Kinetic and spectroscopic characterization of an intermediate peroxynitrite complex in the nitrogen monoxide induced oxidation of oxyhemoglobin[1]

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Abstract Stopped-flow spectroscopy studies of the nitrogen monoxide mediated oxidation of oxyhemoglobin in the pH range 5–10.5 show that an intermediate can be characterized at alkaline pH. The rate of decay of this species to methemoglobin increases significantly with decreasing pH such that it does not accumulate in quantities large enough to be observed under neutral or acidic conditions. Kinetic and spectroscopic properties of this intermediate support its assignment as a methemoglobin peroxynitrite complex.

Key words: Hemoglobin; Nitrogen monoxide; Peroxynitrite; Rate constant

1. Introduction

Nitrogen monoxide plays an essential role in many biological systems and contributes to a variety of physiological and pathophysiological processes [1,2]. Among others, it controls neurotransmission, platelet aggregation, smooth muscle relaxation, and blood pressure. Overproduction of nitrogen monoxide has been implicated to be responsible for pathological situations such as inflammation, diabetes, stroke, neurodegeneration, and sepsis [3]. Nitrogen monoxide reacts at nearly diffusion controlled rate with superoxide to yield peroxynitrite[2] [4], a strong oxidant which can initiate lipid peroxidation [5], oxidize thiols [6], as well as hydroxylate and nitrate aromatic compounds [7,8]. A large amount of nitrogen monoxide rapidly oxidizes oxyhemoglobin to its iron(III) form, methemoglobin, and nitrate [9,10] in a reaction which is considered to be the major route for NO• depletion in vivo [3] and the cause for an increase in blood pressure observed when extracellular hemoglobin-based blood substitutes are administered [10,11]. Despite the physiological importance of the NO• -induced oxidation of oxyhemoglobin, its mechanism is not well understood. As the dioxygen coordinated to the heme in oxyhemoglobin has a superoxide-like character [12,13], an analogy has often been drawn to the reaction of free superoxide with nitrogen monoxide. It has thus been postulated that this reaction might proceed via peroxynitrite, free or coordinated to methemoglobin, which then isomerizes to nitrate [3,9,10,14].

To get a better understanding of the mechanism, we have studied by stopped-flow spectroscopy the kinetics of the NO•-mediated oxidation of oxyhemoglobin in the pH range 5–10.5. We report that an intermediate can be observed when the reaction is carried out at alkaline pH, but its very rapid decay does not allow for its detection under neutral or acidic conditions. The spectroscopic and kinetic data presented suggest that the intermediate species observed is a hemoglobin iron(III)-peroxynitrite complex.

2. Materials and methods

2.1. Reagents

Pure human oxyhemoglobin stock solution HbA_0 (57 mg/ml solution with approximately 1.1% methemoglobin) was a kind gift from APEX Bioscience (NC, USA). Buffer solutions (0.1 M) were prepared from K_2HPO_4/KH_2PO_4 (pH 5–9) and from $Na_2B_4O_7{\cdot}10H_2O$/NaOH (pH 9.5–10.5) (Fluka) with deionized Milli-Q water. Nitrogen monoxide was obtained from Linde and passed through an NaOH solution as well as a column of NaOH pellets to remove higher nitrogen oxides before use.

2.2. Nitrogen monoxide and oxyhemoglobin solutions

Nitrogen monoxide solutions were prepared by degassing the buffer solutions for 30 min with N_2 and then saturating them with NO•. The obtained stock solutions (ca. 2 mM) were diluted with degassed buffer in gastight SampleLock Hamilton syringes. The final nitrogen monoxide concentrations were measured with an ANTEK Instruments nitrogen monoxide analyzer, with a chemiluminescent detector. Oxyhemoglobin solutions were prepared by diluting the stock solution with buffer and concentrations were determined by measuring the absorbance at 415, 541 and/or 577 nm ($\varepsilon_{415} = 125$ mM^{-1} cm^{-1}, $\varepsilon_{541} = 13.8$ mM^{-1} cm^{-1} and $\varepsilon_{577} = 14.6$ mM^{-1} cm^{-1}) [15]. Absorption spectra were collected on a UVIKON 820 spectrophotometer.

2.3. Stopped-flow kinetic analysis

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

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Abbreviations: HbO_2, oxyhemoglobin; metHb, iron(III)hemoglobin

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[2] The recommended IUPAC nomenclature for peroxynitrite is oxoperoxonitrate(1-); for peroxynitrous acid, hydrogen oxoperoxonitrate. The term peroxynitrite is used in the text to refer generically to both oxoperoxonitrate(1-) ($ONOO^-$) and its conjugate acid, hydrogen oxoperoxonitrate (ONOOH).

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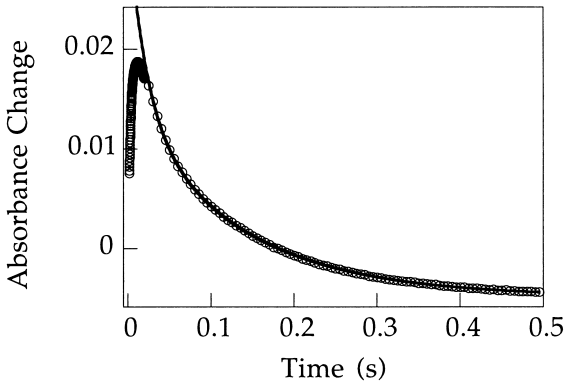

Fig. 1. Time course measured at 406 nm for the reaction of 2.3 μM HbO_2 with ca. 0.2 μM NO• in 0.1 M borate buffer at pH 9.5, 20°C. The solid line corresponds to the best fit for the decay of the hemoglobin peroxynitrite complex resulting in the rate constants $k_1 = 37 \pm 1$ s^{-1} and $k_2 = 7.5 \pm 0.1$ s^{-1}, with the corresponding amplitudes $A_1 = 0.020 \pm 0.001$ and $A_2 = 0.019 \pm 0.001$.

3. Results

3.1. Stopped-flow kinetic studies

Single-wavelength stopped-flow spectroscopy studies of the nitrogen monoxide induced oxidation of oxyhemoglobin to methemoglobin were carried out under pseudo-first order conditions, with HbO_2 in excess, in the pH range 5–10.5 at 20°C. Between pH 5 and 7 the kinetic traces measured at 406 nm could be fitted well to a single exponential expression indicating that the α- and β-subunits of hemoglobin reacted at the same rate. An averaged second order rate constant of 8.5×10^7 M^{-1} s^{-1} (per heme) was obtained, a value slightly larger than what had been previously reported at pH 7.4 [9,10]. At pH ≥ 7.5 the kinetic traces measured at 406 nm showed that an intermediate was rapidly formed and then decayed to methemoglobin (Fig. 1). The first step of the reaction could still be fitted well to a single exponential expression. With increasing pH the second order rate constants increased continuously up to 1.1×10^8 M^{-1} s^{-1} at pH 9.5.

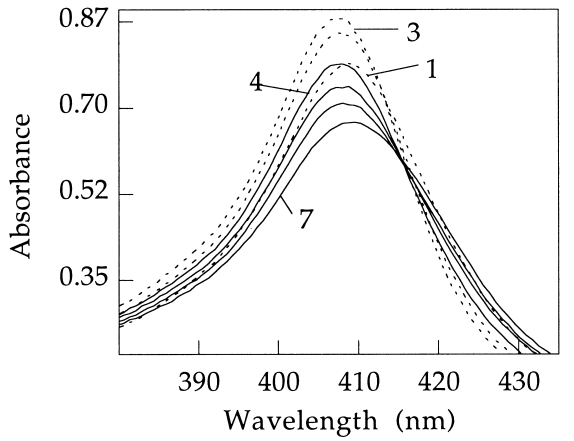

Fig. 2. Rapid-scan UV-VIS spectra of the reaction of 3 μM HbO_2 with ca. 4 μM NO• in 0.1 M borate buffer at pH 9.5, 5°C. Spectra were collected every ms, but to improve the signal to noise ratio, each curve shown represents the average of two measured curves. The formation of the intermediate from HbO_2, dashed traces 1–3, and its decay to metHb, traces 4–7, are presented. Time intervals of the shown spectra are: traces 1–3, every 4 ms; traces 4–6, every 80 ms; trace 7, 240 ms later, for a total of 646 ms.

Table 1
pH dependence of the observed rate constants (s^{-1}) for the decay of the intermediate $HbFe^{III}(OONO)$ complex and peroxynitrite at 20°C

pH	$HbFe^{III}(OONO)$		Peroxynitrite[c]
	Fast	Slow	
7.5[a]	58 ± 5	33 ± 1	0.167
8.1[a]	48 ± 3	16 ± 1	0.048
8.3[a]	42 ± 1	9.7 ± 0.2	0.031
8.9[a]	41 ± 1	8.1 ± 0.1	0.008
9.5[b]	36 ± 5	6.9 ± 0.1	0.110
10.5[b]	17 ± 1	0.19 ± 0.02	0.012

[a]0.1 M phosphate buffer.
[b]0.1 M borate buffer.
[c][4].

The kinetics of the decay of the intermediate were studied within a broad range of nitrogen monoxide (from 0.1 to 50 μM) and oxyhemoglobin (from 1 to 20 μM) concentrations and in the pH range between 7.5 and 10.5. Identical normalized traces were obtained at several wavelengths between 300 and 650 nm. The time courses could be fitted to a two-exponential expression with significantly different rate constants (Fig. 1). As the resulting amplitudes were always almost identical, and as only one process was observed in the case of myoglobin (data not shown), we assigned the two different rates to the reaction of the α- and β-subunits of hemoglobin. The two rates of decay were independent from the NO• as well as the HbO_2 concentrations, and the same kinetic data were obtained when either of the two reagents was used in excess. When nitrogen monoxide was used in large excess an additional reaction was observed on a longer time scale which corresponded to NO• binding the formed methemoglobin. The rates of decay of the intermediate were highly pH-dependent and increased significantly with decreasing pH (Table 1). At pH lower than 7.5 the intermediate decayed so fast that it did not accumulate in quantities large enough to be observed.

To determine whether free peroxynitrite was formed in the course of the NO•-induced oxidation of HbO_2 the reaction was followed by stopped-flow spectroscopy at 302 nm, the

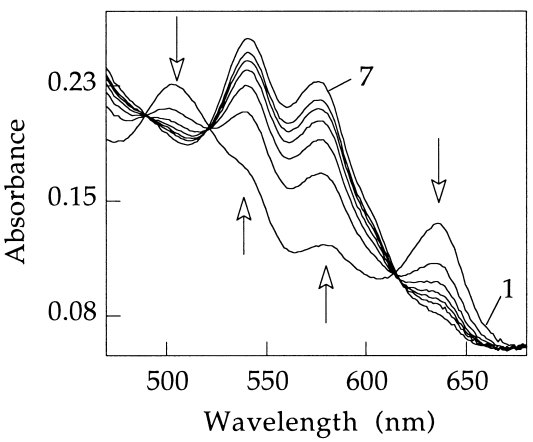

Fig. 3. Rapid-scan UV-VIS spectra of the reaction of 13.5 μM HbO_2 with ca. 50 μM NO• in 0.1 M borate buffer at pH 9.5, 5°C. Averaged spectra were collected (62 scans/s). The decay of the intermediate to metHb is presented. Time intervals of the shown spectra are: traces 1–6, every 160 ms; trace 7, 480 ms later for a total of 1.28 s.

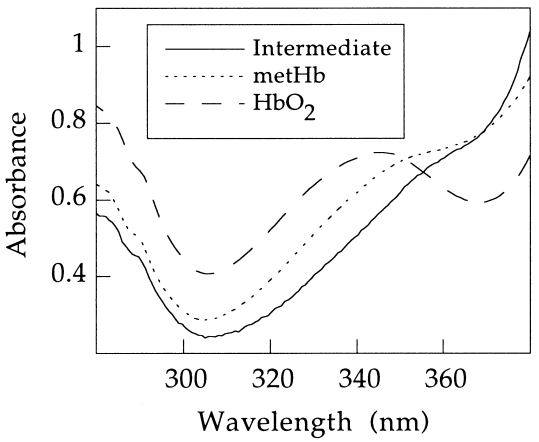

Fig. 4. Spectral changes in the range between 280 and 380 nm during the NO•-mediated oxidation of HbO_2. The spectrum of the intermediate was obtained from a reaction of 13 μM HbO_2 with ca. 30 μM NO• in 0.1 M borate buffer at pH 9.5, 5°C.

wavelength at which the peroxynitrite anion has an absorbance maximum ($\varepsilon_{302} = 1705 \pm 10$ M^{-1} cm^{-1}) [16]. Upon mixing of 11 μM HbO_2 and about 9 μM NO• at pH 8.1 and 20°C a rapid decrease and a slower increase in absorbance were observed in the first 300 ms of the reaction, reflecting the formation of the intermediate and its decay to metHb. No further changes were observed on a longer time scale up to 20 s. The half-life of peroxynitrite under these conditions is about 14 s and the decomposition of 9 μM $ONOO^-$ would have given rise to a decrease of about 0.015 absorbance units. It could thus be excluded that free peroxynitrite was formed.

3.2. Stopped-flow spectral characterization of the intermediate

The NO•-mediated oxidation of oxy- to methemoglobin was studied by rapid-scan UV-VIS spectroscopy between 280 and 680 nm at pH 9.5 and 5°C to obtain a spectrum of the intermediate over the entire wavelength range. As shown in Fig. 2, upon mixing of HbO_2 and NO• the Soret band shifted from 415 nm (HbO_2) to 411 nm (metHb at pH 9.5) via an intermediate species with an absorption maximum at 407 nm and an extinction coefficient of about 165 mM^{-1} cm^{-1}.

The spectrum of the intermediate in the visible region, shown as the first trace in Fig. 3, has two characteristic absorption maxima at 636 nm ($\varepsilon_{636} = 5.4$ mM^{-1} cm^{-1}) and 504 nm ($\varepsilon_{504} = 8.7$ mM^{-1} cm^{-1}). The weak band at 577 nm and the shoulder at 543 nm could derive partly from residual oxyhemoglobin or already formed methemoglobin. As higher concentrations were used in order to reduce the signal to noise ratio, accumulation of the intermediate occurred within the dead time of the instrument.

Fig. 4 shows a spectrum of the intermediate complex in the range between 280 and 380 nm. Below 350 nm its absorbance is always weaker than that of both oxy- and methemoglobin. The minimum is at 305 nm ($\varepsilon_{305} = 9.4$ mM^{-1} cm^{-1}).

Table 2
Absorption maxima (nm) and extinction coefficients (mM^{-1} cm^{-1}), in parentheses, of methemoglobin derivatives [15]

Ligand	Soret	Visible	
H_2O (pH 6.4)	405 (179)	500 (10.0)	631 (4.4)
F^-	403 (144)	483 (10.3)	605 (10.9)
CH_3COO^-	404 (178)	497 (10.5)	620 (5.5)
$HCOO^-$	404 (178)	496 (9.2)	620 (5.8)
$ONOO^-$	407 (165)	504 (8.7)	636 (5.4)

4. Discussion

In the present study we show that, as has often been proposed [3,9,10], an intermediate is indeed formed in the reaction between nitrogen monoxide and oxyhemoglobin. The dioxygen complex in oxyhemoglobin is best described as an intermediate form between an iron(II)-dioxygen and an iron(III)-superoxide complex [12,13] and free superoxide can be generated during the spontaneous autoxidation of oxy- to methemoglobin [17]. Consequently, in analogy to the reaction between nitrogen monoxide and superoxide, which yields peroxynitrite at an almost diffusion controlled rate [4], it is conceivable that the NO•-mediated oxidation of oxyhemoglobin proceeds via peroxynitrite, either free or coordinated to the iron(III). The data presented in this paper support the hypothesis that nitrogen monoxide rapidly adds to oxyhemoglobin to yield an iron(III)-peroxynitrite complex.

Stopped-flow kinetic studies over the pH range from 5 to 10.5 showed that the rate of oxidation of HbO_2 by NO• increases with increasing pH. At alkaline pH the reaction proceeds via a detectable intermediate species which decays with a rate independent from both the NO• and the HbO_2 concentrations. This indicates that it is a unimolecular process that corresponds to the isomerization of the hemoglobin iron(III)-peroxynitrite complex to methemoglobin and nitrate.

The UV-VIS spectrum of the identified intermediate displays very characteristic absorption maxima at 636 and 504 nm (Fig. 3). A comparison with the spectra of other methemoglobin derivatives with anionic ligands such as F^-, $HCOO^-$ or CH_3COO^- [15] supports the assignment of this species as an iron(III) peroxynitrite anion complex (Table 2). All of these derivatives give rise to similar spectra, with absorption maxima around 610 and 500 nm. The position and the intensity of the Soret band (Fig. 2) are also compatible with the listed derivatives. Another species which displays a similar spectrum is the acidic form of methemoglobin ($pH \leq 6.4$), with a water molecule bound to the heme (Table 2). Nevertheless, it can be excluded that the observed decay reaction corresponds to a deprotonation of a coordinated water molecule because this process is expected to occur at a very fast rate [18,19] and to exhibit a pH dependence opposite to what was found (Table 1).

In the wavelength range between 280 and 350 nm the intermediate hemoglobin complex displays an absorbance spectrum very similar to that of methemoglobin but with lower intensity (Fig. 4). The peroxynitrite anion has a weak absorbance maximum at 302 nm, $\varepsilon_{302} = 1705 \pm 10$ M^{-1} cm^{-1} [16], whereas hemoglobin has relatively strong background absorbance in this wavelength range, for instance $\varepsilon_{302}(HbO_2) = \sim 15$ mM^{-1} cm^{-1}. Consequently, the spectrum of the intermediate is not indicative of whether free peroxynitrite is formed or not during the reaction as its contribution to the absorbance would not be significant. Nevertheless, single-wavelength stopped-flow studies at 302 nm showed that free peroxynitrite was not produced during the NO•-mediated oxidation of oxyhemoglobin as no absorbance changes were observed after the first 300 ms of the reaction, that is after the intermediate hemoglobin iron(III)-peroxynitrite complex had rapidly

formed and decayed. Had free peroxynitrite been formed, a significant decrease would have been expected. This observation, as well as the significantly larger rate of oxidation of oxyhemoglobin by nitrogen monoxide compared to that of its autoxidation [17], suggests that nitrogen monoxide directly attacks the coordinated dioxygen to give a peroxynitrite complex which then rearranges to nitrate without prior dissociation. In analogy to peroxynitrous acid, which has much weaker absorbance bands than $ONOO^-$ between 300 and 400 nm [20], the coordinated peroxynitrite is not expected to contribute in a detectable way to the hemoglobin background absorbance.

Peroxynitrite in its anionic form is stable, but rapidly isomerizes to nitrate when protonated ($pK_a = 6.8$) or in the presence of other Lewis acids [4]. Traces of metal ions accelerate the decay of peroxynitrite possibly by neutralizing the negative charge on the peroxide group and thus inducing isomerization. The kinetic data presented in this work show that the rate of decay of peroxynitrite bound to methemoglobin increases with decreasing pH and is significantly larger than that of free peroxynitrite (Table 1). The different rates of decay observed for the α- and β-subunits may reflect an unequivalent environment of the heme group. X-ray crystallographic data have shown that in the β-subunit some amino acid residues overlap with the ligand binding site to a larger extent than in the α-subunit, giving rise to different rate constants for some of the reactions of hemoglobin [10,21,22].

Iron(III)porphyrin complexes have been reported to catalyze the isomerization of peroxynitrite to nitrate at pH 7.4 [23–25]. It has been proposed that the first step of this reaction involves the rapid formation of a iron(III)peroxynitrite complex which then decays to an oxoFe(IV) complex, the compound observed spectroscopically during peroxynitrite decomposition and which is suggested to be the active species for the catalysis [25]. In our system, the formation of the ferryl, oxoFe(IV) complex of hemoglobin can be excluded because its characteristic absorbance maxima at 423, 543 and 580 nm [26] were not observed. Apparently, methemoglobin does not react with peroxynitrite (see also [27]). This difference in reactivity towards peroxynitrite of iron(III)porphyrin complexes and hemoglobin may be caused by the presence of the proximal imidazole bound to the heme.

In summary, we have shown that an intermediate is formed during the $NO^{\bullet}$-induced oxidation of oxyhemoglobin. This species can be observed at alkaline pH, but rapidly decays to methemoglobin under neutral or acidic conditions. Spectroscopic properties and the pH dependence of the rate of decay of this intermediate suggest that it is a hemoglobin iron(III)-peroxynitrite complex. We are currently investigating whether the formed peroxynitrite complex is capable of oxidizing or nitrating any amino acid residue of the protein despite its relative fast rate of decay, in particular under physiological conditions.

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