Pulse Radiolysis Studies of the Reactions of $CO_3^{\bullet-}$ and $NO_2^{\bullet}$ with Nitrosyl(II)myoglobin and Nitrosyl(II)hemoglobin

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The reactions of carbonate radical anion [$CO_3^{\bullet-}$, systematic name: trioxidocarbonate(•1−)] with nitrosyl(II)hemoglobin ($HbFe^{II}NO$) and nitrosyl(II)myoglobin ($MbFe^{II}NO$) were studied by pulse radiolysis in N_2O-saturated 0.25 M sodium bicarbonate solutions at pH 10.0 and room temperature. The reactions proceed in two steps: outer-sphere oxidation of the nitrosyliron(II) proteins to their corresponding nitrosyliron(III) forms and subsequent dissociation of $NO^{\bullet}$. The second-order rate constants measured for the first reaction steps were $(4.3 \pm 0.2) \times 10^8$ and $(1.5 \pm 0.3) \times 10^8$ M^{-1} s^{-1}, for $MbFe^{II}NO$ and $HbFe^{II}NO$, respectively. The reactions between nitrogen dioxide and $MbFe^{II}NO$ or $HbFe^{II}NO$ were studied by pulse radiolysis in N_2O-saturated 0.1 M phosphate buffer pH 7.4 containing 5 mM nitrite. Also for the reactions of this oxidant with the nitrosyliron(II) forms of Mb and Hb a two-step reaction was observed: oxidation of the iron was followed by dissociation of $NO^{\bullet}$. The second-order rate constants measured for the first reaction steps were $(2.9 \pm 0.3) \times 10^7$ and $(1.8 \pm 0.3) \times 10^7$ M^{-1} s^{-1}, for $MbFe^{II}NO$ and $HbFe^{II}NO$, respectively. Both radicals appear to be able to oxidize the iron(II) centers of the proteins directly. Only for the reactions with $HbFe^{II}NO$ it cannot be excluded that, in a parallel reaction, $CO_3^{\bullet-}$ and $NO_2^{\bullet}$ first react with amino acid(s) of the globin, which then oxidize the nitrosyliron(II) center.

Introduction

Activated macrophages produce micromolar concentrations of both radicals nitrogen monoxide and superoxide, which react at a diffusion-controlled rate (1.6×10^{10} M^{-1} s^{-1})[1] to produce peroxynitrite.[2,3] In the presence of physiological concentrations of CO_2 the dominant reaction of this strong oxidizing and nitrating agent is represented by the formation of the adduct $ONOOCO_2^-$ from the interaction of $ONOO^-$ with CO_2.[4,5] Homolytic cleavage of the peroxo-bond in $ONOOCO_2^-$ leads to rapid production of carbonate radical anion ($CO_3^{\bullet-}$)[6,7] and nitrogen dioxide[8,9] in a yield of less than 5%[10] or 30%.[11,12] Recent reports show that another pathway for $CO_3^{\bullet-}$ production under physiological conditions may be the reaction of the Cu(I) form of Cu,Zn-superoxide dismutase with hydrogen peroxide, which generates a very strong oxidant capable to carry out a one-electron oxidation of CO_3^{2-}/HCO_3^-.[13−15] Several additional pathways exist for $NO_2^{\bullet}$ generation in vivo: reaction of $NO^{\bullet}$ with O_2,[16] oxidation of nitrite by the oxoiron(IV) (ferryl) form of hemoproteins,[17,18] and in particular reaction of nitrite with Compound I and/or Compound II of peroxidases.[19] The reactivity of $CO_3^{\bullet-}$ and $NO_2^{\bullet}$ toward biological targets has only recently started to be investigated.[20−27]

Under physiological conditions, nitrosyl(II)hemoglobin ($HbFe^{II}NO$) is present in the red blood cells in a concentration of ca. 200 nM.[28] Inhalation of 80 ppm $NO^{\bullet}$ led to a 10-fold increase of the $HbFe^{II}NO$ concentration in the red blood cells of healthy volunteers, with the appearance of a significant arterial−venous gradient: the $HbFe^{II}NO$ concentration in arterial blood was nearly twice as much as that found in the venous blood.[28] Despite the high concentration of $NO^{\bullet}$ used in this experiment, the gradient may suggest that $HbFe^{II}NO$ represents a biologically active form of stabilized $NO^{\bullet}$. Dissociation of $NO^{\bullet}$ from $HbFe^{II}NO$ is an extremely slow process (the fastest rate is on the order of 10^{-3} s^{-1}),[29,30] whereas dissociation from the oxidized form $HbFe^{III}NO$ is ca. 1000 times faster.[31] Thus, it is conceivable that under physiological conditions release of $NO^{\bullet}$ from $HbFe^{II}NO$ may first require its oxidation.

We have recently shown that peroxynitrite selectively oxidizes $HbFe^{II}NO$ to the corresponding nitrosyliron(III) form, from which $NO^{\bullet}$ dissociates in a second reaction step.[32] The second-order rate constant determined by stopped-flow spectroscopy for the first reaction step is $(6.1 \pm 0.3) \times 10^3$ M^{-1} s^{-1} (at pH 7.2 and 20 °C).[32] Interestingly, in the presence of 1.2 mM CO_2 the value of the second-order rate constant is 1 order of magnitude higher, that is, $(5.3 \pm 0.2) \times 10^4$ M^{-1} s^{-1} (at pH 7.2 and 20 °C).[32] Preliminary studies of the analogous reaction between peroxynitrite and $MbFe^{II}NO$ point to a similar reaction mechanism (F. Boccini and S. Herold, unpublished results). To evaluate the contribution of $CO_3^{\bullet-}$ and $NO_2^{\bullet}$ in the $ONOO^-/CO_2$-mediated oxidation of the nitrosylated proteins, in this paper we have examined by pulse radiolysis the reactivity of these two radicals toward $MbFe^{II}NO$ and $HbFe^{II}NO$. Our data show that the second-order rate constants of the $CO_3^{\bullet-}$-mediated oxidations are on the order of 10^8 M^{-1} s^{-1}. We also report that $NO_2^{\bullet}$ oxidizes $MbFe^{II}NO$ and $HbFe^{II}NO$ at a slower rate ($k \approx 10^7$ M^{-1} s^{-1}).

Experimental Section

Chemicals. All chemicals used were of the highest purity available. Sodium bicarbonate was purchased from Merck and sodium nitrite from Fluka. Horse heart metmyoglobin was purchased from Sigma. Purified human oxyHb stock solution

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HbA_0 (57 mg/mL solution with approximately 1.1% metHb) was a kind gift from APEX Bioscience, Inc.. 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. Aqueous $NO^{\bullet}$-saturated solutions (2 mM) were prepared as described previously.[33] For the preparation of the $MbFe^{II}NO$ solutions, $NO^{\bullet}$ was purified by passing it first through a NaOH solution and then through two traps cooled with dry ice/2-propanol to condense residual traces of N_2O_3 still present in the line.

$HbFe^{II}NO$ and $MbFe^{II}NO$ Stock Solutions. For the preparation of concentrated $HbFe^{II}NO$ solutions, an ice-cooled oxyHb solution (ca. 1 mM in 0.1 M phosphate buffer pH 7.0) was first deoxygenated with a constant flow of Ar over the surface of the solution while gently stirring. Then, approximately 2 equiv of a saturated $NO^{\bullet}$ solution were added to the deoxyHb solution. The excess of $NO^{\bullet}$ was finally removed by purging the solution again with Ar. The final concentration of the $HbFe^{II}NO$ solutions used for the kinetics studies was determined prior to each experiment by measuring the absorbance at 544, 572, and/or 600 nm ($\epsilon_{544} = 11.4\ mM^{-1}\ cm^{-1}$, $\epsilon_{572} = 11.4\ mM^{-1}\ cm^{-1}$, and $\epsilon_{600} = 2.6\ mM^{-1}\ cm^{-1}$).[34] The concentrations of the Hb solutions are always expressed per heme.

Concentrated $MbFe^{II}NO$ stock solutions were prepared via reductive nitrosylation under alkaline conditions (reaction 1).[35]

$$MbFe^{III}OH_2 + 2\ NO^{\bullet} + 2\ HO^- \rightarrow MbFe^{II}NO + NO_2^- + 2\ H_2O \quad (1)$$

Briefly, an alkaline, concentrated metMb solution (500−800 μM in 0.1 M phosphate buffer pH 8.7) was first saturated with Ar by applying alternatively vacuum and an atmosphere of Ar to the solution for at least 8 times while gently stirring. Then, to saturate the protein solution with $NO^{\bullet}$ we applied analogously vacuum and an atmosphere of $NO^{\bullet}$ 3−4 times. The protein solution was allowed to react overnight at room temperature and finally saturated with Ar by applying again eight cycles of vacuum and Ar. To remove the nitrite produced from reductive nitrosylation (reaction 1), the $MbFe^{II}NO$ stock solutions used for the experiments with $CO_3^{\bullet-}$ were purified under Ar by chromatography over a Sephadex G-25 column with degassed 0.1 M phosphate buffer (pH 8.7) as the eluent. This step was essential as nitrite was found to interfere with the reaction and to lead to poorly reproducible results. The final concentration of the $MbFe^{II}NO$ solutions used for the pulse radiolysis experiments was determined prior to each measurement by determining the absorbance at 547, 579, and/or 600 nm ($\epsilon_{544} = 11.8\ mM^{-1}\ cm^{-1}$, $\epsilon_{572} = 10.5\ mM^{-1}\ cm^{-1}$, and $\epsilon_{600} = 3.7\ mM^{-1}\ cm^{-1}$).[34]

The $HbFe^{II}NO$ and $MbFe^{II}NO$ solutions were prepared freshly for each experiment. Because the nitrosyl−proteins are slowly oxidized by oxygen,[29,36] the solutions were always kept under Ar.

Methods. Pulse radiolysis experiments were carried out by irradiation of the samples with a 2 MeV electron accelerator (Febetron 705, Hewlett-Packard) as described earlier.[37] The light source was a Xenon-lamp and the detection system consisted of a SpectraPro-300i monochromator and a Hamamatsu R928 photomultiplier. The optical path length was 2 cm for the reactions of $MbFe^{II}NO$ or $HbFe^{II}NO$ with $CO_3^{\bullet-}$ and 1 cm for the reactions with $NO_2^{\bullet}$. The dose per pulse used varied from 4 to 16 Gy for the 2 cm cell and between 5 and 24 Gy for the 1 cm cell. Dosimetry was conducted by using the thiocyanate dosimeter. All reactions were investigated at different wavelengths in the approximate range 300−600 nm. The kinetics of the reactions of $CO_3^{\bullet-}$ with $MbFe^{II}NO$ or $HbFe^{II}NO$ was studied at 600 nm, whereas the rate constants of the reactions between $MbFe^{II}NO$ or $HbFe^{II}NO$ and $NO_2^{\bullet}$ were determined by following the absorbance changes at 590 or 585 nm, respectively.

Reactions with the Carbonate Radical Anion. $CO_3^{\bullet-}$ was generated as described before[22] by irradiating a N_2O-saturated (24.4 mM) 0.25 M sodium bicarbonate solution containing different amounts of protein (pH 10.0). The experimental conditions chosen ensured that practically all $HO^{\bullet}$ produced from radiolysis of the aqueous solution reacted directly with CO_3^{2-} to produce $CO_3^{\bullet-}$. In analogy to oxyMb[38] and oxyHb,[39] $MbFe^{II}NO$ and $HbFe^{II}NO$ were assumed to react with $HO^{\bullet}$ at a rate of $(1-9) \times 10^9\ M^{-1}\ s^{-1}$. The dose was set to generate between 2 and 9 μM of $CO_3^{\bullet-}$, depending on the protein concentration.

The $MbFe^{II}NO$ or $HbFe^{II}NO$ solutions were prepared by diluting the protein stock solutions (0.5−1 mM) with a N_2O-saturated 0.25 M sodium bicarbonate solution at pH 10.0. In a typical experiment, a 10 mL gastight SampleLock Hamilton syringe was filled with the N_2O-saturated solution. In the same syringe we then added the amount of protein necessary to reach the required concentration (0.2−1.2 mL).

Reactions with Nitrogen Dioxide. $NO_2^{\bullet}$ was generated as described before[23] by irradiating a N_2O-saturated 5 mM sodium nitrite solution containing different amounts of protein (pH 7.4). Radiation doses were set to produce between 3 and 13 μM $NO_2^{\bullet}$. The experimental conditions chosen ensured that ca. 90% of the radicals produced from radiolysis of the aqueous solution were converted to $NO_2^{\bullet}$. Low concentrations of $NO^{\bullet}$ may be produced from the reaction of $H^{\bullet}$ with NO_2^-,[40] and may eventually lead to the formation of N_2O_3 from the reaction of $NO^{\bullet}$ with $NO_2^{\bullet}$. Nevertheless, under our experimental conditions the protein concentration was always significantly higher than that of $NO_2^{\bullet}$ and thus amounts of N_2O_3 produced were negligible. Moreover, a control experiment showed that N_2O_3 does not oxidize $MbFe^{II}NO$ and $HbFe^{II}NO$ (S. Herold, unpublished results).

N_2O-saturated $MbFe^{II}NO$ or $HbFe^{II}NO$ solutions used for the reactions with $NO_2^{\bullet}$ were prepared by adding the required volume of the concentrated protein stock solutions 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 in a second syringe from a 0.2 M nitrite stock solution. The $MbFe^{II}NO$ (or $HbFe^{II}NO$) and the nitrite solutions were then rapidly mixed before irradiating the cell (mixing time ca. 2−3 s).

Statistics. At least 5−10 measurements were carried out to determine the observed rate constants at each protein concentration. The error bars depicted in the Figures represent the standard deviation from the mean value. The second-order rate constants were determined from the linear fits of the plots of k_{obs} versus protein concentration and are given as mean values plus or minus the corresponding standard error.

Results

Reaction of $CO_3^{\bullet-}$ with $MbFe^{II}NO$. The reaction of $CO_3^{\bullet-}$ with $MbFe^{II}NO$ was studied by pulse radiolysis at pH 10.0 and room temperature. To minimize the concurring bimolecular decay of $CO_3^{\bullet-}$,[41,42] we chose to keep the protein concentration at least 6 times higher than that of $CO_3^{\bullet-}$ to maintain pseudo-first-order conditions.

Because the Soret bands of $MbFe^{II}NO$ and $MbFe^{III}NO$ are rather similar ($\epsilon_{420} = 132$ and $\epsilon_{421} = 150\ mM^{-1}\ cm^{-1}$, respectively),[34,43] the reaction was first studied by following the

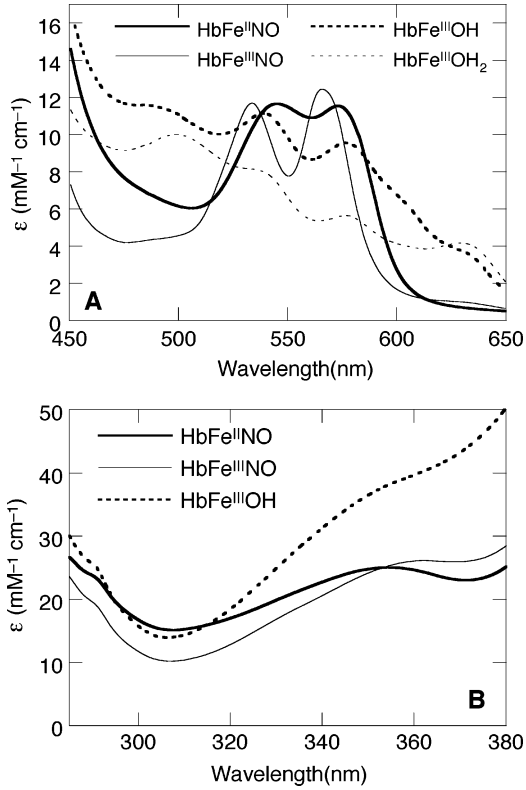

Figure 1. Different regions of the absorbance spectra of $HbFe^{II}NO$, $HbFe^{III}NO$, and of metHb under neutral and basic conditions.

absorbance changes at different wavelengths in the visible region of the spectrum (see Figure 1, the corresponding spectra for Mb are comparable). In all cases, we observed a fast process followed by a significantly slower reaction. At all measured wavelengths (450, 535, and 575 nm) the absorbance changes corresponded to those expected for the oxidation of $MbFe^{II}NO$ to $MbFe^{III}NO$, followed by the dissociation of $NO^{\bullet}$ to produce metMb (reactions 2 and 3).

$$MbFe^{II}NO + CO_3^{\bullet-} \rightarrow MbFe^{III}NO + CO_3^{2-} \qquad (2)$$

$$MbFe^{III}NO + H_2O \rightarrow MbFe^{III}OH_2 + NO^{\bullet} \qquad (3)$$

The kinetics of the reaction was determined by following the absorbance decrease at 600 nm, the maximum of the broad absorbance band of $CO_3^{\bullet-}$ ($\epsilon_{600} = (22 \pm 2) \times 10^2\ M^{-1}\ cm^{-1}$).[42] The reaction time courses of the first reaction step (reaction 2) could all be fitted well to a single-exponential expression. Also at this wavelength, over longer reaction times (up to 500 ms) an increase in absorbance was observed, which corresponded to the dissociation of $NO^{\bullet}$ from $MbFe^{III}NO$ (reaction 3). As shown in Figure 2, the observed rate constants (k_{obs}) for the first reaction step were linearly dependent on the protein concentration and the second-order rate constant obtained from the linear fit was $(4.3 \pm 0.2) \times 10^8\ M^{-1}\ s^{-1}$. The rate of the second step was independent of the protein and the $CO_3^{\bullet-}$ concentrations and was in good agreement with the rate of $NO^{\bullet}$ dissociation measured by stopped-flow after oxidation of $MbFe^{II}NO$ by peroxynitrite under similar conditions (F. Boccini and S. Herold, unpublished results).

As a control, the kinetics of the reaction between $CO_3^{\bullet-}$ and $MbFe^{II}NO$ was also studied by following the absorbance changes at 310 nm, a wavelength at which only the different myoglobin forms contribute to the absorbance. As expected (Figure 1B), at this wavelength we observed a fast decrease

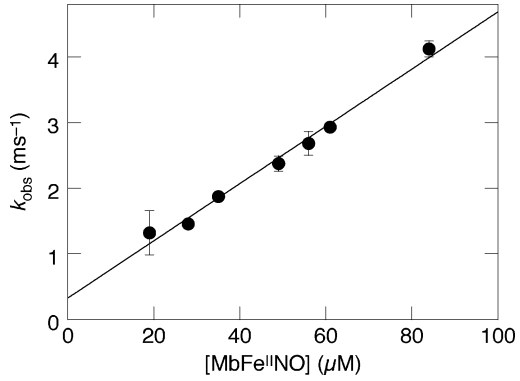

Figure 2. k_{obs} versus $MbFe^{II}NO$ concentration for the $CO_3^{\bullet-}$-mediated oxidation of $MbFe^{II}NO$ to $MbFe^{III}NO$, at pH 10.0 and room temperature. The observed rate constants were determined by following the absorbance decrease at 600 nm. The second-order rate constant resulting from the linear fit depicted is given in Table 1.

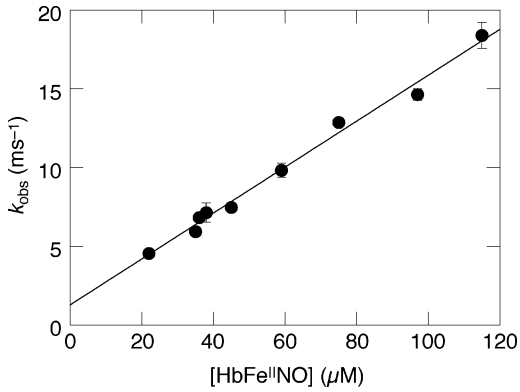

Figure 3. k_{obs} versus $HbFe^{II}NO$ concentration for the $CO_3^{\bullet-}$-mediated oxidation of $HbFe^{II}NO$ to $HbFe^{III}NO$, at pH 10.0 and room temperature. The observed rate constants were determined by following the absorbance decrease at 600 nm. The second-order rate constant resulting from the linear fit depicted is given in Table 1.

followed by a slow increase of the absorbance. The rate constants obtained for the two reaction steps were essentially identical to those measured at 600 nm.

Reaction of $CO_3^{\bullet-}$ with $HbFe^{II}NO$. The reaction of $CO_3^{\bullet-}$ with $HbFe^{II}NO$ was studied under analogous conditions, at pH 10.0 and room temperature. Also for this protein, at different wavelengths (450, 470, 552, and 600 nm) we observed two steps that corresponded to the oxidation of $HbFe^{II}NO$ and the dissociation of $NO^{\bullet}$ from $HbFe^{III}NO$, respectively. The kinetics of the first reaction step was studied by following the absorbance changes at 600, 450, or 310 nm. The traces could all be fitted well to a single-exponential expression and the observed rate constants (k_{obs}) were linearly dependent on the protein concentration. The second-order rate constant obtained from the linear fit shown in Figure 3 was $(1.5 \pm 0.1) \times 10^8\ M^{-1}\ s^{-1}$. The rate of $NO^{\bullet}$ dissociation from $HbFe^{III}NO$ was independent of the protein and the $CO_3^{\bullet-}$ concentrations and was in good agreement with published values.[31,32]

The absorbance changes of the traces collected at 310 nm derived only from the different forms of the protein and thus we could calculate that the approximate yield of the reaction is 60%.

Reaction of $NO_2^{\bullet}$ with $MbFe^{II}NO$. The reaction of $NO_2^{\bullet}$ with $MbFe^{II}NO$ was studied in 0.1 M phosphate buffer pH 7.4 and room temperature. To avoid interferences with the broad absorbance band of $NO_2^{\bullet}$ ($\epsilon_{400} = 201\ M^{-1}\ cm^{-1}$)[44] and to minimize the bimolecular reaction of $NO_2^{\bullet}$ to N_2O_4, we kept the protein concentration at least 6 times higher than that of

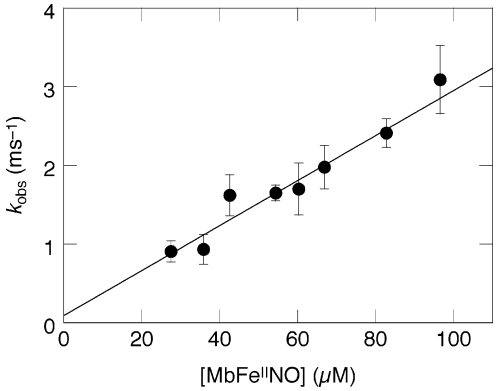

Figure 4. k_{obs} versus MbFe^{II}NO concentration for the $NO_2^{\bullet}$-mediated oxidation of MbFe^{II}NO to MbFe^{III}NO, at pH 7.4 and room temperature. The observed rate constants were determined by following the absorbance decrease at 590 nm. The second-order rate constant resulting from the linear fit depicted is given in Table 1.

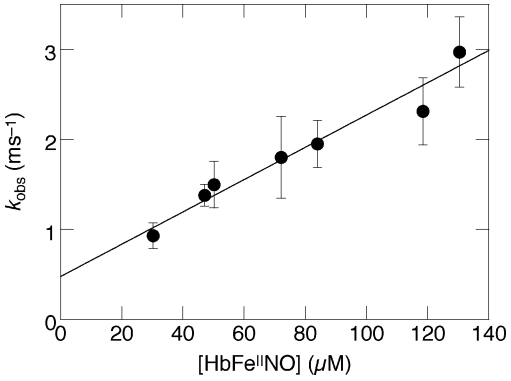

Figure 5. k_{obs} versus HbFe^{II}NO concentration for the $NO_2^{\bullet}$-mediated oxidation of HbFe^{II}NO to HbFe^{III}NO, at pH 7.4 and room temperature. The observed rate constants were determined by following the absorbance decrease at 585 nm. The second-order rate constant resulting from the linear fit depicted is given in Table 1.

$NO_2^{\bullet}$ to maintain pseudo-first-order conditions. The absorbance changes observed at different wavelengths in the visible region of the spectrum (525, 553, 577, and 590 nm) corresponded to those expected for the $NO_2^{\bullet}$-mediated oxidation of MbFe^{II}NO to MbFe^{III}NO, followed by the dissociation of $NO^{\bullet}$ to produce metMb (reactions 4 and 3).

$$\text{MbFe}^{II}\text{NO} + \text{NO}_2^{\bullet} \rightarrow \text{MbFe}^{III}\text{NO} + \text{NO}_2^{-} \qquad (4)$$

The kinetics of the first step of the reaction between $NO_2^{\bullet}$ and MbFe^{II}NO was studied by following the absorbance changes at 590 nm. The traces could all be fitted well to a single-exponential expression and the observed rate constants (k_{obs}) were linearly dependent on the protein concentration. The second-order rate constant obtained from the linear fit shown in Figure 4 was $(2.9 \pm 0.3) \times 10^7$ M^{-1} s^{-1}. The value of the rate of $NO^{\bullet}$ dissociation from MbFe^{III}NO was independent of the protein and the $NO_2^{\bullet}$ concentrations and was on the same order of magnitude as the published values.[45]

Reaction of $NO_2^{\bullet}$ with HbFe^{II}NO. The reaction of $NO_2^{\bullet}$ with HbFe^{II}NO was studied under identical conditions as described above for the reaction with MbFe^{II}NO. Interestingly, at all wavelengths investigated (530, 550, and 585 nm) we observed three reaction steps. The kinetics was studied by following the absorbance changes at 585 nm. At this wavelength, for the first two steps we observed absorbance changes expected for the oxidation of HbFe^{II}NO to HbFe^{III}NO (a very fast decrease followed by a further decrease). The third reaction step (further absorbance decrease) was very slow and had a rate on the order of magnitude anticipated for $NO^{\bullet}$ dissociation from HbFe^{III}NO.[31,32] Because of the small absorbance changes associated with the second reaction step, it was not possible to determine the exact rate constant of this process, which was mostly over after about 5−8 ms. Moreover, it was not possible to find out whether the rate of the second step depends on the protein and/or on the $NO_2^{\bullet}$ concentrations.

To obtain the rate constant of the first reaction step, the first 4 half-lives of the reaction time courses were fitted to a single-exponential expression. The observed rate constants (k_{obs}) were found to be linearly dependent on the protein concentration (Figure 5). The second-order rate constant obtained from the linear fit shown in Figure 5 was $(1.8 \pm 0.3) \times 10^7$ M^{-1} s^{-1}.

Discussion

Interest in the reactivity of carbonate and nitrogen dioxide radicals toward biologically relevant targets has increased because these radicals have been shown to be produced from the decay of peroxynitrite in the presence of CO_2.[20,21] Increasing amounts of data suggest that these radicals may play a role in pathophysiological processes.[20,21] The carbonate radical anion is a stronger [$E°(CO_3^{\bullet-}/CO_3^{2-}) = 1.58$ V][46] oxidant than nitrogen dioxide [$E°(NO_2^{\bullet}/NO_2^{-}) = 1.04$ V)][47] and thus in vivo only few oxidizing agents are strong enough to produce this radical. Under physiological conditions, the major route for $CO_3^{\bullet-}$ formation is probably the homolytic cleavage of the O−O-bond in $ONOOCO_2^{-}$. In contrast, several biochemical processes can lead to $NO_2^{\bullet}$ production, in particular the peroxidase-mediated oxidation of nitrite and $ONOOCO_2^{-}$ decay.[19,21]

We have recently studied the reactions of $CO_3^{\bullet-}$ with the oxygenated and the oxidized forms of myoglobin and hemoglobin.[22] The reaction of $CO_3^{\bullet-}$ with metMb (and metHb) takes place exclusively with amino acid residues, without further oxidation of the iron center. The second-order rate constants are $(4.7 \pm 0.3) \times 10^7$ and $(1.9 \pm 0.3) \times 10^8$ M^{-1} s^{-1}, for metMb and metHb, respectively.[22] In contrast, oxyMb and oxyHb are oxidized by $CO_3^{\bullet-}$ to their corresponding iron(III) forms. Interestingly, the reaction of $CO_3^{\bullet-}$ with the oxygenated forms of the two proteins follows different mechanisms. OxyMb is oxidized directly and the second-order rate constant is $(5.2 \pm 0.3) \times 10^7$ M^{-1} s^{-1}. In contrast, when $CO_3^{\bullet-}$ is generated in the presence of oxyHb first globin radicals are produced ($k = (2.1 \pm 0.1) \times 10^8$ M^{-1} s^{-1}), which then oxidize the iron center intramolecularly ($k = (1.0 \pm 0.2) \times 10^2$ s^{-1}).[22]

In this work we showed that MbFe^{II}NO and HbFe^{II}NO are both oxidized directly by $CO_3^{\bullet-}$ to the corresponding nitrosyl-iron(III) forms. For both proteins we obtained the same rate constant for the disappearance of the absorbance band of $CO_3^{\bullet-}$ (at 600 nm) and for the conversion of the nitrosyl-iron(II) to the nitrosyliron(III) forms (at 310 nm). The second-order rate constants were both on the order of 10^8 M^{-1} s^{-1} (Table 1). Because the second-order rate constant of the reaction of $CO_3^{\bullet-}$ with the amino acid residues of metHb is also on the order of 10^8 M^{-1} s^{-1},[22] it is not possible to exclude that $CO_3^{\bullet-}$ first oxidizes one or more amino acid residues of the globin and, thus, that the iron center is oxidized only in a second reaction step (reactions 5 and 6). However, to explain the kinetics data obtained here, the second reaction step (reaction 6) should proceed at a rate higher than the highest observed rate constant (Figure 3), that is $k_6 > 2 \times 10^4$ s^{-1}. This value is 2 orders of magnitude larger than that determined for the intramolecular oxidation of oxyHb by the radicals generated

TABLE 1: Summary of the Second-Order Rate Constants for the Reactions of $CO_3^{\bullet-}$ and $NO_2^{\bullet}$ with $MbFe^{II}NO$ or $HbFe^{II}NO$, at pH 10.0 ($CO_3^{\bullet-}$) or 7.4 ($NO_2^{\bullet}$) and Room Temperature

reactions	$MbFe^{II}NO$	$HbFe^{II}NO$
$ProteinFe^{II}NO + CO_3^{\bullet-} \rightarrow ProteinFe^{III}NO + CO_3^{2-}$	$(4.3 \pm 0.2) \times 10^8\ M^{-1}\ s^{-1}$	$(1.5 \pm 0.3) \times 10^8\ M^{-1}\ s^{-1}$
$ProteinFe^{II}NO + NO_2^{\bullet} \rightarrow ProteinFe^{III}NO + NO_2^{-}$	$(2.9 \pm 0.3) \times 10^7\ M^{-1}\ s^{-1}$	$(1.8 \pm 0.3) \times 10^7\ M^{-1}\ s^{-1}$

on the globin upon reaction with $CO_3^{\bullet-}$.[22] Obviously, the two mechanisms (reaction 2 or reactions 5 and 6) could also take place in parallel.

$$HbFe^{II}NO + CO_3^{\bullet-} \rightarrow {}^{\bullet}HbFe^{II}NO + CO_3^{2-} \quad (5)$$

$${}^{\bullet}HbFe^{II}NO \rightarrow HbFe^{III}NO \quad (6)$$

Our data indicate that the reaction of $MbFe^{II}NO$ by $CO_3^{\bullet-}$ does not involve prior oxidation of the globin and thus must take place directly at the iron center. Indeed, our previous studies showed that the second-order rate constant for the reaction of $CO_3^{\bullet-}$ with the amino acid residues of metMb is significantly slower, on the order of $10^7\ M^{-1}\ s^{-1}$.[22]

Nitrogen dioxide is a weaker oxidant and second-order rate constants for its reaction with various amino acids are rather small ($10^4-10^5\ M^{-1}\ s^{-1}$).[20,21] We have recently shown that in analogy to the reaction of oxyHb with $NO^{\bullet}$ (reaction 7), oxyHb reacts with $NO_2^{\bullet}$ to generate the intermediate peroxynitrato-complex $HbFe^{III}OONO_2$ (reaction 8).[23] The same mechanism has been suggested to take place upon reaction of $NO_2^{\bullet}$ with oxyMb.[24]

$$(HbFe^{II}O_2 \leftrightarrow HbFe^{III}O_2^{\bullet-}) + NO^{\bullet} \rightarrow HbFe^{III}OONO \quad (7)$$

$$(HbFe^{II}O_2 \leftrightarrow HbFe^{III}O_2^{\bullet-}) + NO_2^{\bullet} \rightarrow HbFe^{III}OONO_2 \quad (8)$$

Reaction of oxyHb with an excess of $NO_2^{\bullet}$ leads to the formation of metHb. We have proposed that cleavage of the O−O-bond in $HbFe^{III}OONO_2$ generates ferrylHb, which has been shown to react with $NO_2^{\bullet}$ to produce the nitrato complex $HbFe^{III}ONO_2$ (reaction 9),[23,48] from which nitrate dissociates rapidly (reaction 10). The same reactions have been reported to take place with the corresponding myoglobin forms.[24]

$$(HbFe^{IV}{=}O \leftrightarrow HbFe^{III}{-}O^{\bullet}) + NO_2^{\bullet} \rightarrow HbFe^{III}ONO_2 \quad (9)$$

$$HbFe^{III}ONO_2 \rightarrow HbFe^{III}OH_2 + NO_3^{-} \quad (10)$$

The second-order rate constants of the reactions of $NO_2^{\bullet}$ with oxyHb and ferrylHb are rather large ($\sim 10^7\ M^{-1}\ s^{-1}$).[23,24,48] These values may be explained by the radical−radical nature of the reactions (reactions 8 and 9). Indeed, reactions of $NO_2^{\bullet}$ with other radical species such as $O_2^{\bullet-}$, Gly−TyrO$^{\bullet}$, or $NO_2^{\bullet}$ also display large values of second-order rate constants, in the range $(5-45) \times 10^8\ M^{-1}\ s^{-1}$.[44,49,50]

In this work we have shown that $NO_2^{\bullet}$ is also capable of carrying out an outer-sphere oxidation of the iron center of $HbFe^{II}NO$ and of $MbFe^{II}NO$. Our kinetics data showed that the reaction of $NO_2^{\bullet}$ with $MbFe^{II}NO$ proceeds in one step via direct oxidation of the iron center. Indeed, the value of the second-order rate constant of this oxidation process (Table 1) is 2−3 orders of magnitude larger than those of the reactions of $NO_2^{\bullet}$ with various amino acid residues under similar conditions.[20,21]

The reaction of $NO_2^{\bullet}$ with $HbFe^{II}NO$ appeared to be more complex: the oxidation of $HbFe^{II}NO$ to $HbFe^{III}NO$ proceeded in two subsequent steps. However, the largest fraction of the protein was oxidized in the fastest process. The second-order rate constant for this reaction was comparable to that of the $NO_2^{\bullet}$-mediated oxidation of $MbFe^{II}NO$ (Table 1). Unfortunately, because of the small absorbance changes associated with the second process, it was not possible to get a complete understanding of the reaction mechanism. The presence of this second process may explain the larger deviation from zero of the intercept of the linear fit shown in Figure 5, compared to those of Figures 2−4. Alternatively, the nonzero intercept may be an indication of a more complex mechanism.

It is conceivable that $NO_2^{\bullet}$ reacts also with amino acid residues of $HbFe^{II}NO$, which then oxidize the iron center in a second intramolecular reaction step. It is tempting to speculate that a reactive cysteine residue, possibly Cysβ93, is responsible for this parallel reaction pathway. Indeed, $NO_2^{\bullet}$ reacts at a slower rate with most other amino acid residues[20,21] and, in addition, horse heart myoglobin used for these studies does not contain cysteine residues. We have recently observed an analogous indirect intramolecular oxidation of the iron center of oxyHb upon reaction with $CO_3^{\bullet-}$.[22] Moreover, in the presence of ca. 100 μM of oxygen, Goldstein and co-workers have observed an intra- as well as an intermolecular oxidation of oxyHb by radicals formed from the reaction of $CO_3^{\bullet-}$ with the globin.[25] Because the $NO_2^{\bullet}$-mediated oxidation of $HbFe^{II}NO$ proceeds largely via direct oxidation of the iron center, in this work it was not possible to distinguish between the two oxidation pathways.

Oxidation of $HbFe^{II}NO$ and of $MbFe^{II}NO$ by the two radicals $CO_3^{\bullet-}$ and $NO_2^{\bullet}$ was followed by slow dissociation of $NO^{\bullet}$ to yield metHb and metMb, respectively. A similar two-step reaction was recently observed for the peroxynitrite-mediated oxidation of $HbFe^{II}NO$.[32] Addition of 1.2 mM CO_2 led to a 10-fold increase of the value of the second-order rate constant for this reaction.[32] The results of the present study suggest that the peroxynitrite-mediated oxidation of $HbFe^{II}NO$ in the presence of CO_2 is largely due to the reaction of the protein with $CO_3^{\bullet-}$.

Nitrosyl hemoglobin ($HbFe^{II}NO$) is considered as a biologically active form of stabilized $NO^{\bullet}$ in the red blood cells from which $NO^{\bullet}$ may be released in the capillaries when needed. If such a mechanism would take place in vivo, a concentration gradient in venous vs arterial blood should be found. It has recently been shown that such a gradient is indeed present in congestive heart failure patients[51] and in a rat model of endotoxemic sepsis.[52] Because dissociation of $NO^{\bullet}$ from $HbFe^{II}NO$ is too slow to be responsible for the observed gradients, it is likely that the iron center of $HbFe^{II}NO$ has to be oxidized before $NO^{\bullet}$ can be liberated. Under the pathophysiological conditions mentioned above, peroxynitrite may be produced and thus liberation of $NO^{\bullet}$ from $HbFe^{II}NO$ may take place after oxidation of the iron center by peroxynitrite and/or $CO_3^{\bullet-}/NO_2^{\bullet}$, produced in the presence of physiological amounts of CO_2.

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References and Notes

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