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Original Contribution

KINETICS OF THE REACTIONS OF NITROGEN MONOXIDE AND NITRITE WITH FERRYL HEMOGLOBIN

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Abstract—Hemoglobin released in the circulation from ruptured red blood cells can be oxidized by hydrogen peroxide or peroxynitrite to generate the highly oxidizing iron(IV)oxo species $HbFe^{IV}$=O. Nitrogen monoxide, produced in large amounts by activated inducible nitric oxide synthase, can have indirect cytotoxic effects, mainly through the generation of peroxynitrite from its very fast reaction with superoxide. In the present work we have determined the rate constant for the reaction of $HbFe^{IV}$=O with $NO^{\bullet}$, 2.4×10^7 $M^{-1}s^{-1}$ at pH 7.0 and 20°C. The reaction proceeds via the intermediate $HbFe^{III}ONO$, which then dissociates to metHb and nitrite. As these products are not oxidizing and because of its large rate, the reaction of $HbFe^{IV}$=O with $NO^{\bullet}$ may be important to remove the high valent form of hemoglobin, which has been proposed to be at least in part responsible for oxidative lesions. In addition, we have determined that the rate constant for the reaction of $HbFe^{IV}$=O with nitrite is significantly lower (7.5×10^2 $M^{-1}s^{-1}$ at pH 7.0 and 20°C), but increases with decreasing pH (1.8×10^3 $M^{-1}s^{-1}$ at pH 6.4 and 20°C). Thus, under acidic conditions as found in ischemic tissues, this reaction may also have a physiological relevance. © 2003 Elsevier Science Inc.

Keywords—Nitric oxide, Hemoglobin, Nitrite, Kinetics, Free radicals

INTRODUCTION

Heme-mediated redox reactions have been suggested to contribute to organ dysfunction and/or tissue damage, which occur in some pathological states characterized by the release of hemoglobin (Hb) and myoglobin (Mb) into the extracellular environment [1,2]. Autoxidation of oxyhemoglobin ($HbFeO_2$) and oxymyoglobin ($MbFeO_2$) to their iron(III)-forms releases superoxide, that dismutates to dioxygen and hydrogen peroxide, which may cause tissue damage [3]. In addition, oxidation of these two proteins generates potentially cytotoxic products such as the oxoiron(IV) species [4,5]. Indeed, $MbFe^{IV}$=O and $HbFe^{IV}$=O are strong oxidants that can promote oxidation, peroxidation [6–8], and epoxidation of various biomolecules in vitro [9,10].

Hemoglobin is normally sequestered inside red blood cells where protective antioxidants such as methemoglobin reductase, superoxide dismutase, catalase, and glutathione peroxidase limit its potentially harmful oxidation reactions and preserve its oxygen-carrying ability. However, large amounts of hemoglobin released in the circulation from ruptured red blood cells are devoid of these antioxidants. Thus, cell-free hemoglobin can overwhelm plasma protective mechanisms and lead to significant damage of the endothelial cells. In analogy, ferryl myoglobin has been proposed to be a key determinant of the oxidative tissue damage caused, for instance, by reperfusion of an ischemic heart [1].

High valent hemoglobin and myoglobin species are mainly generated by their reaction with hydrogen peroxide [11] or with peroxynitrite [12]. Hydrogen peroxide, produced by superoxide dismutation or direct enzymatic production (superoxide dismutase, amine oxidase, or glucose oxidase), can be generated by a variety of mammalian cells, including neutrophils, macrophages, vascular smooth muscle, and endothelial cells. Its production may be increased under pathological conditions such as ischemia-reperfusion [13] and its concentration may be as high as 10 μM in ischemic heart muscle [14]. Hydrogen peroxide reacts with metHb to yield the two-electron oxidized ferryl hemoglobin form that has an additional transient radical on the globin, $^{\bullet}HbFe^{IV}$=O. The protein-based radical has been proposed to be centered on a

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sulfur and/or an oxygen atom [15–17]. Both ferryl hemoglobin [5] and the protein-based radical [18] have been identified in normal human blood. This implies that, despite the presence of catalase and glutathione peroxidase, under normal conditions the steady state concentration of hydrogen peroxide in the blood (reported to be 2×10^{-10} M [19]) is large enough to cause metHb oxidation to the ferryl form and concomitant production of globin-centered radical(s).

One of the species present in vivo that may scavenge high valent Mb and Hb forms is nitrogen monoxide. Indeed, we have recently shown that the rate of the reaction of $NO^{\bullet}$ with $MbFe^{IV}$=O is quite large (1.8×10^7 $M^{-1}s^{-1}$, at pH 7.5 and 20°C [20]). Thus, as has repeatedly been proposed [21–26], $NO^{\bullet}$ may act as an antioxidant and inhibit $MbFe^{IV}$=O-induced oxidative damage. The rate constant for the analogous reaction of $NO^{\bullet}$ with $HbFe^{IV}$=O has not been reported yet.

Except for nitrate generated from the reaction of $NO^{\bullet}$ with $HbFeO_2$, the major end product of the $NO^{\bullet}$ metabolism is nitrite. Thus, local nitrite concentrations in cells are mostly correlated to the extent of $NO^{\bullet}$ production and can reach micromolar levels [27,28]. Nitrite has recently been shown to be oxidized to nitrogen dioxide by peroxidases in the presence of hydrogen peroxide [29,30]. This reaction system can nitrate tyrosine and is currently considered as the major alternative to peroxynitrite-mediated tyrosine nitration in vivo [31–34].

The goal of this work was to determine the second-order rate constants of the reactions of $HbFe^{IV}$=O with $NO^{\bullet}$ and with nitrite. These constants are essential to evaluate the biological relevance of these two reactions.

MATERIALS AND METHODS

Reagents

Buffer solutions were prepared from K_2HPO_4/KH_2PO_4 (Fluka, Buchs, Switzerland) or from $Na_2B_4O_7 \cdot 10H_2O$/NaOH (Fluka) with deionized Milli-Q water. Sodium nitrite, sodium nitrate, sodium dithionite, potassium hexacyanoferrate(III), 3-nitro-L-tyrosine (NO_2-Tyr), and L-tyrosine were obtained from Fluka. Catalase (bovine liver, 17,000 units/mg protein) was purchased from Sigma (St. Louis, MO).

Nitrogen monoxide was obtained from Linde (Höllriegelskreuth, Germany) and passed through a NaOH solution as well as a column of NaOH pellets to remove higher nitrogen oxides before use. Saturated $NO^{\bullet}$ solutions were prepared and diluted as described previously [35]. Hydrogen peroxide (30%) was obtained from Fluka. This solution was diluted with water, and the final concentration was determined spectrophotometrically at 240 nm (ε_{240} = 39.4 $M^{-1}cm^{-1}$ [36]).

A purified human oxyhemoglobin ($HbFeO_2$) stock solution (57 mg/ml solution of HbA_0 with approximately 1.1% metHb) was a kind gift from APEX Bioscience, Inc. (Durham, NC, USA). MetHb solutions were prepared as described elsewhere [37]. $HbFe^{IV}$=O solutions for the stopped-flow experiments were prepared by adding 10 equivalents of H_2O_2 to a metHb solution (about 200 μM in 0.1 M phosphate buffer pH 7.0) at room temperature. Conversion of metHb to $HbFe^{IV}$=O was followed spectrophotometrically and was found to be complete within ca. 4 min. The $HbFe^{IV}$=O solution was diluted with buffer (0.1 M phosphate buffer pH 7.0 or 8.5, or 0.1 borate buffer pH 9.5) to the required concentration (0.25–2 μM) and used within 10 min. Previous studies have shown that, under these conditions, H_2O_2 is consumed within 8 min and less than 10% $HbFe^{IV}$=O is decomposed [38]. The yield of the reaction of metHb with H_2O_2 is approximately 80%, and the estimated extinction coefficients for $HbFe^{IV}$=O are $\varepsilon_{418} = 100 \pm 10\ mM^{-1}cm^{-1}$, $\varepsilon_{544} = 11 \pm 1\ mM^{-1}cm^{-1}$, and $\varepsilon_{574} = 10 \pm 1\ mM^{-1}cm^{-1}$.

Horse heart myoglobin was purchased from Sigma. Purified metMb and $MbFe^{IV}$=O (ferryl Mb) solutions were prepared as described elsewhere [20].

Stopped-flow kinetic analysis

Kinetic studies were carried out at 20°C with an Applied Photophysics (Surrey, UK) SX17MV single-wavelength stopped-flow instrument and an On-Line Instrument Systems Inc. (Bogart, GA, USA) stopped-flow instrument equipped with an OLIS RSM 1000 rapid scanning monochromator. With the Applied Photophysics apparatus, kinetic traces were taken at different wavelengths between 280 and 650 nm and the data were analyzed with the SX17MV operating software or with Kaleidagraph, Versions 3.0.5 and 3.0.8. To determine the rate constants, traces were generally collected at 410 or 418 nm. The results of the fits of the traces (averages of at least 10 single shots) from at least five experiments were averaged to obtain each observed rate constant, given as the mean plus or minus the standard deviation. Care was taken that the absolute absorbance of the reaction mixture was not higher than one absorbance unit. The pH was measured at the end of the reactions.

For the reactions of $HbFe^{IV}$=O with $NO^{\bullet}$, the protein solutions were prepared as described above under aerobic conditions. Because of problems with inhomogeneous mixing at higher protein concentrations, the experiments were carried out with $NO^{\bullet}$ in excess. However, a few control experiments showed that for both systems, the obtained rate constants were not dependent on which of the reagents was used in excess. When high concentrations of $NO^{\bullet}$ were used and the reactions had to be followed over a long time scale (more than 5–10 s), the protein solutions were diluted with

degassed buffer to prevent the formation of higher nitrogen oxides from the reaction of $NO^{\bullet}$ with dioxygen.

For the measurements between pH 6.5 and 9.5 the $HbFe^{IV}=O$ and the $NO^{\bullet}$ solutions were all prepared in 0.1 M buffers of the required pH value. Because of the instability of the $HbFe^{IV}=O$ solution, measurements at pH 6.4 were carried out by mixing a $HbFe^{IV}=O$ solution prepared in a 0.1 M phosphate buffer at pH 7.0 with a $NO^{\bullet}$ solution at pH 4.0.

For the reactions of $HbFe^{IV}=O$ with nitrite high concentrations of nitrite were always used to make sure that the reaction between nitrite and $HbFe^{IV}=O$ was significantly faster than the decomposition of $HbFe^{IV}=O$. All solutions were prepared under aerobic conditions. For the measurements between pH 6.5 and 9.5 the $HbFe^{IV}=O$ and the nitrite solutions were all prepared in 0.1 M buffers of the required pH value. Measurements with hemoglobin at pH 6.4 were carried out by mixing a $HbFe^{IV}=O$ solution prepared in a 1 mM phosphate buffer at pH 7.0 with a nitrite solution in a 0.1 M phosphate buffer at pH 6.4.

UV-visible spectroscopy

Absorbance spectra were collected on a UVIKON 820 spectrophotometer. To determine the yield of the reactions of $NO^{\bullet}$ with $HbFe^{IV}=O$ spectra were measured in sealable cells for anaerobic applications (Hellma). About 3 ml of a ca. 100 μM protein solution (in the iron(III) form) were placed in the cell and mixed with ca. 3 equivalents of H_2O_2 (100 μl of a 0.01 M solution in water). When the formation of the ferryl form of the proteins was complete (see above), the cell was sealed and one equivalent of $NO^{\bullet}$ (about 150 μl of a 2 mM $NO^{\bullet}$ solution) was added by using a gastight Hamilton syringe.

Ion chromatographic product analysis

Product analysis was carried out by anion chromatography with conductivity detection with a Metrohm Instrument (ICSeparation Center 733, ICDetector 732, and IC pump 709) equipped with an Anion SUPER-SEP (6.1009.000) column and an Anion SUPER-SEP (6.1009.010) or an Anion PRP-X100 (6.1005.020) precolumn as described previously [20,39]. Catalase was found to contain nitrate impurities, which were determined separately and subtracted from the experimental data.

RESULTS

Stopped-flow kinetic studies of the $NO^{\bullet}$-mediated reduction of ferryl hemoglobin

The second-order rate constant of the reaction between $NO^{\bullet}$ and $HbFe^{IV}=O$ (human) was measured by single-wavelength stopped-flow spectroscopy in the pH range 6.4–9.5 at 20°C. $HbFe^{IV}=O$ was prepared by allowing metHb to react under aerobic conditions with about 10 equivalents of hydrogen peroxide for ca. 4 min [40]. These solutions were shown to slowly decay to metHb and were thus used within 10–15 min. Because of the large reaction rates, the instability of the $HbFe^{IV}=O$ solutions did not cause any problems for the determination of the rate constant of the reaction with $NO^{\bullet}$. Upon reaction with metHb, the second oxidizing equivalent of H_2O_2 is known to generate a radical on the globin [15–17], which may also react with $NO^{\bullet}$. However, in the presence of a large excess of H_2O_2, used to prepare $HbFe^{IV}=O$, this radical has been shown to be formed in low yield and to decay very rapidly [17]. In addition, as we studied the reaction of $HbFe^{IV}=O$ with $NO^{\bullet}$ by following the absorbance changes arising from the heme, partial scavenging of $NO^{\bullet}$ (always present in large excess) by this radical would not affect the determination of the rate constant. The solutions generated from the reaction of metHb with H_2O_2 may also contain unreacted metHb and/or hemichrome (see below). However, under the conditions of our experiments the rate of $NO^{\bullet}$ binding to metHb is significantly smaller than that of the $NO^{\bullet}$-mediated reduction of $HbFe^{IV}=O$, and the hemichrome does not react with $NO^{\bullet}$. Thus, these minor impurities do not influence the determination of the rate of the reaction of $HbFe^{IV}=O$ with $NO^{\bullet}$.

At protein concentrations higher than 5 μM, large interferences due to inhomogeneous mixing disturbed the measurement of the first 30–50 ms of the reaction. This problem is often observed when highly concentrated viscous protein solutions are mixed in the stopped-flow apparatus with nonviscous solutions, such as $NO^{\bullet}$ or nitrite solutions. Thus, we chose to work with $NO^{\bullet}$ in 8–10-fold excess to maintain pseudo-first-order conditions, and the hemoglobin concentration was mostly kept below 1 μM. Under these conditions Hb is mostly present in its dissociated dimeric form ($\alpha\beta$), thus the rate constants reported are for the reaction with dimeric Hb. The presence of dioxygen in the protein solutions was found not to affect the results, as under the conditions of our experiments $NO^{\bullet}$ reacts at a significant larger rate with $HbFe^{IV}=O$ than with O_2.

In analogy to the reaction with myoglobin [20], upon mixing of $HbFe^{IV}=O$ with $NO^{\bullet}$ we observed the formation of the intermediate $HbFe^{III}ONO$ and its subsequent decay to metHb (Eqn. 1).

$$HbFe^{IV}=O + NO^{\bullet} \xrightarrow{k_1} HbFe^{III}ONO \xrightarrow{k_{2a},k_{2b}} metHb + NO_2^- \quad (1)$$

This was clearly visible from all traces collected at several wavelengths in the Soret region and for all pH values studied. The kinetics of the formation of this intermediate were studied by following the absorbance changes at 410 nm. The traces were fitted to a single-exponential expression (Fig. 1 inset). In some cases, the

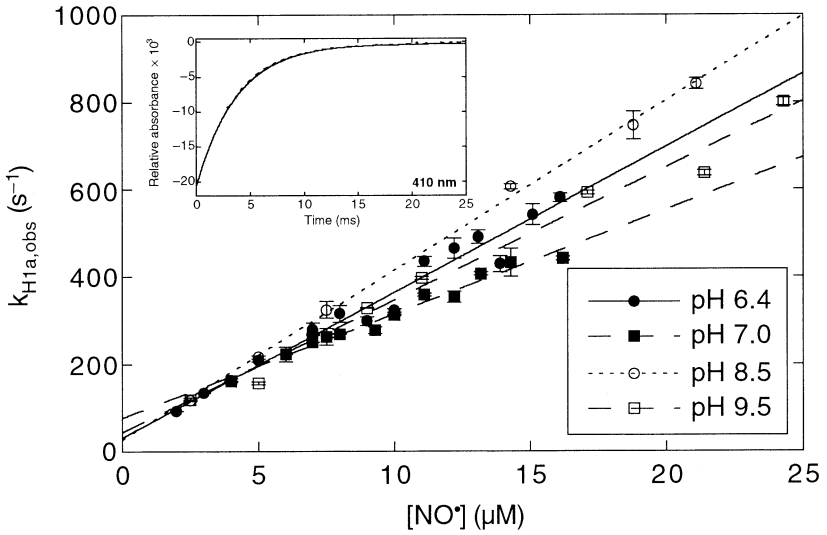

Fig. 1. Plots of $k_{1,obs}$ vs. NO• concentration for the formation of the intermediate $HbFe^{III}ONO$ (from the reaction of $HbFe^{IV}$=O with NO•) in 0.1 M phosphate buffer at pH 6.4, 7.0, 8.5, and 9.5 (20°C). The values of the second-order rate constants obtained from the linear fits are given in Table 1. In the inset: Time course measured at 410 nm for the reaction of $HbFe^{IV}$=O (1.0 μM) with NO• (9.3 μM) in 0.1 M phosphate buffer at pH 7.0 and 20°C. The solid line corresponds to the best fit for the formation of the intermediate $HbFe^{III}ONO$. The resulting rate constant is $k_{1,obs} = 266 \pm 1\ s^{-1}$. The first 3 ms represent the mixing time of the stopped-flow apparatus and are thus omitted.

fits were not perfect but could be improved to some extent by using a two-exponential expression. This result suggests that the two subunits of hemoglobin may react at slightly unequal rates. However, the difference between the two rates is probably not large enough to allow for their exact determination. Thus, we always determined the average rate.

The second-order rate constants for the formation of $HbFe^{III}ONO$ from $HbFe^{IV}$=O and NO• (k_1), obtained from the linear plots of the observed pseudo-first-order rate constants vs. NO• concentration, vary slightly in the pH range studied (Fig. 1 and Table 1). At pH 7.5 we obtained $k_1 = (24 \pm 1) \times 10^6\ M^{-1}s^{-1}$, a value only slightly larger than that for the generation of the corresponding myoglobin complex, $(18 \pm 1) \times 10^6\ M^{-1}s^{-1}$ [20]. As we have previously shown that the corresponding rates for the NO•-mediated reduction of $MbFe^{IV}$=O are pH-independent in the range 5.9–9.5 [20], it is conceivable that the small variations observed among the second-order rate constants of the reaction of $HbFe^{IV}$=O with NO• measured in the pH range 6.5–9.5 are due to the slight difference between the reaction rates of the two subunits of Hb. As already discussed above, the two rates cannot be distinguished and thus some uncertainty is connected with the reported average values. In general, the rate of the reaction of NO• with $HbFe^{IV}$=O is 1.5–2 times larger than that of its reaction with $MbFe^{IV}$=O [20]. This result correlates with the observation that the rate of reaction of NO• with $HbFeO_2$ is also about twice as large as that that for its reaction with $MbFeO_2$ [35].

Table 1. pH-Dependencies of the Second-order Rate Constants (k_1) for the Formation of the Intermediate $HbFe^{III}ONO$ from $HbFe^{IV}$=O and NO•

pH	k_1 ($\mu M^{-1}s^{-1}$)	k_{2a} (s^{-1})	k_{2b} (s^{-1})
6.5	33 ± 2	1.14 ± 0.03	0.32 ± 0.01
7.0	24 ± 1	0.48 ± 0.02	0.12 ± 0.01
8.5	39 ± 1	0.11 ± 0.01	0.025 ± 0.001
9.5	30 ± 2	0.074 ± 0.005	0.017 ± 0.002

Values of the observed rate constants for the decay of the intermediates of the two subunits of $HbFe^{III}ONO$ (k_{2a} and k_{2b}) to metHb at different pH. All rates were measured at 20°C.

The kinetics of the decay of the hemoglobin intermediate to metHb were studied by following the absorbance changes at 418 nm, approximately the wavelength at which the difference in the extinction coefficients of the two species is maximal. Figure 2 (inset) shows a typical trace. It is apparent that the decay of the intermediate proceeds with two distinguishable rates, attributed to the α- and the β-subunits, respectively. Similar values of the two decay rates (k_{2a} and k_{2b}) were obtained within a broad range of NO• and $HbFe^{IV}$=O concentrations and were independent of the NO• as well as of the $HbFe^{IV}$=O concentrations. However, when reactions are carried out with large NO• concentrations care must be taken to exclude oxygen from the solutions. Indeed, as the decay of the $HbFe^{III}ONO$ intermediates to metHb can proceed over 50–100 s, the concurrent formation of higher nitrogen oxides, from the reaction of excess NO• with O_2, and their reaction with hemoglobin may interfere with the

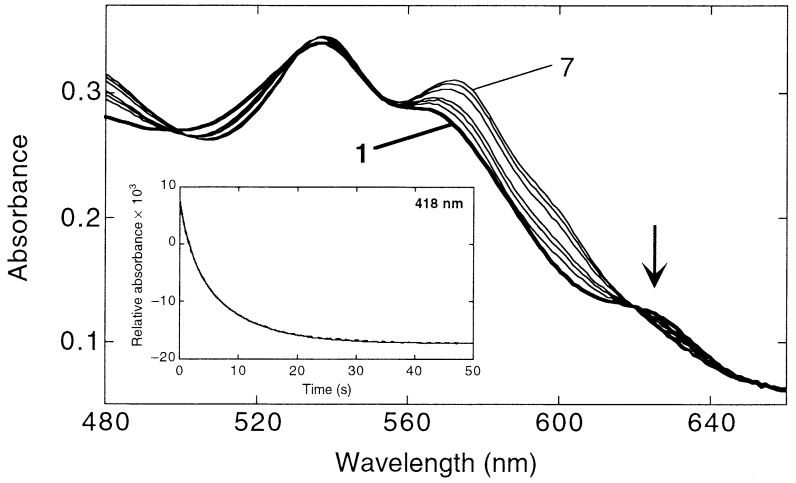

Fig. 2. Rapid-scan UV-vis spectra of the reaction of $HbFe^{IV}$=O (41 μM) with NO• (60 μM) in 0.1 M borate buffer at pH 9.5, 20°C. Traces 1–7, recorded 0, 4.8, 9.6, 14.4, 40.8, 67.2, and 99.4 s after mixing, show the decay of the intermediate $HbFe^{III}ONO$ (bold trace 1) to $HbFe^{III}OH$ (trace 7). In the inset: Time course measured at 418 nm for the reaction of $HbFe^{IV}$=O (1.0 μM) with NO• (10 μM) in 0.1 M phosphate buffer at pH 7.0 and 20°C. The solid line corresponds to the best fit for the decay of the intermediate $HbFe^{III}ONO$ to metHb. The resulting rate constants for the two subunits are $k_{2a,obs} = 0.49 \pm 0.01\ s^{-1}$ and $k_{2b,obs} = 0.120 \pm 0.001\ s^{-1}$, respectively. The two amplitudes are approximately equivalent.

measurements. Under anaerobic conditions, when NO• was used in large excess an additional reaction was observed on a longer time scale which corresponds to NO• binding to the product metHb.

The decay rates of the intermediates of the two hemoglobin subunits are highly pH-dependent and increase with decreasing pH (Table 1). At pH 7.0 we obtained $k_{2a} = 0.48 \pm 0.02\ s^{-1}$and $k_{2b} = 0.12 \pm 0.01\ s^{-1}$, respectively. These values are significantly lower than those of the corresponding decay rates of the myoglobin intermediate ($5.98 \pm 0.06\ s^{-1}$ at pH 7.0 and 20°C [20]). Interestingly, a similar trend was observed for the decay rates of $HbFe^{III}OONO$ and $MbFe^{III}OONO$ to nitrate and metHb or metMb, respectively [35]. Also for $HbFe^{III}OONO$ two separate rates were obtained for the α- and β-subunits. In addition, these Hb intermediates were found to be significantly more stable than $MbFe^{III}OONO$. This observation suggests that, even though the intermediate *O*-nitrito complexes decay at significantly lower rates than the corresponding peroxynitrito complexes, similar factors, probably due to the protein environment, seem to influence their stability.

The observation that the dissociation of nitrite from $HbFe^{III}ONO$ takes place faster at lower pH suggests that a protonated form of the enzymes may facilitate this process. Indeed, as the pK_a of nitrous acid is 3.3, only very low concentrations of HNO_2 are present in our reaction mixtures in the pH range studied. Thus, the observed pH dependence cannot arise from reactions with HNO_2. Therefore, we can assume that the pH-dependence originates from a single ionizable residue of the protein and consequently the relationship given in Eqn. 2 exists between k_2 and the proton concentration.

$$k_2 = (k_{2'} \times [H^+])/(K + [H^+]) \quad (2)$$

In Eqn. 2, $k_{2'}$ stays for the pH-independent first-order dissociation constant and *K* for the dissociation constant of the protonable residue. Even though only few values of rate constants in the narrow pH range 6.5–9.5 were measured, the experimental data could be approximately fitted to Eqn. 2. The best fits for the two nitrite dissociation constants from $HbFe^{III}ONO$ gave values of $pK_a = 6.2 \pm 0.5$ and $pK_b = 6.0 \pm 0.5$ for the dissociation constant and values of $k_{2a'} = 3 \pm 1\ s^{-1}$ and $k_{2b'} = 1.5 \pm 0.5\ s^{-1}$ for the pH-independent first-order rate constants. In analogy to the dissociation of nitrite from $MbFe^{III}ONO$ [20], these data imply a role for the distal histidine. Indeed, in the protonated form—at pH lower than 6.0—this histidine residue swings out of the heme pocket toward the solvent and consequently leaves the active site more accessible [41]. In this open conformation, dissociation of nitrite could be accelerated.

Spectral characterization of the O-nitrito intermediate $HbFe^{III}ONO$

The spectral changes arising from the reaction between NO• and $HbFe^{IV}$=O were monitored by rapid-scan UV-vis spectroscopy between 380 and 680 nm at pH 9.5 and 20°C. Upon mixing, the absorbance maximum of the Soret band of hemoglobin shifted from 418 nm

Table 2. Spectroscopic Data for metMb and metHb Complexes

	Soret	Visible				Reference
	λ_{max} (ε)[a]	λ_{max} (ε)[a]		λ_{max} (ε)[a]		
$MbFe^{III}ONO$	410 (137)	504 (8.7)	534 sh	575 (6.3)	631 (5.1)	[20]
$MbFe^{III}NO_2$	412 (137)	502 (8.4)	537 sh	573 (5.4)	628 (4.2)	[35]
$MbFe^{III}ONO_2$	408 (172)	502 (8.8)		591 (3.1)	629 (3.6)	[35]
$MbFe^{III}OONO$	410 (138)	504 (8.0)	533 sh	580 (5.0)	636sh (3.2)	[35]
$HbFe^{III}ONO$	410 (133)	537 (10.1)		562 sh	617 (3.9)	this work
$HbFe^{III}NO_2$	411 (132)	538 (10.0)		567 sh	623 (3.6)	[35]
$HbFe^{III}ONO_2$	408 (120)	527 (9.9)		561 sh	628 (3.2)	[35]
$HbFe^{III}OONO$	407 (165)	504 (8.7)	533 sh	577 (4.7)	636 (5.4)	[42]

[a] λ_{max} in nm; ε in $mM^{-1}\ cm^{-1}$.

($HbFe^{IV}$=O) to 411 nm (metHb at pH 9.5) via an intermediate species with a maximum at 410 nm and an extinction coefficient of about 133 $mM^{-1}cm^{-1}$ (data not shown). To obtain a spectrum of $HbFe^{III}ONO$ in the visible region we mixed higher concentrations of the reagents to reduce the signal to noise ratio. Thus, accumulation of $HbFe^{III}ONO$ from the rapid reaction of $NO^{\bullet}$ with $HbFe^{IV}$=O occurred within the dead time of the instrument. The best spectrum of the intermediate, shown as the first, bold trace in Fig. 2 displays two characteristic absorbance maxima at 617 (ε_{617} = 3.9 $mM^{-1}cm^{-1}$) and 537 nm (ε_{537} = 10.1 $mM^{-1}cm^{-1}$) and a shoulder at 562 nm. These absorbance features are very similar to those of the *N*-nitrito- and nitrato-metHb complexes (Table 2) but significantly different from those of $MbFe^{III}ONO$. Indeed, the observed maxima for $HbFe^{III}ONO$ are characteristic for a low-spin iron(III) species, whereas $MbFe^{III}ONO$ displays maxima typical for high-spin iron(III) complexes (Table 2). In analogy to myoglobin [20], the spectral data support the assignment of the intermediate as the *O*-nitrito-metHb complex $HbFe^{III}ONO$, generated from the radical/radical reaction of $NO^{\bullet}$ with $HbFe^{III}-O^{\bullet}$, a resonance form of $HbFe^{IV}$=O.

An identical spectrum for $HbFe^{III}ONO$ was obtained when the reaction was carried out at pH 7.0. As shown in Fig. 3A, the characteristic absorbance bands for $HbFe^{III}ONO$ (first, bold trace) were observed immediately after mixing a $HbFe^{IV}$=O solution with a slight excess of $NO^{\bullet}$. $HbFe^{III}ONO$ then decays to metHb (spectrum 5 in Fig. 3A), that, however, displays a slightly altered spectrum because of the side products generated in the course of the formation of $HbFe^{IV}$=O (see next section).

In contrast, when large concentrations of $NO^{\bullet}$ (higher than 80 μM) were mixed with $HbFe^{IV}$=O at pH 7.0, under anaerobic conditions, we observed the direct transformation of $HbFe^{III}ONO$ (first bold trace in Fig. 3B) to $HbFe^{III}NO$ (trace 5 in Fig. 3B, absorbance maxima at 533 and 564 nm [43]). As expected, the kinetics of this reaction were biphasic and the resulting constants coincided with those of nitrite dissociation from $HbFe^{III}ONO$ and subsequent binding of excess $NO^{\bullet}$ to metHb. Under the conditions of the experiment shown in Fig. 3B, the former reaction is the rate-determining step and thus metHb cannot accumulate in concentrations large enough to be detected.

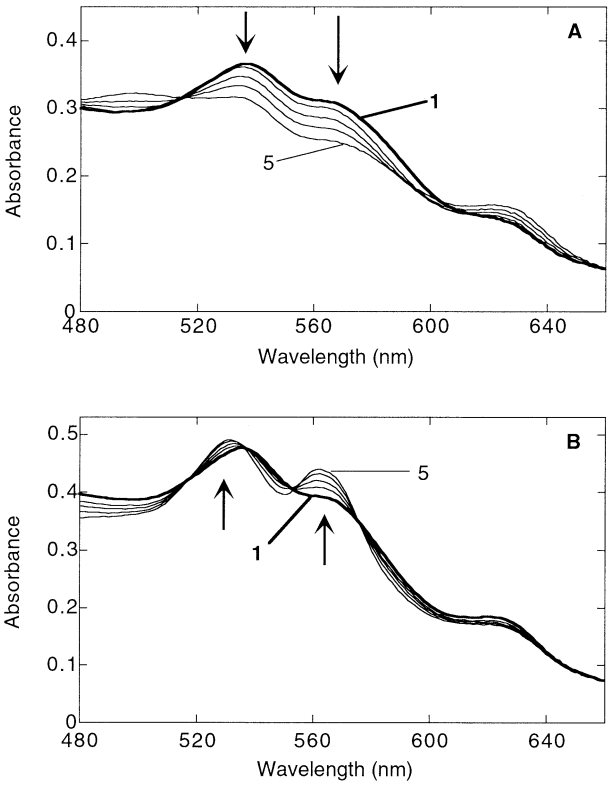

Fig. 3. Rapid-scan UV-vis spectra of the reaction of $HbFe^{IV}$=O (38.3 μM) with $NO^{\bullet}$ (A) (60.0 μM) and (B) (383 μM) in 0.1 M phosphate buffer at pH 7.0 and 20°C. (A) Traces 1–5, recorded 0, 32, 368, 1000, and 2900 ms after mixing, show the decay of the intermediate $HbFe^{III}ONO$ (bold trace 1) to $HbFe^{III}OH$ (trace 5). (B) Traces 1–5, recorded 0, 320, 480, 800, and 1440 ms after mixing, show the transformation of the intermediate $HbFe^{III}ONO$ (trace 1) to $HbFe^{III}NO$ (bold trace 5).

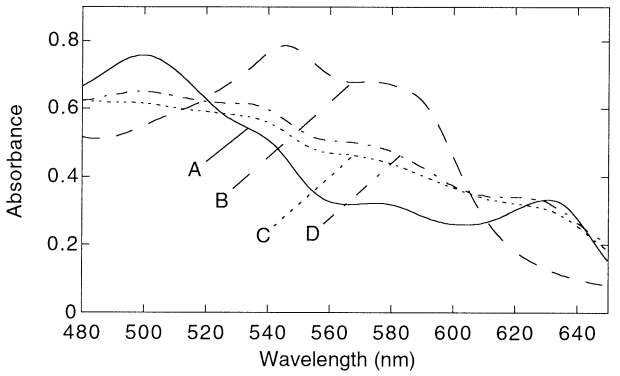

Fig. 4. UV-vis spectra in 0.1 M phosphate buffer at pH 7.0. (A) $HbFe^{III}OH_2$ (70 μM); (B) $HbFe^{IV}$=O from the reaction of $HbFe^{III}OH_2$ (70 μM) + H_2O_2 (700 μM); (C) solution (B) decayed after ca. 2.5 h. (D) Trace 5 from Fig. 3A [$HbFe^{IV}$=O (38.3 μM) + NO• (60 μM) after 2.9 s] multiplied by 70/38.3, to adjust the concentration to that of the other solutions.

UV-vis spectra of the protein product and yield of the reaction

The products of the reaction of a stoichiometric amount of NO• with $HbFe^{IV}$=O were analyzed by UV-vis spectroscopy to determine whether the protein is significantly modified during this reaction. As shown in Fig. 4, reaction of a slight excess of NO• with $HbFe^{IV}$=O (curve B) at pH 7.0 yields a species with an absorbance spectrum (curve D) similar but not identical to that of pure metHb (curve A). In particular, a new absorbance maximum and a shoulder appeared at 532 and 566 nm, respectively, and the bands at 500 and 631 nm were significantly modified as well. However, the spectrum obtained when a $HbFe^{IV}$=O solution was allowed to decay back to metHb (curve C in Fig. 4) showed similar modifications, but was more significantly different from that of pure metHb. This result is in agreement with previous studies that showed that, upon reaction of metHb with H_2O_2, not only $HbFe^{IV}$=O is produced but also the hemichrome and denatured hemoglobin species [38,44]. The hemichrome represents the low-spin iron(III) form of hemoglobin in which the heme binds the nitrogen atom of the distal histidine at the sixth coordination position [45]. Hemoglobin conversion into hemichrome is characterized by the disappearance of the peaks typical for the high-spin state of heme iron(III), with maxima at 500 and 631 nm, and by the simultaneous appearance of a peak with a maximum at 536 nm and a shoulder in the region of 565 nm, representative for a low-spin iron(III) form [45]. The product obtained from the decomposition of the $HbFe^{IV}$=O solution was further characterized by reducing it with sodium dithionite. The resulting spectrum (data not shown) displays a new maximum at 558 nm and a shoulder at 529 nm, characteristic for the reduced hemochrome, that is a bis-histidyl low-spin iron(II) hemoglobin species [45].

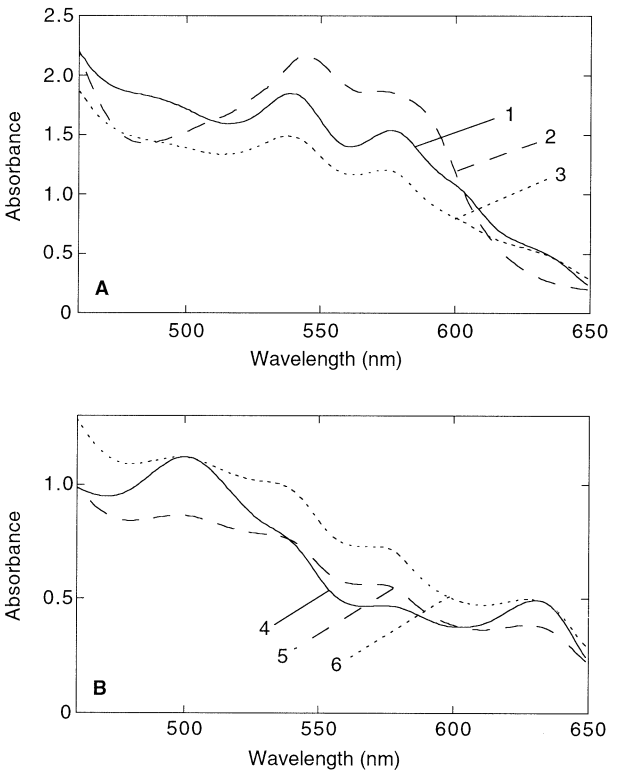

Fig. 5. (A) UV-vis spectra in 0.1 M phosphate buffer at pH 8.5. (1) $HbFe^{III}OH_2$ (226 μM); (2) $HbFe^{IV}$=O from the reaction of $HbFe^{III}OH_2$ (226 μM) + H_2O_2 (2.3 mM); (3) solution (2) decayed after ca. 2.5 h. (B) UV-vis spectra in 0.1 M phosphate buffer at pH 7.0. (4) $HbFe^{III}OH_2$ (113 μM); (5) solution (3) diluted 1:1; (6) solution (5) with concentration adjusted to 113 μM by setting the absorbance at 631 nm equal to that of spectrum (4).

Studies of the formation of $MbFe^{IV}$=O from the reaction between metMb and H_2O_2 have shown that when the reaction is carried out in the pH range 8.0–9.0 significantly smaller amounts of side products are generated [11,46]. Thus, we analyzed the products of the decay of a $HbFe^{IV}$=O solution prepared from metHb and H_2O_2 under alkaline conditions. As shown in Fig. 5A, when $HbFe^{IV}$=O (spectrum 2) was generated at pH 8.5 and was then allowed to decay back to metHb, a species was formed (spectrum 3) with absorbance features rather similar to those of metHb under the same conditions (spectrum 1). However, when this reaction solution was neutralized to pH 7.0 the new species (spectra 5 and 6 in Fig. 5B) displayed the characteristic absorbance features for the hemichrome (532 and 566 nm) together with those of high-spin metHb (500 and 631 nm, spectrum 4 in Fig. 5B). Taken together, these data suggest that the reaction between metHb and H_2O_2 also seems to proceed with fewer side reactions under alkaline conditions. Nevertheless, the Hb hemichrome is also generated under

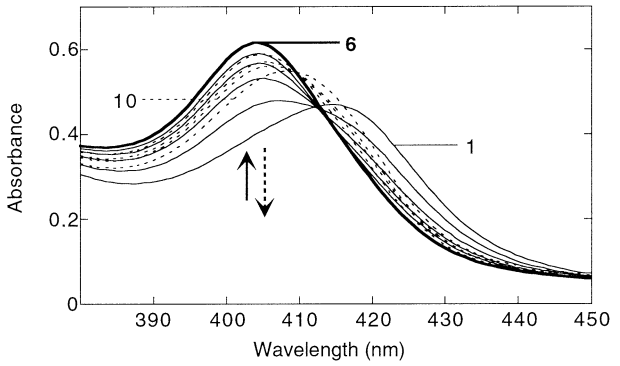

Fig. 6. Rapid-scan UV-vis spectra of the reaction of $HbFe^{IV}=O$ (4.2 μM) with nitrite (12.5 mM) in 0.1 M phosphate buffer at pH 7.0, 20°C. Traces 1–6, recorded 0, 48, 96, 144, 192, and 384 ms after mixing, and traces 7–10, recorded 0.8, 1.3, 1.8, and 6.6 s after mixing, show the formation of metHb (bold trace 6) from $HbFe^{IV}=O$ (spectra 1–5) and its further reaction with nitrite to a mixture of to $HbFe^{III}OH_2$ and $HbFe^{III}NO_2$ (dashed spectra 7–10).

alkaline conditions. In general, when metHb is allowed to react with H_2O_2, the protein is significantly more altered than when metMb is mixed with H_2O_2 under the same conditions.

Stopped-flow kinetic studies of the nitrite-mediated reduction of ferryl hemoglobin

As nitrite is always present as a contaminant in our $NO^{\bullet}$ solutions (variable amounts between 0.1 and 1 equivalents, relative to the $NO^{\bullet}$ concentration), we also studied the kinetics of the reaction between nitrite and $HbFe^{IV}=O$. The spectral changes taking place during this reaction were monitored by rapid-scan stopped-flow spectroscopy between 380 and 650 nm. Upon mixing of a large excess of nitrite with $HbFe^{IV}=O$ at pH 7.0, the absorbance maximum of the Soret band shifted from 418 nm ($HbFe^{IV}=O$, spectrum 1 in Fig. 6) to 405 nm (metHb at pH 7.0, spectrum 6 in Fig. 6). In the visible region of the spectrum, the two absorbance maxima of $HbFe^{IV}=O$ at 544 and 574 nm (spectrum 1 in Fig. 7A) disappeared and two new bands with maxima at 500 and 625 nm appeared (spectrum 7 in Fig. 7A). These absorbance features are characteristic for metHb, despite the fact that the latter band is slightly shifted, as the maximum for pure metHb appears at 631 nm [47]. As mentioned above, the differences between the spectrum of pure metHb and that of the species generated by the reduction of $HbFe^{IV}=O$ with nitrite are probably due to modifications of the protein generated during the synthesis of $HbFe^{IV}=O$. Indeed, the two absorbance features characteristic for the hemichrome generated from the reaction of metHb with H_2O_2, a maximum at 532 nm and a shoulder 566 nm, were also visible in the spectrum of the product of the reaction between $HbFe^{IV}=O$ and nitrite (spectrum 7 in Fig. 7A).

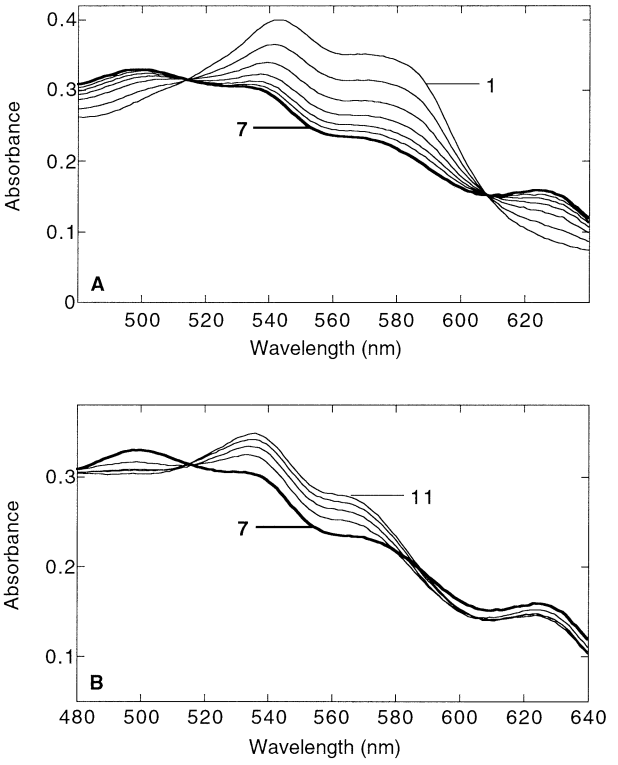

Fig. 7. Rapid-scan UV-vis spectra of the reaction of $HbFe^{IV}=O$ (38.2 μM) with nitrite (12.5 mM) in 0.1 M phosphate buffer at pH 7.0, 20°C. Traces 1–6, recorded 0, 48, 96, 144, 192, and 240 ms after mixing, and traces 7–11, recorded 0.34, 1.2, 2.0, 2.8, and 4.4 s after mixing, show (A) the formation of $HbFe^{III}OH_2$ (bold trace 7) from $HbFe^{IV}=O$ (spectra 1–6) and (B) its further reaction with nitrite to $HbFe^{III}NO_2$ (spectra 7–11).

Because of the instability of the $HbFe^{IV}=O$ solutions, large amounts of nitrite were used in most of our experiments to avoid significant decomposition of $HbFe^{IV}=O$ prior to its reaction with nitrite. Thus, on a longer time scale the subsequent binding of nitrite to metHb was also observed (Eqn. 4). This reaction induces a shift of the maximum of the Hb Soret band from 408 to 411 nm (dashed spectra 7–10 in Fig. 6) and is characterized by the appearance of an absorbance band at 538 nm with a shoulder at 567 nm (spectrum 11 in Fig. 7B).

$$HbFe^{IV}=O + NO_2^- \xrightarrow{k_3} metHb + 0.5\,NO_2^- + 0.5\,NO_3^- \quad (3)$$

$$metHb + NO_2^- \underset{k_{-4a},k_{-4b}}{\overset{k_{4a},k_{4b}}{\rightleftharpoons}} HbFe^{III}NO_2 \quad (4)$$

The second-order rate constants of the reaction between $HbFe^{IV}=O$ and nitrite were determined in the pH range 6.4–8.0 by single-wavelength stopped-flow spectroscopy under pseudo-first-order conditions with nitrite

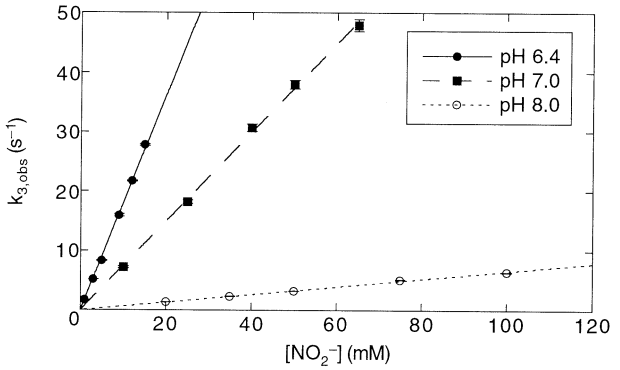

Fig. 8. Plots of $k_{3,obs}$ vs. NO_2^- concentration for the reaction of $HbFe^{IV}=O$ with NO_2^- at pH 6.4, 7.0, and 8.0 (20°C). The values of the second-order rate constants obtained from the linear fits are summarized in Table 3.

in at least 10-fold excess (at 20°C). The kinetic traces, measured by following the absorbance increases at 410 nm, could all be fitted well to a single-exponential expression. This result indicates that the two subunits of $HbFe^{IV}=O$ react at the same rate with nitrite. The second-order rate constants (k_3), obtained from the linear plots of the observed pseudo-first-order rate constants versus nitrite concentration (Fig. 8), are highly pH-dependent (Table 3). At pH 6.4 we obtained $k_3 = (1.8 \pm 0.2) \times 10^3$ $M^{-1}s^{-1}$. With increasing pH the second-order rate constant decreased continuously to $k_3 = (6.5 \pm 0.1) \times 10$ $M^{-1}s^{-1}$ at pH 8.0. These data can also be fitted to Eqn. 2, by assuming that the pH dependence originates from a single ionizable protein residue. The best fit gave values of p$K = 6.4 \pm 0.5$ and $k_{3'} = (3.4 \pm 0.2) \times 10^3$ $M^{-1}s^{-1}$. As for the reaction with Mb, the values obtained for the pK of the protonable residue suggest an important role for the distal histidine. Under acidic conditions when this residue is protonated [41], nitrite can diffuse more rapidly into the active site and react with the heme center of Hb. Alternatively, a mechanism involving the protonation of the oxoiron(IV)-center, analogous to that proposed for oxidation reactions by $MbFe^{IV}=O$, may also be valid for $HbFe^{IV}=O$. Indeed, it has been shown that efficient oxidation by $MbFe^{IV}=O$ requires its conversion to an activated protonated form [48,49]. The pK_a of $MbFe^{IV}=O$ has been estimated to be in the region of 6.0 [50] but no data are available for hemoglobin. Thus, it cannot be excluded that a consequence of the observed increase in the reaction rate at lower pH may arise from the protonation of the ferryl center.

The rate constant for the second step of the reaction, the subsequent binding of nitrite to metHb was measured by following the absorbance changes at 418 nm. The kinetic traces had to be fitted to a two-exponential expression. This result indicates that nitrite binds at a different rate to the two subunits of hemoglobin. The second-order rate constants (k_4) obtained from the linear plots of the observed pseudo-first-order rate constants vs. nitrite concentration (Fig. 9), are also highly pH-dependent and decrease with increasing pH (Table 3). As a control, the rate constant of the reaction between nitrite and metHb at pH 7.0 was also determined in an independent experiment from pure metHb under the same experimental conditions. The values obtained for the two association rate constants, $k_{4a} = (1.6 \pm 0.1) \times 10^2$ $M^{-1}s^{-1}$ and $k_{4b} = 22 \pm 1$ $M^{-1}s^{-1}$, are nearly identical to those determined from the second step of the nitrite-mediated reduction of $HbFe^{IV}=O$ (see Table 3) and are in good agreement with those reported previously ($k_{4a} = 1.4 \times 10^2$ $M^{-1}s^{-1}$ and $k_{4b} = 2.0 \times 10$ $M^{-1}s^{-1}$ at pH 6.85 and 20°C, [47]). The values of the rate constants for nitrite binding to the isolated α- and β-chains of metHb were determined to be $k_{4\alpha} = 15$ $M^{-1}s^{-1}$ and $k_{4\beta} = 80$ $M^{-1}s^{-1}$ (at pH 7.0 and 20°C [47]). Thus, the fast (k_{4a}) and the slow (k_{4b}) steps observed probably correspond to nitrite binding to the β- and α-chain, respectively. A recent work reports lower values for the rate constants for nitrite binding to bovine metHb (84.3 $M^{-1}s^{-1}$ and 7.86 $M^{-1}s^{-1}$ at pH 7.0 and 20°C) [52] but no explanation was given for this discrepancy. Interestingly, the largest value for nitrite binding to metHb is still almost

Table 3. pH-Dependencies of the Second-order Rate Constants for the Reactions of Nitrite with $HbFe^{IV}=O$ (k_3) at 20°C. pH-Dependencies of the Rates of Nitrite Binding to metHb (k_4) and of Nitrite Dissociation from $HbFe^{III}NO_2$ (k_{-4}) at 20°C, Together with the Calculated Equilibrium Constants (K_4)

pH	k_3 ($M^{-1}s^{-1}$)	k_{4a} ($M^{-1}s^{-1}$)	k_{4b} ($M^{-1}s^{-1}$)	k_{-4a} (s^{-1})	k_{-4b} (s^{-1})	K_{4a} (M)	K_{4b} (M)
6.4	$(1.8 \pm 0.2) \times 10^3$	$(3.8 \pm 0.3) \times 10^2$	$(5.4 \pm 0.2) \times 10$	0.6 ± 0.1	0.41 ± 0.01	1.6×10^{-3}	7.6×10^{-3}
6.8[a]		140	20				
7.0	$(7.5 \pm 0.1) \times 10^2$	$(1.4 \pm 0.1) \times 10^2$	$(2.3 \pm 0.1) \times 10$	0.27 ± 0.01	0.12 ± 0.02	1.9×10^{-3}	5.2×10^{-3}
7.4[b]						2.8×10^{-3}	
7.7[a]		16	2.5				
8.0	$(6.5 \pm 0.1) \times 10$	5.5 ± 0.4	2.2 ± 0.2	0.15 ± 0.02	0.04 ± 0.01	2.7×10^{-2}	1.8×10^{-2}

[a] Reference [47].
[b] At 25°C, reference [51].

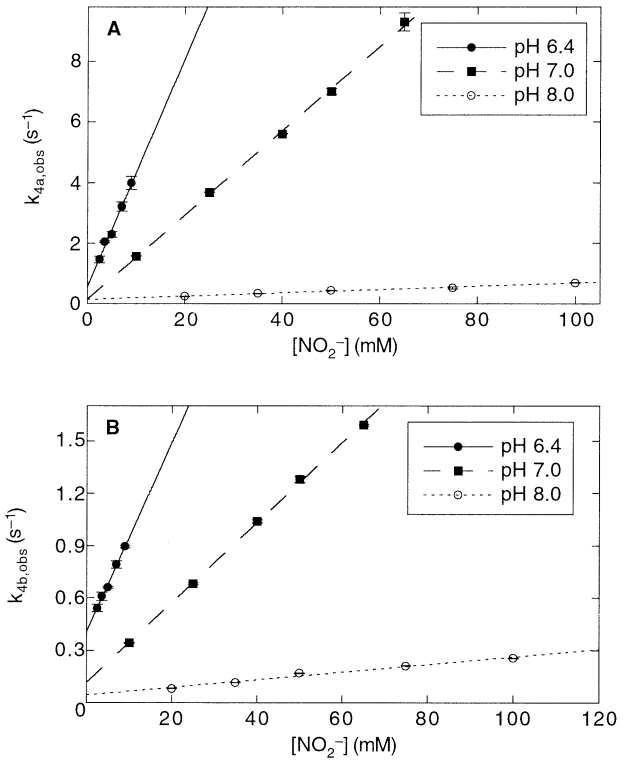

Fig. 9. Plots of $k_{4a,obs}$and $k_{4b,obs}$ vs. NO_2^- concentration for the second part of the reaction of $HbFe^{IV}$=O with NO_2^-, that is binding of NO_2^- to metHb, at pH 6.4, 7.0, and 8.0 (20°C). The values of the second-order rate constants obtained from the linear fits are given in Table 3.

one order of magnitude smaller than the corresponding value for metMb [20].

The rates of dissociation of nitrite from the two subunits of the *N*-nitrito-metHb complex, obtained from the extrapolation of the linear plots, are also pH-dependent and decrease with increasing pH (Table 3). Because of the few numbers of points in the linear plots and the intrinsic error of this mathematic procedure, large uncertainties are clearly associated with the values of the dissociation constants. However, from the association and the dissociation constants we could estimate the equilibrium constant for nitrite binding to metHb at different pH values (Table 3). At pH 7.0 we obtained $K_{4a} = 1.9 \times 10^{-3}$ M and $K_{4b} = 5.2 \times 10^{-3}$ M, respectively. These values are in good agreement with those reported previously (no distinction between the subunits, $K_4 = 2.8 \times 10^{-3}$ M [51]).

Analogously to the dissociation of nitrate from the peroxynitrito- and of nitrite from the *O*-nitrito-intermediate, the dissociation of nitrite from the *N*-nitrito complexes is significantly faster for myoglobin than for hemoglobin. These results confirm the hypothesis discussed above, that the rate of dissociation of nitrite and nitrate from the iron(III)-forms of these proteins is strongly influenced by the surrounding environment of the proteins. In general, hemoglobin seems to prevent dissociation of these ligands and leads to a larger stabilization of the intermediate complexes. Also for the dissociation reaction, two separate values are obtained for the two hemoglobin subunits, and these values are at least one order of magnitude smaller than the corresponding myoglobin values. As a result, at the different pH values studied, the nitrite dissociation equilibrium constants for myoglobin are approximately one order of magnitude larger than those for hemoglobin.

As $HbFe^{IV}$=O solutions are not indefinitely stable, it was not possible to determine the yield of the very slow reaction of nitrite with $HbFe^{IV}$=O. Indeed, the reaction of $HbFe^{IV}$=O with one equivalent of nitrite is slower than its corresponding autoreduction to metHb.

Analysis of the nitrogen-containing products

The amount of nitrite and nitrate ions formed from the reaction of $HbFe^{IV}$=O with one equivalent of $NO^\bullet$ was determined by anion chromatography with conductivity detection. As $HbFe^{IV}$=O is prepared by mixing about 10 equivalents of H_2O_2 with metHb, the excess of H_2O_2 had to be destroyed by addition of catalase before addition of $NO^\bullet$. Indeed, in the presence of hemoglobin, H_2O_2 slowly oxidizes nitrite to nitrate (see below). Under our experimental conditions, the amount of added catalase did not influence the relative concentration of nitrogen-containing products formed. At pH 7.0, reduction of $HbFe^{IV}$=O by $NO^\bullet$ generated nitrite almost quantitatively. In a typical experiment, addition of one equivalent of $NO^\bullet$ to a 44.6 μM $HbFe^{IV}$=O solution led to the formation of 43 $\pm$ 1 μM nitrite and 3 $\pm$ 1 μM nitrate.

To avoid decomposition of $HbFe^{IV}$=O in the course of its very slow reduction by nitrite, the identification of the nature of the nitrogen-containing products of this reaction had to be carried out by using a large excess of nitrite. At pH 7.0, we found that one equivalent of nitrite, relative to the amount of $HbFe^{IV}$=O, was transformed to half an equivalent of nitrite plus half an equivalent of nitrate (columns 6 and 7 in Table 4). In a series of control experiments, we established that formation of nitrate only takes place in the presence of both Hb and H_2O_2, thus in the presence of $HbFe^{IV}$=O.

DISCUSSION

The $NO^\bullet$-mediated reduction of $HbFe^{IV}$=O

Large amounts of nitrogen monoxide ($NO^\bullet$), produced by activated inducible nitric oxide synthase (iNOS), can have cytotoxic effects mainly because of its diffusion-controlled reaction with superoxide to generate the powerful oxidizing and nitrating agent peroxynitrite. It is now well established that a considerable portion of the

Table 4. Amount of Nitrite and Nitrate Formed From the Reaction of $HbFe^{IV}$=O With an Excess Nitrite

1	2	3	4	5	6	7
pH	[$HbFe^{IV}$=O] (μM)	added [NO_2^-] (μM)	ex. [NO_2^-][a] (μM)	found [NO_2^-][b] (μM)	[NO_2^-][c] (μM)	[NO_3^-][d] (μM)
7.0	50.2	217.7 (50.2 + 167.5)	167.5	190 (22.5 + 167.5)	22.5	24
7.0	44.6	301.7 (44.6 + 257.1)	257.1	281 (23.9 + 257.1)	23.9	24
7.0	44.1	397.8 (44.1 + 353.7)	353.7	379 (25.3 + 353.7)	25.3	24

[a] Concentration of nitrite added in excess, by considering that only one equivalent reacts with $HbFe^{IV}$=O.
[b] Total concentration of nitrite ions found in the protein solution after reaction.
[c] Concentration of nitrite ions generated from the reaction of $HbFe^{IV}$=O with one equivalent of nitrite, calculated from the total concentration of nitrite ions found in the protein solution after reaction (column 5) minus the concentration of the excess nitrite ions added (column 4).
[d] Concentration of nitrate ions in the protein solution after reaction with nitrite.

deleterious effects previously attributed to NO• or superoxide alone may in fact be modulated by peroxynitrite [13,53]. Paradoxically, NO• has been proposed to also have a protective role and, under certain conditions, to act as an antioxidant. Indeed, it has been shown that NO• can terminate a common pathological manifestation of oxidant overload to biological membranes, the deleterious process of lipid peroxidation [54], by reacting very rapidly with fatty alkoxyl (RO•) and peroxyl (ROO•) radical intermediates, which would otherwise propagate the membrane damage [55]. In addition, NO• may inactivate catalytically active high valent hemoprotein intermediates [24]. We have recently demonstrated that, as proposed by Gorbunov et al. [22], the reaction between $MbFe^{IV}$=O and NO• may represent a potential antioxidant function of NO•. Indeed, we have determined that the second-order rate constant for this reaction is very large, 1.8×10^7 $M^{-1}s^{-1}$, at pH 7.5 and 20°C. Thus, if large amounts of $MbFe^{IV}$=O are generated, for instance in reperfused—previously ischemic—tissues, its reaction with NO• may represent an important detoxification pathway.

The work described in this paper shows that, in analogy to the reaction between $MbFe^{IV}$=O and NO• [20], the NO•-mediated reduction of $HbFe^{IV}$=O proceeds via the rapid formation of the *O*-nitrito intermediate $HbFe^{III}ONO$, which then decays to metHb and nitrite. The strong similarity between the *N*-nitrito- and the *O*-nitrito-metHb UV-vis spectra may suggest that, after the fast radical/radical reaction between $HbFe^{III}-O^•$ and NO•, in a second very rapid step $HbFe^{III}ONO$ could rearrange to $HbFe^{III}NO_2$, which would then dissociate to nitrite and metHb. In this case, the detected intermediate would be the *N*-nitrito complex $HbFe^{III}NO_2$. Nevertheless, several considerations can be applied to argue against this second possibility. The isomerization of a *O*-nitrito-Co(III) complex ($[(NH_3)_5Co^{III}ONO]^{2+}$) to its *N*-nitrito-Co(III) isomer ($[(NH_3)_5Co^{III}NO_2]^{2+}$) has been reported to proceed intramolecularly at a rather slow rate (0.17 s^{-1} at 20°C) without dissociation of the ligand [56]. Despite the fact that Co(III) complexes are significantly more inert than the corresponding Fe(III) species, this rate implies that a similar concerted isomerization may be too slow to be relevant in our system, in particular for the myoglobin intermediate. In addition, we have recently shown that the spectra of $MbFe^{III}NO_2$, $MbFe^{III}ONO_2$, and $MbFe^{III}OONO$ are also very similar [35]. However, the rates of dissociation of nitrite and nitrate from these three complexes are significantly different [35] and consequently they can clearly be distinguished from each other. The dissociation rate of nitrite from $HbFe^{III}ONO$ (k_2) has been determined directly with very good accuracy (Table 1). In contrast, the values for the dissociation rate of nitrite from $HbFe^{III}NO_2$ (k_{-4}) have been determined indirectly from the intercept of the linear plot of the observed pseudo-first-order rate constant vs. nitrite concentration (Table 3) and may thus be inaccurate. Despite the fact that the nitrite dissociation rates from the two complexes are very similar, dissociation from $HbFe^{III}ONO$ (k_2) always proceeds slightly faster than dissociation from $HbFe^{III}NO_2$ (k_{-4}). As it is known that nitrite preferentially binds via the nitrogen atom to iron, these data support the different nature of the two species and the lower stability of the *O*-nitrito complexes. Taken together, our results strongly suggest that the intermediate observed during the reaction of $HbFe^{IV}$=O and NO• is indeed the *O*-nitrito complex $HbFe^{III}ONO$.

Several rate constants have recently been measured for the reaction of NO• with the ferryl form of a variety of different peroxidases (Compound II). These rates are $7.4–13 \times 10^5$ $M^{-1}s^{-1}$ for horseradish peroxidase (HRP, at pH 7.4 and 20°C [57]), 8×10^3 $M^{-1}s^{-1}$ for myeloperoxidase (MPO, at pH 7.0 and 25°C [58]), 1.7×10^4 $M^{-1}s^{-1}$ for eosinophil peroxidase (EPO, at pH 7.0 and 25°C [59]), and 8.7×10^4 $M^{-1}s^{-1}$ for lactoperoxidase (LPO, at pH 7.0 and 25°C [59]). In this contest, it has recently been reported that catalase can also rapidly scavenge NO• in the presence of hydrogen peroxide. To explain this observation, it has been proposed that cata-

lase Compound I ($^{\bullet}$cat-Fe^{IV}=O) reacts with NO$^{\bullet}$ to yield nitrite and the iron(III) form of the protein [60]. The rate constant for the reaction of NO$^{\bullet}$ with HbFe^{IV}=O is 1–2 orders of magnitude larger than those of its reaction with peroxidases-Compound II. This difference might be due to the strong hydrogen bond present in peroxidases between the proximal histidine and a conserved aspartate residue [61]. No intermediate has been reported to be detectable in the reaction of NO$^{\bullet}$ with HRP-, MPO-, EPO-, and LPO-Compound II. This result suggests that dissociation of nitrite from the iron(III) form of these peroxidases is significantly faster than from metMb and metHb. This difference can again arise from the partial negative charge on the proximal histidine of the peroxidases. Indeed, it can be expected that nitrite dissociates faster from the negatively charged peroxidase-[(His^{-})Fe^{III}ONO] than from the neutral Mb- or Hb-[(His)Fe^{III}ONO]. In fact, it has recently been reported that the rate constant for nitrite dissociation from the low-spin *N*-nitrito complex of MPO (MPO-Fe^{III}NO$_2$) is 60 s^{-1}, at pH 7.0 and 15°C [62], a value several orders of magnitude larger than those for the corresponding dissociation of nitrite from the two subunits of HbFe^{III}NO$_2$ (0.27 and 0.12 s^{-1}, at pH 7.0 and 20°C).

The nitrite-mediated reduction of HbFe^{IV}=O

Local nitrite concentrations in tissues are linked to the amounts of NO$^{\bullet}$ produced. Indeed, except for nitrate generated from the reaction of NO$^{\bullet}$ with HbFeO$_2$, nitrite is the major endproduct of NO$^{\bullet}$ metabolism. Nitrite concentrations have been shown to reach micromolar levels [27,28]. The reaction between high valent forms of peroxidases (Compound I and II) and nitrite has recently attracted the interest of a large number of research groups. It has been shown that these reactions can generate nitrogen dioxide and it has repeatedly been proposed that they may represent an important pathway for tyrosine nitration in vivo [29,30]. In particular, the MPO-catalyzed oxidation of nitrite is considered as the most significant alternative to peroxynitrite-mediated nitration of tyrosine under physiological conditions [31–34].

The rate constants for the reaction of nitrite with MPO-, LPO-, and HRP-Compound II have recently been measured. The values, which vary significantly between the three proteins, are 5.5×10^2 $M^{-1}s^{-1}$(MPO, at pH 7.0 and 15°C [62]), 3.5×10^4 $M^{-1}s^{-1}$ (LPO, at pH 7.0 and room temperature [63]), and 13.3 $M^{-1}s^{-1}$ (HRP, at pH 7.0 and room temperature [63]). A significantly larger rate constant has recently been reported for the reaction of LPO-Compound II and nitrite (3.5×10^5 $M^{-1}s^{-1}$, at pH 7.2 and 20°C [64]) but no explanation has been given for this discrepancy. In addition, nitrite has been shown to enhance the reactivity of LPO, MPO, and HRP towards compounds such as tyrosine [29,30]. This rate enhancement is thought to occur because tyrosine reacts more rapidly with $NO_2^{\bullet}$, generated from the oxidation of nitrite by LPO-Compounds I and II, than with LPO-Compound II [63].

In the present work, we measured the rate constant of the reaction of nitrite with HbFe^{IV}=O, $k_3 = 7.5 \times 10^2$ $M^{-1}s^{-1}$ at pH 7.0 and 20°C. This value is significantly larger than that for the corresponding reaction with myoglobin, 16 $M^{-1}s^{-1}$ at pH 7.5 and 20°C [20] but several orders of magnitude smaller than those of the reactions with peroxidases. Thus, as in vivo other biomolecules may be present at higher concentrations than nitrite and/or react at a faster rate with HbFe^{IV}=O, the reaction with nitrite may not have a physiological relevance. However, the pH dependence in the range 6.4–8.0 shows that, in analogy to the reactions of nitrite with MPO-Compound II [62] and with MbFe^{IV}=O [20], the rates of the reaction of HbFe^{IV}=O with nitrite increase with decreasing pH (Table 3). The faster reaction rate under acidic conditions ($k_3 = 1.8 \times 10^3$ $M^{-1}s^{-1}$ at pH 6.4 and 20°C) may have physiological consequences as the pH of ischemic tissues, characterized by elevated concentrations of H_2O_2 and NO$^{\bullet}$ and thus nitrite, has been shown to be lower than 6.5. Consequently, under these pathologic conditions the reaction between nitrite and HbFe^{IV}=O may obtain a physiological relevance. This conclusion is important as our analysis of the nitrogen-containing products suggest that, in analogy to the corresponding reactions with peroxidases Compound II [23,30,65] and with MbFe^{IV}=O [20], nitrogen dioxide is generated in the first step of the reaction between nitrite and HbFe^{IV}=O (Eqn. 5). Indeed, in the presence of HbFe^{IV}=O, nitrite is converted to half an equivalent of nitrate and half an equivalent of nitrite, produced from the subsequent hydrolysis of N_2O_4 (Eqns. 5 and 6).

$$HbFe^{IV}{=}O + NO_2^- \rightarrow HbFe^{III}OH_2 + NO_2^{\bullet} \quad (5)$$

$$2\,NO_2^{\bullet} \leftrightarrows N_2O_4 \text{ and } N_2O_4 + H_2O \rightarrow NO_2^- + NO_3^- + 2\,H^+ \quad (6)$$

$$HbFe^{IV}{=}O + NO_2^{\bullet} \rightarrow HbFe^{III}OH_2 + NO_3^- \quad (7)$$

Alternatively, the half equivalent of nitrate could be generated from the reaction between HbFe^{IV}=O and $NO_2^{\bullet}$ (Eqn. 7), which we have recently shown to proceed with a rate constant of ca. 10^7 $M^{-1}s^{-1}$ (at pH 9.5 and 20°C) (S. Herold, unpublished results). Generation of $NO_2^{\bullet}$ can have physiological consequences, as preliminary results show that, as described for myoglobin [20,66], in the presence of H_2O_2, the reaction of metHb with nitrite induces nitration of the tyrosine residues of Hb and of free tyrosine.

CONCLUSIONS

In summary, we have determined the rate constant for the reaction of $HbFe^{IV}=O$ with $NO^{\bullet}$ to be 2.4×10^7 $M^{-1}s^{-1}$ (at pH 7.0 and 20°C). This reaction proceeds at a rate comparable to that of the $NO^{\bullet}$-mediated reduction of $MbFe^{IV}=O$ [20] and of those of the reactions of $HbFeO_2$ and $MbFeO_2$ with $NO^{\bullet}$ [35]. Thus, in particular under pathological conditions when the concentration of $HbFe^{IV}=O$ can be significant, this oxidizing species may represent a possible sink for $NO^{\bullet}$. In addition, as the reaction of $HbFe^{IV}=O$ with $NO^{\bullet}$ generates species that are not oxidizing (metHb and nitrite), it may be important to remove the high valent form of hemoglobin, which has been proposed to be at least in part responsible for oxidative lesions. Other one-electron reductants present in vivo can reduce $HbFe^{IV}=O$, but most of these reagents are significantly less efficient [10].

The rate constant for the reaction of $HbFe^{IV}=O$ with nitrite is significantly lower (7.5×10^2 $M^{-1}s^{-1}$ at pH 7.0 and 20°C), but increases with decreasing pH (1.8×10^3 $M^{-1}s^{-1}$ at pH 6.4 and 20°C). Thus, under acidic conditions as found in ischemic tissues, this reaction may also have a physiological relevance. This conclusion is of great consequence as, in the first step of the reaction, $HbFe^{IV}=O$ oxidizes nitrite to $NO_2^{\bullet}$, a potent nitrating agent. Indeed, under pathophysiological conditions characterized by high levels of nitrite, reaction of hemoglobin with nitrite in the presence of hydrogen peroxide may enhance the levels of tyrosine nitration.

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REFERENCES

[1] Galaris, D.; Eddy, L.; Arduini, A.; Cadenas, E.; Hochstein, P. Mechanism of reoxygenation injury in myocardial infarction: implication of a myoglobin redox cycle. *Biochem. Biophys. Res. Commun.* **160:**1162–1168; 1989.

[2] Moore, K. P.; Holt, S. G.; Patel, R. P.; Svistunenko, D. A.; Zackert, W.; Goodier, D.; Reeder, B. J.; Clozel, M.; Anand, R.; Cooper, C. E.; Morrow, J. D.; Wilson, M. T.; Darley-Usmar, V.; Roberts, L. J. II. A causative role for redox cycling of myoglobin and its inhibition by alkalinization in the pathogenesis and treatment of rhabdomyolysis-induced renal failure. *J. Biol. Chem.* **273:**31731–31737; 1998.

[3] Shikama, K. The molecular mechanism of autoxidation for myoglobin and hemoglobin: a venerable puzzle. *Chem. Rev.* **98:**1357–1373; 1998.

[4] Giulivi, C.; Cadenas, E. Heme protein radicals: formation, fate and biological consequences. *Free Radic. Biol. Med.* **24:**269–279; 1998.

[5] Giulivi, C.; Davies, J. A. Hydrogen peroxide-mediated ferrylhemoglobin generation in vitro and in red blood cells. *Methods Enzymol.* **231:**490–496; 1994.

[6] Galaris, D.; Sevanian, A.; Cadenas, E.; Hochstein, P. Ferrylmyoglobin-catalyzed linoleic acid peroxidation. *Arch. Biochem. Biophys.* **281:**163–169; 1990.

[7] Harel, S.; Kanner, J. The generation of ferryl or hydroxyl radicals during interaction of hemeproteins with hydrogen peroxide. *Free Radic. Res. Commun.* **5:**21–33; 1988.

[8] Newman, E. S. R.; Rice-Evans, C. A.; Davies, M. J. Identification of initiating agents in myoglobin-induced lipid peroxidation. *Biochem. Biophys. Res. Commun.* **179:**1414–1419; 1991.

[9] Giulivi, C.; Cadenas, E. Ferrylmyoglobin: formation and chemical reactivity toward electron-donating compounds. *Methods Enzymol.* **233:**189–202; 1994.

[10] Everse, J.; Hsia, N. The toxicities of native and modified hemoglobins. *Free Radic. Biol. Med.* **22:**1075–1099; 1997.

[11] George, P.; Irvine, D. H. The reaction between metmyoglobin and hydrogen peroxide. *Biochem. J.* **52:**511–517; 1952.

[12] Exner, M.; Herold, S. Kinetic and mechanistic studies of the peroxynitrite-mediated oxidation of oxymyoglobin and oxyhemoglobin. *Chem. Res. Toxicol.* **13:**287–293; 2000.

[13] Beckman, J. S.; Koppenol, W. H. Nitric oxide, superoxide, and peroxynitrite: the good the bad, and the ugly. *Am. J. Physiol.* **271:**C1424—C1437; 1996.

[14] Chance, B.; Sies, H.; Boveris, A. Hydroperoxide metabolism in mammalian organs. *Physiol. Rev.* **59:**527–605; 1979.

[15] Maples, K. R.; Kennedy, C. H.; Jordan, S. J.; Mason, R. P. In vivo thiyl free radical formation from hemoglobin following administration of hydroperoxides. *Arch. Biochem. Biophys.* **277:**402–409; 1990.

[16] Svistunenko, D. A.; Patel, R. P.; Wilson, M. T. An EPR investigation of human methemoglobin. Oxdation by hydrogen peroxide: methods to quantify all paramagnetic species observed in the reaction. *Free Radic. Res.* **24:**269–280; 1996.

[17] McArthur, K. M.; Davies, M. J. Detection and reactions of the globin radical in hemoglobin. *Biochim. Biophys. Acta* **1202:**173–181; 1993.

[18] Svistunenko, D. A.; Patel, R. P.; Voloshchenko, S. V.; Wilson, M. T. The globin-based free radical of ferryl hemoglobin is detected in normal human blood. *J. Biol. Chem.* **272:**7114–7121; 1997.

[19] Giulivi, C.; Hochstein, P.; Davies, K. J. A. Hydrogen peroxide production by red blood cells. *Free Radic. Biol. Med.* **16:**123–129; 1994.

[20] Herold, S.; Rehmann, F.-J. K. Kinetic and mechanistic studies of the reactions of nitrogen monoxide and nitrite with ferryl myoglobin. *J. Biol. Inorg. Chem.* **6:**543–555; 2001.

[21] Dee, G.; Rice-Evans, C.; Obeyesekera, S.; Meraji, S.; Jacobs, M.; Bruckdorfer, K. R. The modulation of ferryl myoglobin formation and its oxidative effects on low density lipoproteins by nitric oxide. *FEBS Lett.* **294:**38–42; 1991.

[22] Gorbunov, N. V.; Osipov, A. N.; Day, B. W.; Zayas-Rivera, B.; Kagan, V. E.; Elsayed, N. M. Reduction of ferrylmyoglobin and ferrylhemoglobin by nitric oxide: a protective mechanism against ferryl hemoprotein-induced oxidations. *Biochemistry* **34:**6689–6699; 1995.

[23] Wink, D. A.; Cook, J. A.; Krishna, M. C.; Hanbauer, I.; DeGraff, W.; Gamson, J.; Mitchell, J. B. Nitric oxide protects against alkyl peroxide-mediated cytotoxicity: further insights into the role nitric oxide plays in oxidative stress. *Arch. Biochem. Biophys.* **319:**402–407; 1995.

[24] Jourd'heuil, D.; Mills, L.; Miles, A. M.; Grisham, M. B. Effect of nitric oxide on hemoprotein-catalyzed oxidative reactions. *Nitric Oxide* **2:**37–44; 1998.

[25] Gorbunov, N. V.; Tyurina, Y. Y.; Salama, G.; Day, B. W.; Claycamp, H. G.; Argyros, G.; Elsayed, N. M.; Kagan, V. E. Nitric oxide protects cardiomyocytes against tert-butyl hydroperoxide-induced formation of alkoxyl and peroxyl radicals and peroxidation of phosphatidylserine. *Biochem. Biophys. Res. Commun.* **244:**647–751; 1998.

[26] Grisham, M. B.; Granger, D. N.; Lefer, D. J. Modulation of leukocyte-endothelial interactions by reactive metabolites of oxygen and nitrogen: relevance to ischemic heart disease. *Free Radic. Biol. Med.* **25:**404–433; 1998.

[27] Kaur, H.; Halliwell, B. Evidence for nitric oxide-mediated oxidative damage in chronic inflammation. Nitrotyrosine in serum

and synovial fluid from rheumatoid patients. *FEBS Lett.* **350:**9–12; 1994.
[28] Torre, D.; Ferrario, G.; Speranza, F.; Orani, A.; Fiori, G. P.; Zeroli, C. Serum concentrations of nitrite in patients with HIV-1 infection. *J. Clin. Pathol.* **49:**574–576; 1996.
[29] van der Vliet, A.; Eiserich, J. P.; Halliwell, B.; Cross, C. E. Formation of reactive nitrogen species during peroxidase-catalyzed oxidation of nitrite. *J. Biol. Chem.* **272:**7617–7625; 1997.
[30] Klebanoff, S. J. Reactive nitrogen intermediates and antimicrobial activity: role of nitrite. *Free Radic. Biol. Med.* **14:**351–360; 1993.
[31] Podrez, E. A.; Abu-Soud, H. M.; Hazen, S. L. Myeloperoxidase-generated oxidants and atherosclerosis. *Free Radic. Biol. Med.* **28:**1717–1725; 2000.
[32] Pfeiffer, S.; Lass, A.; Schmidt, K.; Mayer, B. Protein tyrosine nitration in cytokine-activated murine macrophages. Involvement of a peroxidase/nitrite pathway rather than peroxynitrite. *J. Biol. Chem.* **276:**34051–34058; 2001.
[33] Gaut, J. P.; Byun, J.; Tran, H. D.; Lauber, W. M.; Carroll, J. A.; Hotchkiss, R. S.; Belaaouaj, A.; Heinecke, J. W. Myeloperoxidase produces nitrating oxidants in vivo. *J. Clin. Invest.* **109:**1311–1319; 2002.
[34] Brennan, M.-L.; Wu, W.; Fu, X.; Shen, Z.; Song, W.; Frost, H.; Vadseth, C.; Narine, L.; Lenkiewicz, E.; Borchers, M. T.; Lusis, A. J.; Lee, J. J.; Lee, N. A.; Abu-Soud, H. M.; Ischiropoulos, H.; Hazen, S. L. A tale of two controversies. Defining both the role of peroxidases in nitrotyrosine formation in vivo using eosinophil peroxidase and myeloperoxidase-deficient mice, and the nature of peroxidase-generated reactive nitrogen species. *J. Biol. Chem.* **277:**17415–17427; 2002.
[35] Herold, S.; Exner, M.; Nauser, T. Kinetic and mechanistic studies of the $NO^{\bullet}$-mediated oxidation of oxymyoglobin and oxyhemoglobin. *Biochemistry* **40:**3385–3395; 2001.
[36] Østdal, H.; Daneshvar, B.; Skibsted, L. H. Reduction of ferrylmyoglobin by beta-Lactoglobulin. *Free Radic. Res.* **24:**429–438; 1996.
[37] Herold, S.; Shivashankar, K.; Mehl, M. Myoglobin scavenges peroxynitrite without being significantly nitrated. *Biochemistry* **41:**13460–13472; 2002.
[38] Kelder, P. P.; Fischer, M. J. E.; de Mol, N. J.; Janssen, L. H. M. Oxidation of chloropromazine by methemoglobin in the presence of hydrogen peroxide. Formation of chlorpromazine radical cation and its covalent binding to methemoglobin. *Arch. Biochem. Biophys.* **284:**313–319; 1991.
[39] Herold, S. Mechanistic studies of the oxidation of pyridoxalated hemoglobin polyoxyethylene conjugate (PHP) by nitrogen monoxide. *Arch. Biochem. Biophys.* **372:**393–398; 1999.
[40] Giulivi, C.; Davies, J. A. In: Everse, J.; Vandegriff, K. D.; Winslow, R. M., eds. *Methods in enzymology*, Vol. 231. San Diego, CA: Academic Press; 1994:490–496.
[41] Miller, L. M.; Patel, M.; Chance, M. R. Identification of conformational substrates in oxymyoglobin through the pH-dependence of the low-temperature photoproduct yield. *J. Am. Chem. Soc.* **118:**4511–4517; 1996.
[42] Herold, S. Kinetic and spectroscopic characterization of an intermediate peroxynitrite complex in the nitrogen monoxide induced oxidation of oxyhemoglobin. *FEBS Lett.* **443:**81–84; 1999.
[43] Addison, A. W.; Stephanos, J. J. Nitrosyliron(III) hemoglobin: autoreduction and spectroscopy. *Biochemistry* **25:**4104–4113; 1986.
[44] Tomoda, A.; Sugimoto, K.; Suhara, M.; Takeshita, M.; Yoneyama, Y. Haemichrome formation from haemoglobin subunits by hydrogen peroxide. *Biochem. J.* **171:**329–335; 1978.
[45] Rachmiliwitz, E. A.; Preisach, J.; Blumberg, W. E. Studies on the stability of oxyhemoglobin A and its constituent chains and their derivatives. *J. Biol. Chem.* **246:**3356–3366; 1971.
[46] Tajima, G.-I.; Shikama, K. Decomposition of hydrogen peroxide by metmyoglobin: a cyclic formation of the ferryl intermediate. *Int. J. Biochem.* **25:**101–105; 1993.
[47] Antonini, E.; Brunori, M. *Hemoglobin and myoglobin in their reactions with ligands*. Amsterdam: North-Holland; 1971.
[48] Fenwick, C. W.; English, A. M.; Wishart, J. F. pH and driving force dependence of intramolecular oxyferryl heme reduction in myoglobin. *J. Am. Chem. Soc.* **119:**4758–4764; 1997.
[49] Reeder, B. J.; Wilson, M. T. The effects of pH on the mechanism of hydrogen peroxide and lipid hydroperoxide consumption by myoglobin: a role for the protonated ferryl species. *Free Radic. Biol. Med.* **30:**1311–1318; 2001.
[50] Foote, N.; Gadsby, P. M. A.; Greenwood, C.; Thomson, A. J. pH-dependent forms of the ferryl haem in myoglobin peroxide analysed by variable-temperature magnetic circular dichroism. *Biochem. J.* **261:**515–522; 1989.
[51] Smith, R. P. The nitrite methemoglobin complex. Its significance in methemoglobin analyses and its possible role in methemoglobinemia. *Biochem. Pharmacol.* **16:**1655–1664; 1967.
[52] Wanat, A.; Gdula-Argasinska, J.; Rutkowska-Zbik, D.; Witko, M.; Stochel, G.; van Eldik, R. Nitrite binding to metmyoglobin and methemoglobin in comparison to nitric oxide binding. *J. Biol. Inorg. Chem.* **7:**165–176; 2002.
[53] Moncada, S.; Palmer, R. M. J.; Higgs, E. A. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* **43:**109–142; 1991.
[54] Rubbo, H.; Radi, R.; Anselmi, D.; Kirk, M.; Barnes, S.; Butler, J.; Eiserich, J. P.; Freeman, B. A. Nitric oxide reaction with lipid peroxyl radicals spares alpha-tocopherol during lipid peroxidation. Greater oxidant protection from the pair nitric oxide/alpha-tocopherol than alpha-tocopherol/ascorbate. *J. Biol. Chem.* **275:**10812–10818; 2000.
[55] Hogg, N.; Kalyanaraman, B. Nitric oxide and low-density lipoprotein oxidation. *Free Radic. Res.* **28:**593–600; 1998.
[56] Basolo, F.; Hammaker, G. S. Synthesis and isomerization of nitropentammine complexes of rhodium(III), iridium(III), and platinum(III). *Inorg. Chem.* **1:**1–5; 1962.
[57] Glover, R. E.; Koshkin, V.; Dunford, H. B.; Mason, R. P. The reaction rates of NO with horseradish peroxidase compounds I and II. *Nitric Oxide* **3:**439–444; 1999.
[58] Abu-Soud, H. M.; Hazen, S. L. Nitric oxide is a physiological substrate for mammalian peroxidases. *J. Biol. Chem.* **275:**37524–37532; 2000.
[59] Abu-Soud, H. M.; Khassawneh, M. Y.; Sohn, J.-T.; Murray, P.; Haxhiu, M. A.; Hazen, S. L. Peroxidases inhibit nitric oxide (NO) dependent bronchodilation: development of a model describing NO-peroxidase interactions. *Biochemistry* **40:**11866–11875; 2001.
[60] Brunelli, L.; Yermilov, V.; Beckman, J. S. Modulation of catalase peroxidatic and catalytic activity by nitric oxide. *Free Radic. Biol. Med.* **30:**709–714; 2001.
[61] Poulos, T. L. The role of the proximal ligand in heme enzymes. *J. Biol. Inorg. Chem.* **1:**356–350; 1996.
[62] Burner, U.; Furtmüller, P. G.; Kettle, A. J.; Koppenol, W. H.; Obinger, C. Mechanism of reaction of myeloperoxidase with nitrite. *J. Biol. Chem.* **275:**20597–20601; 2000.
[63] Gebicka, L. Kinetic studies on the oxidation of nitrite by horseradish peroxidase and lactoperoxidase. *Acta Biochim. Pol.* **46:**919–927; 1999.
[64] Brück, T. B.; Fielding, R. J.; Symons, M. C. R.; Harvey, P. J. Mechanism of nitrite-stimulated catalysis by lactoperoxidase. *Eur. J. Biochem.* **268:**3214–3222; 2001.
[65] Goss, S. P. A.; Singh, R. J.; Hogg, N.; Kalyanaraman, B. Reactions of NO, NO_2 and peroxynitrite in membranes: physiological implications. *Free Radic. Res.* **31:**597–606; 1999.
[66] Kilinc, K.; Kilinc, A.; Wolf, R. E.; Grisham, M. B. Myoglobin-catalyzed tyrosine nitration: no need for peroxynitrite. *Biochem. Biophys. Res. Commun.* **285:**273–276; 2001.

ABBREVIATIONS

cat—catalase
Compound I—(porphyrin$^{\bullet+}$)oxoiron(IV)
Compound I—(porphyrin)oxoiron(IV)
EPO—eosinophil peroxidase

Hb—hemoglobin
$HbFeO_2$—oxyhemoglobin
$HbFe^{IV}=O$—ferryl, oxoiron(IV)-hemoglobin
$^{\bullet}HbFe^{IV}=O$—ferryl hemoglobin with a transient protein radical
HRP—horseradish peroxidase
LPO—lactoperoxidase
Mb—myoglobin
metHb—iron(III)hemoglobin
metMb—iron(III)hemoglobin
MPO—myeloperoxidase
sh—shoulder