_2$ (●) and $HbFeO_2$ (×) at 20 °C.

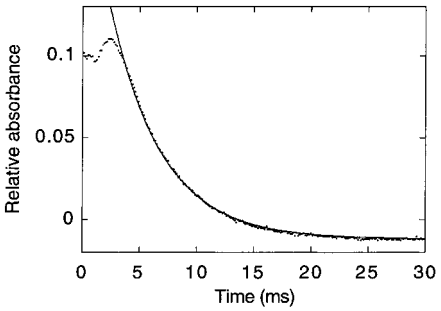

FIGURE 4: Time course measured at 406 nm for the reaction of 23.7 μM $MbFeO_2$ with ca. 10 μM $NO^{\bullet}$ in 0.1 M borate buffer at pH 9.5, 20 °C. The solid line corresponds to the best fit for the decay of $MbFe^{III}OONO$ resulting in the rate constant $k = (205 \pm 5)$ s^{-1}.

linear fit (data not shown) was $(87 \pm 2) \times 10^6$ M^{-1} s^{-1}, identical to that measured in 0.1 M phosphate buffer ($(89 \pm 3) \times 10^6$ M^{-1} s^{-1}).

Stopped-Flow Kinetic Studies of the Decay of the Intermediate Peroxynitrito−Metmyoglobin Complex ($MbFe^{III}$-OONO). In analogy to the $NO^{\bullet}$-induced oxidation of $HbFeO_2$ (*42*), an intermediate peroxynitrito−metmyoglobin complex, $MbFe^{III}OONO$, could be detected when the reaction between $NO^{\bullet}$ and $MbFeO_2$ was studied under alkaline conditions. Kinetic studies of the decay of $MbFe^{III}OONO$ to metmyoglobin were carried out by following the absorbance changes at 406 nm. As shown in Figure 4 the measured traces show a very rapid increase (ca. 3 ms) and a subsequent decrease in absorption. The decay was fitted to a single-exponential expression. As expected, the rate of decay, measured within a broad range of $NO^{\bullet}$ and $MbFeO_2$ concentrations, was independent from the $NO^{\bullet}$ as well as the $MbFeO_2$ concentrations (Figure 5). Moreover, the same rate constant was obtained when either of the two reagents was used in excess. When $NO^{\bullet}$ was used in large excess an additional reaction was observed on a longer time scale which corresponded to $NO^{\bullet}$ binding to metmyoglobin formed as product from the reaction between $MbFeO_2$ and $NO^{\bullet}$.

In analogy to the peroxynitrito−methemoglobin complex, $HbFe^{III}OONO$ (*42*), the rate of decay of $MbFe^{III}OONO$ was highly pH-dependent and increased with decreasing pH. As shown in Table 1, the absolute values of the observed rate constants were significantly larger than those for the α- and β-subunits of Hb. At pH lower than 8.5, $MbFe^{III}OONO$ decayed so rapidly that it did not accumulate in concentrations large enough to be observed.

Spectral Characterization of the Peroxynitrito Intermediate $MbFe^{III}OONO$. The $NO^{\bullet}$-mediated oxidation of oxy- to metmyoglobin was studied by rapid scan UV/vis spectroscopy between 380 and 680 nm at pH 9.5 and 5 °C to obtain a spectrum of the intermediate over the entire wavelength range. As shown in Figure 6, the Soret band of Mb shifted from 417 nm ($MbFeO_2$) to 411 nm ($MbFe^{III}OH$ at pH 9.5) via an intermediate species with an absorption maximum at 410 nm (spectrum 5 in Figure 6) and an extinction coefficient of about 138 mM^{-1} cm^{-1}. The best spectrum obtained for $MbFe^{III}OONO$ in the visible region, shown as the first trace in Figure 7, has two characteristic absorption features: a shoulder at 636 nm ($\epsilon_{636} = 3.2$ mM^{-1} cm^{-1}) and another at 504 nm ($\epsilon_{504} = 8$ mM^{-1} cm^{-1}). As higher concentrations were used to reduce the signal-to-noise ratio, accumulation

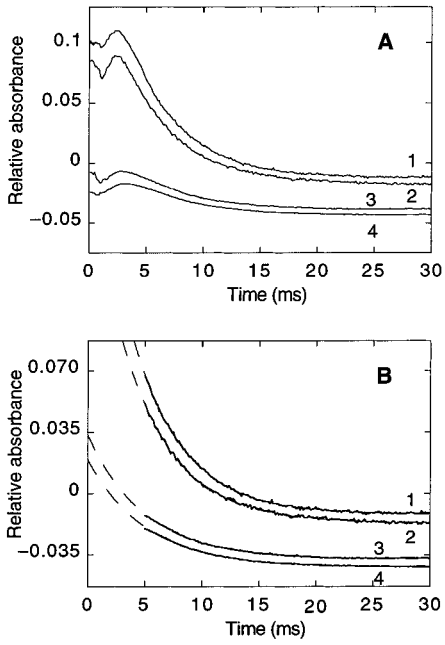

FIGURE 5: (A) Normalized time courses measured at 406 nm for the reaction of $MbFeO_2$ with $NO^{\bullet}$ in 0.1 M borate buffer at pH 9.5, 20 °C: (1) $[MbFeO_2] = 23.7$ μM and $[NO^{\bullet}] = 10$ μM; (2) $[MbFeO_2] = 23.7$ μM and $[NO^{\bullet}] = 5$ μM ((data points × 2) + 0.035); (3) $[MbFeO_2] = 12.2$ μM and $[NO^{\bullet}] = 5$ μM; (4) $[MbFeO_2] = 12.2$ μM and $[NO^{\bullet}] = 1$ μM ((data points × 2) + 0.080). (B) Same traces (solid lines) with the best fits (dashed lines) to the data resulting in the observed rate constants: (1) $k_{obs} = (208 \pm 2)$ s^{-1}; (2) $k_{obs} = (210 \pm 2)$ s^{-1}; (3) $k_{obs} = (208 \pm 1)$ s^{-1}; (4) $k_{obs} = (209 \pm 2)$ s^{-1}.

Table 1: pH Dependence of the Decay Rates of the Intermediate Complexes $MbFe^{III}OONO$, $HbFe^{III}OONO$, and Peroxynitrite at 20 °C

pH	$MbFe^{III}OONO$ (s^{-1})	$HbFe^{III}OONO$[a] (s^{-1})		peroxynitrite[b] (s^{-1})
7.5[c]		58 ± 5	33 ± 1	0.167
8.1[c]		48 ± 3	16 ± 1	0.048
8.3[c,d]	341 ± 20	42 ± 1	9.7 ± 0.2	0.031
8.9[c]	257 ± 7	41 ± 1	8.1 ± 0.1	0.008
9.5[e]	205 ± 5	36 ± 5	6.9 ± 0.1	0.110

[a] Ref *42*. The two values represent the decay rates for the complexes of the α- and β-subunits of Hb. [b] Ref *8*. [c] 0.1 M phosphate buffer. [d] $MbFe^{III}OONO$ measured at pH 8.5. [e] 0.1 M borate buffer.

of $MbFe^{III}OONO$ occurred within the dead time of the instrument. Almost identical absorption maxima were previously reported for $HbFe^{III}OONO$ (Table 2) (*42*). In contrast to $HbFe^{III}OONO$, $MbFe^{III}OONO$ is not very stable under the experimental conditions but rapidly decays to $MbFe^{III}OH$. This instability made it impossible to obtain an accurate spectrum of $MbFe^{III}OONO$ and may thus explain the significant differences in the extinction coefficients between the peroxynitrito complexes of these two proteins.

UV/Vis Spectra of Methemoglobin and Metmyoglobin Nitro and Nitrato Complexes. To prove that the intermediate complexes were peroxynitrito− and not nitro− or nitrato−methemoglobin complexes, derived from contamination of the $NO^{\bullet}$ solution (NO_2^-) or from binding of the product (NO_3^-), we studied via UV/vis the binding of these ligands to the protein in 0.1 M borate buffer. As shown in Figure 8, a very large excess of nitrite (ca. 10^4 equiv) and an even larger excess of nitrate (ca. 10^5 equiv) are needed to convert

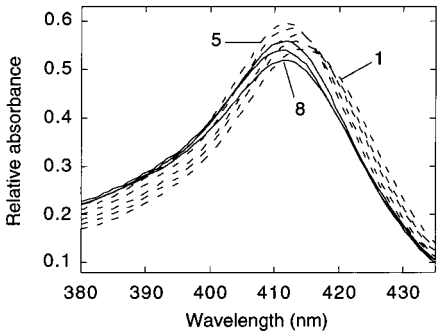

FIGURE 6: Rapid scan UV/vis spectra of the reaction of 2.4 μM $MbFeO_2$ with ca. 5 μM $NO^{\bullet}$ in 0.1 M borate buffer at pH 9.5, 5 °C. Spectra were collected every millisecond, but to improve the signal-to-noise ration, each curve shown represents the average of two measured curves. The formation of $MbFe^{III}OONO$ from $MbFeO_2$, dashed traces 1−5, and its decay to $MbFe^{III}OH$, traces 6−8, are presented. Time intervals of the shown spectra are: traces 1−3, every 4 ms; traces 6−8, every 80 ms, for a total of 140 ms.

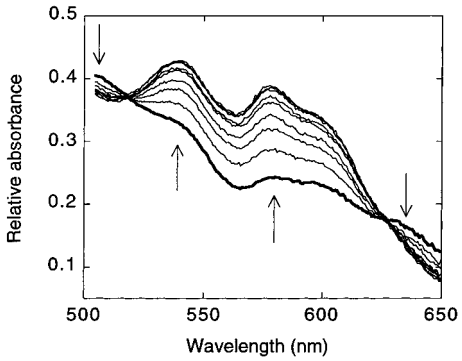

FIGURE 7: Rapid scan UV/vis spectra of the reaction of 24.5 μM $MbFeO_2$ with ca. 30 μM $NO^{\bullet}$ in 0.1 M borate buffer at pH 9.5, 5 °C. Spectra were collected every millisecond, but to improve the signal-to-noise ration, each curve shown represents the average of five measured curves. The decay of $MbFe^{III}OONO$ (bold curve) to $MbFe^{III}OH$ (last trace) is presented. The spectra are shown every 10 ms up to 80 ms.

Table 2: Spectroscopic Data for Metmyoglobin and Methemoglobin Complexes

	Soret	visible		
complex	λ_{max} (ϵ)[a]	λ_{max} (ϵ)[a]	λ_{max} (ϵ)[a]	reference
$MbFe^{III}OONO$	410 (138)	504 (8)	636 (3.2)	this work
$MbFe^{III}OH_2$	408 (188)	502 (10.2)	630 (3.9)	*43*
$MbFe^{III}ONO_2$	404 (172)	502 (8.8)	629 (3.6)	this work
$MbFe^{III}NO_2$	412 (137)	502 (8.4)	628 (4.2)	this work
$HbFe^{III}OONO$	407 (165)	504 (8.7)	636 (5.4)	*42*
$HbFe^{III}OH_2$	405 (179)	500 (10.0)	631 (4.4)	*43*
$HbFe^{III}ONO_2$	408 (120)	527 (9.9)	628 (3.2)	this work
$HbFe^{III}NO_2$	401 (132)	538 (10.0)	623 (3.6)	this work
$HbFe^{III}OC(O)CH_3$	404 (178)	497 (10.5)	620 (5.5)	*43*
$HbFe^{III}OC(O)H$	404 (178)	496 (9.2)	620 (5.8)	*43*

[a] λ_{max} (nm) (ϵ, mM^{-1} cm^{-1}).

methemoglobin to the nitro and nitrato complexes, respectively. The absorbance maxima of $HbFe^{III}NO_2$ and $HbFe^{III}ONO_2$, summarized in Table 2, are significantly different from those of $HbFe^{III}OONO$. Taken together, these data suggest that the complex characterized as an intermediate of the reaction between $HbFeO_2$ is neither $HbFe^{III}NO_2$ nor $HbFe^{III}ONO_2$.

The corresponding nitro− and nitrato−metmyoglobin complexes can also be prepared by adding analogously large excesses of the ligands to metMb. As summarized in Table 2, the absorbance maxima of $MbFe^{III}NO_2$ and $MbFe^{III}ONO_2$ are very similar to those of $HbFe^{III}OONO$, but the large excess required to generate these species makes it very unlikely that the intermediate complex identified in the course of the $NO^{\bullet}$-mediated oxidation of $MbFeO_2$ is one of these species.

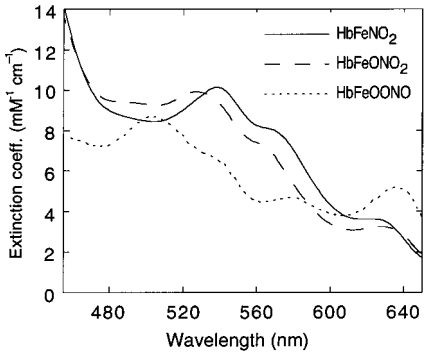

FIGURE 8: Normalized UV/vis spectra in 0.1 M borate buffer of $HbFe^{III}NO_2$ (full line), generated by addition of ca. 2×10^4 equiv of NO_2^- to metHb; $HbFe^{III}ONO_2$ (dashed line), generated by addition of ca. 2×10^5 equiv of NO_3^- to metHb; $HbFe^{III}OONO$ (dotted line), measured by stopped-flow spectroscopy of a reaction between $HbFeO_2$ and 3.5 equiv of $NO^{\bullet}$ as described in ref *42*.

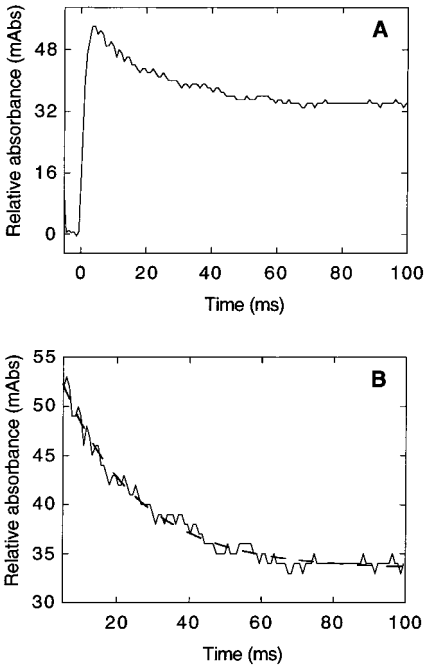

FIGURE 9: (A) Time course measured at 410 nm for the reaction of 1.5 μM $MbFe^{IV}$=O with ca. 150 μM $^{\bullet}NO_2$ in 0.05 M borate buffer at pH 9.5, 20 °C. (B) Same trace (solid line) shown between 10 and 100 ms with the best fit (dashed line) to the data resulting in the observed rate constant of 35 ± 5 s^{-1}.

Reaction between $MbFe^{IV}$=O and Nitrogen Dioxide. To gain further evidence that the intermediary complex identified in the course of the $NO^{\bullet}$-mediated oxidation of $MbFeO_2$ is a peroxynitrito−metmyoglobin ($MbFe^{III}OONO$) and not a nitrato−metmyoglobin ($MbFe^{III}ONO_2$) complex, we studied the reaction between $MbFe^{IV}$=O and nitrogen dioxide, generated by pulse radiolysis from a N_2O-saturated nitrite solution. When an excess of $NO_2^{\bullet}$ was allowed to react with $MbFe^{IV}$=O the intermediate complex $MbFe^{III}ONO_2$ was generated (Figure 9A) with an approximate second-order rate constant of 10×10^6 M^{-1} s^{-1} and decayed (Figure 9B) with

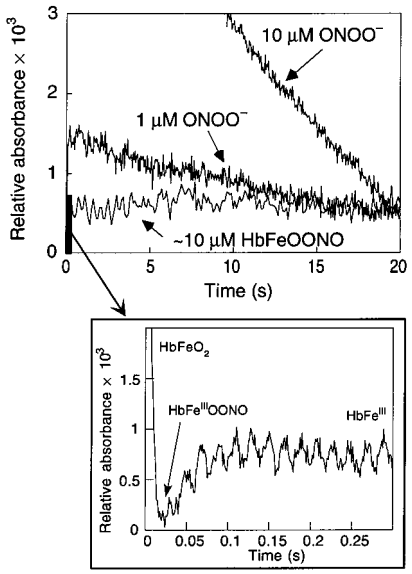

FIGURE 10: Time course measured at 302 nm for the reaction of 11 μM $HbFeO_2$ with 9 μM NO• in 0.1 M phosphate buffer at pH 8.1 and 20 °C. Comparison with the traces measured at 302 nm for the decay of 10 and 1 μM peroxynitrite under the same experimental conditions.

a rate constant of 35 ± 5 s^{-1}, significantly lower than that of the analogous peroxynitrito complex (205 ± 5 s^{-1}).

Analysis of Free Peroxynitrite. To determine whether free peroxynitrite was formed in the course of the NO•-mediated oxidations of $MbFeO_2$ and $HbFeO_2$, we studied these reactions by stopped-flow spectroscopy at 302 nm, the wavelength at which peroxynitrite anion has an absorbance maximum ($\epsilon_{302} = 1705$ M^{-1} cm^{-1}) (*50*). As shown in Figure 10 (bottom), when $HbFeO_2$ was mixed with about 1 equiv of NO• at pH 8.1 a rapid decrease and a slower increase in absorbance were observed in the first 300 ms of the reaction, which reflect the formation of $HbFe^{III}OONO$ and its decay to methemoglobin, respectively. No further changes were observed on a longer time scale (Figure 10, top). The half-life of peroxynitrite under the conditions of this experiment is about 14 s. As shown in Figure 10, if 1 or 0.1 equiv of free peroxynitrite would have been formed, a decrease in the absorbance of about 0.015 or 0.001 absorbance unit, respectively, would clearly have been detected.

Analysis of the Nitrogen-Containing Products. The determination of the amount of nitrite and nitrate ions formed from the reaction of $MbFeO_2$ or $HbFeO_2$ with 1 equiv of NO• at pH 7.0 and 9.0 was carried out by anion chromatography with conductivity detection. As shown in Table 3 (columns 2 and 3), nitrate was always formed quantitatively relative to the proteins. The nitrite ions found in the analyzed protein solutions (column 4 in Table 3) were already present as a contaminant in the NO• solutions utilized for the single experiments. Indeed, separate measurements of the nitrite concentrations in the NO• solutions were always in good agreement with the values obtained from the protein solutions (columns 5 and 6 in Table 3).

Analysis of the Protein. Even though nitrate was generated quantitatively as the only nitrogen-containing product, to prove that the globin did not undergo any reaction, and in particular to determine whether any nitrotyrosine was formed, the proteins were hydrolyzed and analyzed by HPLC. As the yield of nitrotyrosine produced from the reaction between peroxynitrite and $HbFeO_2$ (*51*) as well as $MbFeO_2$ (unpublished results) is very low, we cycled the reaction 10 times before carrying out the amino acid analysis. The oxy-proteins were first allowed to react with slightly less than 1 equiv of NO•, then reduced back to the starting oxy-form by mixing them with an excess of ascorbic acid under anaerobic conditions. Exposure to air after removal of excess ascorbic acid yielded the oxygenated form of the proteins, which was allowed to react again with NO•. As shown in Figures 11 and 12, the HPLC analyses indicate that for both proteins less than 1% nitrotyrosine, relative to the total amount of tyrosine, was formed.

In a further experiment, 0.5 equiv of free tyrosine was added to a $MbFeO_2$ solution prior to its reaction with a stoichiometric amount of NO•. Analysis by HPLC of the solution showed that the intermediate $MbFe^{III}OONO$ was not able to nitrate free tyrosine (Figure 13A). As shown in Figure 13B,C, the HPLC analysis would have been able to detect between 1 and 2% nitrotyrosine.

Table 3: Amount of Nitrate Formed from the Reaction of $HbFeO_2$ or $MbFeO_2$ with 1 equiv of NO•: Comparison between the Amount of Nitrite Obtained during the Reaction and That Present in the NO• Solution

1	2	3	4	5	6
pH	[$HbFeO_2$] (μM)	[NO_3^-][a] (μM)	[NO_2^-][a] (μM)	[NO_2^-] in NO• soln[b] (mM)	measured [NO_2^-] in NO• soln[c] (mM)
7	11.1	10.6	15.3	1.5	1.3
7	11.1	10.8	13.5	1.4	1.4
9	16.1	16.1	4.1	0.75	0.65
9	16.1	15.6	3.9	0.71	0.75
pH	[$MbFeO_2$] (μM)	[NO_3^-][a] (μM)	[NO_2^-][a] (μM)	[NO_2^-] in NO• soln[b] (mM)	measured [NO_2^-] in NO• soln[c] (mM)
7	16.1	16.0	16.8	1.7	1.8
7	16.1	16.4	18.0	1.8	1.8
9	11.5	12.0	17.7	1.8	2.0
9	11.5	11.7	18.6	1.9	2.0

[a] Concentrations of the ions in the protein solutions after reaction with NO•. [b] Values for the nitrite contamination of the NO• solutions calculated from the [NO_2^-] measured in the protein solutions, by taking into account the different dilutions. [c] Nitrite contamination of the NO• solutions determined separately as described in Experimental Procedures.

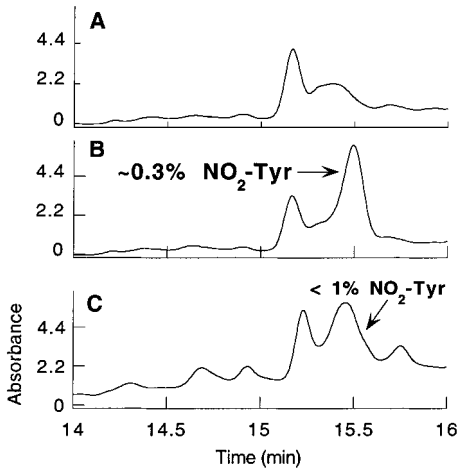

FIGURE 11: HPLC analysis (at 400 nm) of Mb after 10 cycles of reaction with NO• after complete acid hydrolysis as described in Experimental Procedures: (A) 500 μM Mb; (B) 500 μM Mb + 500 μM peroxynitrite; (C) 500 μM Mb after 10 cycles of reaction with NO•.

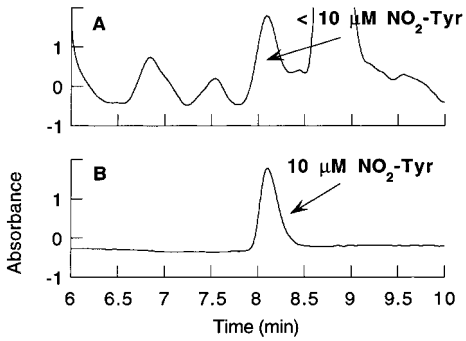

FIGURE 12: HPLC analysis (at 400 nm) of Hb after 10 cycles of reaction with NO• after complete acid hydrolysis as described in Experimental Procedures: (A) 500 μM Hb after 10 cycles of reaction with NO•; (B) 10 μM nitrotyrosine.

DISCUSSION

The interaction between NO• and Hb is currently an area of intense research (*38*, *39*, *52*, *53*). The relevance of the reaction between NO• and $HbFeO_2$, which had repeatedly been proposed to represent the main route for NO• depletion in the blood vessels (*7*, *31*, *33*, *36*, *40*, *41*, *54*), has recently been questioned (*39*). Indeed, it has been proposed that, in particular with low phosphate concentrations, NO• preferentially binds to the very small amount of deoxygenated heme of Hb which is present under physiological conditions (about 1%) to yield an iron(II)−nitrosyl complex (*39*). Our data reveal that the rate of reaction between NO• and $HbFeO_2$ is independent of phosphate concentration and is identical in 0.1 M and 1 mM phosphate buffer. Moreover, the detailed kinetic and mechanistic studies reported in this paper allow for a better understanding of the mechanism of the reaction between NO• and $HbFeO_2$.

pH Dependence of the Second-Order Rate Constant. Stopped-flow kinetic data presented in this work show that the rates of the reactions of NO• with $HbFeO_2$ and $MbFeO_2$ are strongly pH-dependent. In particular, the second-order rate constants for both these reactions are invariable between pH 5 and 7 but significantly increase at higher pH (Figure 3). This result was unexpected. Indeed, at low pH the distal histidine, which is hydrogen-bonded to the coordinated oxygen, is protonated and swings out of the heme pocket toward the solvent, with consequent rupture of the hydrogen bond and opening of the active site (*55*). It has been proposed that this hydrogen bond must be disrupted to allow for the initial entry of NO• into the distal pocket, the process which is considered to be the rate-limiting step for this reaction (*31*). This hypothesis has been confirmed by the significant acceleration of the NO•-mediated oxidation of Mb mutants in which the distal histidine had been substituted with smaller amino acids which cannot form a hydrogen bond to the coordinated oxygen (*31*). Therefore, at acidic pH an *increase* in the rate of oxidation of $HbFeO_2$ and $MbFeO_2$ by NO• would rather have been anticipated. A possible explanation for the observation that the rates of oxidation of $MbFeO_2$ and $HbFeO_2$ by NO• are constant between pH 5 and 7 is that at lower pH some amino acid residues along the route from which NO• approaches the distal pocket could be protonated and thus the diffusion of the hydrophobic NO• could be slowed. Alternatively, conformational changes could take place which would block NO• diffusion by decreasing the pH.

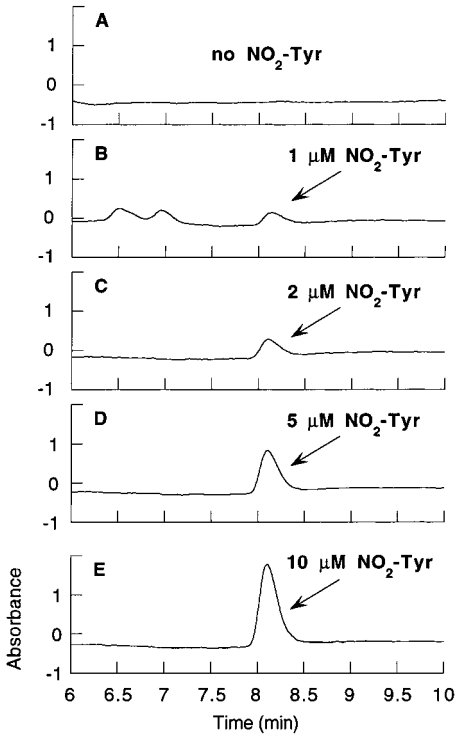

FIGURE 13: HPLC analysis (at 400 nm) of (A) the reaction product between 200 μM NO• and 200 μM $MbFeO_2$ in the presence of 100 μM free tyrosine; (B) 1 μM nitrotyrosine; (C) 2 μM nitrotyrosine; (D) 5 μM nitrotyrosine; (E) 10 μM nitrotyrosine.

The significant increase in the rate of reaction at alkaline pH could be rationalized by assuming that a partial deprotonation of the proximal histidine takes place under basic conditions. This hypothesis is based on the observation that the acidity of the imino proton of imidazole increases on coordination to metal ions (*56*). In particular, it has been reported that the pK_a values of imidazole bound to metmyoglobin and methemoglobin are about 10 and 11, respectively (*57*). Deprotonation of the histidine bound *trans* to the oxygen ligand in the oxy-proteins increases the stabilization of the iron(III)−superoxide form relative to the iron(II)−dioxygen form, weakens the bond between the iron and the oxygen, and thus brings about the observed acceleration of the reaction at alkaline pH. Alternatively, as suggested above, by increasing the pH conformational changes could take place in the protein, which could cause an increase in the rate of diffusion of NO• into the distal pocket.

As can clearly be seen in Figure 3, the rate constants for the reaction of NO• with $HbFeO_2$ are always almost twice as large as those for the reaction with $MbFeO_2$. This difference might arise from the different affinity that these proteins display for oxygen, which may cause Mb to react at a lower rate.

Kinetic and Spectroscopic Characterization of the Intermediate Peroxynitrito Complexes. In the present study, we show that $MbFe^{III}OONO$ and $HbFe^{III}OONO$ complexes are formed as intermediates in the NO•-induced oxidation of $MbFeO_2$ and $HbFeO_2$ and can be characterized spectroscopically at alkaline pH. The presence of such an intermediate has often been proposed in the literature (*7*, *31*, *40*, *41*). As the dioxygen complex of these proteins is best described as an intermediate form between an iron(II)−dioxygen and an iron(III)−superoxide complex (*58*, *59*), it is obvious to draw

an analogy to the reaction between $NO^{\bullet}$ and superoxide, which yields peroxynitrite at an almost diffusion controlled rate (*8*). However, previous groups which have studied these reactions (*31*, *40*, *41*) have not reported any intermediates.

Stopped-flow kinetic studies presented in this paper clearly show that the $NO^{\bullet}$-mediated oxidations of $MbFeO_2$ and $HbFeO_2$ proceed via detectable intermediate species, which decay with a rate independent from both the $NO^{\bullet}$ and protein concentrations (Figure 5). Interestingly, the peroxynitrito complexes of the two subunits of Hb decay with different rates, and the $MbFe^{III}OONO$ is significantly less stable (Table 1). This result might reflect the small difference between the active site pockets of these two proteins. In addition, the different rates of decay observed for the α- and β-subunits may reflect an inequivalent environment of the heme groups in the two subunits. X-ray crystallographic data have shown that in the β-subunit some amino acid residues overlap with the ligand-binding site to a larger extent than in the α-subunit (*60*). Therefore, in some reactions the two subunits of Hb have different rate constants (*61*).

Peroxynitrite in its anionic form is stable but rapidly isomerizes to nitrate when protonated ($pK_a = 6.8$) or in the presence of other Lewis acids (*8*). Traces of metal ions accelerate the decay of peroxynitrite possibly by neutralizing the negative charge on the peroxide group and thus inducing isomerization. The kinetic data presented in this work show that the rate of decay of peroxynitrite bound to methemoglobin and metmyoglobin increases with decreasing pH and is significantly larger than that of free peroxynitrite (Table 1). As already discussed above, at alkaline pH the proximal histidine is at least in part deprotonated, and consequently, the heme is less Lewis acidic than at neutral pH and the peroxynitrito complexes are more stable.

Even though it was not possible to measure an accurate UV/vis spectrum of $MbFe^{III}OONO$, Figures 6 and 7 show that the identified intermediate displays an absorption maximum around 410 and two characteristic absorption features: a shoulder at 504 and another at 636 nm. A comparison with the spectra of the corresponding Hb complex $HbFe^{III}OONO$ (*42*) and other metHb and metMb derivatives with anionic ligands such as $HCOO^-$ or CH_3COO^- (*43*) supports the assignment of this species as an iron(III)−peroxynitrite anion complex (Table 2). All of these compounds give rise to similar spectra, with absorption maxima around 500 and 610 nm. The position and intensity of the Soret band (Figure 6) are also compatible with the listed derivatives. Other species which display similar spectra are the acidic forms of methemoglobin and metmyoglobin (pH ≤ 6.4), which have a water molecule bound to the heme (Table 2). Nevertheless, it can be excluded that the observed decay reactions correspond to a deprotonation of a coordinated water molecule because this process is expected to occur at a very fast rate (*62*, *63*) and to exhibit a pH dependence opposite to what was found (Table 1).

Interestingly, a spectrum very similar to that of $HbFe^{III}$-OONO and $MbFe^{III}OONO$ has been obtained for the intermediate complex formed during the oxygenation of nitrosylmyoglobin ($MbFe^{II}NO$) (*64*). It has been proposed that this reaction proceeds via an initial attack by oxygen on the nitrogen of the nitrosyl and thus forms an intermediate complex in which peroxynitrite is coordinated to the iron through the nitrogen atom ($MbFe^{III}N(O)OO$) (*64*). The N-bound peroxynitrite−metmyoglobin complex has been reported to decay to metMb and nitrate with a rate of $(2.81 \pm 0.04) \times 10^{-3}\ s^{-1}$ at pH 7.0 and 37 °C (*64*). Another set of metmyoglobin complexes which have spectra very similar to that of $HbFe^{III}OONO$ are the nitro−, nitrato−, and nitrito−metmyoglobin complexes. Nitrite and nitrate are not good ligands for metmyoglobin: a very large nitrite excess ($>10^4$ equiv) is needed to obtain a spectrum of $MbFe^{III}NO_2$ (*65*) and an even larger nitrate excess ($>10^5$ equiv) is needed to obtain a spectrum of $MbFe^{III}ONO_2$. The $MbFe^{III}ONO$ is not stable but has been characterized by rapid scan spectroscopy as an intermediate of the $NO^{\bullet}$-mediated reduction of $MbFe^{IV}{=}O$ (*66*). At pH 7.0 and 20 °C $MbFe^{III}ONO$ decays to metMb and nitrite with a rate of $5.2 \pm 0.1\ s^{-1}$ (*66*). In contrast, the comparison of the UV/vis spectra of $HbFe^{III}$-ONO_2 and $HbFe^{III}NO_2$ with that of the intermediate peroxynitrito complex $HbFe^{III}OONO$ (Figure 8 and Table 2) clearly indicates that the peroxynitrito complex has a significantly different spectrum. Taken together these data show that there is no doubt that the intermediate observed in the course of the $NO^{\bullet}$-mediated oxidation of $HbFeO_2$ is a species different from the ones which could be formed by reaction of metHb with nitrite, the unavoidable contaminant of $NO^{\bullet}$ solutions, or nitrate, the final product of the reaction. Therefore, we can assign with confidence the intermediate as $HbFe^{III}OONO$.

The comparison of the nitrato− and peroxynitrito−metmyoglobin spectra does not allow for such a conclusion. Therefore, we determined the dissociation rate of nitrate from $MbFe^{III}ONO_2$ prepared by reacting $MbFe^{IV}{=}O$ with nitrogen dioxide, generated by pulse radiolysis from a N_2O-saturated nitrite solution. The obtained dissociation rate ($35 \pm 5\ s^{-1}$) is significantly lower than that of the peroxynitrito complex under the same conditions ($205 \pm 5\ s^{-1}$) and thus strongly suggests that the two species are not equivalent.

Iron(III)−porphyrin complexes have been reported to catalyze the isomerization of peroxynitrite to nitrate at pH 7.4 (*24*, *67*, *68*). It has been proposed that the first step of this reaction involves the rapid formation of a peroxynitrito−iron(III) complex which then decays to a ferryl, oxoiron-(IV) complex, the active species for the catalysis (*68*). In our system, it can be excluded that $MbFe^{IV}{=}O$ or Hb are formed because their characteristic absorbance maxima, at 421, 548, and 582 nm for $MbFe^{IV}{=}O$ (*69*, *70*) and at 423, 543, and 580 nm for $HbFe^{IV}{=}O$ (*71*), were not observed. Apparently, in contrast to other heme proteins (*27*, *72*), no absorbance changes are observed in the UV/vis spectra of the iron(III) forms of Mb and Hb after reaction with peroxynitrite (*45*, *73*−*75*). However, the reduced iron(II) forms and the oxy-forms of Mb and Hb react with peroxynitrous acid to yield the corresponding ferryl species (*45*). The difference in reactivity toward peroxynitrite of iron−porphyrin complexes and these proteins may be caused by the presence of the proximal imidazole bound to the heme.

Stopped-Flow Studies at 302 nm. In a recent paper, Wade and Castro (*41*) suggested that *free* peroxynitrite is formed in the course of the $NO^{\bullet}$-mediated oxidation of $MbFeO_2$. However, the evidence presented in support to this hypothesis is not very convincing. Indeed, they show that the UV/vis spectrum of the product of the oxidation of $MbFeO_2$ by $NO^{\bullet}$ at pH 9 displays a stronger absorbance at 302 nm compared to that of its oxidation by nitrite, which is known to yield

metMb. This increased absorbance at 302 nm was attributed to the formation of exactly 1 equiv of free peroxynitrite. However, as the extinction coefficients for $MbFeO_2$ and metMb at 302 nm are about 17800 and 14400 M^{-1} cm^{-1}, respectively, about 10 times larger than that of peroxynitrite at this wavelength (ϵ_{302} = 1705 M^{-1} cm^{-1}) (*50*), such a measurement is not likely to be very accurate. Moreover, under basic conditions peroxynitrite is known to decompose partly to nitrite and dioxygen (*76*, *77*), whereas Wade and Castro (*41*) reported the yield of nitrate to be quantitative.

Single-wavelength stopped-flow studies presented in this work demonstrate that free peroxynitrite is *not* produced during the $NO^{\bullet}$-mediated oxidation of $HbFeO_2$. As shown in Figure 10, when the reaction is carried out at pH 8.1 and followed at 302 nm, no absorbance changes are observed after the first 300 ms of the reaction, that is after the intermediate $HbFe^{III}OONO$ complex has rapidly formed and decayed. Had 1 equiv of free peroxynitrite been formed under these conditions, a significant decrease of about 0.015 absorbance unit would have been expected over 20 s (Figure 10, top). This observation, as well as the significantly larger rate of oxidation of $HbFeO_2$ by $NO^{\bullet}$ compared to that of its autoxidation (*78*), suggests that $NO^{\bullet}$ directly attacks the coordinated dioxygen to give a peroxynitrite complex which then rearranges to nitrate without prior dissociation. In analogy to peroxynitrous acid, which has much weaker absorbance bands than $ONOO^-$ between 300 and 400 nm (*79*), the coordinated peroxynitrite is not expected to contribute in a detectable way to the Hb background absorbance.

Product and Protein Analyses. Analysis of the nitrogen-containing species derived from the $NO^{\bullet}$-mediated oxidation of $MbFeO_2$ and $HbFeO_2$ indicates that nitrate is formed quantitatively under both neutral and alkaline conditions (Table 3). As already discussed above, the observation that nitrate is formed quantitatively also when the reaction is carried out at pH 9.0 is further evidence that no free peroxynitrite is formed in the course of the reaction, as peroxynitrite is known to decompose to nitrite and dioxygen under alkaline conditions (*76*, *77*). Furthermore, these results suggest that when peroxynitrite is coordinated to the heme center of Mb or Hb it rapidly isomerizes to nitrate without undergoing any oxidation or nitration reactions.

Further evidence that the peroxynitrito−met-protein complexes do not nitrate any of the two tyrosine residues present in Mb and in the α- and β-subunits of Hb is given by the HPLC analysis of the proteins after reacting them 10 times with $NO^{\bullet}$ (Figures 11 and 12). For both proteins less than 1% nitrated tyrosine (relative to the total tyrosine content) could be detected, which indicated that less than 0.1% tyrosine is nitrated when $NO^{\bullet}$ is allowed to react only once with $MbFeO_2$ or $HbFeO_2$.

CONCLUSIONS AND BIOLOGICAL RELEVANCE

In summary, we have shown that an intermediate complex is formed during the $NO^{\bullet}$-induced oxidation of both $MbFeO_2$ and $HbFeO_2$. These species can be characterized spectroscopically at alkaline pH but rapidly decay to the met-form of the proteins under neutral or acidic conditions. The intermediate Hb complex was assigned as $HbFe^{III}OONO$; its UV/vis spectrum is significantly different from those of $HbFe^{III}NO_2$ and $HbFe^{III}ONO_2$. The Mb intermediate complex was assigned as $Mb^{III}OONO$; its decay rate was significantly larger than that of $MbFe^{III}ONO_2$. The rate of decay of $MbFe^{III}OONO$ is significantly larger than those for the peroxynitrito complexes of the two subunits of Hb. No free peroxynitrite is formed during these reactions, and nitration of the globin does not take place in physiologically significant amounts. The isomerization product of peroxynitrite, nitrate, is always formed quantitatively.

The average lifetime of red blood cells is 120 days, and in vivo about 1% of $HbFeO_2$ is oxidized daily to methemoglobin either by autoxidation or, more likely, by reaction with $NO^{\bullet}$. From these data it can be concluded that each Hb molecule is oxidized once in the course of its existence. If we assume that this is caused exclusively by reaction with $NO^{\bullet}$, our results indicate that maximally 0.1% nitrotyrosine is produced and thus that this nitration reaction is physiologically not relevant. This conclusion is in agreement with the observation that no nitrated tyrosine residues were found in aged red blood cells (*80*).

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