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The Outer-Sphere Oxidation of Nitrosyliron(II)hemoglobin by Peroxynitrite Leads to the Release of Nitrogen Monoxide

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It has been suggested that nitrosyliron(II)hemoglobin may represent a form of stabilized NO• and may be responsible for NO• delivery in the peripheral circulation. In this work, we show that NO• can be released from nitrosyliron(II)hemoglobin through reaction with peroxynitrite. Outer-sphere oxidation of the iron center generates nitrosyliron(III)hemoglobin, from which NO• dissociates at a rate of ca. 1 s^{-1}. The second-order rate constant for the reaction of peroxynitrite with nitrosyliron(II)hemoglobin is $(6.1 \pm 0.3) \times 10^3$ M^{-1} s^{-1} (at pH 7.2 and 20 °C). In the presence of 1.2 mM CO_2, the rather large value of the second-order rate constant, $(5.3 \pm 0.2) \times 10^4$ M^{-1} s^{-1} (at pH 7.2 and 20 °C), indicates that this reaction may take place in vivo. The reactive nitrogen species generated from this reaction, N_2O_3 and/or NO_2, may lead to protein modifications, such as nitration of tyrosine and/or tryptophan residues and nitrosation of cysteine residues.

Nitrogen monoxide participates in a variety of different biologically relevant reactions.[1] Many of these processes are triggered by the reaction of NO• with metalloproteins, in particular with hemoproteins. Endothelium-derived NO•, generated by the endothelial isoform of nitric oxide synthase (eNOS), is a key determinant of blood pressure homeostasis.[2] Paradoxically, oxyhemoglobin (oxyHb) and deoxyHb (HbFe(II)) scavenge NO• producing metHb/nitrate[3−5] or nitrosyliron(II)hemoglobin (HbFe(II)NO),[6] respectively. Both reactions have rate constants[5,6] on the order of 10^7 M^{-1} s^{-1} and, thus, drastically reduce the half-life of NO• in blood, which is about 1 ms.[7] To rationalize the observation that NO• maintains its bioactivity in the blood vessels even in the presence of large amounts of Hb, it has been proposed that NO• is partly stabilized and transported as *S*-nitrosohemoglobin (SNO−Hb), the Hb form in which the cysteine residue β93 is nitrosated.[8] According to this highly controversial hypothesis,[9] the "NO group" may then be transferred from SNO−Hb to other thiols inside and outside the red blood cells, and finally induce vasodilation particularly under hypoxic conditions.[8] Alternatively, it has been suggested that HbFe(II)NO may be responsible for NO• delivery in the peripheral circulation.[10] Indeed, it has been shown that volunteers inhaling 80 ppm NO• gas have a significantly increased concentration of HbFe(II)NO in the blood, but only slightly higher SNO−Hb concentrations.[10] Moreover, HbFe(II)NO has been detected by EPR spectroscopy in the blood from animals affected by pathological conditions known to cause an increase of the NO• concentration in the plasma, that is in shock, in inflammation, or upon administration of cytokines, nitrite, organic nitrates, and NO•-donors.[11−13]

We are currently investigating the biochemical properties of HbFe(II)NO, in order to identify a possible pathway for NO• release from this complex under physiological conditions. In this context, we have considered peroxynitrite[14] as a possible oxidant and have studied its reaction with HbFe(II)NO[15] by rapid-scan UV−vis spectroscopy.[16] As shown in Figure 1 (top), upon addition of an excess of

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(14) The recommended IUPAC nomenclature for peroxynitrite is oxoperoxonitrate(1−); for peroxynitrous acid, it is hydrogen oxoperoxonitrate. The term peroxynitrite is used in the text to refer generically to both $ONOO^-$ and its conjugate acid ONOOH.
(15) (a) HbFe(II)NO was generated as described recently by adding 1 equiv of an aqueous NO• solution to a deoxyHb solution, prepared by thoroughly degassing an oxyHb solution.[15b] The purified human oxyHb solution was a kind gift from APEX Bioscience, Inc. (b) Herold, S.; Röck, G. *J. Biol. Chem.* **2003**, *278*, 6623−6634.
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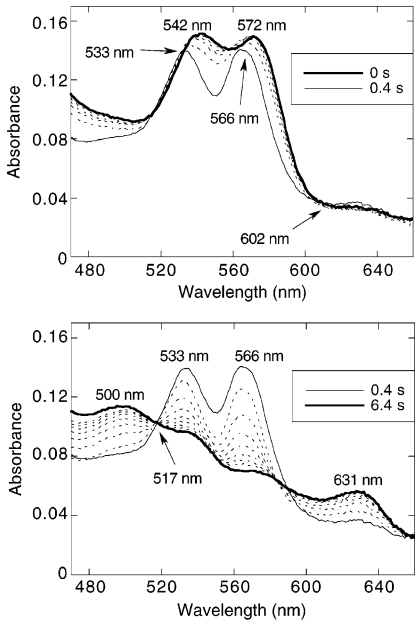

Figure 1. Rapid-scan UV−vis spectra of the reaction of HbFe(II)NO (13 μM) with peroxynitrite (330 μM) in 0.05 M potassium phosphate buffer pH 7.2 at 20 °C. In the first 400 ms (top), oxidation of HbFe(II)NO (bold line) to HbFe(III)NO (thin line). Between 0.4 and 6.4 s (bottom), dissociation of NO• from HbFe(III)NO (thin line) generates metHb (bold line).

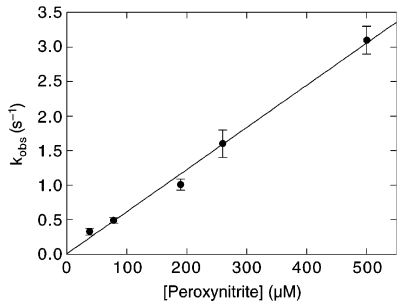

Figure 2. Plot of k_{obs} versus peroxynitrite concentration for the oxidation of HbFe(II)NO (25 μM) to HbFe(III)NO by peroxynitrite in 0.05 M potassium phosphate buffer pH 7.2 at 20 °C. The value of the second-order rate constant obtained from the linear fit shown is $(6.1 \pm 0.3) \times 10^3$ M^{-1} s^{-1}.

peroxynitrite[17] (330 μM) the characteristic spectrum of HbFe(II)NO (thick line), absorbance maxima at 542 and 572 nm,[18] was converted into that of HbFe(III)NO (thin line), absorbance maxima at 533 and 566 nm.[18] Over a longer time scale (Figure 1, bottom), the spectrum of HbFe(III)NO (thin line) changed into that of metHb, absorbance maxima at 500 and 631 nm (thick line).[18] On the time-scale of these processes, no reaction was detected upon mixing HbFe(II)NO with nitrite, a ubiquitous contaminant of our peroxynitrite solutions (up to 50% relative to the peroxynitrite concentration). Two sets of isosbestic points were identified in the course of the reaction, at 533 and 602 nm (Figure 1, top) and at 517 and 583 nm (Figure 1, bottom). Thus, the kinetics of the two reaction steps were determined separately by fitting the traces obtained at 517 and 602 nm for the first and the second process, respectively. The observed rate constant for the oxidation of HbFe(II)NO to HbFe(III)NO was found to depend linearly on the peroxynitrite concentration in the range 40−500 μM (Figure 2). The linear fit of this plot gave a value of the second-order rate constant of $(6.1 \pm 0.3) \times 10^3$ M^{-1} s^{-1} (at pH 7.2 and 20 °C). This value is approximately 1 order of magnitude smaller than that for the peroxynitrite-mediated oxidation of oxyHb.[16b]

In contrast, the rate of the transformation of HbFe(III)NO into metHb was independent of the peroxynitrite concentration in the range 200−500 μM. This observation suggests that the value of ca. 1 s^{-1}, measured for the second reaction step, corresponds to the dissociation rate of NO• from HbFe(III)NO. This number is in rather good agreement with those determined previously: 0.65 and 1.5 s^{-1}, for the dissociation of NO• from the α- and the β-subunit of HbFe(III)NO, respectively.[19] In the presence of lower peroxynitrite concentrations ($<\sim$200 μM), the oxidation of HbFe(II)NO to HbFe(III)NO by peroxynitrite becomes the rate-determining step and, thus, is the only observable process. Moreover, because of the parallel decay of peroxynitrite, addition of only 40 μM peroxynitrite did not lead to the complete conversion of HbFe(II)NO (25 μM) to metHb, and the final product was a mixture of HbFe(II)NO and metHb.

The decay of peroxynitrite in the presence of HbFe(II)NO was observed at 302 nm, the wavelength at which $ONOO^-$ has an absorbance maximum. Under the conditions of our experiments, we found that the rate of peroxynitrite decay was not affected significantly by addition of substoichiometric concentrations of HbFe(II)NO, in agreement with the rather small value of the second-order rate constant of this reaction.

Finally, an attempt was made to detect NO• released during the second step of the reaction, after oxidation of the iron center. For this purpose, a solution of HbFe(II)NO was placed in the analysis vessel of an NO-analyzer,[20] and the reaction was started by addition of a slight excess of peroxynitrite. However, only traces of NO• could be detected (data not shown), probably because of the rapid reaction of peroxynitrite with NO• (see below).[21,22] Slightly higher yields were detected under alkaline conditions.

Interestingly, Gladwin and co-workers recently reported that NO• gas is liberated from HbFe(II)NO upon oxidation with $K_3[Fe(III)(CN)_6]$.[23] This reaction is very likely to proceed according to a similar mechanism, that is, oxidation of HbFe(II)NO to HbFe(III)NO and subsequent dissociation of NO•.

Since the rate constant for the reaction between peroxynitrite and HbFe(II)NO is not very large, in the presence of

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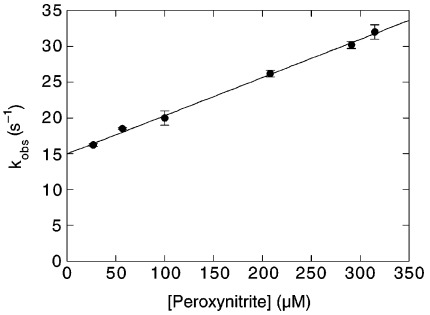


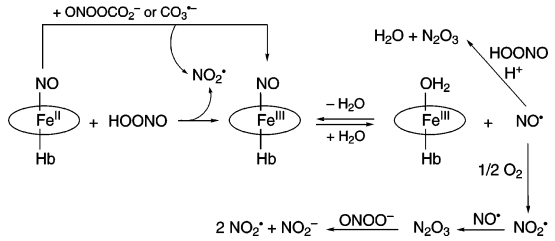
Figure 3. Plot of k_{obs} versus peroxynitrite concentration for the oxidation of HbFe(II)NO (3.5−4 μM) to HbFe(III)NO by peroxynitrite in the presence of 1.2 mM CO_2, in 0.05 M potassium phosphate buffer pH 7.2 at 20 °C. The value of the second-order rate constant obtained from the linear fit shown is $(5.3 \pm 0.2) \times 10^4$ M^{-1} s^{-1}.

physiologically relevant concentrations of CO_2 this reaction is not likely to take place. Indeed, peroxynitrite will rather react with CO_2 (3×10^4 M^{-1} s^{-1} at 24 °C).[24] We have thus investigated the reaction between HbFe(II)NO and peroxynitrite in the presence of 1.2 mM CO_2.[25] Rapid-scan UV−vis spectroscopic studies showed that the reaction proceeds in two steps, exactly as observed in the absence of CO_2 (data not shown): HbFe(II)NO is oxidized to HbFe(III)NO from which NO• is dissociated to generate metHb. Interestingly, the observed rate of the first reaction step was significantly larger than that measured in the absence of CO_2. For instance, the rates of formation of HbFe(III)NO from the reaction of 200 μM peroxynitrite with HbFe(II)NO were approximately 1 and 25 s^{-1}, in the absence and presence of CO_2 (1.2 mM), respectively. In addition, as shown in Figure 3 the observed rate constants increased linearly with increasing peroxynitrite concentration also in the presence of 1.2 mM CO_2. The linear fit of this plot resulted in a value of the second-order rate constant of $(5.3 \pm 0.2) \times 10^4$ M^{-1} s^{-1} (at pH 7.2 and 20 °C), a number 1 order of magnitude larger than that of the peroxynitrite-mediated oxidation of HbFe(II)NO in the absence of added CO_2. The plot of k_{obs} versus peroxynitrite concentrations (Figure 3) showed a positive *y*-axis intercept. Since oxidation of HbFe(II)NO is not likely to be a reversible process, this result may be indicative of a complex mechanism. Indeed, with the data obtained up to now it is not possible to determine whether $ONOOCO_2^-$ or $CO_3^{\bullet-}$ is responsible for HbFe(II)NO oxidation. Interestingly, a similar observation was made for the reaction of oxyMb with peroxynitrite, also only in the presence of 1.2 mM CO_2.[25b]

As expected, the rate of the second reaction step, the dissociation of NO• from HbFe(III)NO, was not affected by addition of CO_2. For all concentrations of peroxynitrite studied we obtained a value of ca. 1 s^{-1}. Finally, also in the presence of CO_2, the decay of peroxynitrite (followed at 302 nm) was not accelerated by the presence of HbFe(II)NO.

A possible mechanism for the reaction of HbFe(II)NO with peroxynitrite is depicted in Scheme 1. In the first step, an outer-sphere electron transfer from HbFe(II)NO to peroxynitrite produces HbFe(III)NO and most likely nitrogen dioxide. In the presence of CO_2, the oxidant is either $ONOOCO_2^-$ or $CO_3^{\bullet-}$ derived from its decomposition. Since in the iron(III) complex NO• is bound only weakly ($K_{d(\alpha)} =$ 4267 M^{-1} and $K_{d(\beta)} = 2631$ M^{-1}),[19] after oxidation of the

Scheme 1. Proposed Pathway for the Peroxynitrite-Mediated Oxidation of HbFe(II)NO, in the Presence and Absence of CO_2



iron center, under the conditions of our experiments, over 90% of NO• is released. This equilibrium is probably shifted toward the dissociated species by the consumption of NO• via its reaction with peroxynitrite and/or with the reactive species generated during peroxynitrite decomposition. It has been shown that, in the presence of oxygen, NO• accelerates the decay of peroxynitrite under neutral conditions.[21,26] This process is probably mediated by N_2O_3, generated from the reaction of NO• with $NO_2^{\bullet}$, formed from the spontaneous homolysis of HOONO.[21,22] The products of the rapid reaction between N_2O_3 and $ONOO^-$ ($k = (3.1 \pm 0.3) \times 10^8$ M^{-1} s^{-1}) are nitrite and $NO_2^{\bullet}$.[21] Obviously, since in vivo the concentration of peroxynitrite is not likely to be significantly larger than that of HbFe(II)NO, these subsequent reactions will not take place. In this context, it has recently been shown that despite the high Hb concentration in the red blood cells, a fraction of NO•, produced from the reaction of nitrite and deoxyHb, can escape and is detected in the plasma.[27]

We have recently shown that HbFe(III)NO is an efficient nitrosating agent. Specifically, the "NO^+" group can be formally transferred from the heme to the cysteine residue β93 yielding SNO−Hb.[15b] Moreover, SNO−Hb may also be produced by the reaction of Cysβ93 with the additional nitrosating agents formed in our system, i.e., N_2O_3 and $NO_2^{\bullet}$.

In conclusion, we have demonstrated that NO• can be released from HbFe(II)NO by peroxynitrite, after outer-sphere oxidation of the iron center. The second-order rate constant measured in the presence of physiological concentrations of CO_2 suggests that this reaction may take place in vivo, in particular, under pathological conditions such as septic shock, characterized by elevated production of NO• and peroxynitrite. The reactive nitrogen species generated from this reaction, N_2O_3 and/or $NO_2^{\bullet}$, may lead to protein modifications, such as nitration of tyrosine and/or tryptophan residues and nitrosation of Cysβ93. We are currently investigating this hypothesis.

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