Mechanistic Studies of the Oxidation of Oxyhemoglobin by Peroxynitrite†

Francesca Boccini and Susanna Herold*

Laboratorium für Anorganische Chemie, Eidgenössische Technische Hochschule, ETH Hönggerberg, CH-8093 Zürich, Switzerland

Received August 17, 2004; Revised Manuscript Received October 1, 2004

ABSTRACT: The strong oxidizing and nitrating agent peroxynitrite has been shown to diffuse into erythrocytes and oxidize oxyhemoglobin (oxyHb) to metHb. Because the value of the second-order rate constant for this reaction is on the order of 10^4 M^{-1} s^{-1} and the oxyHb concentration is about 20 mM (expressed per heme), this process is rather fast and oxyHb is considered a sink for peroxynitrite. In this work, we showed that the reaction of oxyHb with peroxynitrite, both in the presence and absence of CO_2, proceeds via the formation of oxoiron(IV)hemoglobin (ferrylHb), which in a second step is reduced to metHb and nitrate by its reaction with $NO_2^{\bullet}$. In the presence of physiological relevant amounts of CO_2, ferrylHb is generated by the reaction of $NO_2^{\bullet}$ with the coordinated superoxide of oxyHb ($HbFe^{III}O_2^{\bullet-}$). This reaction proceeds via formation of a peroxynitrato−metHb complex ($HbFe^{III}OONO_2$), which decomposes to generate the one-electron oxidized form of ferrylHb, the oxoiron(IV) form of hemoglobin with a radical localized on the globin. $CO_3^{\bullet-}$, the second radical formed from the reaction of peroxynitrite with CO_2, is also scavenged efficiently by oxyHb, in a reaction that finally leads to metHb production. Taken together, our results indicate that oxyHb not only scavenges peroxynitrite but also the radicals produced by its decomposition.

Peroxynitrite,[1] a biological relevant oxidizing and nitrating species, is produced by the nearly diffusion-limited reaction between nitrogen monoxide ($NO^{\bullet}$) and superoxide ($O_2^{\bullet-}$) radicals (*1*, *2*). Hemoproteins are among the targets that are modified by peroxynitrite in vivo. These proteins can be oxidized by peroxynitrite (*3*−*5*) or can catalyze its isomerization to nitrate (*6*, *7*). Moreover, in some cases hemoproteins have been shown to catalyze the peroxynitrite-mediated nitration of externally added tyrosine and/or of their own tyrosine residues (*5*, *8*). The high concentration in which hemoproteins are found in some tissues and/or the large values of the second-order rate constants for their reactions with peroxynitrite lead to in vivo rates comparable to that of the reaction of peroxynitrite with CO_2, a key compound that strongly influences peroxynitrite reactivity in biological systems. The reaction between peroxynitrite anion and CO_2 [6×10^4 M^{-1} s^{-1} at 37 °C (*9*) and 3×10^4 M^{-1} s^{-1} at 20 °C (*10*)] significantly shortens the lifetime of peroxynitrite in biological systems and leads to the production of carbonate radicals [$CO_3^{\bullet-}$, systematic name: trioxidocarbonate (•1-)] and nitrogen dioxide ($NO_2^{\bullet}$).

Myoglobin (Mb)[2] and hemoglobin (Hb) have been shown to react with peroxynitrite according to a variety of different mechanisms, depending on their oxidation state and on the ligands bound to the sixth coordination site. Their iron(III) forms, metMb and metHb, catalyze the isomerization of peroxynitrite to nitrate, albeit not very efficiently (at pH 7.0 and 20 °C, $k_{cat} = (2.9 \pm 0.1) \times 10^4$ and $(1.2 \pm 0.1) \times 10^4$ M^{-1} s^{-1}, respectively) (*6*). The reduced nitrosyl form of Hb ($HbFe^{II}NO$) is oxidized by peroxynitrite to the corresponding iron(III) nitrosyl from ($HbFe^{III}NO$) (*11*). The second-order rate constants for this process are $(6.1 \pm 0.3) \times 10^3$ and $(5.3 \pm 0.2) \times 10^4$ M^{-1} s^{-1}, in the absence and in the presence of 1.2 mM CO_2, respectively (*11*).

OxyMb is oxidized to metMb by an excess of peroxynitrite in a two-step mechanism (eqs 1 and 2). First, oxyMb is oxidized to $MbFe^{IV}$═O [$(5.4 \pm 0.2) \times 10^4$ M^{-1} s^{-1} at pH 7.3 and 20 °C], which then is reduced by peroxynitrite to metMb [$(2.2 \pm 0.1) \times 10^4$ M^{-1} s^{-1} at pH 7.3 and 20 °C] (*12*). In the presence of 1.2 mM CO_2, the overall reaction mechanism is the same but the value of the second-order rate constant for the first reaction step is significantly larger [$(4.1 \pm 0.7) \times 10^5$ M^{-1} s^{-1} at pH 7.5 and 20 °C] (*13*). In contrast, the value of the second-order rate constant for the reduction of $MbFe^{IV}$═O to metMb is only slightly larger [$(3.2 \pm 0.2) \times 10^4$ M^{-1} s^{-1} at pH 7.5 and 20 °C] (*13*).

$$MbFeO_2 + HOONO/ONOO^- \rightarrow MbFe^{IV}{=}O + ... \quad (1)$$

$$MbFe^{IV}{=}O + HOONO/ONOO^- \rightarrow metMb + ... \quad (2)$$

Peroxynitrite has been shown to be able to cross erythrocyte membranes by simple diffusion of the protonated form or via an anion-channel dependent mechanism (for $ONOO^-$) (*14*, *15*). Thus, oxyHb is also an important target for peroxynitrite produced in the intravascular compartment. In

† This work was supported by the ETH Zürich.

* To whom correspondence should be addressed: Laboratorium für Anorganische Chemie, ETH Hönggerberg − HCI, Room H 215, CH-8093 Zürich, Switzerland. E-mail: herold@inorg.chem.ethz.ch. Fax: (41) (1) 632 10 90. Tel: (41)(1) 632 28 58.

[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).

10.1021/bi0482250 CCC: $27.50
Published on Web 12/03/2004

this article, we present a detailed kinetic and mechanistic study of the reaction of peroxynitrite with deoxyHb and oxyHb, both in the presence and absence of added CO_2. Moreover, to support our new mechanistic hypothesis that attributes to $NO_2^{\bullet}$ an important role for the production of the observed ferryl intermediate ($HbFe^{IV}$=O), we also present pulse radiolysis studies of the reaction between oxyHb and $NO_2^{\bullet}$.

EXPERIMENTAL PROCEDURES

Reagents. Buffer solutions were prepared from K_2HPO_4/KH_2PO_4 (Fluka) with deionized Milli-Q water. Sodium dithionite, sodium nitrite, sodium nitrate, potassium superoxide, *N*-ethylmaleimide (NEM), and hydrogen peroxide were obtained from Fluka. Sodium bicarbonate was purchased from Merck. 4,4′-Dithiopyridine (4-PDS) was purchased from Aldrich. Nitrogen monoxide was obtained from Linde and passed through a NaOH solution as well as a column of NaOH pellets to remove higher nitrogen oxides before use.

Peroxynitrite, Carbon Dioxide, and Protein Solutions. Peroxynitrite was prepared from KO_2 and gaseous nitrogen monoxide according to the literature (*16*) 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. Nitrite did not interfere with our studies, since the reactions of $HbFeO_2$ or $HbFe^{IV}$=O with nitrite proceed at a significantly slower rate than the corresponding reactions with peroxynitrite (*6*, *17*, *18*). The peroxynitrite stock solution was diluted with 0.01 M NaOH, and the concentration of the resulting solutions was determined spectrophotometrically prior to each experiment by measuring their absorbance at 302 nm [$\epsilon_{302} = 1705\ M^{-1}\ cm^{-1}$ (*19*)].

For the experiments carried out in the absence of added CO_2, the buffers and the 0.01 M NaOH solutions were prepared fresh daily and thoroughly degassed. Experiments in the presence of CO_2 were carried out by adding the required amount of a freshly prepared 0.5 M sodium bicarbonate 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 taken from ref *20*, by taking in consideration the ionic strength of the solutions. After addition of CO_2 or bicarbonate, the protein solutions were allowed to equilibrate at room temperature for at least 5 min.

A purified human oxyhemoglobin ($HbFeO_2$) stock solution (57 mg/mL solution of HbA0 with approximately 1.1% metHb, no apoHb, and free of catalase) was a kind gift from APEX Bioscience, Inc. MetHb and $HbFe^{IV}$=O (ferrylHb) solutions were prepared as described elsewhere (*6*). Deoxyhemoglobin was prepared by thoroughly degassing a $HbFeO_2$ solution. The concentrations of the solutions containing the different hemoglobin forms were determined spectrophotometrically (*21*). Cysteine*β*93-blocked $HbFeO_2$ was prepared by allowing $HbFeO_2$ (∼2 mM in 0.1 M phosphate buffer pH 7.2) to react with a 10-fold excess of NEM for 1 h. The solution was then purified by chromatography over a Sephadex G-25 column by using a 0.1 M phosphate buffer solution (pH 7.0) as the eluent. The efficiency of the blocking procedure was controlled by analyzing the free Cys*β*93 content with 4,4′-dithiopyridine (4-PDS) (*22*, *23*). The concentration of the NEM-blocked $HbFeO_2$ solution was determined by using the values of the extinction coefficients of the absorbance maxima of unmodified metHb (*21*).

Stopped-Flow Experiments. The protein solutions were prepared by diluting the $HbFeO_2$ and the $HbFe^{IV}$=O stock solutions to the desired concentration with 0.1 M phosphate buffer (pH 5.6−7.3) under aerobic conditions, in the absence or presence of added bicarbonate. Peroxynitrite solutions were prepared by diluting the stock solution immediately before use with 0.01 M NaOH to achieve the required concentration. In general, the protein was dissolved in a 0.1 M buffer at a pH slightly more acidic (0.4−0.7 pH units lower) than the desired final pH, which was always measured at the end of the reactions for control.

Kinetic studies were carried out either with a SX18MV-R or a SX17MV Applied Photophysics single-wavelength stopped-flow instrument and with an On-Line Instrument Systems Inc. stopped-flow instrument equipped with an OLIS RSM 1000 rapid scanning monochromator. The length of the cells in the three spectrophotometers is 1 cm. The mixing time of the instruments is 2−4 ms. All measurements were carried out at 20 °C.

With the Applied Photophysics instrument, kinetic traces were collected at different wavelengths between 300 and 650 nm and the data were analyzed with Kaleidagraph, version 3.52. For the determination of the second-order rate constants, the traces were mostly collected at 586 and 609 nm. For the kinetic studies of the reactions in the presence of CO_2 carried out with the SX18MV-R Applied Photophysics instrument, it was essential to insert a cutoff filter (550 nm) to avoid the interference of stray light due to second-order effects of the strong absorbance band of peroxynitrite (λ_{max} = 302 nm). Indeed, in the absence of the filter the traces measured at 609 nm showed the expected increase in absorbance, followed by a decrease with an amplitude mostly larger than that of the first process. Variation of pH, protein, and peroxynitrite concentrations showed that the observed decrease in absorbance corresponded to the second-order absorbance of the 302 nm band of peroxynitrite. In the presence of the filter, the traces measured at 609 nm displayed only the expected increase in absorbance.

In all cases, the results of the fits of the traces (averages of at least 10 single traces) from at least five experiments were averaged to obtain each observed rate constant, given with the corresponding standard error. Care was taken that the absolute absorbance of the reaction mixture was not higher than one absorbance unit.

For the studies of the reaction between deoxyHb and peroxynitrite, the cooling water of the OLIS stopped-flow instrument was degassed, and the syringes and the lines of the instrument were filled with a concentrated sodium dithionite solution, which was allowed to react with the oxygen present in the lines for several minutes. Immediately before the experiments, the stopped-flow lines were washed with several milliliters of degassed buffer. The two thor-

[2] Abbreviations: EPR, electron paramagnetic resonance; Hb, hemoglobin; $HbFeO_2$, oxyhemoglobin (oxyHb); metHb, iron(III)hemoglobin; $HbFe^{IV}$=O, oxoiron(IV)hemoglobin, ferrylHb; Mb, myoglobin; $MbFeO_2$, oxymyoglobin (oxyMb); metMb, iron(III)myoglobin; $MbFe^{IV}$=O, oxoiron(IV)myoglobin, ferrylMb; $HbFe^{II}$, deoxyhemoglobin; NEM, *N*-ethylmaleimide.

oughly degassed solutions of deoxyHb and peroxynitrite were transferred in two gastight Hamilton SampleLock syringes. Before inserting the syringes in the corresponding connections of the instrument, we filled these connections with water and degassed them for several minutes by using a thin needle. The insertion of the syringes was then performed as fast as possible by contemporaneously removing the needles. A few milliliters of the solutions were used to wash again the lines, and finally several shots were collected as fast as possible. With all these precautions, essentially no $HbFeO_2$ was formed prior to mixing deoxyHb with peroxynitrite.

Determination of the Amount of Peroxynitrite Required to Completely Convert $HbFeO_2$ or $HbFe^{IV}$═O to metHb. Absorption spectra were collected on an Analytik Jena Specord 200 spectrophotometer. The amount of peroxynitrite required to completely convert $HbFeO_2$ to metHb was determined in 0.1 M phosphate buffer pH 7.4 in the absence or presence of 1.2 mM CO_2. Peroxynitrite was either added as a bolus or titrated in the protein solution. In a typical experiment, the $HbFeO_2$ solution (2 mL of a 8 or a 39 μM solution) was placed in a sealable cell, 100−200 μL of a peroxynitrite solution of variable concentrations was added, and the cell was gently shaken. Finally, a spectrum was recorded as fast as possible (in ca. 20 s) to avoid subsequent reaction with the nitrite present as an impurity in the peroxynitrite solution (*13*). If the reaction was not complete, an additional peroxynitrite aliquot was added immediately. The reaction was considered to be finished when addition of peroxynitrite did not induce further changes in the UV/vis spectrum. In the presence of 1.2 mM CO_2, upon addition of more than 5 equiv of peroxynitrite to the 39 μM $HbFeO_2$ solution the protein was found to degrade during the time required to measure the UV/vis spectra. Thus, the amount of peroxynitrite required to completely convert $HbFeO_2$ (8 or 39 μM) to metHb was also determined by mixing equal volumes of the two solutions with the OLIS stopped-flow instrument (both in the absence and presence of added CO_2). The reaction was followed spectrophotometrically and the last spectra were compared with that of pure metHb (collected under analogous conditions). Similar reactions were carried out by mixing $HbFe^{IV}$═O with peroxynitrite. Finally, to study whether the reaction of peroxynitrite with the reactive cysteine residues β93 influenced the number of equivalents required to completely oxidize $HbFeO_2$ to metHb, we carried out analogous experiments also with NEM-blocked $HbFeO_2$. All experiments were carried out at least twice and the accuracy of the results is to be considered ±1 equivalent.

Determination of Nitrite and Nitrate. Product analysis was carried out as described previously (*24*, *25*) by anion chromatography with conductivity detection with a Metrohm instrument (IC Separation Center 733, IC detector 732 and IC pump 709) equipped with an Anion SUPER-SEP (6.1009.000) column and an Anion SUPER−SEP (6.1009.010) precolumn. Calibration curves were obtained by measuring 5−10 standard sodium nitrite and sodium nitrate solutions in 5 mM phosphate buffer. The samples were prepared by mixing 500 μL of $HbFeO_2$ (5 or 10 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 500 μL of an ice-cooled peroxynitrite solution (200 μM in 0.01 M NaOH) while vortexing. The reaction mixture was diluted 1:10 with water and analyzed within ca. 5 min. At least two analyses of three separate experiments were carried out for each protein. The results are given as mean values plus or minus the corresponding standard deviation. The contamination of nitrite and nitrate in peroxynitrite was determined as reported recently (*25*). Usual nitrite and nitrate contaminations were in the range 20−50 and 0−10% of the peroxynitrite concentration, respectively.

Pulse Radiolysis. Pulse radiolysis experiments were carried out with a Febetron 705 (Titan Systems Corp, San Leandro, CA) 2 MeV accelerator as described previously (*26*). The length of the cell was 1 cm. N_2O-saturated $HbFeO_2$ solutions of different concentrations were prepared by adding the required volume of a ca. 3 mM $HbFeO_2$ stock solution to N_2O-saturated 0.1 M phosphate buffer pH 7.4 directly in a gastight SampleLock Hamilton syringe. The 10 mM N_2O-saturated nitrite solution was prepared analogously from a 0.2 M nitrite stock solution. The $HbFeO_2$ and the nitrite solutions were then rapidly mixed before irradiating the cell (mixing time ca. 2−3 s). Under the conditions of our experiments, radiolysis primarily yields hydroxyl radicals which rapidly and selectively react with nitrite to generate nitrogen dioxide. Radiation doses (3−140 Gy) were used to produce between 2 and 75 μM $NO_2^{\bullet}$.

RESULTS

Rapid-Scan UV/vis Spectroscopic Studies of the Reaction Between Oxyhemoglobin and Peroxynitrite in the Absence of Carbon Dioxide. Preliminary studies of the reaction of peroxynitrite with oxyhemoglobin ($HbFeO_2$) showed that it proceeds in two steps, analogously to the corresponding reaction with oxymyoglobin (*12*). First, $HbFeO_2$ is oxidized by peroxynitrite to $HbFe^{IV}$═O, which is subsequently reduced by peroxynitrite to yield the final product, metHb. Because the observed rate constants for the two reaction steps are nearly identical, $HbFe^{IV}$═O does not accumulate in concentrations large enough to be detected directly. However, the presence of $HbFe^{IV}$═O as a reaction intermediate was confirmed by detecting Fe(II)sulfohemoglobin when peroxynitrite was added to $HbFeO_2$ in the presence of Na_2S (*12*, *27*).

As depicted in Figure 1, rapid-scan UV/vis spectroscopic studies of the reaction of $HbFeO_2$ with an excess of peroxynitrite (at pH 7.4 and 20 °C) clearly indicated the presence of two distinct reaction steps, each with a set of isosbestic points corresponding to the transformations $HbFeO_2$ → $HbFe^{IV}$═O (475, 527, and 584 nm) and $HbFe^{IV}$═O → metHb (468, 529, and 610 nm), respectively. To determine the relative concentration of the different Hb species during the course of the reaction, all the spectra shown in Figure 1 were fitted by using a linear combination of the spectra of pure $HbFeO_2$, $HbFe^{IV}$═O, and metHb. The results given in Figure 2 indicate that maximally 20% of $HbFe^{IV}$═O accumulated after about 0.6 s, under the conditions of the experiment depicted in Figure 1. At the end of the reaction (after ca. 4 s), most $HbFeO_2$ was converted to metHb, but approximately 5% of Hb were present in the ferryl form.

pH Dependence of the Oxidation Rate of Oxyhemoglobin by Peroxynitrite in the Absence of Carbon Dioxide. To get a better understanding of the mechanism of the reaction of $HbFeO_2$ with peroxynitrite, we studied the kinetics of this

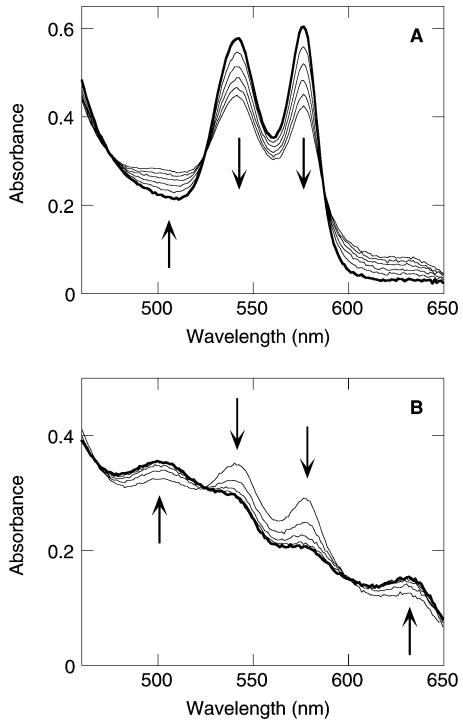

FIGURE 1: Rapid-scan UV/vis spectra of the reaction between $HbFeO_2$ (42 μM) and peroxynitrite (294 μM), in 0.05 M phosphate buffer at pH 7.4 and 20 °C. The traces depicted were recorded in (A) 0, 128, 256, 384, 512, and 640 ms after mixing, and in (B) 1.7, 2.3, 2.9, 3.6, and 4.2 s after mixing. In (A), the spectrum of $HbFeO_2$ is shown in bold. In (B), the final spectrum is shown in bold.

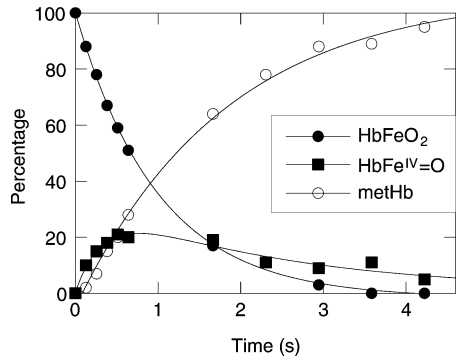

FIGURE 2: Percentage of the different Hb forms present during the reaction of $HbFeO_2$ (42 μM) with peroxynitrite (294 μM) in 0.05 M phosphate buffer pH 7.4 at 20 °C. The relative concentrations of the three Hb forms were determined by fitting the spectra shown in Figure 1 with a linear combination of the spectra of pure $HbFeO_2$, $HbFe^{IV}$=O, and metHb.

reaction at pH 6.4, 7.4, and 8.0 (at 20 °C). In all our experiments, peroxynitrite was present at least in 10-fold excess to maintain pseudo-first-order conditions. As in our previous work with $MbFeO_2$ (*12*), the two steps of the reaction were studied separately. The first step ($HbFeO_2 \rightarrow HbFe^{IV}$=O) was followed at absorbance changes at 609 nm (at pH 7.4, $\Delta\epsilon_{609} = 3.3\ mM^{-1}\ cm^{-1}$), close to one of the isosbestic points of the spectra of $HbFe^{IV}$=O and metHb. The reaction of $HbFe^{IV}$=O to metHb (second step) was studied by following the absorbance changes at 586 nm (at pH 7.4, $\Delta\epsilon_{586} = 4.3\ mM^{-1}\ cm^{-1}$), one of the isosbestic points of the spectra of $HbFeO_2$ and $HbFe^{IV}$=O. For both wavelengths, time courses could be fitted to a single exponential expression (data not shown). Traces for the second reaction

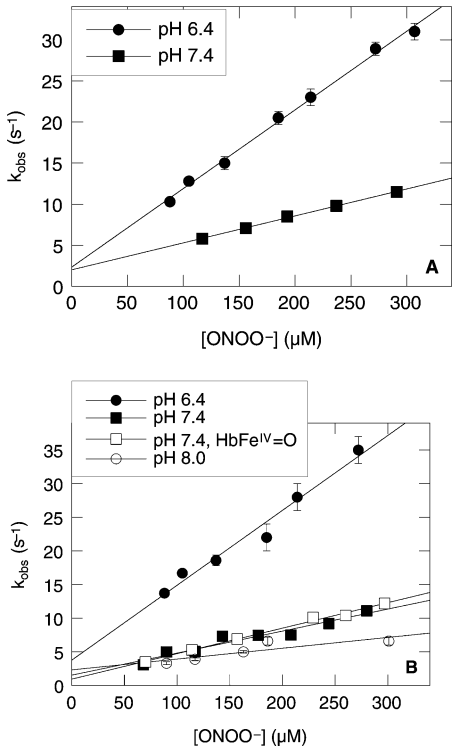

FIGURE 3: Plots of k_{obs} versus peroxynitrite concentration for the two steps (A) $HbFeO_2 \rightarrow HbFe^{IV}$=O and (B) $HbFe^{IV}$=O → metHb of the peroxynitrite-mediated oxidation of $HbFeO_2$ (4 μM) in 0.05 M phosphate buffer at 20 °C. In (B), comparison with the data obtained from the reaction of separately prepared $HbFe^{IV}$=O with peroxynitrite under the same conditions. The second-order rate constants resulting from the linear fits depicted are summarized in Table 1.

step were fitted approximately from the point when the first step was over.

At pH 8.0, no clear isosbestic point was observed for the second step of the reaction, the conversion of $HbFe^{IV}$=O to metHb, which under these conditions is a mixture of $HbFe^{III}OH_2$ and $HbFe^{III}OH$. Thus, no wavelength was found at which we could follow the absorbance changes arising exclusively from the first reaction step. Moreover, because the values of the observed rate constants for the two reaction steps are very similar, it was also not possible to determine the two values by fitting a trace collected at a wavelength at which both reaction steps contribute to the observed absorbance changes. Consequently, at pH 8.0 we could only determine the second-order rate constant for the conversion $HbFe^{IV}$=O → metHb. Indeed, the spectra of $HbFeO_2$ and $HbFe^{IV}$=O are pH independent and, thus, the kinetic traces for this second reaction step could be collected at 586 nm, as under neutral conditions.

As shown in Figure 3, at all pH values studied the observed rate constants increased linearly with increasing peroxynitrite concentration. For both reaction steps, the values of the second-order rate constants decreased with increasing pH (Table 1). The observed pH dependence suggests that HOONO [$pK_a = 6.8$ (*28*)], or the products of its decomposition, are the species that react with $HbFeO_2$. Alternatively, the larger second-order rate constant obtained at lower pH may be a consequence of conformational changes occurring in the protein after protonation of histidine residues, in particular, of the distal histidine. Interestingly, the same pH dependence was observed for the analogous reaction of

Table 1: pH-Dependence of the Second-Order Rate Constants (M^{-1} s^{-1}) of the Two Steps of the Reaction between $HbFeO_2$ and Peroxynitrite Obtained at 20 °C

pH	$[CO_2]$ (mM)	$HbFeO_2 \rightarrow HbFe^{IV}{=}O$	$HbFe^{IV}{=}O \rightarrow$ metHb
6.4	0	$(9.6 \pm 0.3) \times 10^4$	$(9 \pm 1) \times 10^4$
6.4	1.2	$(1.7 \pm 0.4) \times 10^5$	$(2.3 \pm 0.2) \times 10^5$
6.4	2.4	$(2.6 \pm 0.2) \times 10^5$	n.d.[b]
7.4	0	$(3.3 \pm 0.1) \times 10^4$	$(3.3 \pm 0.4) \times 10^4$
7.4[a]	0		$(3.8 \pm 0.2) \times 10^4$
7.4	1.2	$(3.5 \pm 0.3) \times 10^5$	$(1.1 \pm 0.1) \times 10^5$
7.4[a]	1.2		$(2.5 \pm 0.2) \times 10^5$
7.4	2.4	$(6.1 \pm 0.7) \times 10^5$	n.d.[b]
8.0	0	n.d.[b]	$(1.6 \pm 0.5) \times 10^4$
8.0	1.2	$(2.2 \pm 0.1) \times 10^5$	$(5.2 \pm 0.6) \times 10^4$
8.0	2.4	$(5.1 \pm 0.3) \times 10^5$	n.d.[b]

[a] Reaction with separately prepared $HbFe^{IV}{=}O$. [b] Not determined.

peroxynitrite with $MbFeO_2$ (*12*).

At all pH studied, the values of the observed rate constants for the first and the second steps of the reaction between $HbFeO_2$ and peroxynitrite were always very similar. However, for the analogous reaction with $MbFeO_2$ the value of the first reaction step was always 2−3 times larger than that of the second step (*12*).

To further support the assignment of the second step of the peroxynitrite-mediated oxidation of $HbFeO_2$ as the reduction of $HbFe^{IV}{=}O$ to metHb, we investigated the kinetics of the reaction of separately prepared $HbFe^{IV}{=}O$ with peroxynitrite by following the absorbance changes at 586 nm (at pH 7.4). As shown in Figure 3B, the second-order and the observed rate constants measured for the reaction of $HbFe^{IV}{=}O$ with different peroxynitrite concentrations are essentially identical to those of the second step of the peroxynitrite-mediated oxidation of $HbFeO_2$ under identical conditions.

Dependence of the Rate Constants for the Peroxynitrite-Mediated Oxidation of Oxyhemoglobin on the Protein Concentration. We have previously reported values of $(8.8 \pm 0.4) \times 10^4$ and $(9.4 \pm 0.7) \times 10^4$ M^{-1} s^{-1} for the second-order rate constants for the first and the second steps of the peroxynitrite-mediated oxidation of $HbFeO_2$ at pH 7.0 and 20 °C, respectively (*12*). At first sight, these values are not consistent with those reported in this work (Table 1). However, all the kinetic studies reported here were carried out with a protein concentration of 4 μM, whereas previous studies were carried out with 2 μM protein. As shown in Figure 4, the observed rate constants of the peroxynitrite-mediated oxidation of $HbFeO_2$ increase with decreasing protein concentration. Interestingly, a similar trend was previously observed for the analogous reaction between peroxynitrite and $MbFeO_2$ (*13*).

A possible explanation for these results is that the reaction of peroxynitrite with the dimeric form of the protein is faster because in tetrameric $HbFeO_2$ the direct interaction of peroxynitrite with the heme center is partly hindered. As discussed in our previous work (*13*), aggregation of the protein may also be responsible for the observed decrease of the rate of the reaction with higher concentrations of $MbFeO_2$.

Reaction of Deoxyhemoglobin with Peroxynitrite in the Absence of Carbon Dioxide. The reaction of deoxyHb with peroxynitrite was investigated by rapid-scan UV/vis spectroscopy at pH 7.4 and 20 °C. As shown in Figure 5, when deoxyHb (2.5 μM) was mixed with thoroughly degassed peroxynitrite (100 μM), the characteristic absorbance maximum of the Soret band of deoxyHb (430 nm) decreased whereas a new band appeared with a maximum at 405 nm (metHb). At first sight, the reaction seemed to proceed without the formation of intermediates. However, as shown from the trace extracted at 416 nm (Figure 5, below), also the reaction of deoxyHb with peroxynitrite first yields an intermediate, very likely $HbFe^{IV}{=}O$, that is converted to the final product (metHb) only in a second stage of the reaction. $HbFe^{IV}{=}O$ cannot be detected directly because its spectrum shows an absorbance maximum at 418 nm, a wavelength that lies between those of deoxyHb (430 nm) and metHb

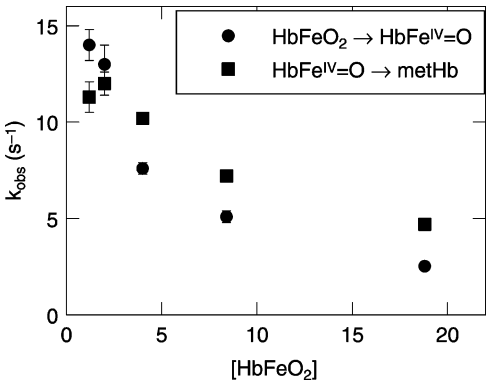

FIGURE 4: Dependence on the protein concentration of the observed pseudo-first-order rate constants for the two steps of the reaction between peroxynitrite (250 μM) and $HbFeO_2$ (in 0.05 M phosphate buffer pH 7.4 and 20 °C).

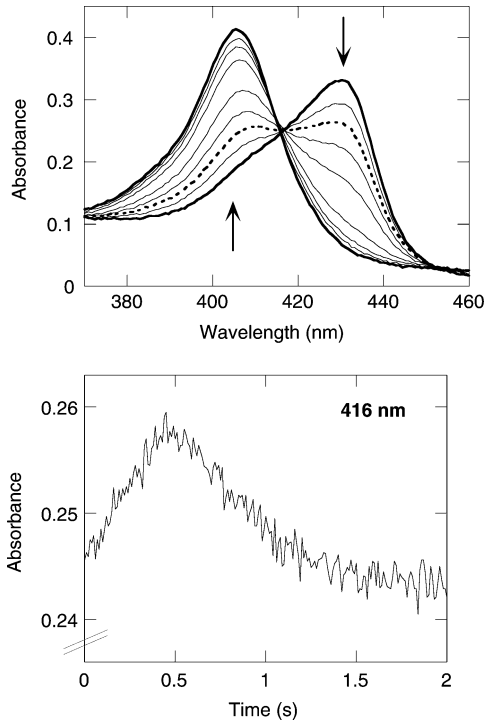

FIGURE 5: (Top) Rapid-scan UV/vis spectra of the reaction between deoxyHb (2.5 μM) and peroxynitrite (100 μM) in 0.05 M phosphate buffer at pH 7.4, 20 °C. The traces depicted were recorded 0, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1, and 1.6 s after mixing. Spectra were collected each millisecond, but to improve the signal-to-noise ratio, each curve shown represents the average of 10 measured curves. The spectra of deoxyHb and metHb are shown in bold (line), and the spectrum recorded after 0.2 s is shown as a dotted bold line. (Bottom) Kinetic trace at 416 nm.

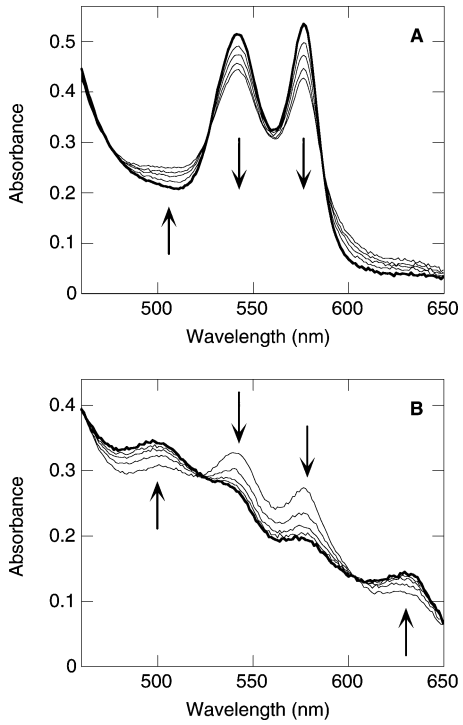

FIGURE 6: Rapid-scan UV/vis spectra of the reaction between $HbFeO_2$ (36 μM) and peroxynitrite (360 μM) in the presence of CO_2 (1.2 mM), in 0.05 M phosphate buffer at pH 7.4 and 20 °C. The traces depicted were recorded in (A) 0, 4, 8, 12, and 16 ms after mixing, and in (B) 72, 108, 144, 180, and 252 ms after mixing. In (A), the spectrum of $HbFeO_2$ is shown in bold. In (B), the final spectrum is shown in bold.

(405 nm). Moreover, the rates of formation and decay of $HbFe^{IV}$=O are rather similar (approximately 3.1 and 2.1 s^{-1}, under the conditions of the experiment depicted in Figure 5) and thus its steady-state concentration is expected to be very small. Taken together, these results show that the order of magnitude of the second-order rate constant for the formation of $HbFe^{IV}$=O from the reaction of deoxyHb with peroxynitrite is also 10^4 M^{-1} s^{-1}. Interestingly, we have previously shown that the reaction of deoxyMb with peroxynitrite proceeds at a significantly faster rate (ca. 10^6 M^{-1} s^{-1} (*12*)), despite the similarity between the two proteins.

Rapid-Scan UV/vis Spectroscopic Studies of the Reaction Between Oxyhemoglobin and Peroxynitrite in the Presence of Carbon Dioxide. Carbon dioxide, which rapidly reacts with ONOO− to yield the adduct $ONOOCO_2^-$ which partly decomposes to $CO_3^{\bullet-}$ and $NO_2^{\bullet}$ (*9*, *10*, *29*), represents one of the most important targets of peroxynitrite in biological systems. Thus, we have investigated the influence of CO_2 on the reaction between $HbFeO_2$ and peroxynitrite. As shown in Figure 6, in the presence of 1.2 mM CO_2 the changes observed in the rapid-scan UV/vis spectra of the reaction between $HbFeO_2$ and peroxynitrite were qualitatively identical to those observed in its absence (Figure 1). In the first part of the reaction (Figure 6A), $HbFeO_2$ is oxidized to $HbFe^{IV}$=O with isosbestic points at 525 and 586 nm. The last spectra (Figure 6B) show the conversion of $HbFe^{IV}$=O to metHb, with isosbestic points at 523 and 605 nm. Taken together, these observations suggest that addition of CO_2 does not significantly alter the overall reaction mechanism. The formation of $HbFe^{IV}$=O as an intermediate of the reaction between $HbFeO_2$ and peroxynitrite in the presence of

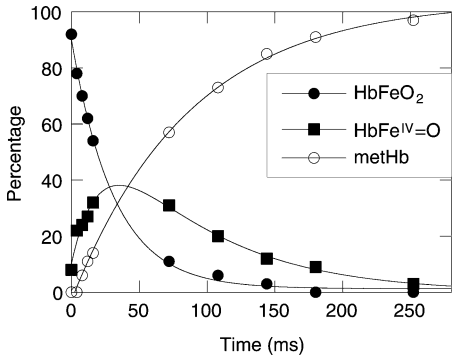

FIGURE 7: Percentage of the different Hb forms present during the reaction of $HbFeO_2$ (36 μM) with peroxynitrite (360 μM)) in the presence of CO_2 (1.2 mM), in 0.05 M phosphate buffer at pH 7.4 and 20 °C. The relative concentrations of the three Hb forms were determined by fitting the spectra shown in Figure 6 with a linear combination of the spectra of pure $HbFeO_2$, $HbFe^{IV}$=O, and metHb.

1.2 mM CO_2 has previously been demonstrated by detection of Fe(II)sulfohemoglobin upon reaction of $HbFeO_2$ and peroxynitrite in the presence of Na_2S (*30*).

Also for the experiment depicted in Figure 6, we fitted all the spectra with a linear combination of the spectra of pure $HbFeO_2$, $HbFe^{IV}$=O, and metHb, to determine the relative concentration of the different Hb species during the course of the reaction. As shown in Figure 7, the amount of $HbFe^{IV}$=O that accumulated under the conditions of this experiment was maximally around 40%, approximately 30 ms after mixing. Interestingly, this amount is significantly higher than that accumulated in a similar experiment in the absence of added CO_2 (Figure 2).

The kinetics of the two steps of the reaction between $HbFeO_2$ and peroxynitrite in the presence of different amounts of CO_2 were studied separately by single-wavelength stopped-flow spectroscopy under pseudo-first-order conditions with peroxynitrite in excess, analogously to the studies in the absence of CO_2. At pH 7.4, we found that the observed rate constants for the two reaction steps increased nearly linearly with increasing CO_2 concentration in the range 0.6−4.8 mM (Figure S1, Supporting Information). This result indicates that $HbFeO_2$ must react at a larger rate with the products formed from the reaction of $ONOO^-$ with CO_2 than with peroxynitrite.

Interestingly, our data showed that addition of CO_2 had a more prominent effect on the value of the rate constant of the first step of the reaction between $HbFeO_2$ and peroxynitrite. Indeed, in contrast to the experiments in the absence of added CO_2, the values of observed rate constants for the first step were always significantly larger than those of the second step. At higher CO_2 concentrations, the errors associated with the values of the observed rate constants were very large, because the values of the amplitudes decreased with increasing CO_2 concentrations.

pH Dependence of the Oxidation Rate of Oxyhemoglobin by Peroxynitrite in the Presence of Carbon Dioxide. To get a better understanding of the influence of CO_2, we investigated the reaction between peroxynitrite and $HbFeO_2$ at pH 6.3, 7.4, and 8.0 in the presence of 1.2 mM CO_2. As shown in Figure 8, at all three pH values studied the observed rate constants linearly increased with increasing peroxynitrite concentration. Interestingly, we did not observe a clear dependence on the pH, either for the pseudo-first-order or

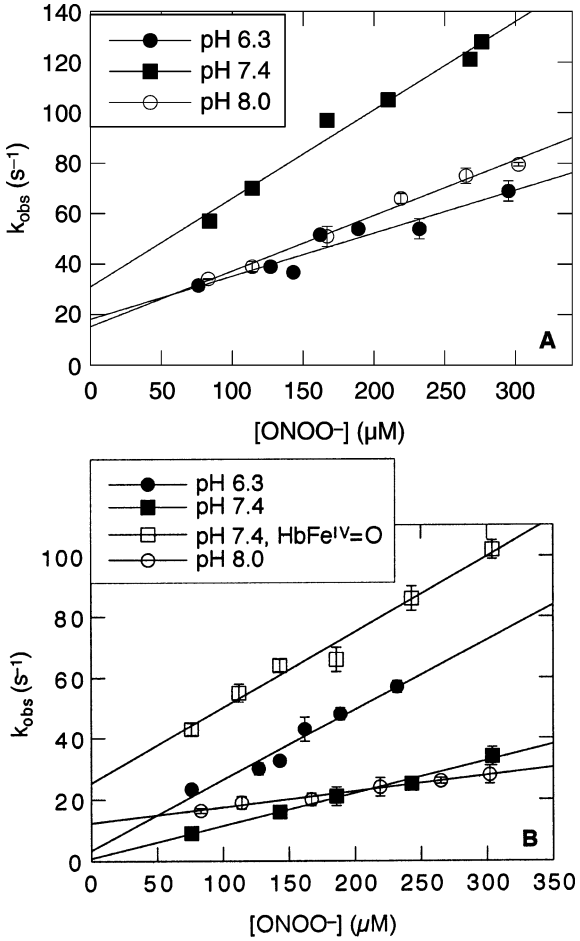

FIGURE 8: Plots of k_{obs} versus peroxynitrite concentration for the two steps (A) $HbFeO_2 \rightarrow HbFe^{IV}{=}O$ and (B) $HbFe^{IV}{=}O \rightarrow$ metHb of the peroxynitrite-mediated oxidation of $HbFeO_2$ (4 μM) in the presence of 1.2 mM CO_2 (in 0.05 M phosphate buffer at 20 °C). Comparison with the data obtained from the reaction of separately prepared $HbFe^{IV}{=}O$ and peroxynitrite under the same conditions. The second-order rate constants resulting from the linear fits depicted are summarized in Table 1.

for the second-order rate constants. For the first reaction step, the reaction proceeded at a similar rate at pH 6.3 and 8.0, but was significantly faster at pH 7.4 (Figure 8A). In contrast, for the second step the values of the pseudo-first-order and the second-order rate constants were larger at pH 6.3 than at pH 7.4 (Figure 8B). At pH 8.0, the value of the second-order rate constant was even smaller. In general, at all pH studied the second-order rate constants for both reaction steps in the presence of 1.2 mM CO_2 were significantly larger than those determined in the absence of CO_2 (Table 1). Interestingly, Mb and Hb do not show an analogous pH dependence of the second-order rate constants in the presence of 1.2 mM CO_2. Indeed, the second-order rate constants for the first step of the reaction between $MbFeO_2$ and peroxynitrite (in the presence of 1.2 mM CO_2) continuously increase with increasing pH, whereas no clear pH dependence was found for the second reaction step (*13*).

The half-life of peroxynitrite in the presence of 1.2 mM CO_2 (at pH 7.4 and 20 °C, $t_{1/2} \approx 28$ ms) is significantly shorter than in its absence (at pH 7.4 and 20 °C, $t_{1/2} \approx 3$ s). Thus, the second step of the reaction between $HbFeO_2$ and peroxynitrite in the presence of CO_2 must consist of the reduction of $HbFe^{IV}{=}O$ to metHb by products derived from the decay of $ONOOCO_2^-$, possibly $NO_2^\bullet$. We have previously shown that $HbFe^{IV}{=}O$ is rapidly reduced by $NO_2^\bullet$ to metHb and nitrate (*31*). As shown in Figure 8, at pH 7.4 and in the presence of 1.2 mM CO_2 the observed rate constants of the reaction of peroxynitrite with separately prepared $HbFe^{IV}{=}O$ were considerably larger than those obtained for the second step of the reaction with $HbFeO_2$. This result can be explained by the observation that when $HbFe^{IV}{=}O$ is mixed with peroxynitrite higher amounts of $NO_2^\bullet$ will be available for reaction. In contrast, when $HbFeO_2$ is mixed with peroxynitrite in the presence of added CO_2, $HbFe^{IV}{=}O$ first has to be generated and in this time a large fraction of $NO_2^\bullet$ will have been consumed through other reactions. Among others, $NO_2^\bullet$ oxidizes $HbFeO_2$ (see below) and may react with tyrosine, tryptophan, and cysteine residues (*32*). Interestingly, this result is again in contrast to what was observed for the corresponding reaction with $MbFeO_2$, for which essentially identical second-order rate constants were measured for the reaction of peroxynitrite with $MbFe^{IV}{=}O$ and for the second step of the reaction with $MbFeO_2$ (*13*).

Table 2: Number of Equivalents of Peroxynitrite Required to Completely Oxidize $HbFeO_2$ under Different Conditions

addition procedure	$[HbFeO_2] = 8\ \mu M$		$[HbFeO_2] = 39\ \mu M$	
	no CO_2	1.2 mM CO_2	no CO_2	1.2 mM CO_2
1[a]	4	7	4	dec[b]
1-NEM[a,c]	5	8	5	7
2[d]	6	12	5	dec[b]
2-NEM[c,d]	5	10	4	>11
3[e]	6	7	5	10

[a] Peroxynitrite was added 1 equiv at a time. [b] The protein decomposed before the reaction was complete. [c] Experiments were carried out with NEM-$HbFeO_2$. [d] Peroxynitrite was added as a bolus from a concentrated solution. [e] Equal volumes of a peroxynitrite and a $HbFeO_2$ solution were mixed with the OLIS stopped-flow instrument.

The first step of the reaction between $HbFeO_2$ and peroxynitrite was studied at pH 6.3, 7.4, and 8.0 also in the presence of 2.4 mM CO_2 (Figure S2, Supporting Information). Under these conditions, the values of the pseudo-first-order and the second-order rate constants were all higher than those obtained in the presence of 1.2 mM CO_2 (Table 1). However, the same pH dependence was observed for the values of the second-order rate constants, which followed the order pH 6.3 < pH 7.4 > pH 8.0.

Amount of Peroxynitrite Required to Completely Convert $HbFeO_2$ or $HbFe^{IV}{=}O$ to metHb, in the Absence and Presence of CO_2. Because the reaction of peroxynitrite with $HbFeO_2$ is not very fast, under the conditions of our experiments, the parallel decay of peroxynitrite also takes place. Therefore, in most cases more than 2 equiv of peroxynitrite were required to completely oxidize $HbFeO_2$ to metHb. As summarized in Table 2, UV/vis spectroscopic studies showed that when $HbFeO_2$ (8 μM) was titrated with subsequent additions of 1 equiv of peroxynitrite (procedure 1), 4 equiv were required to completely oxidize $HbFeO_2$ in the absence of added CO_2. Interestingly, the absorbance maximum of the last spectrum, which remained unchanged upon addition of further equivalents of peroxynitrite, was always at 407 nm instead of 405 nm, as expected for metHb (*21*). This observation suggests that during the reaction with excess peroxynitrite the protein may be partly modified.

Approximately the same number of equivalents of peroxynitrite was needed when a more concentrated protein solution was used (39 μM) for a similar experiment. In this case, the last spectrum corresponded to that expected for essentially quantitative formation of metHb.

To find out whether the method used to add peroxynitrite had an influence on the amount required to completely convert $HbFeO_2$ to metHb, we carried out further experiments in which we added peroxynitrite as a bolus either from a concentrated solution (procedure 2) or by mixing equal volumes of the solutions with the OLIS stopped-flow instrument (procedure 3). In both cases, approximately 6 equiv of peroxynitrite were required to completely oxidize the diluted (8 μM) $HbFeO_2$ solutions, whereas only 5 equiv were needed for the 39 μM $HbFeO_2$ solution. Taken together, these results suggest that the mixing procedure does not influence considerably the outcome of the reaction between $HbFeO_2$ and peroxynitrite. A slightly larger excess of peroxynitrite is required to completely oxidize $HbFeO_2$ when it is added as a bolus, since in this case larger quantities of peroxynitrite decay without interacting with the protein. Alternatively, when peroxynitrite is titrated in the protein solution the slow reaction between nitrite (always present as an impurity in the peroxynitrite solution) and $HbFeO_2$ may contribute to metHb formation, apparently lowering the number of equivalents of peroxynitrite needed to completely oxidize $HbFeO_2$.

It has been shown that the reactive Cys residues β93 also react with peroxynitrite when it is mixed with $HbFeO_2$ (*33*). Thus, we carried out a similar set of experiments with NEM-$HbFeO_2$ (the protein form in which the Cysβ93 residues were blocked with NEM), to find out whether this concurring reaction was responsible for the large excess of peroxynitrite needed to completely oxidize $HbFeO_2$. As summarized in Table 2, no significant difference was observed among the reactions of peroxynitrite with $HbFeO_2$ and with NEM-$HbFeO_2$.

Because of the faster decay of peroxynitrite, in the presence of 1.2 mM CO_2 larger quantities of peroxynitrite were required to completely oxidize $HbFeO_2$, independently of the protein concentration and the addition procedure used (Table 2). Interestingly, for the experiments with high protein concentrations it was not possible to follow the reaction by UV/vis spectroscopy because the protein was apparently modified before complete conversion of $HbFeO_2$ to metHb. The spectrum of the decomposed protein lacked of the characteristic maxima for metHb at 500 and 631 nm and showed a general increase in the absorbance between these two wavelengths. Analogous reaction with NEM-$HbFeO_2$ suggested that the Cysβ93 residues were involved in this decomposition process. Indeed, addition of 7 equiv of peroxynitrite in aliquots of 1 equiv each to NEM-$HbFeO_2$ led to the formation of metHb without significant modification of the protein.

The amount of peroxynitrite needed to quantitatively reduce $HbFe^{IV}$=O to metHb was determined analogously by mixing equal volumes of the solutions with the OLIS stopped-flow instrument (procedure 3). When a $HbFe^{IV}$=O solution (8 μM) was treated with an excess of peroxynitrite, the reaction was complete approximately after addition of 3 and 4 equiv, for the reaction in the absence and in the presence of CO_2, respectively. Taken together, these results show that under our experimental conditions, because of the parallel decay of peroxynitrite and the reaction with Cysβ93, both the peroxynitrite-mediated oxidation of $HbFeO_2$ to metHb and the reduction of $HbFe^{IV}$=O to metHb are not stoichiometric processes.

Analysis of the Nitrogen-Containing Products Obtained From the Reaction of $HbFeO_2$ with Peroxynitrite in the Absence and in the Presence of Carbon Dioxide. The products of the reaction of $HbFeO_2$ with an excess of peroxynitrite were analyzed by ion chromatography. The reactions were carried out with a large excess of peroxynitrite, to make sure that $HbFeO_2$ was oxidized quantitatively to metHb. Indeed, unreacted $HbFeO_2$ and $HbFe^{IV}$=O would lead to artifactual production of nitrate from their reaction of nitrite (present as an impurity in the peroxynitrite solutions) (*6*, *17*, *18*). These processes are significantly slower than the corresponding reactions with peroxynitrite, and thus do not take place under the experimental conditions chosen here.

In agreement with previous reports (*34*), in the absence of added CO_2 spontaneous decay of 100 μM peroxynitrite at pH 7.0 and 20 °C yielded 67 ± 2% nitrate and 34 ± 3% nitrite. In the presence of 5 or 10 μM $HbFeO_2$, the nitrate yields were 82 ± 2 and 84 ± 3%, respectively. The amount of nitrite found after these reactions was 15 ± 4 and 12 ± 5%, respectively.

As expected, in the presence of 1.2 mM CO_2 the yield of nitrate formed from spontaneous decay of 100 μM peroxynitrite was significantly larger. At pH 7.0 and 20 °C, we obtained 85 ± 3% nitrate and 17 ± 2% nitrite. In the presence of 5 or 10 μM $HbFeO_2$, the nitrate yields were 90 ± 3 and 95 ± 2%, respectively. The amount of nitrite found after these reactions was 9 ± 3 and 4 ± 2%, respectively.

Taken together, these product analysis data suggest that the reaction between $HbFeO_2$ and peroxynitrite, both in the presence and absence of CO_2, does not lead to formation of nitrite. Thus, in contrast to our initial proposal (*12*), these data indicate that $HbFe^{IV}$=O cannot be generated by decay of $HbFe^{II}OONO$ to $HbFe^{IV}$=O and NO_2^-.

Pulse Radiolysis Studies of the Reaction of $HbFeO_2$ with Nitrogen Dioxide. The reaction of $HbFeO_2$ with $NO_2^\bullet$ was studied by pulse radiolysis of a N_2O-saturated nitrite solution. Because $HbFeO_2$ reacts with nitrite, albeit at a slow rate (*17*, *18*), the N_2O-saturated $HbFeO_2$ and NO_2^- solutions were mixed in the cell only a few seconds before radiolysis. Preliminary experiments were carried out by following the reaction at 541, 576, and/or 600 nm both in the presence of an excess of $NO_2^\bullet$ or of $HbFeO_2$. The amplitudes of the traces suggested that the reaction of $HbFeO_2$ with an excess of $NO_2^\bullet$ leads to the formation of metHb, whereas the reaction of $HbFeO_2$ with substoichiometric amounts of $NO_2^\bullet$ yields mostly $HbFe^{IV}$=O (data not shown). To confirm these results, we measured a difference spectrum 10 ms after allowing 41 μM $HbFeO_2$ to react with 5 μM $NO_2^\bullet$. As shown in Figure 9A, the measured spectrum corresponded to that expected after conversion of approximately 5 μM $HbFeO_2$ to ca. 4 μM $HbFe^{IV}$=O and 1 μM metHb. Reaction of 41 μM $HbFeO_2$ with 10 μM $NO_2^\bullet$ led to the formation of ca. 6 μM $HbFe^{IV}$=O and 4 μM metHb (Figure 3S, Supporting Information). In contrast, when $HbFeO_2$ (12 μM) was allowed to react with an excess of $NO_2^\bullet$ (67 μM) the spectrum

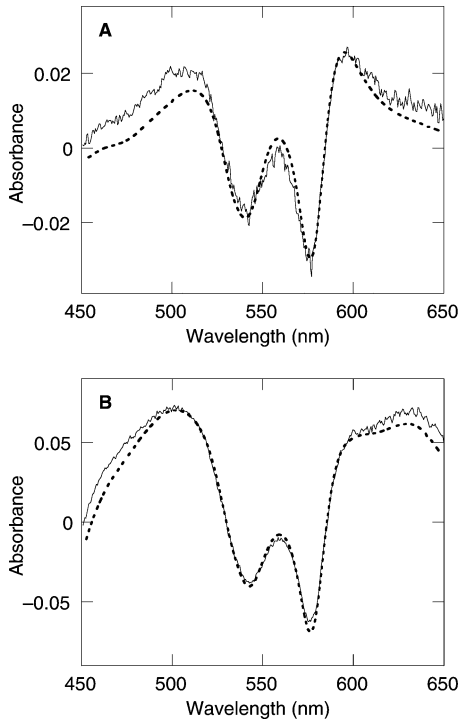

FIGURE 9: Difference spectra (thin lines) measured 10 ms after irradiating N_2O-saturated $HbFeO_2$ solutions containing 5 mM nitrite (in 0.1 M phosphate buffer pH 7.4). The concentrations of $HbFeO_2$ and $NO_2^\bullet$ were (A) 41 and 5 μM and (B) 12 and 67 μM, respectively. Calculated difference spectra (dotted bold line) corresponding to the conversion of (A) 5 μM $HbFeO_2$ to ca. 4 μM $HbFe^{IV}$=O and 1 μM metHb and of (B) 10 μM $HbFeO_2$ to 10 μM metHb.

measured after 10 ms showed the conversion of ca. 10 μM $HbFeO_2$ to metHb (Figure 9B).

The reaction of $MbFeO_2$ with $NO_2^\bullet$ to yield metMb has previously been suggested to proceed via reaction of $MbFeO_2$ with nitrite, rapidly produced from the hydrolysis of N_2O_4 (*35*). However, Wade and Castro (*35*) carried out the reaction by adding gaseous $NO_2^\bullet$ to an aerobic $MbFeO_2$ solution and followed the reaction with a UV/vis spectrometer by measuring spectra each min. Obviously, under these experimental conditions the authors could not observe the very fast reaction described in this pulse radiolysis study.

DISCUSSION

It is now established that in the presence of CO_2 the reaction of peroxynitrite with different substrates mainly involves their modification by $CO_3^{\bullet-}$ and/or $NO_2^\bullet$, the radicals generated from the decay of $ONOOCO_2^-$, the adduct formed from the reaction of $ONOO^-$ with CO_2 (*36*−*38*). The data presented in this paper suggest that in the presence of 1.2 mM CO_2 also the peroxynitrite-mediated oxidation of $HbFeO_2$ to metHb is dominated by reactions of the protein with $CO_3^{\bullet-}$ and $NO_2^\bullet$. The stopped-flow spectroscopic studies presented here clearly show that $HbFe^{IV}$=O is produced up to 40% when $HbFeO_2$ is mixed with an excess of peroxynitrite in the presence of 1.2 mM CO_2. The pulse radiolysis study of the reaction of $NO_2^\bullet$ with $HbFeO_2$ are consistent with this reaction being the main source of $HbFe^{IV}$=O formation. We have recently shown that, in analogy to the nearly diffusion-controlled reaction of superoxide and $NO^\bullet$ (*2*), the reaction of $HbFeO_2$ with $NO^\bullet$ generates a peroxynitrito-metHb complex ($HbFe^{III}OONO$), possibly via the fast radical−radical reaction between the superoxide coordinated to the Fe(III) ($Fe^{II}O_2 \leftrightarrow Fe^{III}O_2^{\bullet-}$) and $NO^\bullet$ (*31*, *39*). The reaction of $HbFeO_2$ with $NO_2^\bullet$ seems to proceed according to a similar mechanism: the superoxide coordinated to the Fe(III) reacts with $NO_2^\bullet$ to generate a peroxynitrato−metHb complex ($HbFe^{III}OONO_2$) (eq 3). Interestingly, a similar peroxynitrato complex has recently been shown to be generated from the reaction of $NO_2^\bullet$ with a superoxochromium(III) complex, $[Cr_{aq}^{III}OO]^{2+}$ (*40*).

$$HbFeO_2 \leftrightarrow HbFe^{III}O_2^{\bullet-} + NO_2^\bullet \rightarrow HbFe^{III}OONO_2 \quad (3)$$

Peroxynitrate ($HOONO_2$) is a potent oxidizing agent, which has been shown to rapidly react with I^-, Br^-, Cl^-, N_3^-, and VO^{2+} (*41*). Thus, one possible decomposition pathway for $HbFe^{III}OONO_2$ represents the formation of $NO_3^\bullet$ and $HbFe^{IV}$=O (eq 4a). $NO_3^\bullet$ is a very strong oxidant that could then react with amino acid residues of the globin and form nitrate, in agreement with our results of the analyses of the nitrogen-containing species. However, the calculated bond dissociation energy for O−O bond homolysis in $HOONO_2$ is very high (39 kcal/mol (*42*)), an observation that renders this pathway rather unlikely. Moreover, at pH 7 the one-electron reduction potential of $HOONO_2$ to yield $NO_3^\bullet$ and H_2O is only 0.74 V (*43*), whereas the reduction potential of $HbFe^{IV}$=O is ca. 1 V (*44*). In contrast, the two-electron reduction potential of $HOONO_2$ to yield NO_3^- and H_2O at pH 7 is 1.59 V (*43*). Taken together, these data suggest that a more likely decomposition pathway for $HbFe^{III}OONO_2$ may lead to the direct formation of NO_3^- and a one-electron oxidized form of $HbFe^{IV}$=O (eq 4b). $^\bullet HbFe^{IV}$=O may correspond to the same product observed from the two-electron oxidation of metHb by H_2O_2, that is the ferryl form of the protein with a radical centered on a sulfur and/or an oxygen atom (*45*−*47*). This reaction has also been suggested to proceed via an Fe(III)-peroxide complex. The optical features of $^\bullet HbFe^{IV}$=O are indistinguishable from those of $HbFe^{IV}$=O. Moreover, the formation of cysteine and tyrosine radicals from reaction between $HbFeO_2$ and peroxynitrite has previously been demonstrated by EPR spectroscopy, both in the presence and absence of CO_2 (*33*, *48*).

$$HbFe^{III}OONO_2 \rightarrow HbFe^{IV}{=}O + NO_3^\bullet \quad (4a)$$

$$HbFe^{III}OONO_2 \rightarrow {}^\bullet HbFe^{IV}{=}O + NO_3^- \quad (4b)$$

In the presence of an excess of peroxynitrite (or $NO_2^\bullet$) $HbFe^{IV}$=O is reduced to metHb (eq 5) and nitrate, as demonstrated previously (*31*) and as clearly shown by the difference spectrum obtained after reaction of $HbFeO_2$ with a large excess of $NO_2^\bullet$ (Figure 9B). Thus, both reaction steps lead to the formation of nitrate, in agreement with our results of the analyses of the nitrogen-containing species produced from this reaction.

$$HbFe^{IV}{=}O + NO_2^\bullet \rightarrow metHb + NO_3^- \quad (5)$$

The results of the pulse radiolysis study of the reaction between $NO_2^\bullet$ and an excess of $HbFeO_2$ suggest that the second-order rate constant for the second reaction step (eq

5) is larger than that for the first step (eq 3). Indeed, from the fit of the resulting difference spectrum it is clear that the final product is not pure $HbFe^{IV}$=O, but a mixture of $HbFe^{IV}$=O and metHb.

In a recent pulse radiolysis study, we have shown that $CO_3^{\bullet-}$ oxidizes $HbFeO_2$ to metHb in a two-step reaction (*49*). First $CO_3^{\bullet-}$ generates radical(s) in the globin which then, over a longer time scale, oxidize the iron center to finally produce ~40% of metHb. The rate constants obtained for the two steps in 0.25 M sodium bicarbonate solutions at pH 10.0 and room temperature are $(2.1 \pm 0.1) \times 10^8$ M^{-1} s^{-1} and $(1.0 \pm 0.2) \times 10^2$ s^{-1}, respectively. At lower pH, reaction of $CO_3^{\bullet-}$ with the globin may proceed with a rate constant 1 order of magnitude lower, as observed for the reactions of $CO_3^{\bullet-}$ with Tyr and Cys (*32*). If a Trp residue is involved in the reaction of Hb with $CO_3^{\bullet-}$, the rate at pH 7.5 would be comparable to that at pH 10 (*50*). Finally, the rate of the second step, the electron transfer reaction that leads to oxidation of the iron center of Hb, may also be slightly slower, as observed for intramolecular electron-transfer reaction between Trp radicals and Tyr (*51*). Taken together, it is conceivable that the reaction of $CO_3^{\bullet-}$ with $HbFeO_2$ also contributes to metHb formation when an excess of peroxynitrite is mixed with $HbFeO_2$ in the presence of CO_2 (eq 6).

$$HbFeO_2 + CO_3^{\bullet-} \rightarrow \text{metHb} + CO_3^{2-} + O_2 \qquad (6)$$

Interestingly, we have recently reported that to be able to simulate the results of the reaction of an excess of peroxynitrite with $MbFeO_2$ in the presence of CO_2, the analogous reaction between $MbFeO_2$ and $CO_3^{\bullet-}$ had to be introduced to reproduce the quantitative metMb formation obtained experimentally (*13*). In conclusion, our results suggest that in the presence of CO_2, the peroxynitrite-mediated oxidation of $HbFeO_2$ to metHb proceeds according to eqs 3−6.

The proposed mechanism may explain the lack of a clear pH dependence of the observed and the second-order rate constants of the two steps of the reaction of $HbFeO_2$ with peroxynitrite in the presence of added CO_2. Indeed, several factors may influence this reaction rate in opposite ways. At lower pH, it has been shown that the distal histidine is protonated and swings out of the heme pocket (*52*). Thus, the reaction of $NO_2^{\bullet}$ with the iron center in $HbFeO_2$ may be facilitated. However, at lower pH the concentration of the anion $ONOO^-$, the species that reacts with CO_2, is lower. Under basic conditions, larger amounts of peroxynitrite will decay through the reaction with CO_2, produce larger amounts of $NO_2^{\bullet}$, and thus one would expect that the reaction becomes faster. However, when larger amounts of $NO_2^{\bullet}$ are generated, the concurring dimerization of $NO_2^{\bullet}$ to N_2O_4 with subsequent rapid hydrolysis to nitrite and nitrate may become more significant. Thus, the observed reaction rate does not increase with increasing pH.

The reaction of peroxynitrite with $HbFeO_2$ in the absence of added CO_2 has recently been studied under experimental conditions close to those found in vivo, that is, in the presence of a large excess of the protein (*33*). Also under these conditions, the reaction has been shown to yield stoichiometric amounts of nitrate and metHb, together with ca. 50% oxygen and hydrogen peroxide (*33*). A small amount of protein radicals were trapped (6%) and the reaction was shown to produce ca. 15% $HbFe^{IV}$=O (*33*). The reaction was proposed to proceed according to eqs 7−8, by substitution of $O_2^{\bullet-}$ by peroxynitrite, formation of the peroxynitrito complex $HbFe^{III}OONO$, and subsequent decay to 90% metHb/NO_3^- and 10% $HbFe^{IV}$=O/$NO_2^{\bullet}$.

$$HbFeO_2 \leftrightarrow HbFe^{III}O_2^{\bullet-} + HOONO \rightarrow HbFe^{III}OONO + O_2^{\bullet-} + H^+ \qquad (7)$$

$$HbFe^{IV}{=}O + NO_2^{\bullet} \xleftarrow{\sim 10\%} HbFe^{III}OONO \xrightarrow{\sim 90\%} \text{metHb} + NO_3^- \qquad (8)$$

However, we have previously demonstrated that the peroxynitrito complex $HbFe^{III}OONO$, produced from the reaction of $HbFeO_2$ with $NO^{\bullet}$, quantitatively decays to metHb and nitrate, without formation of detectable intermediates (*31*, *39*). Treatment of $HbFeO_2$ with 1 equiv of $NO^{\bullet}$ in the presence of a large excess of Na_2S did not lead to detectable amounts of Fe(II)sulfohemoglobin (*27*), suggesting that $HbFe^{IV}$=O is not formed in this process (data not shown).

In analogy to the reaction of $HbFeO_2$ with peroxynitrite/CO_2, we propose that also in the absence of added CO_2 $HbFe^{IV}$=O is generated from the reaction of $NO_2^{\bullet}$ with $HbFeO_2$. Peroxynitrous acid is a strong oxidant with a one-electron reduction potential of ca. 1.6 V at pH 7 (*53*, *54*). Thus, HOONO may oxidize $HbFeO_2$ and produce metHb, O_2, and $NO_2^{\bullet}$ (eq 9). As discussed above, $NO_2^{\bullet}$ then reacts with $HbFeO_2$ to generate $HbFe^{IV}$=O (eqs 3 and 4b). In the presence of an excess of peroxynitrite, $NO_2^{\bullet}$ reacts with $HbFe^{IV}$=O to metHb and nitrate (eq 5). Thus, the overall reaction of $HbFeO_2$ with peroxynitrite may proceed according to eqs 3−5 and 9. Nevertheless, more studies are needed, and are in progress in our laboratory, to elucidate the exact mechanism of the reaction between $HbFeO_2$ and peroxynitrite in the absence of added CO_2.

$$HbFeO_2 \leftrightarrow HbFeO_2^{\bullet-} + HOONO + H^+ \rightarrow HbFe^{III}OH_2 + NO_2^{\bullet} + O_2 \qquad (9)$$

Finally, the reaction of deoxyHb with peroxynitrite is likely to proceed as previously proposed for the corresponding reaction with deoxyMb, via the formation of $HbFe^{II}OONO$, which then decays to $HbFe^{IV}$=O and nitrite (eqs 10 and 11). In the second step, $HbFe^{IV}$=O is converted to metHb and nitrate by reaction with $NO_2^{\bullet}$ (eqs 5).

$$HbFe^{II} + HOONO \rightarrow HbFe^{II}OONO + H^+ \qquad (10)$$

$$HbFe^{II}OONO \rightarrow HbFe^{IV}{=}O + NO_2^- \qquad (11)$$

The mechanism proposed here for the reaction of peroxynitrite with $HbFeO_2$ is consistent with the pH dependence of the observed and the second-order rate constants obtained experimentally. The increase in the rate at lower pH confirms that HOONO is the species that reacts with $HbFeO_2$.

The observation that a larger number of equivalents is required to completely oxidize $HbFeO_2$ to metHb in the presence of added CO_2 reflects the higher $HbFe^{IV}$=O yield obtained under these conditions. Indeed, if more $HbFe^{IV}$=O is formed, a larger amount of $NO_2^{\bullet}$, and thus of peroxynitrite, will be required to convert it to metHb and

nitrate. Moreover, formation of $HbFe^{IV}$═O is accompanied by generation of a radical on the globin only in the presence of CO_2. Thus, these mechanisms are consistent with a significantly larger extent of decomposition of the protein observed in the presence of added CO_2. The radical generated on the globin may be partly localized on a tyrosine residue. Thus, $NO_2^{\bullet}$ may not only react with $HbFe^{IV}$═O but also recombine with this $Tyr^{\bullet}$ to produce nitrotyrosine. Consequently, the mechanism proposed here is in good agreement with our recent results that show that $HbFeO_2$ is nitrated by peroxynitrite to a significantly larger extent in the presence of added CO_2 (*55*). Finally, the experiments with the Cysβ93-blocked NEM-$HbFeO_2$ suggest that also this residue is modified upon reaction of the protein with peroxynitrite. This result is in agreement with the EPR spectroscopic studies of the reaction of $HbFeO_2$ with peroxynitrite in the absence of CO_2 (*33*), which showed the formation of cysteinyl-derived radicals.

In conclusion, we have shown that $HbFeO_2$ reacts rapidly with peroxynitrite in the presence and in the absence of CO_2. The reactions proceed via the formation of $HbFe^{IV}$═O, which in the second step is reduced to metHb by its reaction with $NO_2^{\bullet}$. In the presence of physiological relevant amounts of CO_2, $HbFe^{IV}$═O is generated by reaction of $NO_2^{\bullet}$ with the coordinated superoxide of $HbFeO_2$, via formation of a peroxynitrato−metHb complex. Moreover, oxyHb efficiently scavenges $CO_3^{\bullet-}$, in a reaction that finally also leads to metHb production. Thus, our results indicate that oxyHb not only scavenges peroxynitrite, but also the radicals produced by its decomposition.

ACKNOWLEDGMENT

We thank Shivashankar Kalinga for carrying out the nitrogen-containing product analyses, Dr. Thomas Nauser for technical assistance in the pulse radiolysis experiment, and APEX Bioscience, Inc., for the supply of purified human hemoglobin.

SUPPORTING INFORMATION AVAILABLE

Observed rate constants for the two steps of the reaction between peroxynitrite and $HbFeO_2$ in the presence of different CO_2 concentrations (Figure S1). Plot of k_{obs} versus peroxynitrite concentration for the first step of the peroxynitrite-mediated oxidation of $HbFeO_2$ (4 μM) in the presence of 2.4 mM CO_2 and at different pH values (Figure S2). Difference spectrum measured 10 ms after reaction of a 41 μM $HbFeO_2$ with 10 μM $NO_2^{\bullet}$ (in 0.1 M N_2O-saturated phosphate buffer pH 7.4 containing 10 mM nitrite) (Figure S3). This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., and Freeman, B. A. (1990) Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide, *Proc. Natl. Acad. Sci. U.S.A. 87*, 1620−1624.
2. Nauser, T., and Koppenol, W. H. (2002) The rate constant of the reaction of superoxide with nitrogen monoxide: Approaching the diffusion limit, *J. Phys. Chem. A. 106*, 4084−4086.
3. Floris, R., Piersma, S. R., Yang, G., Jones, P., and Wever, R. (1993) Interaction of myeloperoxidase with peroxynitrite. A comparison with lactoperoxidase, horseradish peroxidase and catalase, *Eur. J. Biochem. 215*, 767−775.
4. Thomson, L., Trujillo, M., Telleri, R., and Radi, R. (1995) Kinetics of cytochrome c^{2+} oxidation by peroxynitrite: implications for superoxide measurements in nitric oxide-producing biological systems, *Arch. Biochem. Biophys. 319*, 491−497.
5. Mehl, M., Daiber, A., Herold, S., Shoun, H., and Ullrich, V. (1999) Peroxynitrite reaction with heme proteins, *Nitric Oxide 3*, 142−152.
6. Herold, S., and Rehmann, F.-J. K. (2003) Kinetics of the reactions of nitrogen monoxide and nitrite with ferryl hemoglobin, *Free Radical Biol. Med. 34*, 531−545.
7. Herold, S., Shivashankar, K., Matsui, T., and Watanabe, Y. (2004) Mechanistic studies of the isomerization of peroxynitrite to nitrate catalyzed by distal histidine metmyoglobin mutants, *J. Am. Chem. Soc. 126*, 6945−6955.
8. Daiber, A., Herold, S., Schöneich, C., Namgaladze, D., Peterson, J. A., and Ullrich, V. (2000) Nitration and activation of cytochrome P450BM-3 by peroxynitrite. Stopped-flow measurements prove ferryl intermediates, *Eur. J. Biochem. 267*, 6729−6739.
9. Lymar, S. V., and Hurst, J. K. (1995) Rapid reaction between peroxynitrite ion and carbon dioxide: Implications for biological activity, *J. Am. Chem. Soc. 117*, 8867−8868.
10. Denicola, A., Freeman, B. A., Trujillo, M., and Radi, R. (1996) Peroxynitrite reaction with carbon dioxide/bicarbonate: Kinetics and influence on peroxynitrite-mediated oxidations, *Arch. Biochem. Biophys. 333*, 49−58.
11. Herold, S. (2004) The outer-sphere oxidation of nitrosyliron(II)-hemoglobin by peroxynitrite leads to the release of nitrogen monoxide, *Inorg. Chem. 43*, 3783−3785.
12. Exner, M., and Herold, S. (2000) Kinetic and mechanistic studies of the peroxynitrite-mediated oxidation of oxymyoglobin and oxyhemoglobin, *Chem. Res. Toxicol. 13*, 287−293.
13. Herold, S., Exner, M., and Boccini, F. (2003) The mechanism of the peroxynitrite-mediated oxidation of myoglobin in the absence and in the presence of carbon dioxide, *Chem. Res. Toxicol. 16*, 390−402.
14. Denicola, A., Souza, J. M., and Radi, R. (1998) Diffusion of peroxynitrite across erythrocyte membranes, *Proc. Natl. Acad. Sci. U.S.A. 95*, 3566−3571.
15. Romero, N., Denicola, A., Souza, J. M., and Radi, R. (1999) Diffusion of peroxynitrite in the presence of carbon dioxide, *Arch. Biochem. Biophys. 368*, 23−30.
16. Koppenol, W. H., Kissner, R., and Beckman, J. S. (1996) Syntheses of peroxynitrite: to go with the flow or on solid grounds? *Methods Enzymol. 269*, 296−302.
17. Kosaka, H., Imaizumi, K., and Tyuma, I. (1982) Mechanism of autocatalytic oxidation of oxyhemoglobin by nitrite. An intermediate detected by electron spin resonance, *Biochim. Biophys. Acta 702*, 237−241.
18. Spagnuolo, C., Rinelli, P., Coletta, M., Chiancone, E., and Ascoli, F. (1987) Oxidation reaction of human oxyhemoglobin with nitrite: a reexamination, *Biochim. Biophys. Acta 911*, 59−65.
19. Bohle, D. S., Glassbrenner, P. A., and Hansert, B. (1996) Synthesis of pure tetramethylammonium peroxynitrite, *Methods Enzymol. 269*, 302−311.
20. Harned, H. S., and Bonner, F. T. (1945) The first ionization of carbonic acid in aqueous solutions of sodium chloride, *J. Am. Chem. Soc. 67*, 1026−1031.
21. Antonini, E., and Brunori, M. (1971) *Hemoglobin and Myoglobin in Their Reactions with Ligands*, North-Holland, Amsterdam.
22. Herold, S., and Röck, G. (2003) Reactions of deoxy-, oxy-, and methemoglobin with nitrogen monoxide. Mechanistic studies of the S-nitrosothiol formation under different mixing conditions, *J. Biol. Chem. 278*, 6623−6634.
23. Grassetti, D. R., and Murray, J. F., Jr. (1967) Determination of sulfhydryl groups with 2,2′- or 4,4′-dithiodipyridine, *Arch. Biochem. Biophys. 119*, 41−49.
24. Herold, S. (1999) Mechanistic studies of the oxidation of pyridoxalated hemoglobin polyoxyethylene conjugate (PHP) by nitrogen monoxide, *Arch. Biochem. Biophys. 372*, 393−398.
25. Herold, S., and Shivashankar, K. (2003) Metmyoglobin and methemoglobin catalyze the isomerization of peroxynitrite to nitrate, *Biochemistry 42*, 14036−14046.
26. Daiber, A., Nauser, T., Takaya, N., Kudo, T., Weber, P., Hultschig, C., Shoun, H., and Ullrich, V. (2002) Isotope effects and intermediates in the reduction of NO by P450NOR, *J. Inorg. Biochem. 88*, 343−352.
27. Berzofsky, J. A., Peisach, J., and Blumberg, W. E. (1971) Sulfheme proteins I. Optical and magnetic properties of sulfmyoglobin and its derivatives, *J. Biol. Chem. 246*, 3367−3377.

28. Koppenol, W. H., Moreno, J. J., Pryor, W. A., Ischiropoulos, H., and Beckman, J. S. (1992) Peroxynitrite, a cloaked oxidant formed by nitric oxide and superoxide, *Chem. Res. Toxicol. 5*, 834−842.
29. Meli, R., Nauser, T., and Koppenol, W. H. (1999) Direct observation of intermediates in the reaction of peroxynitrite with carbon dioxide, *Helv. Chim. Acta 82*, 722−725.
30. Minetti, M., Scorza, G., and Pietraforte, D. (1999) Peroxynitrite induces long-lived tyrosyl radical(s) in oxyhemoglobin of red blood cells through a reaction involving CO_2 and a ferryl species, *Biochemistry 38*, 2078−2087.
31. Herold, S., Exner, M., and Nauser, T. (2001) Kinetic and mechanistic studies of the $NO^{\bullet}$-mediated oxidation of oxymyoglobin and oxyhemoglobin, *Biochemistry 40*, 3385−3395.
32. Augusto, O., Bonini, M. G., Amanso, A. M., Linares, E., Santos, C. C. X., and de Menezes, S. L. (2002) Nitrogen dioxide and carbonate radical anion: two emerging radicals in biology, *Free Radical Biol. Med. 32*, 841−859.
33. Romero, N., Radi, R., Linares, E., Augusto, O., Detweiler, C. D., Mason, R. P., and Denicola, A. (2003) Reaction of human hemoglobin with peroxynitrite: Isomerization to nitrate and secondary formation of protein radicals, *J. Biol. Chem. 278*, 44049−44057.
34. Kissner, R., and Koppenol, W. H. (2002) Product distribution of peroxynitrite decay as a function of pH, temperature, and concentration, *J. Am. Chem. Soc. 124*, 234−239.
35. Wade, R. S., and Castro, C. E. (1996) Reactions of oxymyoglobin with NO, NO_2, and NO_2^- under argon and in air, *Chem. Res. Toxicol. 9*, 1382−1390.
36. Goldstein, S., Czapski, G., Lind, J., and Merényi, G. (2001) Carbonate radical ion is the only observable intermediate in the reaction of peroxynitrite with CO_2, *Chem. Res. Toxicol. 14*, 1273−1276.
37. Hodges, G. R., and Ingold, K. U. (1999) Cage-escape of geminate radical pairs can produce peroxynitrate from peroxynitrite under a wide variety of experimental conditions, *J. Am. Chem. Soc. 121*, 10695−10701.
38. Meli, R., Nauser, T., Latal, P., and Koppenol, W. H. (2002) Reaction of peroxynitrite with carbon dioxide: intermediates and determination of the yield of $CO_3^{\bullet-}$ and $NO_2^{\bullet}$, *J. Biol. Inorg. Chem. 7*, 31−36.
39. Herold, S. (1999) Kinetic and spectroscopic characterization of an intermediate peroxynitrite complex in the nitrogen monoxide induced oxidation of oxyhemoglobin, *FEBS Lett. 443*, 81−84.
40. Pestovsky, O., and Bakac, A. (2003) Generation of a peroxynitrato metal complex from nitrogen dioxide and coordinated superoxide, *Inorg. Chem. 42*, 1744−1750.
41. Appelman, E. H., and Gosztola, D. J. (1995) Aqueous peroxynitric acid ($HOONO_2$): a novel synthesis and some chemical and spectroscopic properties, *Inorg. Chem. 34*, 787−791.
42. Olson, J. S., Bartberger, M. D., and Houk, K. N. (2003) Peroxynitrate and peroxynitrite. A complete basis set investigation of similarities and differences between these NOx species, *J. Am. Chem. Soc. 125*, 3999−4006.
43. Goldstein, S., Czapski, G., Lind, J., and Merényi, G. (1998) Mechanism of decomposition of peroxynitric ion (O_2NOO^-): evidence for the formation of O_2^- and NO_2 radicals, *Inorg. Chem. 37*, 3943−3947.
44. Koppenol, W. H., and Liebman, J. F. (1984) The oxidizing nature of the hydroxyl radical. A comparison with the ferryl ion (FeO_2^+), *J. Phys. Chem. 88*, 99−101.
45. Maples, K. R., Kennedy, C. H., Jordan, S. J., and Mason, R. P. (1990) In vivo thiyl free radical formation from hemoglobin following administration of hydroperoxides, *Arch. Biochem. Biophys. 277*, 402−409.
46. Svistunenko, D. A., Patel, R. P., and Wilson, M. T. (1996) An EPR investigation of human methemoglobin. Oxidation by hydrogen peroxide: methods to quantify all paramagnetic species observed in the reaction, *Free Rad. Res. 24*, 269−280.
47. McArthur, K. M., and Davies, M. J. (1993) Detection and reactions of the globin radical in hemoglobin, *Biochim. Biophys. Acta 1202*, 173−181.
48. Minetti, M., Pietraforte, D., Carbone, V., Salzano, A. M., Scorza, G., and Marino, G. (2000) Scavenging of peroxynitrite by oxyhemoglobin and identification of modified globin residues, *Biochemistry 39*, 6689−6697.
49. Boccini, F., Domazou, A., and Herold, S. (2004) Pulse radiolysis studies of the reactions of carbonate radical anion with myoglobin and hemoglobin, *J. Phys. Chem. A. 108*, 5800−5805.
50. Chen, S., and Hoffman, M. Z. (1974) Reactivity of the carbonate radical in aqueous solution. Tryptophan and its derivatives, *J. Phys. Chem. 78*, 2099−202102.
51. Butler, J., Land, E. J., Prütz, W. A., and Swallow, A. J. (1982) Charge transfer between tryptophan and tyrosine in proteins, *Biochim. Biophys. Acta 705*, 150−162.
52. Brantley, R. E., Jr., Smerdon, S. J., Wilkinson, A. J., Singleton, E. W., and Olson, J. S. (1993) The mechanism of auto-oxidation of myoglobin, *J. Biol. Chem. 268*, 6995−7010.
53. Koppenol, W. H. (1998) Peroxynitrite uncloaked? *Chem. Res. Toxicol. 11*, 716−717.
54. Lymar, S. V., and Poskrebyshev, G. A. (2003) Rate of ON−OO-bond homolysis and the Gibbs energy of formation of peroxynitrite, *J. Phys. Chem. A. 107*, 7991−7996.
55. Herold, S., Shivashankar, K., and Mehl, M. (2002) Myoglobin scavenges peroxynitrite without being significantly nitrated, *Biochemistry 41*, 13460−13472.

BI0482250