Doctoral Thesis

Mechanistic studies of the nitrogen monoxide- and nitrite-mediated reduction of ferryl myoglobin and ferryl hemoglobin

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Mechanistic studies of the
nitrogen monoxide- and nitrite-mediated reduction of
ferryl myoglobin and ferryl hemoglobin

A Dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH

for the Degree of
DOCTOR OF NATURAL SCIENCES

presented by

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Curriculum Vitae
Abstract

Nitrogen monoxide generated endogenously has a variety of different properties. Among others it regulates blood pressure and transmission of nerve impulses. It has been shown that it exerts specific toxic effects and, paradoxically, acts as an antioxidant. Recent studies suggested that nitrogen monoxide can protect against the highly oxidizing ferryl myoglobin, which has been proposed to be at least in part responsible for the oxidative damage caused by the reperfusion of ischemic tissues. In the present work, we determined the rate constants for the reactions of nitrogen monoxide with ferryl myoglobin and ferryl hemoglobin, \((18 \pm 1) \times 10^6 \text{ M}^{-1}\text{s}^{-1}\) at pH 7.5 and 20 °C and \((24 \pm 2) \times 10^6 \text{ M}^{-1}\text{s}^{-1}\) at pH 7.0 and 20 °C, respectively. These fast rates imply that the nitrogen monoxide-mediated reductions of ferryl myoglobin and ferryl hemoglobin are very likely to take place \textit{in vivo} and might indeed represent a detoxifying pathway for these oxidizing forms of myoglobin and hemoglobin as well as for nitrogen monoxide.

In addition, we have shown that this reaction proceeds \textit{via} the intermediate \(O\)-nitrito complexes, \(\text{Mb}[\text{Fe}^{III}\text{ONO}]^{2+}\) and \(\text{Hb}[\text{Fe}^{III}\text{ONO}]^{2+}\), respectively, which subsequently decay to the met forms of the proteins and nitrite. The rate of formation of the intermediate does not depend on the pH whereas the decay of the intermediate is highly pH dependent.

In the second part of this work, we have measured the rate of the reaction between ferryl myoglobin and nitrite \((16 \pm 2 \text{ M}^{-1}\text{s}^{-1}\) at pH 7.5 and 20 °C). As this rate is significantly lower than the corresponding reaction with nitrogen monoxide, this reaction probably plays a role only when nitrogen monoxide has
Abstract

been consumed completely and large concentrations of nitrite are still present. The rate of the nitrite-induced reduction of ferryl hemoglobin (7.5±0.4) × 10^2 M⁻¹s⁻¹ at pH 7.0 and 20 °C) is higher than that found for ferryl myoglobin but still too low as that it could play a significant physiological role. However, as the rate constant increases with decreasing pH, it may be relevant at low tissue pH found under conditions of ischemia and subsequent reperfusion.

In contrast to the protecting role of nitrogen monoxide, the reaction with nitrite generates nitrogen dioxide which can lead to tyrosine nitration. Indeed, in this work it has been demonstrated that nitrite can nitrate protein-bound and free tyrosine in the presence of metmyoglobin or methemoglobin and hydrogen peroxide. However, yield of nitrated tyrosine residues or nitrated free tyrosine is very low. As the maximum of nitrated free tyrosine is reached at acidic pH at lower nitrite concentrations, this reaction may be relevant under conditions of ischemia and subsequent reperfusion.

The nitrogen monoxide-induced oxidation of oxyhemoglobin proceeds via formation of oxoperoxonitrato methemoglobin which subsequently decays to methemoglobin and nitrate. We attempted to prepare a model complex for oxoperoxonitrato methemoglobin. The reaction between nitrogen monoxide and the dioxygen iron porphyrin complex [Fe(TpivPP)(1-MeIm)(O₂)] proceeds in two reaction steps. In the first step, the oxoperoxonitrato iron(III)-porphyrin complex may be formed which subsequently decomposes.

The reaction between iron(III)-tetraphenylporphyrin and an excess of oxoperoxonitrate(1-) proceeds in three reaction steps. It may be assumed that in the first step, the oxoperoxonitrato iron(III) complex is formed which subsequently generates the oxoiron(IV) complex as an intermediate. As decay rate of oxoperoxonitrate(1-) is significantly increased in the presence of the iron-porphyrin complex, it may be assumed that the iron(IV) complex represents a catalyst for oxoperoxonitrate(1-) decomposition.
Zusammenfassung

Endogen gebildetes Stickstoffmonoxid besitzt eine Reihe verschiedener Eigenschaften. Unter anderem reguliert es den Blutdruck und die Übertragung von Nervenimpulsen. Es wurde gezeigt, dass es spezifische toxische Wirkungen besitzt und paradoxerweise auch als Antioxidant fungiert. Neuere Studien legten nahe, dass Stickstoffmonoxid als Antioxidant gegen das stark oxidierende Ferrylmyoglobin schützen kann, welches zumindest zum Teil verantwortlich gemacht wird für die bei Ischämie und anschliessender Reperfusion auftretenden oxidativen Gewebeschäden. In der vorliegenden Arbeit bestimmten wir die Geschwindigkeitskonstanten der Reaktionen von Stickstoffmonoxid mit Ferrylmyoglobin und Ferrylhämoglobin, \((18 \pm 1) \times 10^6 \text{M}^{-1}\text{s}^{-1}\) bei pH 7.5 und 20 °C bzw. \((24 \pm 2) \times 10^6 \text{M}^{-1}\text{s}^{-1}\) bei pH 7.0 und 20 °C. Diese grossen Geschwindigkeitskonstanten deuten an, dass die Stickstoffmonoxid-induzierte Reduktionen von Ferrylmyoglobin und Ferrylhämoglobin sehr wahrscheinlich in vivo stattfinden und tatsächlich einen entgiftenden Weg für die oxidierenden Formen von Myoglobin und Hämoglobin sowie für Stickstoffmonoxid darstellen können. Zusätzlich haben wir gezeigt, dass diese Reaktion über die O-Nitrito Komplexe, \(\text{Mb}[\text{Fe}^{III}\text{ONO}]^{2+}\) bzw. \(\text{Hb}[\text{Fe}^{III}\text{ONO}]^{2+}\), führt, die anschliessend zu den Metformen der Proteine und Nitrit zerfallen. Die Geschwindigkeitsrate der Bildung des Intermediates hängt nicht vom pH ab, während der Zerfall des Intermediates stark pH abhängig ist.

Im zweiten Abschnitt der Arbeit haben wir die Reaktionsrate von Ferrylmyoglobin mit Nitrit bestimmt \((16 \pm 2 \text{M}^{-1}\text{s}^{-1}\) bei pH 7.5 und 20 °C). Da die
Zusammenfassung

Geschwindigkeitskonstante deutlich niedriger ist als die entsprechende Reaktion mit Stickstoffmonoxid, spielt diese Reaktion wahrscheinlich nur eine Rolle, wenn Stickstoffmonoxid vollständig verbraucht worden ist und grosse Mengen an Nitrit noch präsent sind. Die Rate der Nitrit-induzierten Reduktion von Ferrylhämoglobin [(7.5±0.4) x 10^2 M^-1s^-1 bei pH 7.0 und 20 °C] ist höher als die für Ferrylmyoglobin gefundene, aber immer noch zu niedrig, als dass sie eine bedeutende physiologische Rolle spielen könnte. Da aber die Konstante bei niedrigerem pH ansteigt, kann sie relevant sein bei niedrigem Gewebe pH, der unter Bedingungen von Ischämie und anschliessender Reperfusion gefunden wird.


Die Reaktion zwischen Eisen(III)-tetraphenylporphyrin und einem Überschuss an Oxoperoxonitrat(1-) führt über drei Reaktionsschritte. Es kann
angenommen werden, dass im ersten Reaktionsschritt der Oxoperoxonitrato Eisen(III) Komplex gebildet wird, der anschließend den Oxoeisen(IV) Komplex als Intermediat generiert. Da die Zerfallsrate von Oxoperoxonitrat(1-) in der Gegenwart des Eisen-porphyrin Komplexes deutlich erhöht ist, kann angenommen werden, dass der Oxoeisen(IV) Komplex einen Katalysator für den Zerfall von Oxoperoxonitrat(1-) darstellt.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tr>
<td>BH₄</td>
<td>tetrahydrobiopterin</td>
</tr>
<tr>
<td>CaM</td>
<td>calmodulin</td>
</tr>
<tr>
<td>Compound I</td>
<td>(porphyrin++)oxoiron(IV)</td>
</tr>
<tr>
<td>Compound II</td>
<td>(porphyrin)oxoiron(IV)</td>
</tr>
<tr>
<td>18-crown-6</td>
<td>1,4,7,10,13,16-hexaoxacyclooctadecane</td>
</tr>
<tr>
<td>cyst</td>
<td>cysteine</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FMN</td>
<td>flavin mononucleotide</td>
</tr>
<tr>
<td>Hb</td>
<td>hemoglobin</td>
</tr>
<tr>
<td>HbFe⁺⁺</td>
<td>deoxyhemoglobin</td>
</tr>
<tr>
<td>HbFe⁺⁺⁺</td>
<td>iron(III) hemoglobin, methemoglobin</td>
</tr>
<tr>
<td>HbFe⁺⁺⁺⁺O</td>
<td>ferryl [oxoiron(IV)] hemoglobin</td>
</tr>
<tr>
<td>HbFeO₂</td>
<td>oxyhemoglobin</td>
</tr>
<tr>
<td>*HbFe⁺⁺⁺⁺O</td>
<td>ferryl hemoglobin with a transient protein radical</td>
</tr>
<tr>
<td>his</td>
<td>histidine</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>Mb</td>
<td>myoglobin</td>
</tr>
<tr>
<td>MbFe⁺⁺</td>
<td>deoxymyoglobin</td>
</tr>
<tr>
<td>MbFe⁺⁺⁺</td>
<td>iron(III) myoglobin, metmyoglobin</td>
</tr>
<tr>
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</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>MbFeO₂</td>
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<tr>
<td>*MbFeIV=O</td>
<td>ferryl myoglobin with a transient protein radical</td>
</tr>
<tr>
<td>metHb</td>
<td>methemoglobin</td>
</tr>
<tr>
<td>metMb</td>
<td>metmyoglobin</td>
</tr>
<tr>
<td>MeCN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>MeIm</td>
<td>methylimidazole</td>
</tr>
<tr>
<td>NADPH</td>
<td>reduced nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>TMPS</td>
<td>5,10,15,20-tetrakis(2,4,6-trimethyl-3,5-sulfonato-phenyl)-porphyrin</td>
</tr>
<tr>
<td>TMPyP</td>
<td>5,10,15,20-tetrakis(N-methyl-4'-pyridyl)-porphyrin</td>
</tr>
<tr>
<td>TpivPP</td>
<td>meso-tetra-(α,α,α,α-o-pivalamidophenyl)-porphyrin</td>
</tr>
<tr>
<td>TPP</td>
<td>meso-tetrphenylporphyrin</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
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</tbody>
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Chapter 1

General Introduction

1.1 Nitrogen monoxide

1.1.1 Chemical and physical properties of nitrogen monoxide

Nitrogen monoxide is a colourless, paramagnetic gas with a boiling point of -151.8 °C. The formal bond multiplicity of nitrogen monoxide is 2.5, as this diatomic molecule has three electron pairs in bonding orbitals and an unpaired electron located in an antibonding $\pi^*$-orbital. The bond distance is 1.14 Å, a value between an N-O-double bond (1.16 Å) and the bond length found for nitrosyl cation (1.06 Å) [1].

In the gaseous form, nitrogen monoxide is monomeric. Dimerization does not result in a significant gain in stability because dinitrogen dioxide has a total of five bonds, as two separate nitrogen monoxide molecules. In contrast, dimerization is accompanied by a decrease in entropy. Thus, dinitrogen dioxide can be formed by high concentrations of nitrogen monoxide in the liquid and especially in the solid form, where the temperature-dependent entropy factor $-T\Delta S$ is reduced [1].

Due to the small difference in electronegativity between nitrogen and oxygen, nitrogen monoxide is quite unpolar. Therefore, like dioxygen, it has
General Introduction

only a very small solubility in water, 1.9 mM at 25 °C (calculated from data
given in reference [2]).

One-electron oxidation of nitrogen monoxide gives the nitrosyl cation
which is isoelectronic with carbon monoxide, whereas one-electron reduction
yields the dioxygen isoelectronic species oxonitrate(1-). Reduction potentials for
nitrosyl cation and nitrogen monoxide are given in Table 1 [3].

Table 1 Reduction potentials for nitrosyl cation and nitrogen monoxide. Values are
given for 1 M solutions at pH 7 and 25 °C [3].

<table>
<thead>
<tr>
<th>Couple</th>
<th>E°(V)</th>
</tr>
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<tbody>
<tr>
<td>NO(^+)/ NO(^-)</td>
<td>1.21</td>
</tr>
<tr>
<td>NO(^-)/ NO(^-)</td>
<td>0.39(^a)</td>
</tr>
</tbody>
</table>

\(^a\) Deduced from higher pK\(_a\) values found for oxonitric acid in recent studies, the correct value for the reduction potential of nitrogen monoxide is expected to be smaller than this calculated value [4, 5].

Nitrogen monoxide can bind to a variety of biologically relevant transition
metals, such as iron(II), iron(III), and copper(II) [6]. In these metal complexes,
nitrogen monoxide can adopt a linear or a bent geometry which reflects the
different metal-nitrogen monoxide bonding interactions [7]. In the linear
complexes, nitrogen monoxide formally donates an electron to the metal centre
and therefore binds as an nitrosyl cation-ligand, that is as a three-electron donor.
M-N-O bond angles are in the range between 175° and 180°. In addition to the
metal-nitrogen monoxide sigma bond, a strong \(\pi\)-backbonding can lead to
further stabilization of these complexes. In the bent structure, nitrogen monoxide
formally accepts an electron from the metal and therefore binds as an
oxonitrate(1-)-ligand, corresponding to a one-electron donor. M-N-O bond
angles found for these complexes are in the range between 120° and 125°.
Intermediate complexes show bond angles between these two extremes [7].
1.1.2 Nitrogen monoxide in vivo

The identification of the endothelium-derived-relaxing-factor as nitrogen monoxide [8] opened a new research area. Besides its potent vasodilatory effects, under certain circumstances nitrogen monoxide was found to be responsible for the killing of microorganisms and tumor cells by activated macrophages, and to act as a novel type of neurotransmitter. In 1992, nitrogen monoxide was selected as the “Molecule of the Year” by Science [9] and in 1998, Ferid Murad, Robert F. Furchgott, and Louis J. Ignarro were awarded with the Nobel Prize in Physiology and Medicine for their pioneering work that led to the discovery of some of the physiological functions of nitrogen monoxide [10-12].

1.1.2.1 Biosynthesis of nitrogen monoxide

In vivo, nitrogen monoxide is primarily generated by the class of enzymes called nitric oxide synthase. Three isoforms of mammalian nitric oxide synthase have been identified: endothelial nitric oxide synthase, inducible nitric oxide synthase, and brain or neuronal nitric oxide synthase [13].

The active form of all nitric oxide synthase isoforms is a homodimer. Each subunit consists of a carbon-terminal reductase and a nitrogen-terminal oxygenase domain (Figure 1). The reductase domain contains each one molecule of bound flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), and a binding side for reduced nicotinamide adenine dinucleotide phosphate (NADPH). The oxygenase domain includes a heme-thiolate centre and one binding site each for tetrahydrobiopterin (BH₄) and the substrate L-arginine. Between the reductase and the oxygenase domains is a binding site for calmodulin (CaM). In addition, a zinc ion is tetrahedrally coordinated to two cysteine residues of the oxygenase domains of each subunit, suggesting that the
General Introduction

zinc centre increases dimer stabilization [13]. The activated forms of neuronal nitric oxide synthase and endothelial nitric oxide synthase require the calcium-loaded form of calmodulin to be bound. In contrast, inducible nitric oxide synthase binds calmodulin so tightly that its activity is independent of cellular levels of calcium and calmodulin. It is upregulated by cytokines or endotoxin. During the catalytic reaction, electron transfer from the electron donor reduced nicotinamide adenine dinucleotide phosphate to the heme group proceeds with the involvement of flavin mononucleotide, flavin adenine dinucleotide and tetrahydrobiopterin. Calmodulin-binding is essential for an efficient electron transfer. Interestingly, electron flow follows a cross-over from the reductase domain of one subunit to the oxidase domain of the other subunit. This feature explains why dimerization is essential for protein activity [13].

![Diagram](https://example.com/diagram.png)

**Figure 1** Domains and cofactors of nitric oxide synthase. The dashed arrow shows the path of electron transfer.
All isoforms of nitric oxide synthase catalyze the conversion of the amino acid L-arginine to nitrogen monoxide and L-citrulline under incorporation of oxygen (Figure 2).

\[
\begin{align*}
\text{L-arginine} & \quad \text{N-hydroxy-L-arginine} & \quad \text{L-citrulline} \\
+H & +H & +H \\
\text{H}_3\text{N} & \text{COO}^- & \text{COO}^- \\
\text{HN} & \text{NH}_2 & \text{HN} \\
\text{H}_2\text{N} & & \text{H}_2\text{N} \\
\text{O}_2 & & \text{O}_2 \\
\text{1.0 NADPH} & & \text{0.5 NADPH} \\
\end{align*}
\]

\[ \text{O}_2 + \text{NO}^\cdot \]

**Figure 2** Formation of nitrogen monoxide.

The reaction takes place in two steps at the heme-thiolate centre of the oxygenase domain. In the first reaction step, hydroxylation of enzyme-bound L-arginine generates N-hydroxy-L-arginine. In the second reaction step, N-hydroxy-L-arginine is oxidized to L-citrulline under simultaneous generation of nitrogen monoxide. During conversion of L-arginine to nitrogen monoxide and L-citrulline, 1.5 equivalents of reduced nicotinamide adenine dinucleotide phosphate and two equivalents of dioxygen are consumed. Subsequent formation of a nitrosyl complex by binding of nitrogen monoxide to the heme iron can regulate activity of nitric oxide synthase [13].

Estimated physiological levels of nitrogen monoxide vary between nanomolar concentrations formed by neuronal nitric oxide synthase and endothelial nitric oxide synthase and up to 10 μM generated by inducible nitric oxide synthase as an answer of the immune system [14].
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Recently, another isoform of nitric oxide synthase has been found which is located in the mitochondria, and thus has been named as mitochondrial nitric oxide synthase [15-17]. Nitrogen monoxide formed by mitochondrial nitric oxide synthase is discussed to be involved in regulation of mitochondrial respiration via binding to cytochrome c oxidase [18].

1.1.2.2 Physiological role of nitrogen monoxide

Activation of soluble guanylyl cyclase
Nitrogen monoxide activates soluble guanylyl cyclase via binding to its ferrous heme group. When nitrogen monoxide binds, rupture of the trans-bond to proximal histidine generates a 5-coordinate nitrosyl iron(II) porphyrin complex. Activated soluble guanylyl cyclase catalyzes the conversion of guanosine 5′-triphosphate to 3′,5′-cyclic guanosine monophosphate [19].

Increased concentrations of 3′,5′-cyclic guanosine monophosphate lead to lower cytosolic calcium concentrations. Lower cytosolic concentrations of calcium cause vascular smooth muscle to relax. In addition, reduced intracellular concentrations of calcium are related with the inhibition of platelet aggregation and nitrogen monoxide-mediated signal transduction in the nervous systems [19, 20].

Nitrogen monoxide formation as an immuno response
Pathological induction by cytokotines and bacterial endotoxins leads to increased formation of inducible nitric oxide synthase [21]. Inducible nitric oxide synthase can produce nitrogen monoxide up to a concentration level of 10 μM [14]. Nitrogen monoxide inhibits the metabolic energy production of the pathogens. In addition, reactive nitrogen species formed from nitrogen monoxide can damage DNA (see section 1.1.2.3) [21]. However, there is no
possibility for nitrogen monoxide to discriminate between pathogenic and host DNA. Therefore, overstimulation of inducible nitric oxide synthase can also cause cell and tissue damage. Under extreme conditions, overproduction of nitrogen monoxide in the endothelial cells can lead to dilation of the blood vessels and thus to a life-threatening decrease of the blood pressure, the so-called septic shock [21].

Antioxidative role of nitrogen monoxide

Nitrogen monoxide has been proposed to play a role as an effective antioxidant. It has been shown that it inhibits lipid peroxidation of low density lipoproteins by scavenging lipid alkoxy and peroxyl radicals which would propagate membrane damage [22, 23]. Nitrogen monoxide also scavenges free or protein bound tyrosyl radicals [24]. It has been shown that tyrosyl radicals generated by myeloperoxidase in the presence of hydrogen peroxide can initiate peroxidation of low density lipoproteins [25].

1.1.2.3 Formation of reactive nitrogen species from nitrogen monoxide in vivo

1.1.2.3.1 Oxidation of nitrogen monoxide by dioxygen

Nitrogen monoxide reacts with dioxygen to form nitrogen dioxide (Scheme 1) [1]. The reaction is second-order in nitrogen monoxide and thus the half-life is given by the equation $t_{1/2} = 1/(k[O_2][NO^\bullet])$ with $k = 8 \times 10^6$ M$^{-2}$s$^{-1}$ at 20 °C [26]. In the presence of an excess of nitrogen monoxide, nitrogen dioxide most likely reacts with nitrogen monoxide to yield dinitrogen trioxide which in aqueous solutions hydrolyses to yield nitrite (Scheme 1).
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**Scheme 1** Nitrogen monoxide oxidation by dioxygen in aqueous solutions.

\[
\begin{align*}
2 \text{NO}^* + \text{O}_2 & \rightarrow 2 \text{NO}_2^* \\
\text{NO}_2^* + \text{NO}^* & \leftrightarrow \text{N}_2\text{O}_3 \\
\text{N}_2\text{O}_3 + \text{H}_2\text{O} & \rightarrow 2 \text{NO}_2^- + 2 \text{H}^+
\end{align*}
\]

Because of the low normal nitrogen monoxide concentration present *in vivo* (10-400 nM), the reaction between nitrogen monoxide and dioxygen is very slow. In the presence of 10 nM nitrogen monoxide and 20 μM dioxygen, the calculated half-life of nitrogen monoxide would be about 12 days [27], and thus is not expected to have a physiological relevance. As the molecule is quite unpolar, nitrogen monoxide preferentially partitions into lipid membranes with consequently high local concentrations of nitrogen monoxide in the membranes [28]. Therefore, and due to high concentrations of nitrogen monoxide formed during inflammatory processes, reaction of nitrogen monoxide with dioxygen may be of physiological relevance. Due to the high reduction potential of nitrogen dioxide (\(E^{\circ} = 0.99\) V) [27] and the capability of dinitrogen trioxide to act as a nitrosyl cation-donor, both species can react with biomolecules. Nitrogen dioxide is known to react with thiols [29, 30], to cause lipid peroxidation [31], and to damage DNA [32]. It has been shown that dinitrogen trioxide effectively nitrosates phenols, primary amines (followed by subsequent deamination), and secondary amines which yield nitrosamines [14]. Furthermore, reaction of dinitrogen trioxide with substrates containing thiol-groups yields nitrosothiols [14]. Nitrosated cysteine thiol-groups have been found in proteins such as albumin, p21\(^{ras}\), glyceraldehyde 3-phosphate dehydrogenase, caspases, and hemoglobin *in vivo*. Nitrosothiol groups have also been detected in the low molecular weight thiols, cysteine and glutathione [33]. Sulfur-nitrosation can modulate the function of the proteins. Therefore, it has been proposed that it plays a regulatory role *in vivo*. For example, nitrosation of glyceraldehyde 3-
phosphate dehydrogenase, a protein which is involved in the glycolytic pathway, leads to a reduction of its activity [33]. Nitrosation of caspase 3, a cysteine protease which is involved in the apoptotic pathway, inhibits apoptosis in hepatocytes [33]. Formation of nitrosothiols is also mediated by nitrosyl complexes which serve as nitrosyl cation-donors under reduction of the metal ion [6]. As protein-nitrosothiols are more stable than low molecular weight nitrosothiols, it has been proposed that transnitrosation may occur in vivo [14]. The proposed physiological role of Cysβ93-nitrosated hemoglobin will be discussed in detail in chapter 1.2.3.

1.1.2.3.2 Formation of oxoperoxonitrate(1-)

Simultaneous generation of nitrogen monoxide and superoxide favours the production of the powerful oxidizing and nitrating agent oxoperoxonitrate(1-) (peroxynitrite). Superoxide is mostly formed during mitochondrial electron transport [34]. In addition, autoxidation of oxyhemoglobin (HbFeO₂) to methemoglobin (HbFe³⁺) generates superoxide in red blood cells [34]. Another source for superoxide is the reduction of dioxygen by xanthine oxidase after ischemia/reperfusion [35]. Furthermore, superoxide is also formed by reduced nicotinamide adenine dinucleotide phosphate oxidase in phagocytes such as macrophages, monocytes, and neutrophils as a defense against invading organisms [36]. Superoxide reacts with nitrogen monoxide at a nearly diffusion-controlled rate \( k = 1.6 \times 10^{10} \text{ M}^{-1}\text{s}^{-1} \) [37] to form oxoperoxonitrate(1-). Oxoperoxonitrate(1-) is very stable at alkaline conditions. In contrast, oxoperoxonitric acid \( (p_{\text{ka}} = 6.8) \) rapidly isomerizes to nitrate with a rate constant of \( 1.2 \text{ s}^{-1} \) at 25 °C [38]. Thus, oxoperoxonitrate(1-) cannot be directly detected under physiological conditions. There is a controversy about the mechanism of its isomerization to nitrate. The main argument consists in whether or not
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hydroxyl radical and nitrogen dioxide are generated during this process. Several groups have proposed that oxoperoxonitric acid undergoes homolysis to form about 30% hydroxyl radical and nitrogen dioxide [39, 40]. Indeed, oxoperoxonitrate(1-) often shows a reactivity towards other substrates similar to that of hydroxyl radical and nitrogen dioxide [41].

However, the observation that the only or main product from reaction between hydroxyl radical and nitrogen dioxide is oxoperoxonitric acid and not nitrate [42] argues against this radical mechanism. In addition, the small rate constant expected for homolysis on thermodynamic grounds \((1 \times 10^{-2}\ s^{-1})\) is not consistent with the measured overall rate constant of \(1.2\ s^{-1}\) for oxoperoxonitrate(1-) isomerization to nitrate [43]. Therefore, it has been proposed that the observed reactivity of oxoperoxonitric acid is due to an intermediate which is close to the transition state of the isomerization [43, 44].

The half-life of oxoperoxonitrate(1-) is much longer than that of the hydroxyl radical, and both, oxoperoxonitrate(1-) and oxoperoxonitric acid are able to diffuse across biological membranes. Therefore, oxoperoxonitrate(1-) and oxoperoxonitric acid can react with substrates away from their point of generation [45]. Oxoperoxonitrate(1-) and its conjugate acid can effect one- and two-electron oxidation reactions. They react with a variety of biologically relevant molecules such as thiols, selenium compounds, and with transition metal centres of proteins (Table 2). They initiate lipid peroxidation [46, 47], and can damage DNA [48, 49].
Table 2 Second-order rate constants for reactions of oxoperoxonitrate(1-) with biologically and pharmacologically relevant target molecules.

<table>
<thead>
<tr>
<th>Target</th>
<th>k (M⁻¹s⁻¹)</th>
<th>T (°C)</th>
<th>pH</th>
<th>Lit.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxyl radical</td>
<td>4.8 x 10⁹</td>
<td>21-24</td>
<td>12</td>
<td>[50]</td>
</tr>
<tr>
<td>Ebselen⁴</td>
<td>2 x 10⁶</td>
<td>25</td>
<td>&gt;8</td>
<td>[51]</td>
</tr>
<tr>
<td>Aconitase</td>
<td>1.4 x 10⁵</td>
<td>25</td>
<td>7.6</td>
<td>[52]</td>
</tr>
<tr>
<td>Oxyhemoglobin</td>
<td>8.8 x 10⁴</td>
<td>20</td>
<td>7.0</td>
<td>[53]</td>
</tr>
<tr>
<td>Ferryl hemoglobin</td>
<td>9.4 x 10⁴</td>
<td>20</td>
<td>7.0</td>
<td>[53]</td>
</tr>
<tr>
<td>Cytochrome c²⁺</td>
<td>2.5 x 10⁴</td>
<td>37</td>
<td>7.4</td>
<td>[54]</td>
</tr>
<tr>
<td>Carbon dioxide</td>
<td>2.9 x 10⁴</td>
<td>24</td>
<td>ind⁵</td>
<td>[55]</td>
</tr>
<tr>
<td>Cu,Zn-SOD³</td>
<td>1 x 10³-10⁵</td>
<td>37</td>
<td>8</td>
<td>[56]</td>
</tr>
<tr>
<td>Cysteine</td>
<td>5.9 x 10³</td>
<td>37</td>
<td>7.4</td>
<td>[57]</td>
</tr>
<tr>
<td>Albumin⁴</td>
<td>3.8 x 10³</td>
<td>37</td>
<td>7.4</td>
<td>[58]</td>
</tr>
<tr>
<td>Glutathione</td>
<td>1.35 x 10³</td>
<td>37</td>
<td>7.4</td>
<td>[3]</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.7 x 10²</td>
<td>25</td>
<td>7.4</td>
<td>[59]</td>
</tr>
</tbody>
</table>

⁴ ebselen: 2-phenyl-1,2-benzisoselenazol-3(2H)-one.
⁵ pH-independent rate constant.
⁶ SOD: superoxide dismutase.
⁷ The rate for the reaction between the cysteine thiol-group and oxoperoxonitrate(1-) has been determined.

Reaction between oxoperoxonitrate(1-) and tyrosine leads to formation of nitrotyrosine. Detection of nitrotyrosine has been proposed to be a marker for oxoperoxonitrate(1-) formation in vivo [41, 44]. Nitrotyrosine has been found in tissues of patients affected by diseases such as heart diseases [60], cancer [48] and Alzheimer’s disease [61]. It is known that tyrosine nitration leads to inactivation of several enzymes [62, 63].

However, it has recently been shown that also reaction of nitrite and hydrogen peroxide in the presence of peroxidases may contribute to formation of nitrotyrosine in vivo [64]. It has been shown that in the course of the reaction nitrogen dioxide is generated, which is known to nitrate phenolic compounds.
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Therefore, it can be assumed that nitration of tyrosine is rather a marker for the formation of reactive nitrogen species than exclusively generation of oxoperoxonitrate(1-).

Due to the high physiological concentrations of carbon dioxide (> 1 mM in plasma), the carbon dioxide-catalyzed isomerization of oxoperoxonitrate(1-) to nitrate is expected to be one of the main routes for oxoperoxonitrate(1-) consumption in vivo [41]. The reaction yields the adduct 1-carboxylato-2-nitrosodioxidane (ONOOOCO$_2$~). Due to its weak oxygen-oxygen bond, 1-carboxylato-2-nitrosodioxidane rapidly decays to the radicals nitrogen dioxide and trioxocarbonate(•1-) (CO$_3$•~). Trioxocarbonate(•1-) has been detected by electron spin resonance spectroscopy by several groups [65, 66]. However, the detected yield of formed trioxocarbonate(•1-) is quite low, 4±1 % [67]. An intermediate has been observed during rapid-scan UV-visible spectroscopic investigations with an absorption maximum at 650 nm. This intermediate with a lifetime of about 4 ms (at 10-12 °C) has been assigned to be the adduct 1-carboxylato-2-nitrosodioxidane [67]. In contrast, it has been argued that 1-carboxylato-2-nitrosodioxidane cannot accumulate as it would have a lifetime of only 0.5 ms or less [68].

The ability of 1-carboxylato-2-nitrosodioxidane to act as an oxidizing agent is reduced relative to that of oxoperoxonitrate(1-). In contrast, 1-carboxylato-2-nitrosodioxidane is a more efficient nitrating agent than oxoperoxonitrate(1-) [41].

Strategies for prevention of pathological damages caused by oxoperoxonitrate(1-)

There are several different strategies that are currently investigated to reduce damages which are related to oxoperoxonitrate(1-) production under pathophysiological conditions. For instance, inhibitors of nitric oxide synthase and mimics of superoxide dismutase have been developed to reduce
concentrations of nitrogen monoxide and superoxide, respectively, and thus also the amount of formed oxoperoxonitrate(1-) [41].

Alternatively, oxoperoxonitrate(1-) can effectively be scavenged by ebselen [51] or catalytically isomerized to nitrate by iron porphyrin complexes such as iron(III)meso-teta(2,4,6-trimethyl-3,5-disulfonato)porphyrin chloride [69]. However, the high valent iron intermediate detected in the catalytic cycle may lead to secondary toxic effects. The reaction between iron(III)-tetraphenylporphyrin and oxoperoxonitrate(1-) will be discussed in detail in chapter 4.

1.2 Hemoglobin and myoglobin

Hemoglobin is the carrier protein which transports oxygen from the lung to tissues. Myoglobin acts as an oxygen storage protein and transports oxygen intracellularly.

1.2.1 Structures of hemoglobin and myoglobin

Human hemoglobin consists of four subunits, two α- and two β-subunits. The subunits are held together by hydrophobic interactions, by hydrogen bonds, and by salt bridges. The polypeptide chains of the α- and the β-subunit are formed by 141 and 146 amino acid residues, respectively [70]. The tertiary structures of the two subunits are quite similar. The β-chain is formed by 8 helices (named A to H, starting from the amino-terminus), whereas the α-chain is formed by 7 helices (the D helix of the β-chain is missing). Nonhelical segments between two helices are named according to the helices they connect, such as CD, EF etc. Location of a specific amino acid residue in the polypeptide chain is given by
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...name of its helix and the number in the helix chain [71]. According to another nomenclature, the specific amino acid residue is given with its number within the polypeptide chain, starting from the amino-terminus.

Each subunit contains a heme (iron coordinated to protophorphyrin IX) as the prosthetic group [70]. The heme is connected to the globin via a histidine amino acid residue (histidine-F8) which is coordinated to the iron ion on the so-called proximal side of the porphyrin plane (Figure 3). On the so-called distal side of the porphyrin plane there is another histidine residue (histidine-E7) which stabilizes coordination of dioxygen to the iron ion (Figure 3) [70].

Myoglobin is a monomeric protein with structural features rather similar to those of the hemoglobin subunits, in particular to the β-subunit [70].

![Figure 3](image_url) Structure of dioxygen coordinated to the iron ion of hemoglobin.
1.2.2 Binding of dioxygen to hemoglobin and myoglobin

Deoxyhemoglobin (HbFe^{II}) has a pentacoordinated iron(II) centre in which the metal ion is situated about 0.6 Å out of the porphyrin plane, directed towards the proximal histidine. Upon binding of dioxygen, the iron ion moves into the porphyrin plane [70]. The hydrogen bond between the coordinated dioxygen and the imidazole amino proton of the distal histidine stabilizes the bent Fe-O-O structure (Figure 3) [71].

Carbon monoxide has a much higher equilibrium association constant for the iron ion of the heme than dioxygen [72]. With the free heme, heme-coordinated carbon monoxide, generated during degradation of the heme, would hinder sufficient supply of the cells with dioxygen [72]. It has long been assumed that steric hindrance caused by the distal histidine does not allow for a linear coordination of carbon monoxide and therefore reduces affinity of hemoglobin for carbon monoxide [73]. However, new investigations demonstrate that the affinity for carbon monoxide is regulated by a water molecule which is hydrogen-bonded to the distal histidine in deoxyhemoglobin [72]. Before a ligand can coordinate to the iron ion of the heme, this water molecule has to be displaced. Energy which is needed for removal of this water molecule is compensated when coordinated dioxygen forms a strong hydrogen-bond with the distal histidine. In contrast, carbon monoxide can only form very weak electrostatic interactions with the distal histidine [72].

The dioxygen saturation curve of hemoglobin is sigmoidal (S-shaped), characteristic for a cooperative interaction between the four dioxygen binding sites of the protein [70]. With no oxygen bound, hemoglobin is in the low oxygen affinity tense-state. Binding of dioxygen causes conformational changes in the protein, which convert it to the high affinity relaxed-state. Thus, the sigmoidal saturation curve is a composite of a low and a high affinity curve. The differences in dioxygen affinity between the tense- and the relaxed-state can be
explained by changes in the tertiary and quaternary structure, which take place when dioxygen binds to the iron. Indeed, upon binding of dioxygen, the iron ion moves into the porphyrin plane and as a consequence, also the proximal histidine moves. This movement partially alters the tertiary structure of the entire subunit. Changes at the surface of the subunit lead to breakage of salt bridges and hydrogen-bonds between adjacent subunits. Subsequently, the other subunits of hemoglobin are forced to change conformation as well and facilitate binding of dioxygen to the unoccupied binding sites [70].

As myoglobin is monomeric, its dioxygen saturation curve shows a normal exponential curve [70]. Comparison of the saturation curves for myoglobin and hemoglobin shows that as expected myoglobin has always a higher affinity for dioxygen than hemoglobin. In tissues, myoglobin must have a higher affinity for dioxygen, to be able to take over dioxygen from hemoglobin [70]. Release of dioxygen from oxyhemoglobin (HbFeO₂) in cells is controlled by different factors. Active cells produce carbon dioxide which reacts with water to yield hydrogen carbonate(1-) and a hydron (equation 1). When the pH is decreased, the affinity of hemoglobin for dioxygen is reduced and dioxygen is liberated. This feature is called the Bohr effect [70]. Formation of carbamate from the reaction of carbon dioxide with amino groups at the beginning of each globin chain (equation 2) also causes a decrease in dioxygen affinity [71]. In addition, some of the physiologically formed carbon dioxide is transported by hemoglobin to the lungs where it is removed from the body [71]. Another way of regulation of dioxygen affinity of hemoglobin is the binding of 2,3-diphosphoglycerate to hemoglobin, which causes a decrease in dioxygen affinity [70].

\[
\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{HCO}_3^- + \text{H}^+ \quad (1)
\]

\[
\text{Hb-NH}_2 + \text{CO}_2 \rightleftharpoons \text{Hb-NH-COO}^- + \text{H}^+ \quad (2)
\]
1.2.3 Reactions of nitrogen monoxide with hemoglobin and myoglobin

In contrast to dioxygen and carbon monoxide, nitrogen monoxide reacts with both, the ferrous (HbFe$^{II}$) and the ferric (HbFe$^{III}$) oxidation states of hemoglobin [6]. When nitrogen monoxide reacts with ferrous hemoglobin, the ferrous nitrosyl hemoglobin (Hb[Fe$^{II}$NO]$^{2+}$) is generated. Reaction of nitrogen monoxide with ferric hemoglobin yields the nitrosyl methemoglobin (Hb[Fe$^{III}$NO]$^{3+}$) complex which effects as a nitrosyl cation donor or is hydrolyzed, under reduction of the iron, to ferrous hemoglobin and nitrite. In the presence of an excess of nitrogen monoxide, ferrous hemoglobin formed by this way is nitrosylated to yield also the ferrous nitrosyl complex [6].

It has been shown that the nitrogen monoxide-induced oxidation of oxyhemoglobin is quite fast ($89 \times 10^6$ M$^{-1}$s$^{-1}$ at pH 7.0 and 20 °C) and proceeds via a oxoperoxonitrato methemoglobin complex, which subsequently decays to methemoglobin and nitrate [74]. Methemoglobin (HbFe$^{III}$) is reduced to deoxyhemoglobin by methemoglobin reductase [75]. Given the high reaction rate between nitrogen monoxide and oxyhemoglobin, this reaction is expected to be the main route for depletion of endothelium nitric oxide synthase-derived nitrogen monoxide in the blood stream [76]. However, as the reaction is so fast, it has been argued that bioavailability of nitrogen monoxide would be too small to effectively activate its target enzyme in the smooth muscle cells, soluble guanylyl cyclase [76]. There are different hypothesis how sufficient concentration of nitrogen monoxide is maintained in vivo [76-80].

It has been observed that the blood flow creates a red-blood-cell-free zone at the endothelial surface of larger blood vessels which limits the probability for nitrogen monoxide to reach red blood cells [77]. It has also been shown that nitrogen monoxide may partly be prevented from entering the red blood cells by limitation of the diffusion to the cell membrane [76, 78]. Before nitrogen
monoxide can react with oxyhemoglobin, it has to cross the membrane of the red blood cells. It has been proposed that the submembrane of red blood cells includes an effective diffusion barrier for influx of nitrogen monoxide [79]. The barrier is formed by a protein network which consists of methemoglobin as well as nitrogen monoxide-inert proteins. The rate constant of the reaction of nitrogen monoxide with oxyhemoglobin in the red blood cells has been determined to be $5.2 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ [79], significantly lower than the rate constant of the reaction between nitrogen monoxide and free oxyhemoglobin.

According to another hypothesis, it has been proposed that under low nitrogen monoxide concentrations generated by the endothelial cells of the blood vessels, nitrogen monoxide does not react irreversibly with oxyhemoglobin. Instead, nitrogen monoxide would react with vacant hemes of partially deoxygenated hemoglobin to form nitrosylhemoglobin \([\text{Hb(FeO}_2\text{)}_3(\text{Fe}^{II}\text{NO})]\) [80]. It order to explain this reaction it has to be assumed that the observed rate for nitrosylation of the vacant heme iron is higher than the observed rate for the reaction of nitrogen monoxide with oxyhemoglobin [80]. That means that partially oxygenated hemoglobin must have a similar cooperativity effect with nitrogen monoxide as with dioxygen and the rate constant of the reaction between \(\text{Hb(FeO}_2\text{)}_3(\text{Fe}^{II})\) and nitrogen monoxide would be 100–1000 times higher than that of the reaction between \(\text{Hb(Fe}^{II})_4\) and nitrogen monoxide. Subsequently, the “NO group” would be transferred in a yet unknown mechanism intramolecularly from the iron ion to the thiol group of the cysteine \(\beta93\) residue [80]. The generated nitrosothiol hemoglobin has been proposed to function as nitrogen monoxide storage [81]. It has been proposed that export of the “NO group” from the red blood cells proceeds \textit{via} transnitrosation to a cysteine residue of the band 3 anion-exchange protein at the red blood cell membrane [81]. The “NO group” is subsequently released and transported out of the red blood cell by a yet not understood mechanism [81]. Thus, hemoglobin
would serve as a transporter not only for dioxygen and carbon dioxide, but also for nitrogen monoxide [81].

However, there is a controversy about the formation and decomposition of nitrosothiol hemoglobin under physiological conditions. Other studies have not shown any cooperativity effect in binding of nitrogen monoxide to hemoglobin [82]. The difference between these results has been explained mainly by the different hemoglobin preparations [82]. A cooperativity effect has only been observed when purified hemoglobin preparations were used and only at a phosphate buffer concentration of 0.01 M, but not at 0.1 M [80]. In contrast, investigations with the more physiologically relevant hemolysate and whole blood do not show any cooperativity effect, and are independent of the buffer concentration in the case of the hemolysate [82]. Furthermore, also no arterial-venous blood gradient in nitrosothiol hemoglobin concentrations could be observed [83]. Such a gradient would be expected if nitrogen monoxide was liberated after dissociation of dioxygen in the capillary vessels [83].

Analogously to oxyhemoglobin, nitrogen monoxide reacts with oxymyoglobin in a fast reaction to yield metmyoglobin and nitrate \((4.4 \times 10^7 \text{ M}^{-1}\text{s}^{-1} \text{ at pH 7.0 and 20 °C})\) [74]. Due to this high reaction rate, it has recently been proposed that oxymyoglobin, besides its function as an oxygen storage protein, also plays a role as a scavenger for nitrogen monoxide. For example, under conditions of elevated concentration levels of nitrogen monoxide, reaction of nitrogen monoxide with oxymyoglobin is proposed to prevent from nitrogen monoxide-induced inhibition of cytochrome c oxidase [84-86].
1.2.4 Spectroscopic features of hemoglobin and myoglobin

Spectroscopic characteristics of heme proteins are a strong absorption band in the wavelength range 400-440 nm, the so-called Soret band and the α- and β-bands with maxima in the visible region [69]. The position of the absorption maxima of these bands depends on the oxidation state of the iron ion and on the ligands bound to the metal centre. Figure 4 shows the Soret bands (A) and the visible spectra (B) of the different oxidation states of horse heart myoglobin, oxymyoglobin (MbFeO₂), metmyoglobin (MbFeⅢOH₂), and ferryl myoglobin (MbFeⅣ=O). Spectroscopic data of these species are presented in Table 3.

![Figure 4 A Soret band region and B, visible spectra of horse heart oxymyoglobin, metmyoglobin and ferryl myoglobin (9.5 μM, 0.1 M phosphate buffer at pH 7.0).](image-url)
Table 3 Spectroscopic data for oxymyoglobin, metmyoglobin, and ferryl myoglobin.

<table>
<thead>
<tr>
<th></th>
<th>Soret</th>
<th>Visible</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\lambda_{\text{max}}$ (nm) $^a$</td>
<td>$\lambda_{\text{max}}$ (nm) $^a$</td>
<td>$\lambda_{\text{max}}$ (nm) $^a$</td>
</tr>
<tr>
<td>MbFe$^{\text{III}}$OH$_2$</td>
<td>408 (188)</td>
<td>502 (10.2)</td>
<td>630 (3.9)</td>
</tr>
<tr>
<td>MbFeO$_2$</td>
<td>417 (128)</td>
<td>542 (13.9)</td>
<td>580 (14.4)</td>
</tr>
<tr>
<td>MbFe$^{\text{IV}}$=O</td>
<td>421 (111)</td>
<td>549 (10.5)</td>
<td>588 (10.1)</td>
</tr>
</tbody>
</table>

$^a$ $\lambda_{\text{max}}$ (nm), (ε, mM$^{-1}$cm$^{-1}$)
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1.3 Goals of the present work

It has been proposed that nitrogen monoxide may play a protective role against oxidative damage caused by ferryl myoglobin by reducing it to metmyoglobin.

In order to determine the physiological importance of this reaction, it was important to know:

- What is the rate of the reaction between nitrogen monoxide and ferryl myoglobin?
- Are there any reactive intermediates formed in the course of this reaction and what are the products of the reaction?
- What is the rate and what are the products of the reaction between nitrite and ferryl myoglobin and can this reaction contribute to nitration of tyrosine under physiological conditions?

Analogous reactions were carried out also with hemoglobin. In addition, we wanted to investigate whether we can use iron porphyrin model complexes to study these reactions.

1.4 References

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Chapter 2

Kinetic and mechanistic studies of the reactions of nitrogen monoxide and nitrite with ferryl myoglobin

2.1 Introduction

The mechanism of tissue damage caused by reperfusion of an ischemic tissue is not well understood yet. It has been proposed to be triggered by the formation of reactive oxygen and nitrogen species such as superoxide, hydrogen peroxide, hydroxyl radical and oxoperoxonitrate(1-) [1-4].

The reaction between hydrogen peroxide (present in concentrations as high as 10 μM in ischemic heart muscle [5]) and myoglobin has been suggested to be a key determinant of oxidative damage in the ischemic and then reoxygenated heart [6]. Hydrogen peroxide reacts with deoxymyoglobin (MbFeII) (also present in elevated concentration in ischemic oxygen-poor tissues) to generate the highly oxidizing species ferryl myoglobin (MbFeIV=O). In addition, the reaction of metmyoglobin (MbFeIII) with hydrogen peroxide generates the one-electron oxidized form of ferryl myoglobin, which has an additional transient radical on the globin, •MbFeIV=O [7]. The radical species generated from this reaction can damage the cell membrane, induce the release of myoglobin from ruptured myocytes and thus lead to destabilization and subsequent liberation of

free iron, providing the potential of hydroxyl radical formation [2, 8]. There is
direct evidence that the high iron(IV) oxidation state of myoglobin is formed in vivo. Indeed, it has been identified, through derivatization with sodium sulfide
to form sulfomyoglobin [9], in isolated ischemic rat hearts [10].

Ferryl myoglobin is a strong oxidant that can promote oxidation,
peroxidation [8, 11, 12], and epoxidation of various biomolecules in vitro [7].
The reaction of several antioxidant species such as β-carotene, ascorbate, thiols,
and vitamin E with ferryl myoglobin has been the subject of various studies [7].
The reduction rates are mostly not very high [13]. For instance, the rate constant
for the reaction of ferryl myoglobin with ascorbate (present in μM concentrations in several cells) is 2.7±0.8 M⁻¹s⁻¹ at pH 7.0 and 25 °C [13].
However, it has been proposed that the presence of these one-electron reductants may be essential to avoid the accumulation of ferryl myoglobin and
thus to prevent cell damage in reperfused tissues.

It has repeatedly been reported that nitrogen monoxide may also act as an
antioxidant and inhibit ferryl myoglobin-induced oxidative damage [3, 14-19].
Indeed, nitrogen monoxide can reduce ferryl myoglobin to metmyoglobin [14, 16], modulate ferryl myoglobin-mediated oxidation of low density lipoproteins
[14], and inhibit ferryl myoglobin protein radical-catalyzed oxidation reactions
[16]. Despite the physiological importance attributed to the reaction between
nitrogen monoxide and ferryl myoglobin, its rate constant has never been
determined. This information is indispensable in order to evaluate the relevance
of this reaction in vivo.

As nitrite is one of the major end products of nitrogen monoxide
metabolism, its local concentration reflects that of nitrogen monoxide.
Increased nitrite levels are thus found under pathophysiological conditions such
as inflammation, when nitrogen monoxide production is elevated. For instance,
high levels of nitrite have been found in synovial fluids of patients with
rheumatoid arthritis [20] and nitrite concentrations as high as 36 μM have been
measured in human serum of immunodeficiency virus-infected patients with interstitial pneumonia [21].

In contrast to the protective properties of nitrogen monoxide against hydrogen peroxide-mediated tissue injuries, nitrite dramatically enhances hydrogen peroxide toxicity, in particular in the presence of hemoproteins [22]. It has been suggested that the increased toxicity is due to the formation of nitrogen dioxide from the reaction of nitrite with the ferryl form of proteins such as myoglobin and peroxidases. Nitrogen dioxide is a toxic substance known to react with thiols [23], to cause lipid peroxidation [17, 24], and to damage deoxyribonucleic acid (DNA) [17, 22]. Moreover, the peroxidase-catalyzed oxidation of nitrite has been proposed to represent an alternative source of tyrosine nitration [25]. In addition to the oxoperoxonitrate(1-)-mediated nitration [26-28], it may contribute to cell and tissue injury under conditions of increased nitrogen monoxide production. The biological significance of tyrosine nitration is a subject of great interest, because extensive evidence supports the formation of 3-nitrotyrosine in vivo under diverse pathological conditions (for review see [28]).

In this paper we present detailed kinetic and mechanistic studies on the reactions of ferryl myoglobin with nitrogen monoxide and with nitrite. The rate constant determined for the nitrogen monoxide-mediated reduction of ferryl myoglobin is very large, \((18\pm1) \times 10^6 \text{ M}^{-1}\text{s}^{-1}\) at pH 7.5 and 20 °C. Thus, we conclude that this reaction is very likely to represent a plausible route for inhibition of ferryl myoglobin-mediated oxidative damage. In contrast, the reaction between nitrite and ferryl myoglobin is relatively slow, \(16\pm2 \text{ M}^{-1}\text{s}^{-1}\) at pH 7.5 and 20 °C. This reaction probably plays a role only when nitrogen monoxide has been completely depleted and no oxoperoxonitrate(1-) is present. The results reported are important for a better understanding of the interaction of nitrogen monoxide with hemoproteins with oxidase activity under inflammatory or ischemic conditions, when generation of both nitrogen monoxide and hydrogen peroxide is elevated.
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2.2 Materials and methods

Reagents
Buffer solutions were prepared from K$_2$HPO$_4$/KH$_2$PO$_4$ (Fluka) or from Na$_2$B$_4$O$_7$·10H$_2$O/NaOH (Fluka) with deionized Milli-Q water. Sodium nitrite, sodium nitrate, and hydrogen peroxide were supplied from Fluka. Catalase (bovine liver, 17'000 units/mg protein) was obtained from Sigma.

Nitrogen monoxide was obtained from Linde and passed through a degassed sodium hydroxide solution as well as a column of sodium hydroxide pellets to remove higher nitrogen oxides before use. A saturated nitrogen monoxide solution was prepared by degassing water for 45 minutes with nitrogen and then saturating it with nitrogen monoxide. The obtained stock solution (about 2 mM) was diluted with degassed buffer in gas-tight SampleLock Hamilton syringes. The final nitrogen monoxide concentrations were measured with an ANTEK Instruments nitrogen monoxide analyzer, with a chemiluminescence detector.

Horse heart metmyoglobin was purchased from Sigma and purified over a Sephadex G-25 column. The concentration of hydrogen peroxide was determined spectrophotometrically at 240 nm (ε$_{240}$ = 39.4 M$^{-1}$cm$^{-1}$) [29]. Ferryl myoglobin was prepared by adding 7–15 equivalents of hydrogen peroxide to a metmyoglobin solution at room temperature. After a reaction time of 5–20 minutes, depending on the pH, the ferryl myoglobin solutions were stored on ice and used within an hour. In some cases catalase was added prior to reaction with nitrogen monoxide to destroy excess hydrogen peroxide. However, this procedure proved not to be necessary as identical kinetic results were obtained with or without addition of catalase. The concentration of the metmyoglobin solutions was determined by measuring the absorbances at 408, 502, and 630 nm (ε$_{408}$ = 188 mM$^{-1}$cm$^{-1}$, ε$_{502}$ = 10.2 mM$^{-1}$cm$^{-1}$, and ε$_{630}$ = 3.9 mM$^{-1}$cm$^{-1}$) at neutral pH as well as at acidic pH and at 411, 539 and 585 nm (ε$_{411}$ = 119 mM$^{-1}$cm$^{-1}$, ε$_{539}$ = 8.8 mM$^{-1}$cm$^{-1}$, and ε$_{585}$ = 7.8 mM$^{-1}$cm$^{-1}$) at pH 9.5 [30].
concentration of the ferryl myoglobin solutions were determined by measuring the absorbance at 421 nm ($\varepsilon_{421} = 111 \text{ mM}^{-1}\text{cm}^{-1}$) [31].

**Stopped-flow kinetic analysis**

Kinetic studies were carried out with an On-Line Instrument System stopped flow instrument equipped with an OLIS RSM 1000 rapid scanning monochromator and with an Applied Photophysics SX17MV single-wavelength stopped-flow instrument. The pathlengths of the cells in the two spectrophotometers are 2 and 1 cm, respectively. With the Applied Photophysics apparatus, kinetic traces were taken at different wavelengths between 400 and 635 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, given as the mean ± 2 × standard deviation. Care was taken that the absolute absorbance of the reaction mixture was not higher than one absorbance unit.

For the measurements between pH 6.5 and 9.5 the ferryl myoglobin and nitrogen monoxide solutions were both prepared in 0.1 M buffers of the required pH value. Measurements at pH 5.9 were carried out by mixing a ferryl myoglobin solution prepared in a 0.1 M phosphate buffer at pH 6.5 with a nitrogen monoxide solution at pH 4.0. The pH was measured at the end of the reactions for control.

**UV-visible spectra**

UV-visible spectra were collected on a UVIKON 820 spectrophotometer. To determine the yield of the reaction between nitrogen monoxide and ferryl myoglobin spectra were measured in sealable cells for anaerobic applications. About 3 mL of a 100 μM metmyoglobin solution were placed in the cell and mixed with about 3 equivalents of hydrogen peroxide (100 μL of a 0.01 M
When the ferryl myoglobin had completely formed the cell was sealed and about 150 μL of nitrogen monoxide-saturated solution were added.

**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) precolumn as described previously in reference [32]. A phtalic acid solution (2.5 mM phtalic acid, 5% acetonitrile, pH 4.2, TRIS) was used as eluent. Calibration curves were obtained by measuring 5–10 standard sodium nitrite and sodium nitrate solutions in 1 mM sodium phosphate buffer.

The protein samples were prepared by adding to 20 mL of a metmyoglobin solution (25–50 μM in 2 mM phosphate or 1 mM borate buffer) 7–15 equivalents hydrogen peroxide. After a reaction time of 5–20 minutes, that is when the UV-visible spectrum indicated that ferryl myoglobin had been formed completely, excess hydrogen peroxide was destroyed by addition of about 100 μL of a solution of catalase in water (1 mg/mL). After 10 minutes the solution was placed in a 20 mL Schlenk flask sealed with a rubber septum and the required amount of nitrogen monoxide (250–500 μL of a saturated 2 mM nitrogen monoxide-solution) was added with a gas-tight Hamilton syringe under constant stirring. A long needle was used in order to add the nitrogen monoxide solution at the bottom of the flask to avoid any contact of nitrogen monoxide with the oxygen present in the head space of the flask. After about five minutes the reaction mixture was diluted with buffer and analyzed. The amount of contaminating nitrite and nitrate present in the nitrogen monoxide-solution was determined prior to each experiment by injecting 1 mL of the saturated nitrogen monoxide-solution with a gas-tight syringe into 9 mL of dioxygen-saturated water placed in a sealed 10 mL Schlenk flask. After stirring for about ten minutes the reaction mixture was diluted and analyzed. With this procedure
nitrite is quantitatively formed from the reaction of nitrogen monoxide with dioxygen in water. Thus, the amount of nitrite found in excess relative to the nitrogen monoxide used for these experiments corresponded to the amount of nitrite already present in the nitrogen monoxide solution. In some cases also nitrate was found as a contaminant and probably derived from oxygen impurities in the apparatus. The protein samples of the reactions with nitrite were prepared analogously by adding 100–200 μL of a 40–60 mM sodium nitrite solution in 0.1 M phosphate buffer.

Analysis of the free 3-nitrotyrosine content generated by the reaction of nitrite with ferryl myoglobin in the presence of added free tyrosine

Ferryl myoglobin, prepared by mixing 1.5 mL of a 295 μM metmyoglobin solution with one equivalent of hydrogen peroxide (44 μL of a 0.01 M solution), was allowed to react with a solution containing 4 equivalents of tyrosine and different amounts of nitrite (between 4 and 50 equivalents). The final solution, which contained 250 μM protein, was analyzed by high performance liquid chromatography (Table 6, entries 1–3) with a Hewlett Packard Series 1050 apparatus with a Series 1100 UV/Vis-Detector, equipped with a VYDAC 218TP54 Protein&Peptide C18-Column (250 x 4.6 mm). Solvent A was 0.07% trifluoracetic acid in water and solvent B 0.07% trifluoracetic acid in acetonitrile. 3-Nitrotyrosine was eluted (about 8 minutes after injection) by keeping the amount of B constant (5%) in the first two minutes and then by using an increasing linear gradient of B from 5 to 10 % between 2 and 10 minutes, and from 10 to 80 % between 10 and 15 minutes. It was detected simultaneously at 220, 280, 350, and 400 nm and was quantified by measuring a calibration curve of 5–10 3-nitrotyrosine standard solutions.

Alternatively, reactions were carried out by mixing 1.5 mL of a 295 μM metmyoglobin solution containing 4 equivalents of tyrosine and different amounts of nitrite (between 4 and 50 equivalents) with a solution containing one equivalent of hydrogen peroxide (relative to the protein). The resulting
protein solution, which contained 250 µM protein, was analyzed by high performance liquid chromatography as described above (Table 6, entries 4–7).

*Analysis of the 3-nitrotyrosine content after reaction of ferryl myoglobin with nitrogen monoxide and nitrite*

A ferryl myoglobin protein radical solution in 0.1 M phosphate buffer pH 7.0 was prepared by adding one equivalent of hydrogen peroxide to metmyoglobin (320 µM) [33, 34]. After 80 s, the time needed for generation of the ferryl myoglobin protein radical, different amounts of nitrogen monoxide (0.1 or 0.5 equivalents) were added under stirring. When the reaction was complete (in less than a minute), nitrite was removed by washing the solution through a 10’000 MW cut-off filter (Centriplus YM-10, Amicon, Switzerland) at 3000 g until the ultrafiltrate did not show any qualitative reaction (pink colouring) with the Griess reagent [35]. The resulting protein was hydrolyzed by treating it (0.5 mL of a 320 µM solution) for 24 h with 0.3 mL 6 M HCl at 110 °C in a closed vial. The solution was allowed to dry on air by maintaining the temperature at 110 °C and by opening the vial. The residual was redissolved in 50 µl water and analyzed by high performance liquid chromatography as described above.

The 3-nitrotyrosine content in myoglobin after reaction of ferryl myoglobin with nitrite was measured analogously. A ferryl myoglobin solution in 0.1 M phosphate buffer pH 7.0, prepared by adding two equivalents of hydrogen peroxide to a metmyoglobin solution (160 µM), was allowed to react with nitrite (either 0.2 or 10 equivalents). When the reaction was over (in less than a minute), nitrite was washed by centrifugation (see above), the resulting protein was hydrolyzed, and the amino acids were analyzed by high performance liquid chromatography as described above.
2.3 Results

2.3.1 Stopped-flow kinetic studies of the nitrogen monoxide-mediated reduction of ferryl myoglobin

The reaction between nitrogen monoxide and ferryl myoglobin (horse heart) was studied by single-wavelength stopped-flow spectroscopy in the pH range 5.9 to 9.5 at 20 °C. In order to avoid the difficulties associated with the accurate determination of the concentration of nitrogen monoxide solutions during the measurements, the protein was present in 8–10-fold excess to maintain pseudo first-order conditions. The kinetic traces were measured by following the absorbance changes at several wavelengths in the Soret region. Over the entire pH range studied the reaction proceeds via an intermediate that decays to the final product metmyoglobin. As depicted in Figure 1, the kinetic trace collected at pH 9.5 at 411 nm clearly shows a rapid increase in the absorbance followed by a slower decrease. At pH 7.0 the trace measured at 410 nm shows a biphasic increase in absorbance which could be fitted well to two single exponential expressions (Figure 2). In both cases the two reactions correspond to the formation of the intermediate and its subsequent decay. The second-order rate constants (k_i) for the formation of the intermediate, obtained from the linear plots of the observed pseudo first-order rate constants versus ferryl myoglobin concentration (Figure 3), did not change significantly in the pH range studied (Figure 3 and Table 1). At pH 7.5 we obtained k_i = (18±1) \times 10^6 \text{ M}^{-1}\text{s}^{-1} for the nitrogen monoxide-mediated reduction of ferryl myoglobin. The same value for the second-order rate constant was obtained when the reaction was studied with nitrogen monoxide as the reagent used in 10-fold excess. Moreover, the same rate constant was also found when ferryl myoglobin was prepared at pH 8.5 and then mixed with a pH 6.5 nitrogen monoxide-solution to yield a final pH of 7.0. Alkaline conditions are known to generate the lowest amount of side products.
in the course of the reaction between ferryl myoglobin and hydrogen peroxide [36, 37].

**Figure 1** Time course measured at 411 nm for the reaction of ferryl myoglobin (8.1 μM) with nitrogen monoxide (0.9 μM) in 0.1 M borate buffer at pH 9.5 and 20 °C.

**Figure 2** Time course measured at 410 nm for the reaction of ferryl myoglobin (4.7 μM) with nitrogen monoxide (0.6 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 and the decay of the intermediate O-nitrito metmyoglobin. The resulting rate constants are $k_{1,\text{observed}} = 80 \text{ s}^{-1}$ and $k_{2,\text{observed}} = 5.8 \text{ s}^{-1}$, respectively.
Figure 3 Plots of $k_{1,\text{observed}}$ versus ferryl myoglobin concentration for the formation of the intermediate $O$-nitrito metmyoglobin from the reaction of ferryl myoglobin with nitrogen monoxide at pH 7.0 (---) and 9.5 (- o -) (20 °C). The values of the second-order rate constants obtained from the linear fits are given in Table 1.

Table 1 pH-dependencies of the second-order rate constants for the formation ($k_1$) of the intermediate $O$-nitrito metmyoglobin and of the observed rate constants for its decay ($k_2$) to metmyoglobin at 20 °C.

<table>
<thead>
<tr>
<th>pH</th>
<th>$k_1$ ($\mu M^{-1}s^{-1}$)</th>
<th>$k_2$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.9</td>
<td>17±1</td>
<td>21±6</td>
</tr>
<tr>
<td>6.5</td>
<td>18.7±0.4</td>
<td>11±2</td>
</tr>
<tr>
<td>7.0</td>
<td>17.1±0.6</td>
<td>6.0±0.4</td>
</tr>
<tr>
<td>7.5</td>
<td>18±1</td>
<td>3.4±0.4</td>
</tr>
<tr>
<td>8.5</td>
<td>19.5±0.2</td>
<td>1.3±0.2</td>
</tr>
<tr>
<td>9.5</td>
<td>16.6±0.8</td>
<td>0.35±0.04</td>
</tr>
</tbody>
</table>

As expected, the rate constant for the decay of the intermediate to metmyoglobin ($k_2$), measured within a broad range of nitrogen monoxide and ferryl myoglobin concentrations, was independent from the nitrogen monoxide
Chapter 2

as well as the ferryl myoglobin concentrations. Moreover, the same rate constant was 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 nitrogen monoxide binding to the product metmyoglobin. The rate of decay of O-nitrito metmyoglobin is highly pH-dependent and increases with decreasing pH (Table 1). At pH 7.5 we obtained $k_2 = 3.4 \pm 0.4$ s$^{-1}$ for the rate of decay of the intermediate to metmyoglobin.

2.3.2 Spectral characterization of the intermediate O-nitrito metmyoglobin

The nitrogen monoxide-mediated reduction of ferryl myoglobin to metmyoglobin was studied by rapid-scan UV-visible spectroscopy between 380 and 680 nm at pH 9.5 and 20 °C. As shown in Figure 4, the Soret band of myoglobin shifted from 421 nm (ferryl myoglobin, spectrum 1) to 411 nm (metmyoglobin at pH 9.5, spectrum 10) via an intermediate species with an absorption maximum at 410 nm and an extinction coefficient of about 137 mM$^{-1}$cm$^{-1}$ (spectrum 6 in Figure 4). The best spectrum obtained for the intermediate in the visible region is shown as the first trace in Figure 5. As higher concentrations were used to reduce the signal to noise ratio, accumulation of the intermediate occurred within the dead time of the instrument. Two characteristic absorption maxima were found at 631 nm ($\varepsilon_{631} = 5.1$ mM$^{-1}$cm$^{-1}$) and 504 nm ($\varepsilon_{504} = 8.7$ mM$^{-1}$cm$^{-1}$). These maxima are very similar to those of the recently characterized oxoperoxonitrato methemoglobin complex (Mb[Fe$^{III}$OONO]$^{2+}$) [38] and are typical for high-spin methemoglobin and metmyoglobin derivatives with anionic oxygen-ligands such as
carboxylates [30], nitrite [39] and nitrate (Table 2). We thus assign this species as the $O$-nitrito metmyoglobin complex $\text{Mb}[\text{Fe}^{III}\text{ONO}]^{2+}$ (Scheme 1).

Figure 4 Rapid-scan UV-visible spectra of the reaction of ferryl myoglobin (3.7 µM) with nitrogen monoxide (4.7 µM) in 0.1 M borate buffer at pH 9.5, 20 °C. The formation of the intermediate $O$-nitrito metmyoglobin (trace 6) from ferryl myoglobin, dashed traces 1 to 5, and its decay to metmyoglobin, traces 6 to 10, are presented. Time intervals of the shown spectra are: traces 1 to 5, every 16 ms; trace 6 after 320 ms; traces 7 to 9 every 1.6 s, and trace 10 after a total of 9.84 s.
Figure 5 Rapid-scan UV-visible spectra of the reaction of ferryl myoglobin (14.7 μM) with nitrogen monoxide (50 μM) in 0.1 M borate buffer at pH 9.5, 20 °C. The decay of the intermediate O-nitrito metmyoglobin to metmyoglobin is presented. Time intervals of the shown spectra are: traces 1 to 6, every 400 ms; trace 7, 8 s later for a total of 10 seconds.

Table 2 Spectroscopic data for metmyoglobin complexes.

<table>
<thead>
<tr>
<th></th>
<th>Soret</th>
<th>Visible</th>
<th>Ref.</th>
</tr>
</thead>
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<tr>
<td></td>
<td>$\lambda_{\text{max}}$ (ε)$^a$</td>
<td>$\lambda_{\text{max}}$ (ε)$^a$</td>
<td>$\lambda_{\text{max}}$ (ε)$^a$</td>
</tr>
<tr>
<td>Mb[Fe$^{III}$ONO]$^{2+}$</td>
<td>410 (137)</td>
<td>504 (8.7)</td>
<td>534sh</td>
</tr>
<tr>
<td></td>
<td></td>
<td>575 (6.3)</td>
<td>631 (5.1)</td>
</tr>
<tr>
<td>Mb[Fe$^{III}$NO$_2$]$^{2+}$</td>
<td>412 (137)</td>
<td>502 (8.4)</td>
<td>537sh</td>
</tr>
<tr>
<td></td>
<td></td>
<td>573 (5.4)</td>
<td>628 (4.2)</td>
</tr>
<tr>
<td>Mb[Fe$^{III}$ONO$_2$]$^{2+}$</td>
<td>408 (172)</td>
<td>502 (8.8)</td>
<td>629 (3.6)</td>
</tr>
<tr>
<td>Mb[Fe$^{III}$OONO]$^{2+}$</td>
<td>410 (138)</td>
<td>504 (8)</td>
<td>636 (3.2)</td>
</tr>
<tr>
<td>Mb[Fe$^{III}$OH$_2$]$^{3+}$</td>
<td>408 (188)</td>
<td>502 (10.2)</td>
<td>630 (3.9)</td>
</tr>
</tbody>
</table>

$^a \lambda_{\text{max}}$ (nm), ε (mM$^{-1}$cm$^{-1}$)

Scheme 1

\[ \text{MbFe}^{IV}=O \rightleftharpoons \text{MbFe}^{III}-O^* + \text{NO}^* \xrightarrow{k_1} \text{Mb[Fe}^{III}\text{ONO}]^{2+} \xrightarrow{k_2} \text{MbFe}^{III}\text{OH}_2 + \text{NO}_2^- \]
2.3.3 UV-visible spectra of the protein product and yield of the reaction

In order to determine the stoichiometry of the reaction of nitrogen monoxide with ferryl myoglobin and the purity of the metmyoglobin formed, the reaction products were analyzed by UV-visible spectroscopy. As shown in Figure 6, when about one equivalent of nitrogen monoxide was mixed with ferryl myoglobin at pH 7.0 a species was formed (spectrum C) with an absorbance spectrum similar but not identical to that of metmyoglobin (spectrum A). In particular, a new absorbance band with a maximum around 590 nm appeared and the band at 630 nm was modified as well. However, when a ferryl myoglobin solution was allowed to decay back to metmyoglobin a very similar altered spectrum was obtained (spectrum D in Figure 6) (as in [40]). The new absorbance band at 590 nm is characteristic for a heme-protein cross-linked form. This species is known to be partly generated in the course of the reaction of hydrogen peroxide with metmyoglobin under acidic as well as neutral conditions [41] and is thus already present in the ferryl myoglobin solution.

It has previously been shown [36, 37] that the reaction between metmyoglobin and hydrogen peroxide proceeds with the minimum amount of side reactions between pH 8.0 and 9.0. Ferryl myoglobin was thus generated by mixing metmyoglobin and hydrogen peroxide at pH 8.5. When this solution was allowed to react with one equivalent of a pH 6.5 nitrogen monoxide-solution, the spectrum of the product obtained at a final pH of 7.0 was identical to that of metmyoglobin (data not shown). Furthermore, as shown in Figure 7, when about one equivalent of nitrogen monoxide was mixed with ferryl myoglobin at pH 9.5 a species was formed (spectrum C) with an absorbance spectrum almost identical to that of metmyoglobin under the same conditions (spectrum A). When this reaction solution was neutralized to pH 7.0 the new spectrum
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(spectrum D in Figure 7) was identical to that of metmyoglobin at pH 7.0 (spectrum A in Figure 6).

Figure 6 UV-visible spectra in 0.1 M phosphate buffer at pH 7.0. A: metmyoglobin (100 μM); B: ferryl myoglobin (100 μM); C: ferryl myoglobin (100 μM) + 110 μM nitrogen monoxide; D: ferryl myoglobin (100 μM) decayed to metmyoglobin after about 2.5 h.

Figure 7 UV-visible spectra in 0.1 M borate buffer at pH 9.5. A: metmyoglobin (100 μM); B: ferryl myoglobin (100 μM); C: ferryl myoglobin (100 μM) + 110 μM nitrogen monoxide; D: Spectrum C at pH 7.0.
2.3.4 Reaction between the ferryl myoglobin protein radical and nitrogen monoxide

It has been reported that in concentrated solutions (300 μM – 1 mM) metmyoglobin reacts with equimolar amounts of hydrogen peroxide to yield a two-electron oxidized form with a radical on the protein (*MbFe$^{IV}$=O) [33, 34]. The radical in *MbFe$^{IV}$=O is probably localized on a tyrosine and/or a tryptophan residue and is stable up to several minutes [33, 34]. An attempt was thus made, by using the sequential stopped-flow technique, to study the reaction between the ferryl myoglobin protein radical and nitrogen monoxide. In particular, we were interested to find out whether nitrogen monoxide reacts with the oxoiron(IV) center or with the radical on the protein.

A ferryl myoglobin protein radical-solution was first generated in the stopped-flow apparatus by premixing equimolar amounts of metmyoglobin and hydrogen peroxide (about 350 μM). After a delay time between 10 and 500 seconds the ferryl myoglobin protein radical solution was mixed with a nitrogen monoxide solution. The reaction was studied by following the absorbance changes at 540 and 590 nm. Unfortunately, we could not get any useful information from the first 50 ms of the measured traces because of inhomogeneous mixing. This problem is often observed when highly concentrated viscous protein solutions are mixed in the stopped-flow apparatus. Thus, it was not possible to identify whether there was a difference in the absorbance changes arising from the reaction of nitrogen monoxide with ferryl myoglobin and those from the reaction with the ferryl myoglobin protein radical.

It has recently been shown that the reaction of nitrogen monoxide with the tyrosyl radicals of prostaglandin H synthase [42], prostaglandin endoperoxide H synthase [43], and photosystem II [44, 45] generates 3-nitrotyrosine. Therefore, we looked for 3-nitrotyrosine in the protein after the reaction of the ferryl
myoglobin protein radical with nitrogen monoxide. However, high performance liquid chromatography-analysis of the amino acids obtained after complete acid hydrolysis indicated that no 3-nitrotyrosine was generated (data not shown).

2.3.5 Stopped-flow kinetic studies of the nitrite-mediated reduction of ferryl myoglobin

The reaction between nitrite and ferryl myoglobin was studied by rapid-scan stopped-flow spectroscopy between 380 and 650 nm. As shown in Figure 8, when a large excess of nitrite was mixed with ferryl myoglobin at pH 7.0 the Soret band shifted from 421 nm (ferryl myoglobin, spectrum 1) to 408 nm (metmyoglobin at pH 7.0, spectrum 9). In addition, the two characteristic absorption maxima at 547 and 584 nm disappeared and the characteristic maxima for metmyoglobin at 502 and 630 nm appeared (Figure 9). However, an additional band was observed at 586 nm, that is at a slightly lower wavelength than that of the maximum arising from the cross-linked species generated during the synthesis of ferryl myoglobin (590 nm, see above). As N-nitrito metmyoglobin has an absorbance maximum at 573 nm (Figure 10), the new absorbance band at 586 nm suggests the formation of a mixture of metmyoglobin, the cross-linked species and N-nitrito metmyoglobin.
Figure 8 Rapid-scan UV-visible spectra of the reaction of ferryl myoglobin (5.5 μM) with nitrite (9.6 mM) in 0.1 M phosphate buffer at pH 7.0, 20 °C. Time intervals of the shown spectra are: traces 1 to 5, every 800 ms; traces 6 to 8 every 1.6 s and trace 9 after a total of 25.6 s.

Figure 9 Rapid-scan UV-visible spectra of the reaction of ferryl myoglobin (78 μM) with nitrite (9.6 mM) in 0.1 M phosphate buffer at pH 7.0, 20 °C. Time intervals of the shown spectra are: traces 1 to 5, every 800 ms; traces 6 to 8 every 1.6 s and trace 9 after a total of 25.6 s.
Figure 10 Comparison of the UV-visible spectra of the intermediate $O$-nitrito metmyoglobin and of $N$-nitrito metmyoglobin in 0.1 M phosphate buffer at pH 7.0.

Kinetic measurements were carried out by single-wavelength stopped-flow spectroscopy under pseudo first-order conditions with nitrite in at least 10-fold excess in the pH range 6.1 to 8.5 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 (Figure 11). The second-order rate constants ($k_3$) obtained from the linear plots of the observed pseudo first-order rate constants versus nitrite concentration (data not shown), are highly pH-dependent (Table 3). At pH 6.1 we obtained $k_3 = (3.7 \pm 0.8) \times 10^2$ M$^{-1}$s$^{-1}$. With increasing pH the second-order rate constant decreased continuously to $k_3 = 1.4 \pm 0.1$ M$^{-1}$s$^{-1}$ at pH 8.5. The large standard deviation of the value at pH 6.1 is due to the instability of the ferryl myoglobin solution under acidic conditions [46-48]. This instability did not cause any problems for the determination of the rate constant of the reaction with nitrogen monoxide because this latter reaction proceeds at a significantly faster rate. In addition, at low pH and in the presence of a large excess of nitrite a second reaction was observed in the course of the stopped-flow measurements. The presence of this reaction, which corresponds to the subsequent binding of nitrite to metmyoglobin, also interfered with the exact
determination of the rate constant of the reaction between nitrite and ferryl myoglobin.

![Graph](image)

**Figure 11** Time course measured at 410 nm for the reaction of ferryl myoglobin (6 μM) with nitrite (2.0 mM) in 0.1 M phosphate buffer at pH 6.7 and 20 °C. The solid line corresponds to the best fit which results in the observed rate constant of $14.4 \times 10^{-2}$ s$^{-1}$.

**Table 3** pH-dependencies of the second-order rate constants for the reactions of nitrite with ferryl myoglobin ($k_3$) at 20 °C. pH-dependencies of the association ($k_4$) and dissociation ($k_{-4}$) rates of the reaction of nitrite with metmyoglobin at 20 °C together with the calculated equilibrium constants ($K_4$).

<table>
<thead>
<tr>
<th>pH</th>
<th>$k_3$ (M$^{-1}$s$^{-1}$)</th>
<th>$k_4$ (M$^{-1}$s$^{-1}$)</th>
<th>$k_{-4}$ (s$^{-1}$)</th>
<th>$K_4$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1</td>
<td>$(3.7\pm0.8) \times 10^2$</td>
<td>$(2.4\pm0.2) \times 10^3$</td>
<td>19±4</td>
<td>$(0.8\pm0.2) \times 10^{-2}$</td>
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<td>6.7</td>
<td>65±6</td>
<td>$(8.1\pm0.4) \times 10^2$</td>
<td>9.8±0.6</td>
<td>$(1.21\pm0.04) \times 10^{-2}$</td>
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<tr>
<td>7.0$^a$</td>
<td>-</td>
<td>470</td>
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<tr>
<td>7.5</td>
<td>16±2</td>
<td>325±4</td>
<td>4.0±0.4</td>
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<tr>
<td>8.5</td>
<td>1.4±0.1</td>
<td>n.d.$^b$</td>
<td>n.d.$^b$</td>
<td>n.d.$^b$</td>
</tr>
<tr>
<td>9.15$^a$</td>
<td>-</td>
<td>42</td>
<td>1.1</td>
<td>$2.6 \times 10^2$</td>
</tr>
</tbody>
</table>

$^a$Ref [30]

$^b$n.d. = not determined

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In order to be able to confirm that the second reaction observed was indeed that between nitrite and metmyoglobin, we determined its rate constant in an independent experiment under the same experimental conditions. The kinetics were studied by stopped-flow spectroscopy with nitrite in at least 10-fold excess by following the absorbance changes at 410 or 575 nm. The plot of the observed pseudo first-order rate constant versus nitrite concentration showed a saturation behaviour at nitrite concentrations above 100 mM (data not shown). However, a second-order rate constant could be obtained from the linear part of the plots in the range 5–40 mM. As shown from the data summarized in Table 3, the binding rate of nitrite to metmyoglobin decreases with increasing pH and is always faster than the corresponding rate of reaction of nitrite with ferryl myoglobin. At pH 6.7 and 20 °C the binding rate is \( k_4 = (8.1 \pm 0.4) \times 10^2 \text{ M}^{-1}\text{s}^{-1} \), a value comparable to that reported in the literature (\( 4.7 \times 10^2 \text{ M}^{-1}\text{s}^{-1} \) at pH 7.0 and 21–23 °C, [30]). The dissociation rate, obtained from the intercept of the linear plot is \( k_{-4} = 9.8 \pm 0.6 \text{ s}^{-1} \) at pH 6.7 and decreases with increasing pH (Table 3). This value is also in agreement with that reported in the literature (6.4 s\(^{-1}\) at pH 7.0 and 21–23 °C, [30]).

As the reaction of ferryl myoglobin with nitrite is very slow and the ferryl myoglobin solutions are not indefinitely stable [46-48], it proved not possible to determine the yield of the nitrite-mediated reduction of ferryl myoglobin. Indeed, the reaction of one equivalent of nitrite with ferryl myoglobin is slower than the autoreduction of ferryl myoglobin to metmyoglobin.

### 2.3.6 Analysis of the nitrogen-containing products

The determination of the amount of nitrite and nitrate ions formed from the reaction of ferryl myoglobin with one equivalent of nitrogen monoxide at pH 7.0 and 9.5 was carried out by anion chromatography with conductivity
detection. As shown in Table 4 (columns 2 and 3), nitrite was always formed quantitatively from the nitrogen monoxide-mediated reduction of ferryl myoglobin. The concentration of nitrate remained approximately unchanged in the course of the reaction (columns 4 and 5 in Table 4).

**Table 4** Amount of nitrite formed from the reaction of ferryl myoglobin with one equivalent of nitrogen monoxide. Comparison between the amount of nitrate found after the reaction and that present in the nitrogen monoxide-solution.

<table>
<thead>
<tr>
<th>pH</th>
<th>[MbFe^{IV}=O] (μM)</th>
<th>[NO_2^-]^a (μM)</th>
<th>measured [NO_3^-] in NO* sol.^b (μM)</th>
<th>[NO_3^-] in NO* sol.^c (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0</td>
<td>47.3</td>
<td>46.6</td>
<td>4.3</td>
<td>4.3</td>
</tr>
<tr>
<td>7.0</td>
<td>47.3</td>
<td>47.6</td>
<td>4.3</td>
<td>3.0</td>
</tr>
<tr>
<td>9.5</td>
<td>37.2</td>
<td>38.3</td>
<td>4.1</td>
<td>0.2</td>
</tr>
<tr>
<td>9.5</td>
<td>37.2</td>
<td>39.6</td>
<td>4.1</td>
<td>0.4</td>
</tr>
</tbody>
</table>

^a Concentration of nitrite ions generated from the reaction of ferryl myoglobin with nitrogen monoxide, calculated from the concentration of nitrite ions found in the reaction solution minus the concentration of nitrite already present in the added nitrogen monoxide-solution.

^b Nitrate contamination of the nitrogen monoxide solutions determined separately as described in the experimental procedures.

^c Concentration of nitrate ions generated from the reaction of ferryl myoglobin with nitrogen monoxide, calculated from the concentration of nitrate ions found in the reaction solution minus the concentration of nitrate already present in the added nitrogen monoxide solution (Column 4).

As the nitrite-mediated reduction of ferryl myoglobin is very slow, the identification of the nature of the nitrogen-containing products had to be carried out by mixing an excess of nitrite with ferryl myoglobin. At pH 7.0, we found that one equivalent of nitrite, relative to the amount of ferryl myoglobin, was transformed to half an equivalent of nitrite plus half an equivalent of nitrate (columns 6 and 7 in Table 5).
Table 5 Amount of nitrite and nitrate formed from the reaction of ferryl myoglobin with an excess of nitrite.

<table>
<thead>
<tr>
<th>pH</th>
<th>[MbFe^{IV}=O] (µM)</th>
<th>added [NO_2^-] (µM)</th>
<th>ex. [NO_2^-]^a (µM)</th>
<th>found [NO_2^-]^b (µM)</th>
<th>[NO_2^-]^c (µM)</th>
<th>[NO_3^-]^d (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0</td>
<td>26</td>
<td>217</td>
<td>191</td>
<td>204</td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>(26 + 191)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.0</td>
<td>43</td>
<td>249</td>
<td>206</td>
<td>227</td>
<td>21</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>(43 + 206)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^a Concentration of nitrite added in excess, by considering that only one equivalent reacts with ferryl myoglobin.
^b Total concentration of nitrite ions found in the protein solution after reaction.
^c Concentration of nitrite ions generated from the reaction of ferryl myoglobin 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.

2.3.7 Analysis of 3-nitrotyrosine generation in the course of the reaction between ferryl myoglobin and nitrite in the presence or in the absence of added free tyrosine

Nitrogen dioxide may be generated from the reaction of ferryl myoglobin with nitrite. As it is known that nitrogen dioxide is a potent nitrating agent [17, 24], it was of interest to find out whether 3-nitrotyrosine is generated in the course of the nitrite-mediated reduction of ferryl myoglobin. We thus analyzed the amino acids obtained after complete acid hydrolysis of the reacted protein by high performance liquid chromatography, but no 3-nitrotyrosine was detected. In contrast, when free tyrosine was added to nitrite prior to its reaction with ferryl myoglobin about 1% 3-nitrotyrosine, relative to the protein, was
generated. As shown in Table 6 (entries 1–3) the yield of 3-nitrotyrosine slightly increased with increasing nitrite-concentrations.

Finally, we were interested to find out whether 3-nitrotyrosine was generated when the ferryl myoglobin protein radical was mixed with nitrite in the presence of tyrosine. As summarized in Table 6 (entries 4–7), when hydrogen peroxide was added to a mixture of nitrite, tyrosine, and metmyoglobin, significantly larger amounts of 3-nitrotyrosine were formed. The yield of 3-nitrotyrosine increased with increasing nitrite concentrations.

Table 6 Entries (1–3): yield of 3-nitrotyrosine (% relative to metmyoglobin) from the reaction of ferryl myoglobin (generated by allowing to react a solution of metmyoglobin (250 μM) with 1 equivalent of hydrogen peroxide (250 μM) for 6 minutes) with a solution containing 4 equivalents of tyrosine (1 mM) and variable amounts of nitrite. Entries (4–7): yield of 3-nitrotyrosine (% relative to metmyoglobin) from the reaction of a solution of metmyoglobin (250 μM), 4 equivalents of tyrosine (1 mM), and variable amounts of nitrite with a solution containing 1 equivalent of hydrogen peroxide (250 μM).

<table>
<thead>
<tr>
<th>Entry</th>
<th>[NO₂⁻] (mM)</th>
<th>yield 3-nitrotyrosine (% relative to metmyoglobin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0</td>
<td>0.7±0.1</td>
</tr>
<tr>
<td>2</td>
<td>2.5</td>
<td>0.78±0.04</td>
</tr>
<tr>
<td>3</td>
<td>7.5</td>
<td>0.92±0.04</td>
</tr>
<tr>
<td>4</td>
<td>1.0</td>
<td>3.1±0.4</td>
</tr>
<tr>
<td>5</td>
<td>2.5</td>
<td>5.1±0.6</td>
</tr>
<tr>
<td>6</td>
<td>5.0</td>
<td>7.8±0.4</td>
</tr>
<tr>
<td>7</td>
<td>19</td>
<td>13±1</td>
</tr>
</tbody>
</table>
2.4 Discussion

Nitrogen monoxide is currently a species of extreme biological interest because of the variety of physiological and pathophysiological functions which have been found to be associated with this inorganic messenger molecule [49-51]. The cytotoxic effects of nitrogen monoxide originate mainly from its reaction with superoxide to generate the powerful oxidizing and nitrating agent oxoperoxonitrate(1-). In contrast, nitrogen monoxide has also been reported to display antioxidant effects [3, 15, 17-19] either by scavenging free radicals to generate less reactive nonradical species [17, 52] or by inactivating catalytically active high valent hemoprotein intermediates [15, 18].

In a recent work [16] Gorbunov et al. reported that the oxoiron(IV) myoglobin species ferryl myoglobin and the ferryl myoglobin protein radical react with nitrogen monoxide to generate metmyoglobin. In addition, they showed by electron spin resonance spectroscopy and electrospray mass spectrometry analysis that in the course of the reaction no covalent adducts were generated between nitrogen monoxide and the globin or the heme [53]. The reaction of nitrogen monoxide with high valence myoglobin species has thus been proposed to represent a potential antioxidant role for nitrogen monoxide. However, the determination of the rate constant is essential in order to evaluate the biological relevance of this reaction. In the present work, we carried out detailed kinetic and mechanistic analyses of the nitrogen monoxide-mediated reduction of ferryl myoglobin.

The reaction between ferryl myoglobin and nitrogen monoxide is shown to proceed via the rapid formation of an intermediate, which then decays to metmyoglobin and nitrite. At pH 7.5 and 20 °C the second-order rate constant for the formation of this intermediate is $(18 \pm 1) \times 10^6$ M$^{-1}$s$^{-1}$ and that for its decay is $3.4 \pm 0.4$ s$^{-1}$. The spectrum of the intermediate species displays very characteristic absorption maxima at 631 and 504 nm. A comparison with the
spectra of other metmyoglobin derivatives with anionic ligands such as the \(N\)nitrito- and nitrato metmyoglobin complexes supports the assignment of this species as a high-spin iron(III) complex (Table 2). The position and the intensity of the Soret band are also comparable with the listed derivatives. Because of the radical-like character of the oxo-ligand in ferryl myoglobin [54-57], it is reasonable to assume that the first step of the reaction is a fast radical recombination (Scheme 1). The observed intermediate may thus be assigned as the \(O\)-nitrito metmyoglobin complex \(\text{Mb}[\text{Fe}^{III}\text{ONO}]^{2+}\). However, the UV-visible spectra of the \(N\)-nitrito- and the \(O\)-nitrito metmyoglobin complexes are almost identical. This observation may suggest that in a second very rapid step \(O\)-nitrito metmyoglobin rearranges to \(N\)-nitrito metmyoglobin. In this case, the observed intermediate would be \(N\)-nitrito metmyoglobin, from which nitrite dissociates in the last step of the reaction.

Nevertheless, several observations can be used to argue against this second possibility. We have recently shown that the spectra of \(N\)-nitrito metmyoglobin, nitrato metmyoglobin and oxoperoxonitrato metmyoglobin are also very similar [58]. These three complexes can clearly be distinguished from each other because the rates of dissociation of nitrite and nitrate, respectively, are significantly different [58]. Thus, these results imply that when an anionic ligand is coordinated to metmyoglobin, the spectrum is not strongly influenced by the type of atom directly bound to the high-spin iron(III). Unfortunately, as shown in Tables 1 and 3, the rates of dissociation of nitrite from \(O\)-nitrito metmyoglobin (\(k_2\)) and from \(N\)-nitrito metmyoglobin (\(k_4\)) are very similar and cannot be used to distinguish between the two complexes. This result was unexpected as it is known that nitrite preferentially binds \textit{via} the nitrogen atom to iron. Indeed, no \(O\)-nitrito-iron-complexes are known whereas several \(N\)-nitrito iron(III) complexes, and in particular \(N\)-nitrito iron(III)porphyrin complexes have been synthesized and structurally characterized [59-64]. However, the similarity between the two dissociation rates \(k_2\) and \(k_4\) might also be a coincidence. The dissociation rate from \(O\)-nitrito metmyoglobin (\(k_2\)) has
been determined directly with a very good accuracy. In contrast, the values for
the dissociation rate from N-nitrito metmyoglobin ($k_4$) have been determined
indirectly either from the intercept of the linear plot of the observed pseudo
first-order rate constant versus nitrite concentration (Table 3) or from the
product of the equilibrium constant ($K_4$) and the association constants [30].
These values may thus be associated with a large errors.

The observation that the values of the dissociation constants increase with
decreasing pH imply that a protonated form of the enzyme facilitates nitrite
dissociation. The protonated form of nitrite, hydrogendioxonitrate, cannot play
a role in this reaction. Indeed, the pK$_a$ value for hydrogendioxonitrate is 3.3 and
hydrogendioxonitrate is thus present only in trace amounts in the pH range
studied. With the assumption that the pH-dependence originates from a single
ionizable residue of the protein the following relationship between $k_2$ and the
proton concentration can be derived.

$$k_2 = \frac{k'_2[H^+]}{(K_a + [H^+])}$$

In equation 1 $k'_2$ represents the pH-independent first-order dissociation
constant and $K_a$ the dissociation constant of the protonable residue. Despite the
fact that the reaction was studied at only few pH-values and thus large errors are
associated with the results, the experimental data could be fitted to equation 1.
The best fit gave a value of pK$_a = 6.5 \pm 0.5$ and $k'_2 = 21 \pm 5$ s$^{-1}$. These results
suggest the involvement of a histidine residue, very likely the distal histidine,
which has been shown to be protonated at pH lower than 6 [65]. In the
protonated form this histidine swings out of the heme pocket toward the solvent
with consequent opening of the active site [65] and possible acceleration of the
dissociation rate. Interestingly, the rate constants measured for the rate of decay
of oxoperoxonitrato metmyoglobin to metmyoglobin and nitrate are
significantly larger but show the same pH-dependence as those obtained for the decay of $O$-nitrito metmyoglobin to metmyoglobin and nitrite.

The rate constant for the reaction of nitrogen monoxide with ferryl myoglobin is one order of magnitude larger than that with the corresponding horseradish peroxidase-Compound II ($7.4-13 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ at pH 7.4 and 20 °C [66]) and three orders of magnitude larger than that with myeloperoxidase-oxoiron(IV) ($8 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ at pH 7.0 and 25 °C, [67]). This difference might arise from the negatively charged character of the proximal histidine. The deprotonated histidine is likely to shift the electron density in the direction of the higher valence structure (ferryl myoglobin) relative to the radical structure ($\text{MbFe}^{III}-\text{O}^*$) and thus slow down the radical recombination (Scheme 1). No intermediate has been reported to be generated in the reaction of nitrogen monoxide with horseradish peroxidase- as well as with myeloperoxidase-Compound II. This result suggests that dissociation of nitrite from iron(III) is much faster for horseradish peroxidase and myeloperoxidase than for myoglobin. This difference is not unexpected as it is conceivable that nitrite dissociates faster from the negatively charged myeloperoxidase- or horseradish peroxidase-{$\text{(His}^-)\text{[Fe}^{III}\text{NO}_2]^2+}$ than from the neutral myoglobin-{$\text{(His)[Fe}^{III}\text{NO}_2]^2+}$.

Nitrite is one of the major end products of nitrogen monoxide metabolism. It has been shown to be oxidized by peroxidases such as horseradish peroxidase, myeloperoxidase, and lactoperoxidase in the presence of hydrogen peroxide to most likely generate nitrogen dioxide, which can nitrate tyrosine residues free or incorporated into proteins [22, 25]. The rate constants for the reaction of nitrite with myeloperoxidase-, lactoperoxidase- and horseradish peroxidase-Compound II have recently been reported to vary significantly between the three proteins and to be $(5.5 \pm 0.1) \times 10^2 \text{ M}^{-1}\text{s}^{-1}$ (at pH 7.0 and 15 °C, [68]), $(3.5 \pm 0.1) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ and $13.3 \pm 0.1 \text{ M}^{-1}\text{s}^{-1}$, respectively (at pH 7.0 and room temperature, [69]). In the present work we have measured by stopped-flow
spectroscopy the rate constant of the reaction between ferryl myoglobin and nitrite. The second-order rate constant at pH 7.5 and 20 °C is 16±2 M⁻¹s⁻¹, which is of the same order of magnitude as that of the reaction between nitrite and horseradish peroxidase-Compound II [69], but 1–3 orders of magnitude slower than the corresponding reactions with myeloperoxidase- and lactoperoxidase-Compounds II [68, 69]. The pH-dependence in the range 6.1–8.5 shows that, as was reported for the reaction of nitrite with myeloperoxidase-Compound II [68], the rate of reaction between ferryl myoglobin and nitrite decreases with increasing pH (Table 3). The same trend has been found for the rate of reaction of ferryl myoglobin with reduced nicotinamide adenine dinucleotide [70] and that of autoreduction of ferryl myoglobin [46]. Also for the reaction of ferryl myoglobin with nitrite it can be assumed that the pH-dependence originates from a single ionizable residue and thus the experimental data can be fitted to equation 1. The best fit gave a value of pKᵦ = 6.0±0.5 and k'₃ = (52±9) x 10 M⁻¹s⁻¹. Again, the large errors of these numbers are due to the small number of pH-values studied. The value obtained for the pKᵦ of the protonable residue reveals that when the distal histidine is protonated and, thus, turned out toward the solvent, nitrite can diffuse more rapidly into the active site. Alternatively, it has been shown that ferryl myoglobin, in order to be able to oxidize substrates efficiently, requires the conversion to an activated form in a pH-dependent process [31]. The pKᵦ of ferryl myoglobin has been estimated to be in the region of 6 [71]. Thus, protonation of the ferryl might as well be an explanation for the observed increase in the rate of reaction at lower pH values.

Analysis of the nitrogen-containing products of the reaction between ferryl myoglobin and nitrite reveals that, as has previously been suggested for the corresponding reactions with peroxidases Compound II [17, 22, 24], nitrogen dioxide may be generated in the first step (Scheme 2). In the absence of added substrates nitrogen dioxide rapidly dimerizes to dinitrogen tetroxide, which then
hydrolyzes to nitrite and nitrate. Alternatively, nitrate could be generated from the fast reaction between ferryl myoglobin and nitrogen dioxide [58].

In the presence of added tyrosine nitrogen dioxide leads to the production of small amounts of 3-nitrotyrosine (Table 6, entries 1–3), which are likely to be formed from the fast recombination of nitrogen dioxide with a tyrosyl radical [23]. Two sources are available to generate tyrosyl radicals from tyrosine. Nitrogen dioxide can react with tyrosine to yield tyrosyl radical at a rate of about $10^5 \text{M}^{-1}\text{s}^{-1}$ [23] or, alternatively, ferryl myoglobin can oxidize tyrosine. As the latter reaction proceeds at a rate of about $40 \text{M}^{-1}\text{s}^{-1}$ (data not shown), this pathway is probably less relevant. The reaction is thus likely to proceed as shown in Scheme 3.

**Scheme 2**

\[ \text{MbFe}^{IV}=\text{O} + \text{NO}_2^- \xrightarrow{k_3} \text{MbFe}^{III}\text{OH}_2 + \text{NO}_2^* \]

\[ 2 \text{NO}_2^* \xrightarrow{\text{N}_2\text{O}_4 \text{ and } \text{N}_2\text{O}_4 + \text{H}_2\text{O}} \xrightarrow{\text{NO}_2^- + \text{NO}_3^- + 2 \text{H}^+} \]

or \[ \text{MbFe}^{IV}=\text{O} + \text{NO}_2^* \xrightarrow{\text{MbFe}^{III}\text{OH}_2 + \text{NO}_3^-} \]

**Scheme 3**

\[ 2 \text{MbFe}^{IV}=\text{O} + 2 \text{NO}_2^- \xrightarrow{\text{2 MbFe}^{III}\text{OH}_2 + 2 \text{NO}_2^*} \]

\[ \text{NO}_2^* + \text{Tyr} \xrightarrow{\text{NO}_2^- + \text{Tyr}^*} \]

\[ \text{NO}_2^* + \text{Tyr}^* \xrightarrow{\text{NO}_2^-\text{Tyr}} \]

Significantly larger amounts of 3-nitrotyrosine were obtained when nitrite was reacted *in situ* with the ferryl myoglobin protein radical in the presence of added free tyrosine (Table 6, entries 4–7). These results indicate that, in order to generate higher yields of 3-nitrotyrosine, a further source of tyrosyl radicals must be present in the system. Indeed, tyrosyl radical may be first generated.
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from the reaction of tyrosine with the ferryl myoglobin protein radical and then rapidly recombine with nitrogen dioxide (Scheme 4). At higher nitrite concentrations larger amounts of nitrogen dioxide are formed and trap tyrosyl radicals more efficiently resulting larger yields of 3-nitrotyrosine. This in situ reaction should represent a better model for the physiological conditions, as it is more likely that all the reagents are present simultaneously. Thus, our data imply that the reaction of hydrogen peroxide with metmyoglobin in the presence of nitrite may represent an alternative route, in addition to oxoperoxonitrate(1-)-mediated nitration [26-28], to generate 3-nitrotyrosine in vivo.

Scheme 4

\[
\begin{align*}
\text{MbFe}^\text{III}OH_2 + H_2O_2 & \rightarrow '\text{MbFe}^\text{IV}=O \\
'\text{MbFe}^\text{IV}=O + \text{Tyr} & \rightarrow \text{MbFe}^\text{IV}=O + \text{Tyr}^* \\
\text{MbFe}^\text{IV}=O + \text{NO}_2^- & \rightarrow \text{MbFe}^\text{III}OH_2 + \text{NO}_2^* \\
\text{NO}_2^* + \text{Tyr}^* & \rightarrow \text{NO}_2^-\text{Tyr}
\end{align*}
\]

2.5 Conclusions

In the present work we have determined the rate constant for the reaction between ferryl myoglobin and nitrogen monoxide to be \((18\pm1) \times 10^6 \text{ M}^{-1}\text{s}^{-1}\) at pH 7.5 and 20 °C. This reaction proceeds at a rate comparable to that of the reaction between oxymyoglobin and nitrogen monoxide ((43.6±0.5) \times 10^6 \text{ M}^{-1}\text{s}^{-1} at pH 7.5 and 20 °C [58]). Thus, it may represent a valid alternative reaction between myoglobin and nitrogen monoxide. The high valence form of myoglobin has been proposed to be at least in part responsible for oxidative lesions found on ischemic/reperfused tissues [6]. Other one-electron reductants
present \textit{in vivo} such as ascorbate react with ferryl myoglobin at a significantly lower rate (2.7±0.8 M$^{-1}$s$^{-1}$ at pH 7.0 and 25 °C) [13]. Therefore, as the products generated from the reaction of ferryl myoglobin with nitrogen monoxide are not strong oxidizing species, this reaction might represent a pathway for detoxification of ferryl myoglobin \textit{in vivo}.

In contrast, the reaction between nitrite and ferryl myoglobin proceeds at a significantly lower rate (16±2 M$^{-1}$s$^{-1}$ at pH 7.5 and 20 °C) and generates the nitrating agent nitrogen dioxide. Our results suggest that this reaction may play a role only in the absence of oxoperoxonitrate(1-), which also reacts significantly faster with ferryl myoglobin ((2.2±0.1) × 10$^4$ M$^{-1}$s$^{-1}$ at pH 7.3 and 20 °C, [72]), or when nitrogen monoxide has been completely converted to nitrite. However, we have also shown that, in the presence of nitrite and added free tyrosine, the reaction of metmyoglobin with hydrogen peroxide can yield significant amounts of 3-nitrotyrosine. Under pathophysiological conditions in which the nitrite level can be elevated, this reaction may thus contribute to nitration of tyrosine residues free or incorporated into proteins. The results reported are important for a better understanding of the interaction of nitrogen monoxide with hemoproteins with oxidase activity under inflammatory or ischemic conditions, when generation of nitrogen monoxide, nitrite, and hydrogen peroxide is elevated.

2.6 References

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Chapter 3

Nitrogen monoxide- and nitrite-mediated reduction of ferryl hemoglobin

3.1 Introduction

Ferryl hemoglobin and ferryl myoglobin have been proposed to be partly responsible for oxidative damage observed when ischemic tissues are reperfused [1]. Indeed, it has been shown that these high valent protein forms can oxidize a variety of biomolecules such as liposomes, low density lipoprotein, cholesterol, and unsaturated fatty acids [2–4].

Ferryl hemoglobin and ferryl myoglobin are formed from one-electron oxidation of methemoglobin and metmyoglobin (HbFe^{III}OH_{2} and MbFe^{III}OH_{2}, at neutral pH) with hydrogen peroxide. In vivo, hydrogen peroxide is generated by superoxide dismutation or via enzymatic production by superoxide dismutase. One source of superoxide is the autoxidation of oxymyoglobin or oxyhemoglobin (MbFeO_{2} and HbFeO_{2}, respectively) to metmyoglobin and methemoglobin, respectively [5]. The second oxidizing equivalent of hydrogen peroxide generates a globin-based transient radical (^{*}HbFe^{IV}=O and ^{*}MbFe^{IV}=O). On the globin system of both, myoglobin and hemoglobin, the

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1 Publication: Herold S, Rehmann FJK (2002), to be submitted
radical is located most likely on a tyrosyl residue and on a peroxyl group which is generated by reaction of oxygen with a tryptophan residue radical [3]. In contrast to myoglobins from other species, human myoglobin contains a cysteine residue at position 110. It has been found that oxidation of human metmyoglobin by hydrogen peroxide also generates a transient radical on the thiyl group of the cysteine residue [6, 7]. Both, ferryl hemoglobin and the protein-based radical have been detected in normal human blood [2, 3, 8].

It has recently been shown that in the first step the reaction between metmyoglobin and hydrogen peroxide generates a short-lived species analogous to Compound I of peroxidases, that is the porphyrin π-cation ferryl myoglobin radical (Por^+MbFe^IV=O) [9]. Because of its short lifetime, this intermediate is not detectable in water. However, when experiments were performed in deuterated water, formation of the porphyrin π-cation ferryl myoglobin radical could be observed by the spectrum of an intermediate with a reduced intensity of the Soret band at 409 nm and a band at 648 nm [9]. These absorption features are characteristics for Compound I forms of other heme proteins such as horseradish peroxidase and catalase. The increased stability of the Compound I species has mainly been explained by the reduced rate of the hydrogen peroxide-mediated degradation of Compound I to methemoglobin, water and dioxygen [9]. In deuterated water, hydrogen peroxide is also deuterated. Therefore, due to the isotope effect, the rate of the two-electron oxidation of deutero peroxide to dioxygen is reduced [9]. The instability of the porphyrin π-cation radical in myoglobin compared to that of Compound I in peroxidases is probably due to the close vicinity of the distal histidine residue (histidine-64) to the iron ion in metmyoglobin. It is expected that the transfer of the radical to the protein proceeds via this histidine residue [10]. In accordance, formation of Compound I has also been observed during m-chloroperbenzoic acid-mediated oxidation of site-directed mutants of sperm whale metmyoglobin. In these mutants, the
histidine-64 residue was replaced by amino acid residues such as alanine, leucine, or serine [10, 11].

We have recently shown that the nitrogen monoxide-induced reduction of ferryl myoglobin is very fast at physiological pH ($1.8 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ at pH 7.5 and 20 °C) [12]. Therefore, under pathological conditions characterized by the presence of high concentrations of ferryl myoglobin, this reaction may play an important role for detoxification of the highly oxidizing ferryl myoglobin species. An additional antioxidative function of nitrogen monoxide has been proposed to be the inhibition of propagation of lipid peroxidation. Nitrogen monoxide reacts with lipid alkoxyl and peroxyl radicals to form the less reactive ester products of oxonitric acid and oxoperoxonitric acid, respectively [13-15].

Except for the nitrogen monoxide-induced oxidation of oxyhemoglobin which forms methemoglobin and nitrate [16], nitrite is the major end product of nitrogen monoxide metabolism. Therefore, local concentrations of nitrite correlate with those of nitrogen monoxide. It has been shown that nitrite reacts with oxyhemoglobin to form methemoglobin and nitrate [17]. In order to explain the complex reaction mechanism most authors suggest that the reaction proceeds via formation of the ferryl hemoglobin protein radical as an intermediate [18]. The reactions starts with an initial lag period when methemoglobin, hydrogen peroxide, and nitrogen dioxide would be formed (equation 1). Hydrogen peroxide oxidizes methemoglobin to the ferryl hemoglobin protein radical (equation 2). Subsequently, nitrite may react with the ferryl hemoglobin protein radical to yield two equivalents of nitrogen dioxide (equation 3). One equivalent of nitrogen dioxide would react with oxyhemoglobin to form methemoglobin, nitrite, and one equivalent of dioxygen (equation 4). The other equivalents of nitrogen dioxide react with water to yield nitrite and nitrate (equation 5) [18]. Although the overal stoichiometry of this reaction scheme (equation 6) has been confirmed [17], the detailed mechanism of the nitrite-mediated oxidation of oxyhemoglobin is under discussion.
Nitrite can also be oxidized by high valent forms of peroxidases such as horseradish peroxidase, myeloperoxidase [19, 20], lactoperoxidase [20] and soybean peroxidase [21]. Indeed, it has been shown that, in the presence of hydrogen peroxide and nitrite, these proteins generate nitrogen dioxide. Nitrogen dioxide is a toxic substance known to react with thiols [22, 23], to cause lipid peroxidation [24], and to damage deoxyribonucleic acid (DNA) [25]. In addition, the peroxidase-catalyzed oxidation of nitrite has been proposed to represent a major source of tyrosine nitration [26]. We have shown that also ferryl myoglobin reacts with nitrite, and nitrogen dioxide is generated in the course of the reaction. The reaction is rather slow (16 M⁻¹s⁻¹ at pH 7.5 and 20 °C) [12]. However, in the presence of the ferryl myoglobin protein radical nitration of free tyrosine can be observed.

Here we report a detailed kinetic and mechanistic study of the nitrogen monoxide- and nitrite-mediated reduction of human ferryl hemoglobin. The reaction between nitrogen monoxide and ferryl hemoglobin is quite fast [(24±2) × 10⁶ M⁻¹s⁻¹ at pH 7.0] whereas nitrite-induced reduction of ferryl hemoglobin is significantly slower [(7.5±0.4) × 10² M⁻¹s⁻¹ at pH 7.0]. However, we show that the reaction of the ferryl hemoglobin protein radical with nitrite leads to tyrosine nitration.
3.2 Materials and methods

Reagents

Buffer solutions were prepared from K$_2$HPO$_4$/KH$_2$PO$_4$ (Fluka) or from NaB$_4$O$_7 • 10$H$_2$O/NaOH (Fluka) with deionized Milli-Q water. Sodium nitrite, sodium nitrate, tyrosine and hydrogen peroxide were supplied from Fluka. Catalase (bovine liver, 17'000 units/mg protein) was obtained from Sigma.

Nitrogen monoxide was obtained from Linde and passed through a degassed sodium hydroxide solution as well as a column of sodium hydroxide pellets to remove higher nitrogen oxides before use. A saturated nitrogen monoxide solution was prepared by degassing water for 45 minutes with nitrogen, followed by saturation with nitrogen monoxide. The obtained stock solution (about 2 mM) was diluted with degassed buffer in gas-tight SampleLock Hamilton syringes. The final nitrogen monoxide concentrations were measured with an ANTEK Instruments nitrogen monoxide analyzer, with a chemiluminescence detector.

The concentration of hydrogen peroxide was determined spectrophotometrically at 240 nm (ε$_{240}$ = 39.4 M$^{-1}$cm$^{-1}$) [27].

Purified human oxyhemoglobin stock solution HbA$_0$ (57 mg/mL solution with approximately 1.1% methemoglobin) was a kind gift from APEX Biosience, Inc. (NC). Methemoglobin solutions were prepared by oxidizing oxyhemoglobin with K$_3$[Fe(CN)$_6$] and purified chromatographically on a Sephadex G-25 column with 0.1 M phosphate buffer solution (pH 7.0) as the eluent. The concentration of the methemoglobin solutions (always expressed per heme) was determined by measuring the absorbances at 405, 500 and/or 631 nm (ε$_{405}$ = 179 mM$^{-1}$cm$^{-1}$, ε$_{500}$ = 10.0 mM$^{-1}$cm$^{-1}$, ε$_{631}$ = 4.4 mM$^{-1}$cm$^{-1}$) at pH 7.0 [28].

Ferryl hemoglobin solutions were prepared by adding about 10 equivalents of hydrogen peroxide to a methemoglobin solution (pH 7.0) at room
temperature. After a reaction time of about 4 minutes, the ferryl hemoglobin solutions were used within 15 minutes.

Lyophilized horse heart myoglobin was purchased from Sigma. Purified metmyoglobin solutions were prepared as described above for methemoglobin. The concentration of the metmyoglobin solutions was determined by measuring the absorbances at 408, 502 and/or 630 nm ($\varepsilon_{408} = 188 \, \text{mM}^{-1}\text{cm}^{-1}$, $\varepsilon_{502} = 10.2 \, \text{mM}^{-1}\text{cm}^{-1}$, $\varepsilon_{630} = 3.9 \, \text{mM}^{-1}\text{cm}^{-1}$) at pH 7.0 [28].

Ferryl myoglobin solutions were prepared as described previously [15]. The concentration of the ferryl myoglobin solutions were determined by measuring the absorbance at 421 nm ($\varepsilon_{421} = 111 \, \text{mM}^{-1}\text{cm}^{-1}$) [29].

*Stopped-flow kinetic analysis*

Kinetic studies were carried out with an On-Line Instrument System stopped flow equipped with an OLIS RSM 1000 rapid scanning monochromator and with an Applied Photophysics SX17MV single-wavelength stopped-flow instrument. The pathlengths of the cells in the two spectrophotometers were 1 cm. With the Applied Photophysics apparatus, kinetic traces were taken between 404 and 420 nm and the data were analyzed with the SX17MV operating software or with Kaleidagraph, version 3.0.5. The results of the fits of the traces (averages of at least 10 single traces) from at least five experiments were averaged to obtain each observed rate constant, given as the mean ± 2 × standard deviation.

Ferryl hemoglobin solutions were prepared by adding hydrogen peroxide to a methemoglobin solution (about 200 μM). After about 4 minutes, that is when the protein radical was expected to have decayed completely, the obtained solution was diluted with buffer to the required concentration (about 0.5–2.0 μM).

For the measurements between pH 7.0 and 9.5, the ferryl hemoglobin and the nitrogen monoxide solutions were prepared in 0.1 M buffers of the required
pH value. Measurements with nitrogen monoxide at pH 6.4 were carried out by mixing a ferryl hemoglobin solution prepared in a 0.1 M phosphate buffer at pH 7.0 with a nitrogen monoxide solution prepared in a 0.1 M phosphate buffer at pH 4.0.

For measurements at pH 7.0, the ferryl hemoglobin and the nitrite solutions were prepared in 0.1 M phosphate buffer. Measurements with nitrite at pH 6.4 were carried out by mixing a ferryl hemoglobin solution prepared in a 1 mM phosphate buffer at pH 7.0 with a nitrite solution at pH 6.4 in a 0.1 M phosphate buffer. For measurements at pH 8.0, the ferryl hemoglobin and nitrite solutions were both prepared in 0.5 M phosphate buffer at pH 8.0. Control experiments were carried out to assure that changes in phosphate buffer concentration did not influence the reaction rates. The pH was measured at the end of each reaction for control.

UV-visible spectra
UV-visible spectra were collected on a UVIKON 820 spectrophotometer in a 1 cm quartz cuvette.

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 PRP-X100 (6.1005.020) precolumn as described previously [30]. A phthalic acid solution (2.5 mM phthalic acid, 5 % acetonitrile, pH 4.2, Tris) was used as eluent. Calibration curves were obtained by measuring 5–10 standard sodium nitrite and sodium nitrate solutions in 1 mM sodium phosphate buffer.

The protein samples were prepared by adding 10 equivalents of hydrogen peroxide to 4 ml of a methemoglobin solution (about 50 μM in 1 mM phosphate
buffer). After a reaction time of 3 minutes, that is when the UV-visible spectra indicated that ferryl hemoglobin had formed completely, excess hydrogen peroxide was destroyed by addition of 100 µL of a solution of catalase in water (1 mg/mL) and the vial was sealed with a gas-tight rubber septum. After 5 minutes, one equivalent of nitrogen monoxide (about 100 µL of a saturated 2 mM nitrogen monoxide solution) was added with a gas-tight Hamilton syringe under vigorous stirring. After about 5 minutes, the reaction mixture was diluted with water and analyzed.

The amount of contaminating nitrite and nitrate present in the nitrogen monoxide solution was determined prior to each experiment by injecting 100 µL of the saturated nitrogen monoxide solution with a gas-tight syringe into 4.9 mL of water placed in a 5 mL sealed vial. After stirring for about 20 minutes, the reaction mixture was diluted and analyzed. With this procedure, nitrite is quantitatively formed from the reaction of nitrogen monoxide with dioxygen in water. Thus, the amount of nitrite found in excess relative to the nitrogen monoxide used for these experiments corresponded to the amount of nitrite already present in the nitrogen monoxide solution (typically about 0.5–2.0 mM). The nitrate found is probably a contaminant derived from oxygen impurities in the apparatus. The protein samples of the reactions with nitrite were prepared analogously by adding 100–200 µL of a about 9 mM sodium nitrite solution in water. Catalase was found to contain nitrate impurities. The exact amount of nitrate was measured separately for each catalase solution (typically about 400 µM).

Analysis of the 3-nitrotyrosine content of the proteins after in situ reaction of metmyoglobin or methemoglobin, nitrite and hydrogen peroxide

Reactions were carried out by mixing 1–1.5 mL of a solution of metmyoglobin or methemoglobin (final concentration about 270 µM in 0.1 M phosphate buffer) and different concentrations of nitrite (final concentration between 135
μM and 27 mM) with a solution containing one equivalent of hydrogen peroxide (relative to the protein concentration). After about 20 minutes nitrite was removed by washing the solution through a 10'000 MW cut-off filter (Centriplus YM-10, Amicon, Switzerland) at 3000g until the ultrafiltrate did not show any qualitative reaction (pink coloring) with the Griess reagent [31]. The resulting protein (reduced to 0.5 mL, ~540–810 μM protein) was hydrolyzed by treatment for 16 h with 0.3 mL 6 M HCl at 110 °C in a closed vial. The solution was allowed to dry by opening the vial and maintaining the temperature at 110 °C. The residual was redissolved in 45-50 μL water and analyzed by high performance liquid chromatography with a Hewlett Packard Series 1050 apparatus with a Series 1100 UV/Vis-Detector, equipped with a VYDAC 218TP54 Protein&Peptide C18-Column (250×4.6 mm). Solvent A was 0.07% trifluoracetic acid in water and solvent B was 0.07% trifluoracetic acid in acetonitrile. 3-Nitrotyrosine was eluted (about 8 minutes after injection) by keeping the amount of B constant (5%) in the first 2 minutes and then by using an increasing linear gradient of B from 5 to 10% between 2 and 10 minutes, and from 10 to 80% between 10 and 30 minutes. 3-Nitrotyrosine was detected simultaneously at 220, 280, 350, and 400 nm and was quantified by measuring a calibration curve of 5–10 3-nitrotyrosine standard solutions.

Analysis of the free 3-nitrotyrosine content generated by in situ reaction of metmyoglobin or methemoglobin, nitrite, free tyrosine, and hydrogen peroxide
Tyrosine stock solutions (8–50 mM) were prepared by dissolving tyrosine in aqueous sodium hydroxide at pH ~10. Reactions were carried out by mixing 1–1.5 mL of a solution of metmyoglobin or methemoglobin (in 0.1 M phosphate buffer), nitrite, and tyrosine with a solution containing one equivalent of hydrogen peroxide (relative to the protein concentration). The pH was measured at the end of the reactions for control. The resulting protein solution was analyzed by high performance liquid chromatography as described above.
Although tyrosine has a maximum solubility of 2.5 mM in water (at 25 °C) [32], we were able to prepare supersaturated solutions of tyrosine at pH 7.0 in 0.1 M phosphate buffer. UV-control experiments were carried out to assure that no tyrosine had precipitated in solutions when higher concentrations of tyrosine were added. From a tyrosine stock solution (50 mM) we prepared samples of 1.0 mM and 8.0 mM. From the 1.0 mM solution (absorbance maximum ε274 = 1300 M⁻¹cm⁻¹) [33] we determined the extinction coefficient at 289 nm to be ε289 = 191 M⁻¹cm⁻¹. The resulting absorbance of the 8.0 mM solution (A = 1.544) confirmed that no tyrosine precipitated over a time period of at least 3 h.

### 3.3 Results

#### 3.3.1 Stopped-flow kinetic studies of the nitrogen monoxide-mediated reduction of ferryl hemoglobin

The second-order rate constants of the reaction between nitrogen monoxide and ferryl hemoglobin (human) were obtained by single-wavelength stopped-flow spectroscopy in the pH range 6.4–9.5 at 20 °C. The kinetic traces were measured mostly by following the absorbance changes at 410 and 418 nm. During mixing of high concentrations of ferryl hemoglobin (> 4 µM), we observed a strong perturbation in the kinetic trace which is caused by reflections of the light beam at zones with different concentrations. Therefore, nitrogen monoxide was used in a 8- to 10-fold excess to maintain pseudo-first-order conditions. The reaction proceeds via formation of an intermediate that subsequently decays to the final product methemoglobin.

As shown in Figure 1, when ferryl hemoglobin (0.5 µM) is mixed with nitrogen monoxide (4.0 µM), the kinetic trace at 410 nm shows an increase in
the absorbance within the first 25 ms. The traces were fitted with a single-exponential expression. In some cases the fits were not perfect, and could be improved by using a two-exponential expression. This result suggests that the α- and β-subunits of hemoglobin may react at slightly different rates. However, the difference between the two rates is probably not large enough to allow for their exact determination. Thus, we determined the averaged rate. The second-order rate constants (k₁) for the formation of the intermediate, obtained from the linear plots of the observed pseudo-first-order rate constants versus nitrogen monoxide concentration, vary slightly in the pH range studied. At pH 7.0 we obtained k₁ = (24±2) × 10⁶ M⁻¹s⁻¹ and at pH 8.5 k₁ = (39±2) × 10⁶ M⁻¹s⁻¹ (Figure 2, Table 1). The observation that the rate constant do not show a clear pH dependence may indicate that the small variations observed are due to the slight difference between the reaction rates of the two subunits.

![Figure 1](image_url)

**Figure 1** Time course measured at 410 nm of the reaction of ferryl hemoglobin (0.5 μM) with nitrogen monoxide (4.0 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 O-nitrito methemoglobin, which results in the observed rate constant of k₁, observed = 166 s⁻¹.
Figure 2 Plots of $k_{1,\text{observed}}$ versus nitrogen monoxide concentration for the formation of the intermediate $O$-nitrito methemoglobin from the reaction of ferryl hemoglobin with nitrogen monoxide 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.

As shown in Figure 3, the trace measured at 418 nm for the reaction between ferryl hemoglobin (1.1 μM) and nitrogen monoxide (10 μM) shows a decrease in absorption over a longer time scale, which corresponds to the decay of the intermediate $O$-nitrito methemoglobin to methemoglobin. In order to fit the decay of the intermediate, a two-exponential expression was needed. Thus, the decay rates of the intermediate complexes of the two subunits are not identical. The decay rates are highly pH dependent and increase with decreasing pH (Table 1). At pH 7.0 we obtained $k_{2a} = 0.5\pm0.1$ s$^{-1}$ and $k_{2b} = 0.12\pm0.04$ s$^{-1}$, respectively. The rate constants for the decay of the intermediate proved to be independent from the nitrogen monoxide as well as the ferryl hemoglobin concentration.
Figure 3 Time course measured at 418 nm of the reaction of ferryl hemoglobin (1.1 μM) with nitrogen monoxide (10.0 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 O-nitrito methemoglobin. The resulting rate constants for the two subunits are $k_{2a,\text{observed}} = 0.46$ s$^{-1}$ and $k_{2b,\text{observed}} = 0.11$ s$^{-1}$.

Table 1 pH dependencies of the second-order rate constants for the formation ($k_1$) of the intermediate O-nitrito methemoglobin. Values of the observed rate constants for the decay of the two subunits of O-nitrito methemoglobin ($k_2$) to methemoglobin at different pH. All rates were measured at 20 °C.

<table>
<thead>
<tr>
<th>pH</th>
<th>$k_1$ (μM$^{-1}$s$^{-1}$)</th>
<th>$k_{2a}$ (s$^{-1}$)</th>
<th>$k_{2b}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
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<td>6.4</td>
<td>35±2</td>
<td>1.1±0.2</td>
<td>0.32±0.04</td>
</tr>
<tr>
<td>7.0</td>
<td>24±2</td>
<td>0.5±0.1</td>
<td>0.12±0.04</td>
</tr>
<tr>
<td>8.5</td>
<td>39±2</td>
<td>0.11±0.04</td>
<td>0.025±0.004</td>
</tr>
<tr>
<td>9.5</td>
<td>31±2</td>
<td>0.07±0.02</td>
<td>0.017±0.006</td>
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</table>
3.3.2 Spectral characterization of the O-nitrito methemoglobin

The spectral changes arising from the reaction between nitrogen monoxide and ferryl hemoglobin were monitored by rapid-scan UV-visible spectroscopy between 380 and 650 nm at pH 9.5 and 20 °C. As shown in Figure 4, the Soret band of hemoglobin shifted from 417 nm (ferryl hemoglobin) to 411 nm (methemoglobin at pH 9.5, spectrum 8) via an intermediate species with an absorption maximum at 410 nm and an extinction coefficient of about 133 mM⁻¹cm⁻¹ (spectrum 3).

![Figure 4](image)

**Figure 4** Rapid-scan UV-visible spectra of the reaction of ferryl hemoglobin (6.0 μM) with nitrogen monoxide (9.0 μM) in 0.1 M borate buffer at pH 9.5 and 20 °C. The formation of the intermediate O-nitrito methemoglobin (trace 3) from ferryl hemoglobin (dashed traces 1–2) and its decay to methemoglobin (traces 4–8) are presented. Traces 1–3 recorded 0, 16, 32 ms after mixing, traces 4–7 recorded after 4, 12, 20, 28 s, and trace 8 recorded after a total of 100 s.

The spectrum of the intermediate in the visible region, shown as the first trace in Figure 5, shows absorption maxima at 537 (ε₅₃₇ = 10.1 mM⁻¹cm⁻¹), 562 (ε₅₆₂ = 8.7 mM⁻¹cm⁻¹) and 617 (ε₆₁₇ = 3.9 mM⁻¹cm⁻¹) nm (spectrum 1). Compared with spectra of similar hemoglobin complexes (Table 2) and in
analogy to the $O$-nitrito metmyoglobin complex which is formed by the reaction between ferryl myoglobin and nitrogen monoxide [12], the UV-visible spectrum of the intermediate can be assigned as the $O$-nitrito methemoglobin complex Hb[Fe$^{III}$ONO]$^{2+}$.

![Figure 5](image)

**Figure 5** Rapid-scan UV-visible spectra of the reaction of ferryl hemoglobin (38 μM) with nitrogen monoxide (60 μM) in 0.1 M borate buffer at pH 9.5 and 20 °C. The decay of the intermediate $O$-nitrito methemoglobin (trace 1) to methemoglobin (trace 6) is presented. Time intervals of the shown spectra are: traces 1–5, every 20.0 s, and trace 6 after a total of 100.0 s.

**Table 2** Spectroscopic data for hemoglobin complexes.

<table>
<thead>
<tr>
<th></th>
<th>Soret</th>
<th>Visible</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
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<td>$\lambda_{\text{max}}$ ($\varepsilon$)$^a$</td>
<td>$\lambda_{\text{max}}$ ($\varepsilon$)$^a$</td>
<td>$\lambda_{\text{max}}$ ($\varepsilon$)$^a$</td>
<td></td>
</tr>
<tr>
<td>HbFe$^{IV}$=O</td>
<td>417 (105)</td>
<td>545 (10.9)</td>
<td>574 (9.5)</td>
</tr>
<tr>
<td>Hb[Fe$^{III}$ONO$_2$]$^{2+}$</td>
<td>408 (120)</td>
<td>527 (9.9)</td>
<td>628 (3.2)</td>
</tr>
<tr>
<td>Hb[Fe$^{III}$NO$_2$]$^{2+}$</td>
<td>411 (132)</td>
<td>538 (10.0)</td>
<td>623 (3.6)</td>
</tr>
<tr>
<td>Hb[Fe$^{III}$ONO]$^{2+}$</td>
<td>410 (133)</td>
<td>537 (10.1)</td>
<td>562 (8.7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>617 (3.9)</td>
</tr>
</tbody>
</table>

$^a \lambda_{\text{max}}$ (nm), $\varepsilon$ (mM$^{-1}$cm$^{-1}$)
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The intermediate O-nitrito methemoglobin was also detected when the reaction between nitrogen monoxide and ferryl hemoglobin was carried out at pH 7.0. However, when nitrogen monoxide was used in a large excess, decay of O-nitrito methemoglobin to methemoglobin could not be observed. Instead, we detected the formation of nitrosyl methemoglobin (Hb\[^{III}\text{Fe}^{\text{II}}\text{NO}^3\text{+}\]) with absorption maxima at 533 and 566 nm [28] (spectrum 9 in Figure 6). Methemoglobin did not accumulate in concentrations large enough to be detectable as under the conditions of this experiment the rate determining step is the decay of O-nitrito methemoglobin. The rates for the binding of nitrogen monoxide to methemoglobin are \(k_\alpha = 1.71 \times 10^3 \text{ M}^{-1}\text{s}^{-1}\) and \(k_\beta = 6.4 \times 10^3 \text{ M}^{-1}\text{s}^{-1}\) (at pH 7.0 and 20 °C) [34]. Therefore, in the presence of 340 \(\mu\text{M}\) nitrogen monoxide (after one equivalent of nitrogen monoxide has reacted with ferryl hemoglobin), the calculated rate constants would be \(k_{\alpha,\text{observed}} = 0.58 \text{ s}^{-1}\) and \(k_{\beta,\text{observed}} = 2.2 \text{ s}^{-1}\). Nitrosyl methemoglobin is under our conditions, in the presence of dioxygen, not stable and subsequently decays to methemoglobin (spectrum 10 in Figure 6).
Figure 6 Rapid-scan UV-visible spectra of the reaction of ferryl hemoglobin (38 μM) with nitrogen monoxide (380 μM) in 0.1 M phosphate buffer at pH 7.0 and 20 °C. The formation of the intermediate nitroso methemoglobin (trace 9) from O-nitrito methemoglobin (traces 1–8) and the trace of the final product methemoglobin (dashed trace 10) are presented. Time intervals of the shown spectra are: traces 1–8, every 240 ms; trace 9 after 1.88 s, and trace 10 after a total of 50.0 s.

The kinetic of the reaction between nitrogen monoxide and methemoglobin is biphasic, confirming that the binding rate of nitrogen monoxide to methemoglobin is different for the α- and β-subunit. Traces are different when reactions are performed with degassed protein solutions. As no dinitrogen trioxide can be formed by reaction of nitrogen monoxide with dioxygen, all nitrogen monoxide binds to methemoglobin (Fig 7B). Over a longer time scale, the decay of the complex is much faster in the presence (Figure 7A) than in the absence of dioxygen (Figure 7B). In the absence of dioxygen, nitrosyl methemoglobin undergoes slow reductive nitrosylation to iron(II) hemoglobin [34]. Whereas in the presence of dioxygen, formed dinitrogen trioxide may react with nitrosyl methemoglobin.
Figure 7 Time courses measured at 418 nm for the reaction of ferryl hemoglobin (1.1 μM) with nitrogen monoxide (80 μM) in 0.1 M phosphate buffer at pH 7.0 and 20 °C. A under aerobic and B under anaerobic conditions.

3.3.3 UV-visible spectra of the protein product after decay of ferryl hemoglobin and reduction

As shown in Figure 8, the spectrum obtained from the decay of ferryl hemoglobin (spectrum C) is different from that of pure methemoglobin (spectrum A). Due to the absence of the band at 630 nm in spectrum C it might be assumed that partial formation of the hemichrome species occurs during
oxidation of methemoglobin to ferryl hemoglobin [35]. In the hemichrome species the distal histidine is coordinated to the iron ion. In order to confirm this proposition, sodium dithionite was added to the solution obtained after decay of ferryl hemoglobin to reduce the hemoglobin species. As shown in spectrum D in Figure 8, the resulting spectrum shows a shoulder at 530 nm and an absorption maximum at 558 nm. In contrast, pure deoxyhemoglobin has an absorption maximum at 555 nm [28]. In comparison with the spectrum of hemochrome (the reduced form of hemichrome) which has absorption maxima at 529 nm and 558 nm [35], it can be concluded that during formation of ferryl hemoglobin and its subsequent decay some hemoglobin is transformed to the hemichrome species. Therefore, differences between pure methemoglobin and methemoglobin obtained after reaction of ferryl hemoglobin and about one equivalent of nitrogen monoxide (spectrum E in Figure 8) are probably caused by the hydrogen peroxide-induced oxidation of methemoglobin to the ferryl species.

**Figure 8** UV-visible spectra in 0.1 M phosphate buffer at pH 7.0. A methemoglobin (79 μM); B ferryl hemoglobin formed after addition of 5 equivalents hydrogen peroxide; C solution B decayed after 36 h; D spectrum obtained after addition of sodium dithionite to the solution of C; E spectrum obtained after reaction of ferryl hemoglobin (79 μM) with nitrogen monoxide (90 μM).
3.3.4 Stopped-flow kinetic studies of the nitrite-mediated reduction of ferryl hemoglobin

As nitrite is always present as a contaminant in our nitrogen monoxide solutions, we studied also the reaction between nitrite and ferryl hemoglobin. Spectral changes taking place during this reaction were monitored by rapid-scan UV-visible spectroscopy between 380 and 650 nm at pH 7.0 and 20 °C. As shown in Figure 9, when ferryl hemoglobin was mixed with a large excess of nitrite, the Soret band shifted from 417 nm (ferryl hemoglobin, spectrum 1) to 405 nm (methemoglobin, spectrum 5). In the presence of high concentrations of nitrite, the subsequent binding of nitrite to methemoglobin was also observed. Partial formation of the N-nitrito methemoglobin complex (Hb[Fe\text{III}NO_2]^2+) (spectrum 9 in Figure 9) is accompanied by a shift of the Soret band to 408 nm.

![Figure 9](image)

**Figure 9** Rapid-scan UV-visible spectra of the reaction of ferryl hemoglobin (4.2 µM) with nitrite (12.5 mM) in 0.1 M phosphate buffer at pH 7.0 and 20 °C. The formation of methemoglobin (trace 5) from ferryl hemoglobin (dashed traces 1–4) and the subsequent partial binding of nitrite to form a mixture of N-nitrito methemoglobin and methemoglobin (traces 7–9) are presented. Traces 1–5 recorded 0, 80, 160, 240 and 320 ms after mixing, traces 6-8 recorded after 1.2, 3.6, 6.0 s, and trace 9 recorded after a total of 16.0 s.
In the visible region of the spectra, the nitrite-mediated reduction of ferryl hemoglobin (Figure 10A, spectrum 1) to methemoglobin (spectrum 6) can be followed by the formation of the absorption bands at 501 and 625 nm. The differences between the spectrum of pure methemoglobin and that found after reaction between ferryl hemoglobin and nitrite are probably caused during hydrogen peroxide-mediated oxidation of methemoglobin to ferryl hemoglobin (see Figure 8). On a longer time scale, subsequent partial binding of excess nitrite to methemoglobin yields a mixture of N-nitrito methemoglobin and methemoglobin, with absorption maxima at 536, 563 and 624 nm (spectrum 5 in Figure 10B).

![Figure 10](image)

**Figure 10** Rapid-scan visible spectra of the reaction of ferryl hemoglobin (38.2 µM) with nitrite (12.5 mM) in 0.1 M phosphate buffer at pH 7.0 and 20 °C. (A) The formation of methemoglobin (trace 6) from ferryl hemoglobin (traces 1–5) is presented. Traces 1–6 recorded 0, 64, 128, 192, 256, and 480 ms after mixing. (B) The partial binding of excess nitrite to methemoglobin to form a mixture of N-nitrito methemoglobin and methemoglobin is presented. Traces 1–4 recorded after 0.48, 1.44, 2.4, 3.36 s, and trace 5 recorded after a total of 15.0 s.
The second-order rate constants of the reaction between nitrite and ferryl hemoglobin were determined by single-wavelength stopped-flow spectroscopy in the pH range 6.4–8.0 at 20 °C. The reaction was studied under pseudo-first-order conditions with nitrite in at least 10-fold excess. The kinetic traces, measured by following the absorbance increases at 410 nm, could all be fitted to a single-exponential expression. The second-order rate constants ($k_3$), obtained from the linear plots of the observed pseudo-first-order rate constants versus nitrite concentration, are highly pH dependent and decrease with increasing pH (Figure 11, Table 3). At pH 7.0 we obtained a second-order rate constant of $(7.5\pm0.4) \times 10^2$ M$^{-1}$s$^{-1}$. This first reaction is followed by absorption changes which were measured at 418 nm. The kinetic traces had to be fitted to a two-exponential expression. From the linear plots of the observed pseudo-first-order rate constants we obtained second-order rate constants ($k_4$) which are also pH-dependent, with values of $k_{4a} = 140\pm6 \times 10^2$ M$^{-1}$s$^{-1}$ and $k_{4b} = 23.8\pm0.8$ M$^{-1}$s$^{-1}$ at pH 7.0 for the $\alpha$- and $\beta$-subunits of hemoglobin. These reactions probably correspond to the binding of nitrite to methemoglobin. In order to confirm the nature of these reactions, we determined the reaction rates of pure
methemoglobin with nitrite at pH 7.0 to be \((1.6\pm0.1) \times 10^2\) M\(^{-1}\)s\(^{-1}\) and \(21.9\pm0.8\) M\(^{-1}\)s\(^{-1}\). These values are in good agreement with reaction rates found when the binding of nitrite to methemoglobin was studied under similar conditions [28, 36].

It is not possible to determine the yield of the reaction between ferryl hemoglobin and nitrite. Ferryl hemoglobin solutions are not indefinitely stable and the reaction is very slow. Therefore, decay of ferryl hemoglobin would be faster than the reaction between one equivalent of nitrite with one equivalent of ferryl hemoglobin.

![Figure 11](image)

**Figure 11** Plots of \(k_{3,\text{observed}}\) versus nitrite concentration for the reaction between ferryl hemoglobin and nitrite at pH 6.4, 7.0 and 8.0 at 20 °C. The values of the second-order rate constants obtained from the linear fits are given in Table 3.
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Table 3 pH dependencies of the second-order rate constants for the reactions of nitrite with ferryl hemoglobin \((k_3)\) at 20 °C. pH dependencies of the association rates \((k_4)\) of reaction of nitrite with methemoglobin after nitrite-mediated reduction of ferryl hemoglobin.

<table>
<thead>
<tr>
<th>pH</th>
<th>(k_3) ((M^{-1}s^{-1}))</th>
<th>(k_{4a}) ((M^{-1}s^{-1}))</th>
<th>(k_{4b}) ((M^{-1}s^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.4</td>
<td>((1.86±0.06)\times10^3)</td>
<td>((3.8±0.6)\times10^2)</td>
<td>54±4</td>
</tr>
<tr>
<td>7.0</td>
<td>((7.5±0.4)\times10^2)</td>
<td>140±6</td>
<td>23.8±0.8</td>
</tr>
<tr>
<td>8.0</td>
<td>65±4</td>
<td>5.5±0.8</td>
<td>2.2±0.4</td>
</tr>
</tbody>
</table>

3.3.5 Analysis of the nitrogen-containing products

The amount of nitrite and nitrate formed during the reaction of ferryl hemoglobin with one equivalent of nitrogen monoxide at pH 7.0 was determined by anion chromatography with conductivity detection. As shown in Table 4, nitrite was always formed quantitatively.

Table 4 Amount of nitrite formed from the reaction of ferryl hemoglobin with one equivalent of nitrogen monoxide. Comparison between the concentrations of nitrate found after the reaction and that present in the nitrogen monoxide solution.

<table>
<thead>
<tr>
<th>[HbFe(^{IV}=O})</th>
<th>([\text{NO}_2^-]a) Measured [\text{NO}_3^-] in NO* soln. (b)</th>
<th>Measured [\text{NO}_3^-] in NO* soln. (c) ((\mu M))</th>
</tr>
</thead>
<tbody>
<tr>
<td>((\mu M))</td>
<td>((\mu M))</td>
<td>((\mu M))</td>
</tr>
<tr>
<td>44.6</td>
<td>44±8</td>
<td>4.5±0.8</td>
</tr>
<tr>
<td>44.6</td>
<td>42±1</td>
<td>4.5±0.8</td>
</tr>
<tr>
<td>44.6</td>
<td>44.0±0.4</td>
<td>4.5±0.8</td>
</tr>
</tbody>
</table>

\(a\) Concentration of nitrite ions generated from the reaction of ferryl hemoglobin with nitrogen monoxide, calculated from the concentration of nitrite ions found in the reaction solution minus the concentration of nitrite already present in the added nitrogen monoxide solutions.

\(b\) Nitrate contamination of the nitrogen monoxide solutions.

\(c\) Concentration of nitrate ions generated from the reaction of ferryl hemoglobin with nitrogen monoxide, calculated from the concentration of nitrate ions found in the reaction solution minus the concentration of nitrate already present in the added nitrogen monoxide solutions and the nitrate contamination of the catalase solution (about 400 \(\mu M\)).
The nitrite-induced reduction of ferryl hemoglobin is very slow. Therefore, analysis of the nitrogen-containing products was carried out by mixing an excess of nitrite with ferryl hemoglobin. At pH 7.0, we found that one equivalent of nitrite (relative to the amount of ferryl hemoglobin) was transformed to half an equivalent of nitrate plus half an equivalent of nitrite (Table 5). Control experiments have shown that no nitrate is formed when nitrite (in 0.1 M phosphate buffer at pH 7.0) is mixed with hydrogen peroxide.

**Table 5** Amount of nitrite and nitrate formed from the reaction of ferryl hemoglobin with an excess of nitrite.

<table>
<thead>
<tr>
<th>[HbFe$^{IV}=O$] (µM)</th>
<th>Added [NO$_2^-$] (µM)</th>
<th>Ex [NO$_2^-$]$^a$ (µM)</th>
<th>Found [NO$_2^-$]$^b$ (µM)</th>
<th>[NO$_2^-$]$^c$ (µM)</th>
<th>[NO$_3^-$]$^d$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>218 (50+168)</td>
<td>168</td>
<td>191±12 (23+168)</td>
<td>23±12</td>
<td>24±4</td>
</tr>
<tr>
<td>45</td>
<td>302 (45+257)</td>
<td>257</td>
<td>281±4 (24+257)</td>
<td>24±4</td>
<td>24±2</td>
</tr>
<tr>
<td>44</td>
<td>398 (44+354)</td>
<td>354</td>
<td>379±24 (25+354)</td>
<td>25±24</td>
<td>24±8</td>
</tr>
</tbody>
</table>

$^a$ Concentration of nitrite added in excess, by considering that only one equivalent reacts with ferryl hemoglobin.

$^b$ Total concentration of nitrite ions found in the protein solution after reaction.

$^c$ Concentration of nitrite ions generated from the reaction of ferryl hemoglobin with one equivalent of nitrite, calculated from the total concentration of nitrite ions found in the protein solution after reaction minus the concentration of the excess nitrite ions added.

$^d$ Concentration of nitrate ions in the protein solution after reaction with nitrite, minus nitrate contamination of catalase (about 400 µM).

### 3.3.6 Analysis of the nitrogen-containing products formed by the
*in situ* reaction of metmyoglobin, nitrite, and hydrogen peroxide

When hydrogen peroxide reacts with metmyoglobin, the transient ferryl myoglobin protein radical is formed. According to the literature, yield of the protein radical is highest, when metmyoglobin is allowed to react with about an equal amount of hydrogen peroxide [37-39].
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As shown in Figure 12 (spectrum C), reaction of one equivalent of hydrogen peroxide with metmyoglobin does not yield ferryl myoglobin quantitatively. By comparison with the spectra of metmyoglobin (spectrum A in Figure 12) and ferryl myoglobin (spectrum B in Figure 12), which has been generated by addition of an excess of hydrogen peroxide, we estimate the yield of ferryl myoglobin formed by one equivalent hydrogen peroxide to be about 60%.

![Figure 12 UV-visible spectra in 0.1 M phosphate buffer at pH 7.0. A metmyoglobin (247 µM); B ferryl myoglobin formed after addition of 5 equivalents hydrogen peroxide to metmyoglobin (247 µM); C Mixture of metmyoglobin and ferryl myoglobin formed after addition of one equivalent hydrogen peroxide to metmyoglobin (247 µM).](image)

We determined the yield of nitrate when the reaction was carried out by addition of one equivalent of hydrogen peroxide to a solution containing metmyoglobin and nitrite (1 or 4 equivalents relative to protein concentration). With this procedure, about 0.75 equivalents nitrate relative to myoglobin concentration were obtained (Table 6). However, we observed a significant loss of nitrogen-containing products in the range of 10% relative to the myoglobin concentration.
Table 6 Amount of nitrite and nitrate generated from the reaction of a solution of metmyoglobin and nitrite with a solution containing one equivalent of hydrogen peroxide (relative to protein concentration).

<table>
<thead>
<tr>
<th>[metMb] (μM)</th>
<th>Add. [NO₂⁻] (μM)</th>
<th>Measured [NO₂⁻] after reaction (μM)</th>
<th>[NO₃⁻] (μM)</th>
<th>% (NO₃⁻) relative to protein</th>
<th>Δ N-balance (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>238</td>
<td>238</td>
<td>31±2</td>
<td>(1.8±0.2)×10²</td>
<td>75</td>
<td>-29</td>
</tr>
<tr>
<td>208</td>
<td>830</td>
<td>(6.5±0.1)×10²</td>
<td>(1.7±0.2)×10²</td>
<td>80</td>
<td>-18</td>
</tr>
</tbody>
</table>

We also determined the yield of nitrate when first one equivalent of hydrogen peroxide was allowed to react with metmyoglobin and after about 7 minutes, when the protein radical was expected to have decayed completely, nitrite (1 or 4 equivalents relative to protein concentration) was added. Under these conditions, only about 0.25 equivalents nitrate relative to the protein concentration were obtained (Table 7).

Table 7 Amount of nitrite and nitrate generated from reaction of nitrite with a solution containing ferryl myoglobin [generated by allowing to react as solution of metmyoglobin with one equivalent hydrogen peroxide (relative to the protein concentration) for 7 minutes].

<table>
<thead>
<tr>
<th>[metMb] (μM)</th>
<th>Add. [NO₂⁻] (μM)</th>
<th>Measured [NO₂⁻] after reaction (μM)</th>
<th>[NO₃⁻] (μM)</th>
<th>% (NO₃⁻) relative to protein</th>
<th>Δ N-balance (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>238</td>
<td>238</td>
<td>171±4</td>
<td>59±4</td>
<td>25</td>
<td>-9</td>
</tr>
<tr>
<td>208</td>
<td>830</td>
<td>787±8</td>
<td>50±4</td>
<td>24</td>
<td>+7</td>
</tr>
</tbody>
</table>
3.3.7 Nitration of protein-bound tyrosine by *in situ* reaction of metmyoglobin or methemoglobin with nitrite and hydrogen peroxide

The formation of equimolar amounts of nitrite and nitrate from the nitrite-mediated reduction of ferryl myoglobin and ferryl hemoglobin suggests that nitrogen dioxide ($E^\circ \approx 0.99$ V) [40] may be generated as the first product of this reaction [19]. As it is known that nitrogen dioxide is a potent nitrating agent [24, 41], it was of interest to find out whether the tyrosine residues of the proteins are nitrated in the course of the nitrite-mediated reduction of ferryl myoglobin and ferryl hemoglobin. We have recently shown that no 3-nitrotyrosine residues are found when the reaction was carried out in the presence of preformed ferryl myoglobin [12]. In contrast, when a solution of metmyoglobin or methemoglobin and variable concentrations of nitrite was mixed with one equivalent of hydrogen peroxide (relative to protein concentration), partial nitration of the tyrosine residues could be detected. After reaction, excess nitrite was removed by centrifugation through a size exclusion membrane. Subsequently, the protein was cleaved by acid hydrolysis at 110 °C for 18 hours. We analyzed the obtained amino acids by high performance liquid chromatography. As shown in Figure 13 and 14, the yield of 3-nitrotyrosine was very low and decreased with increasing concentration of added nitrite. We also determined the formation of 3-nitrotyrosine when metmyoglobin was hydrolyzed in the presence of nitrite. As it is shown in Table 8, significant amounts of 3-nitrotyrosine were generated in the presence of high concentrations of nitrite. Therefore, excess nitrite had to be removed from the solutions after *in situ* reaction of metmyoglobin or methemoglobin, nitrite, and hydrogen peroxide.
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Figure 13  % Yield of 3-nitrotyrosine relative to the amount of tyrosine present in the protein (2 tyrosine/heme) generated by adding one equivalent of hydrogen peroxide (relative to protein) to a mixture of metmyoglobin (about 270 μM) and variable amounts of nitrite at pH 7.0 or pH 6.0.

Table 8  % Yield of 3-nitrotyrosine relative to the amount of tyrosine present in the protein (2 tyrosine/heme) formed when pure metmyoglobin (270 μM) was heated at 110 °C in hydrochloric acid in the presence of variable amounts of nitrite.

<table>
<thead>
<tr>
<th>Nitrite (mM)</th>
<th>% Yield 3-nitrotyrosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>13.5</td>
<td>14±6</td>
</tr>
<tr>
<td>20.3</td>
<td>27±14</td>
</tr>
<tr>
<td>27.0</td>
<td>32±2</td>
</tr>
</tbody>
</table>

It has been shown that in the presence of low concentrations of metmyoglobin (5 μM) and an excess of nitrite, free tyrosine, and hydrogen peroxide (each 50 μM), generation of 3-nitrotyrosine depended on the pH [42]. A maximum of 3-nitrotyrosine formation has been observed at pH 6.0 [42]. Therefore, we also determined the yield of protein-bound 3-nitrotyrosine when a
solution of metmyoglobin and variable concentrations of nitrite was mixed with one equivalent of hydrogen peroxide (relative to protein concentration) at pH 6.0 (Figure 13). However, we did not detect a significant change in 3-nitrotyrosine formation compared with when the reaction was carried out at pH 7.0 (Figure 13).

![Graph](image)

**Figure 14** % Yield of 3-nitrotyrosine relative to the amount of tyrosine present in the protein (3 tyrosine/heme) generated by adding one equivalent of hydrogen peroxide (relative to protein) to a mixture of methemoglobin (about 270 μM) and variable amounts of nitrite at pH 7.0.

### 3.3.8 Nitration of free tyrosine during *in situ* reaction of metmyoglobin or methemoglobin with nitrite and hydrogen peroxide

We have recently shown that when a solution of tyrosine and nitrite is allowed to react with ferryl myoglobin, very small yields of 3-nitrotyrosine are detected by high performance liquid chromatography analysis [12]. In contrast, when one equivalent of hydrogen peroxide (relative to protein concentration) was added to
a mixture of metmyoglobin or methemoglobin, tyrosine, and variable concentrations of nitrite, significantly larger amounts of 3-nitrotyrosine were formed. As shown in Figure 15, the yield of 3-nitrotyrosine increased with increasing nitrite concentration, but reached a maximum in the presence of 25–30 mM nitrite (about 16 % for myoglobin and 12 % for hemoglobin, relative to the protein concentrations). When the reaction in the presence of metmyoglobin is carried out at pH 6.0, the saturation level is reached at significantly lower concentrations of nitrite (about 1.5 mM) (Figure 16). However, the maximum yield of 3-nitrotyrosine (about 11 % relative to protein concentration) did not change significantly compared with the yield obtained at pH 7.0.

**Figure 15** % Yield of 3-nitrotyrosine relative to the protein concentration generated by adding one equivalent of a hydrogen peroxide (relative to protein) to a mixture of metmyoglobin or methemoglobin (about 250 μM), 4 equivalents of tyrosine (1 mM), and variable amounts of nitrite at pH 7.0.
A control experiment showed that no nitration takes place in the absence of the protein. Moreover, the order of addition was found not to be relevant, even though considerable amounts of N-nitrito metmyoglobin are formed in the presence of high nitrite concentrations. Our data showed that the same yields of 3-nitrotyrosine were obtained when a mixture of nitrite and hydrogen peroxide was allowed to react with a solution of metmyoglobin and tyrosine. Indeed, when hydrogen peroxide (250 μM) was added to a solution of metmyoglobin (250 μM), tyrosine (1.0 mM) and nitrite (31 mM), yield of 3-nitrotyrosine was 16±1 % (relative to metmyoglobin concentration) compared with 16±1 % when nitrite and hydrogen peroxide was given to a solution of metmyoglobin and tyrosine.

We also determined the yield of 3-nitrotyrosine when a solution of metmyoglobin, nitrite, and variable concentrations of tyrosine were allowed to react with one equivalent hydrogen peroxide (relative to the protein
concentration). The yield of 3-nitrotyrosine (relative to the protein) decreased with increasing concentrations of tyrosine (Table 9).

**Table 9** % Yield of 3-nitrotyrosine relative to the protein concentration generated by adding 1 equivalent of hydrogen peroxide (relative to protein) to a mixture of metmyoglobin (about 250 μM), 4 equivalents of nitrite (1 mM), and variable amounts of tyrosine at pH 7.0.

<table>
<thead>
<tr>
<th>[tyrosine] mM</th>
<th>Yield 3-nitrotyrosine (% relative to metMb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>3.1±0.6</td>
</tr>
<tr>
<td>2.5</td>
<td>2±1</td>
</tr>
<tr>
<td>5.0</td>
<td>1.6±0.1</td>
</tr>
<tr>
<td>7.5</td>
<td>0.9±0.1</td>
</tr>
</tbody>
</table>

In addition, we determined the yield of 3-nitrotyrosine when one equivalent hydrogen peroxide (relative to the protein concentration) was allowed to react with a mixture of nitrite, tyrosine, and variable concentrations of metmyoglobin. Yield of 3-nitrotyrosine (relative to the protein) decreased with increasing concentrations of myoglobin (Table 10).
Table 10 Yield of 3-nitrotyrosine generated by adding 1 equivalent of hydrogen peroxide (relative to protein) to a mixture of nitrite (1.0 mM), tyrosine (1.0 mM), and variable concentrations of metmyoglobin at pH 7.0.

<table>
<thead>
<tr>
<th>[metmyoglobin] μM</th>
<th>Yield 3-nitrotyrosine (μM)</th>
<th>Yield 3-nitrotyrosine (% relative to metMb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>111</td>
<td>4.6±0.1</td>
<td>4.2±0.1</td>
</tr>
<tr>
<td>173</td>
<td>5.4±0.4</td>
<td>3.1±0.1</td>
</tr>
<tr>
<td>250</td>
<td>8±1</td>
<td>3.1±0.6</td>
</tr>
<tr>
<td>385</td>
<td>7.4±0.1</td>
<td>1.92±0.02</td>
</tr>
<tr>
<td>472</td>
<td>11±1</td>
<td>2.3±0.2</td>
</tr>
<tr>
<td>629</td>
<td>10±1</td>
<td>1.5±0.2</td>
</tr>
</tbody>
</table>

3.4 Discussion

It has often been proposed that nitrogen monoxide may play a physiological role as antioxidant [13-15, 43]. We have recently shown that the reaction between ferryl myoglobin and nitrogen monoxide is very fast at physiological pH. Therefore, it may indeed play an important role for the detoxification of the highly oxidizing species ferryl myoglobin under pathological conditions with elevated concentrations of ferryl myoglobin [12].

3.4.1 Stopped-flow kinetic studies of the nitrogen monoxide-mediated reduction of ferryl hemoglobin

The work described in this chapter shows that, in analogy to the reaction between ferryl myoglobin and nitrogen monoxide, the nitrogen monoxide-mediated reduction of ferryl hemoglobin proceeds via the rapid formation of the
intermediate $O$-nitrito methemoglobin ($\text{Hb}[\text{Fe}^{\text{III}}\text{ONO}]^{2+}$), which subsequently decays to methemoglobin and nitrite. At pH 7.0 and 20 °C, the second-order rate constant for the formation of this intermediate is $k_1 = (2.4 \pm 0.2) \times 10^7 \text{ M}^{-1}\text{s}^{-1}$. The rate is in about the same range as that of the reaction between ferryl myoglobin with nitrogen monoxide, which is $(1.71 \pm 0.06) \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ at pH 7.0 [12]. In analogy to the nitrogen monoxide-induced oxidation of oxyhemoglobin [16], the $\alpha$- and $\beta$-subunits react with the same rate constant. In addition, nitrogen monoxide and oxyhemoglobin also react with a very high rate constant $(8.9 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ at pH 7.0 and 20 °C) [16].

In contrast to the formation of the intermediate, the $O$-nitrito complexes of the two hemoglobin subunits decay at different rates, $k_{2a} = 0.5 \pm 0.1 \text{ s}^{-1}$ and $k_{2b} = 0.12 \pm 0.04 \text{ s}^{-1}$ at pH 7.0, respectively. The values of the decay rates of the intermediates of the two hemoglobin subunits are about one order of magnitude lower than that of the corresponding myoglobin intermediate [12]. This difference is very similar to that of the decay rate of oxoperoxonitrato methemoglobin ($\text{Hb}[\text{Fe}^{\text{III}}\text{ONO}]^{2+}$) to methemoglobin and nitrate, which is also about one order of magnitude lower than that found for the decay of oxoperoxonitrato metmyoglobin. In analogy, oxoperoxonitrato methemoglobin decays with different rate constants for the two hemoglobin subunits [16]. The difference in the decay of the intermediate complexes between $\alpha$- and $\beta$-subunits may be caused by the different environment of the heme groups. In the $\beta$-subunit some amino acid residues overlap with the ligand binding site to a larger extent than in the $\alpha$-subunit, with the consequence of different rate constants for some of the reactions of hemoglobin [34, 44, 45]. In analogy to oxoperoxonitrato metmyoglobin and oxoperoxonitrato methemoglobin, dissociation rate constants of $O$-nitrito methemoglobin to methemoglobin and nitrite increase with decreasing pH. This trend could be explained by the observation that under acidic pH the distal histidine swings out of heme pocket
toward the solvent, opens the active site [46] and, thus, possibly favours the
dissociation of nitrite.

The reaction between nitrogen monoxide and ferryl hemoglobin is about
three orders of magnitude faster than the nitrogen monoxide-mediated reduction
of the Compound II forms of eosinophil peroxidase and lactoperoxidase with
rate constants of $1.7 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ and $8.7 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, respectively, at pH 7.0
and 25 °C [47]. In peroxidases, the generation of an intermediate O-nitrito
complex cannot be observed. The negative charge on the deprotonated distal
histidine residue $\{(\text{His}^-)[\text{Fe}^{III}\text{ONO}]^{2+}\}$ may be responsible for the significant
increase of nitrite dissociation in peroxidases [48]. In this context, the
dissociation rate of nitrite from the N-nitrito complex of myeloperoxidase (60 s\(^{-1}\)
at pH 7 and 15 °C) [48] is much higher than that found for N-nitrito
methemoglobin (0.15 s\(^{-1}\) and 0.018 s\(^{-1}\) for the both subunits at pH 7.4 and 15 °C)
[36].

3.4.2 Spectral characterization of the O-nitrito methemoglobin
intermediate

The visible spectrum of the intermediate formed during nitrogen monoxide-
induced reduction of ferryl hemoglobin displays absorption maxima at 537, 562,
and 617 nm. In comparison with the N-nitrito- and nitrato methemoglobin
complexes (Table 2) and in analogy to O-nitrito metmyoglobin which is formed
during nitrogen monoxide-induced reduction of ferryl myoglobin [12], the
observed intermediate can be assigned as the low-spin O-nitrito methemoglobin
complex (Hb[Fe^{III}\text{ONO}]^{2+}) (equation 7). The spectrum obtained for O-nitrito
methemoglobin is identical to that of N-nitrito methemoglobin. Therefore, we
cannot exclude that O-nitrito methemoglobin intramolecularly rearranges to N-
nitrite methemoglobin before it subsequently decays to methemoglobin and nitrite.

\[
\text{HbFe}^{IV}=O \xleftrightarrow{} \text{HbFe}^{III}-O^* \xrightarrow{\text{NO}^*} \text{Hb}[\text{Fe}^{III}\text{ONO}]^{2+} \quad (7)
\]

Nitrogen monoxide reacts stoichiometrically with ferryl hemoglobin to yield methemoglobin and nitrite. Ion chromatography analysis of the nitrogen-containing products confirm that exclusively nitrite is formed in the course of the reaction.

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

Beside the nitrogen monoxide-induced oxidation of oxyhemoglobin which forms methemoglobin and nitrate [16], nitrite is the major end product of nitrogen monoxide metabolism. It has been shown that the Compound II forms of peroxidases react with nitrite, most likely under intermediary formation of nitrogen dioxide. The determined reaction rates vary from 13.3 M$^{-1}$s$^{-1}$ for horseradish peroxidase at pH 7.0 and room temperature [49] and $5.5 \times 10^2$ M$^{-1}$s$^{-1}$ for myeloperoxidase at pH 7.0 and 15 °C [48] to $3.5 \times 10^4$ M$^{-1}$s$^{-1}$ for lactoperoxidase at pH 7.0 and room temperature [49]. In the present work we have measured by stopped-flow spectroscopy the rate constant of the reaction between ferryl hemoglobin and nitrite. The second-order rate constant at pH 7.0 and 20 °C is $(7.5\pm0.4) \times 10^2$ M$^{-1}$s$^{-1}$, which is about one magnitude larger than that for the corresponding reaction with myoglobin ($65\pm6$ M$^{-1}$s$^{-1}$ at pH 6.7 [12]). In analogy to myoglobin, the reaction rate decreases with increasing pH. The same pH-dependence has been observed for the reaction of nitrite with myeloperoxidase-Compound II [48]. This effect again can be explained by the opening of the protein active side when the distal histidine is protonated at lower
pH. Consequently, access for nitrite to reach the ferryl center is facilitated. In addition, the ferryl group has been shown to be more reactive when protonated at acidic pH [50]. The pKₐ of the ferryl group (pKₐ ~6, in ferryl myoglobin) [51] has about the same value as the pKₐ of the distal histidine residue (pKₐ = 6.0) [52]. Therefore, both, the opening of the protein active site as well as the protonation of the ferryl group may contribute to the increased rate constants at lower pH. Due to the small second-order rate constant of the reaction of nitrite with ferryl hemoglobin, the reaction does not affect the accuracy of the determination of the significantly faster nitrogen monoxide-induced reduction of ferryl hemoglobin.

Analysis of the nitrogen-containing products of the reaction between nitrite and ferryl hemoglobin shows that nitrite is converted to half an equivalent of nitrate and half an equivalent of nitrite. This result suggests that, in analogy to the mechanism proposed for ferryl myoglobin [12], in a first step nitrite is oxidized to nitrogen dioxide (equation 8). In the absence of any substrate, nitrogen dioxide rapidly dimerizes to dinitrogen tetroxide at a rate of $9 \times 10^8$ M⁻¹s⁻¹ [53] which subsequently hydrolyzes to yield nitrite and nitrate at a rate of $1 \times 10^3$ M⁻¹s⁻¹ [54] (equation 9). Alternatively, the half equivalent of nitrate could be generated from the fast oxidation of nitrogen dioxide by ferryl hemoglobin (equation 10) [16].

$$\text{HbFe}^{IV}=O + \text{NO}_2^- \xrightarrow{H_2O} \text{Hb}[\text{Fe}^{III}\text{OH}_2]^3+ + \text{NO}_2^* \quad (8)$$

$$2 \text{NO}_2^* \xrightarrow{N_2O_4} \text{N}_2\text{O}_4 \xrightarrow{H_2O} \text{NO}_2^- + \text{NO}_3^- + 2 \text{H}^+ \quad (9)$$

$$\text{HbFe}^{IV}=O + \text{NO}_2^* \xrightarrow{H_2O} \text{Hb}[\text{Fe}^{III}\text{OH}_2]^3+ + \text{NO}_3^- \quad (10)$$

When only one equivalent of hydrogen peroxide is allowed to react with metmyoglobin to generate ferryl myoglobin as in the experiments in the presence of tyrosine discussed below, only about 25% nitrate (relative to metmyoglobin) is generated. This result suggests that oxidation of
metmyoglobin by one equivalent of hydrogen peroxide does not yield ferryl myoglobin quantitatively. Indeed, we have found that nitrite-induced reduction of ferryl myoglobin generates half an equivalent of nitrate and half an equivalent of nitrite [12]. In addition, comparison of the UV-visible spectrum of ferryl myoglobin formed by an excess of hydrogen peroxide and the spectrum obtained after reaction of metmyoglobin and one equivalent of hydrogen peroxide indicate that only about 60% of metmyoglobin is oxidized to ferryl myoglobin (Figure 9). Therefore, 25% yield of nitrate reflects about half the concentration of formed ferryl myoglobin.

In contrast, when nitrite is allowed to react in situ with the ferryl myoglobin protein radical (\(\text{\textsuperscript{\textbullet}MbFe}^{\text{IV}}=\text{O}\)) generated from the reaction of one equivalent of hydrogen peroxide with metmyoglobin, about 75% nitrate (relative to metmyoglobin) are generated. The increased yield of nitrate is probably due to the reaction between nitrite and the protein radical which also may generate nitrogen dioxide (equation 11). As discussed above, dimerization of nitrogen dioxide and subsequent hydrolysis (equation 9) or reaction with the ferryl group (equation 10) lead to the formation of nitrate.

\[\text{\textsuperscript{\textbullet}MbFe}^{\text{IV}}=\text{O} + \text{NO}_2^- \rightarrow \text{MbFe}^{\text{IV}}=\text{O} + \text{NO}_2^\text{\textbullet}\]  \hspace{1cm} (11)

As it has been shown that in the first step the reaction between metmyoglobin and hydrogen peroxide generates a short-lived species analogous to Compound I of peroxidases [9], we cannot exclude that nitrite also reacts with the intermediary formed porphyrin \(\pi\)-cation radical. Indeed, the rate of the reaction between Compound I of myeloperoxidase and nitrite (2.0 \(\times\) 10\(^6\) M\(^{-1}\)s\(^{-1}\) at pH 7 and 15 °C) is significantly higher than that for the reaction between Compound II of myeloperoxidase and nitrite (5.5 \(\times\) 10\(^2\) M\(^{-1}\)s\(^{-1}\) at pH 7 and 15 °C) [48].
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3.4.4 Nitration of protein-bound tyrosine by in situ reaction of metmyoglobin or methemoglobin with nitrite and hydrogen peroxide

3-Nitrotyrosine has been found in tissues of patients affected by diseases such as heart diseases [55], cancer [56] and also Alzheimer’s disease [57]. It is known that tyrosine nitration leads to inactivation of several enzymes [58, 59]. As reaction between oxoperoxonitrate(1-) and tyrosine leads to formation of 3-nitrotyrosine, detection of 3-nitrotyrosine has been proposed to be a marker for oxoperoxonitrate(1-) formation in vivo [60].

However, it has recently been shown that reaction of nitrite and hydrogen peroxide with peroxidases such as myeloperoxidase or eosinophil peroxidase may also contribute to formation of 3-nitrotyrosine in vivo [26]. Therefore, we have investigated whether myoglobin and hemoglobin, two proteins that have been shown to display peroxidase-like activity [61], can also nitrate tyrosine in the presence of nitrite and hydrogen peroxide. We have previously shown that no 3-nitrotyrosine is generated from the reactions of nitrite with ferryl myoglobin [12]. In contrast, addition of hydrogen peroxide to a solution of metmyoglobin and nitrite (at pH 7.0) yields small amounts of 3-nitrotyrosine (Figure 13). The yield of nitrated tyrosine residues decreases with increasing nitrite concentrations. Oxidation of metmyoglobin with hydrogen peroxide yields the ferryl myoglobin protein radical (equation 12). It has been shown that the protein radical is partly located on a tyrosine residue [7]. Thus, protein-bound 3-nitrotyrosine is most likely formed by reaction of the protein bound tyrosyl radical and nitrogen dioxide (equation 14). Nitrogen dioxide can be generated by reduction of the ferryl group (equation 13) or from the reaction of the protein radical with nitrite (equation 11). In the presence of higher nitrite concentrations, the nitrite-mediated reduction of the protein radical reduces the possible reaction between nitrogen dioxide and the protein radical, and thus the...
amount of formed 3-nitrotyrosine. We did not observe any significant changes when the reaction was carried out at pH 6.0 (Figure 13).

\[
\text{MbFe}^{III}\text{OH}_2 + \text{H}_2\text{O}_2 \rightarrow \text{Tyr-MbFe}^{IV}=\text{O} \tag{12}
\]

\[
\text{MbFe}^{IV}=\text{O} + \text{NO}_2^- \stackrel{\text{H}_2\text{O}}{\longrightarrow} \text{Mb}[\text{Fe}^{III}\text{OH}_2]^{3+} + \text{NO}_2^* \tag{13}
\]

\[
\text{NO}_2^* + \text{Tyr}^-\text{MbFe}^{III}\text{OH}_2 \rightarrow \text{NO}_2^-\text{Tyr-MbFe}^{III}\text{OH}_2 \tag{14}
\]

We also studied the formation of 3-nitrotyrosine by in situ reaction of methemoglobin, nitrite and hydrogen peroxide. In the presence of methemoglobin, yield of 3-nitrotyrosine was slightly higher than that found in the respective reaction with metmyoglobin, but also decreased with increasing concentration of nitrite (Figure 14).

3.4.5 Nitration of free tyrosine by in situ reaction of metmyoglobin or methemoglobin, nitrite, hydrogen peroxide and tyrosine

We have recently shown that when ferryl myoglobin is allowed to react with nitrite in the presence of free tyrosine, 3-nitrotyrosine is generated only in very low yields. In contrast, addition of hydrogen peroxide to a solution of metmyoglobin, nitrite and tyrosine generates higher amounts of 3-nitrotyrosine [12]. We therefore concluded that the ferryl myoglobin protein radical, which is present when ferryl myoglobin is generated in situ, is essential to get a significant yield of 3-nitrotyrosine.

Here we show that the relative yield of 3-nitrotyrosine increases with increasing nitrite concentration when the concentrations of the protein, hydrogen peroxide, and tyrosine are kept constant. The amount of formed 3-nitrotyrosine increases up to a saturation value of about 16 % (Figure 15).
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In this system, formation of 3-nitrotyrosine most likely proceeds via reaction of nitrogen dioxide and a tyrosine radical (equation 18). Tyrosyl radical can be formed by the reaction of nitrogen dioxide with tyrosine ($E^\circ = 0.93$ V) [62] (equation 17), which proceeds with a second-order rate constant of about $10^5 \text{ M}^{-1}\text{s}^{-1}$ [22]. Another possibility for tyrosyl radical formation is the reaction of tyrosine with ferryl myoglobin (equation 16). The rate constant for this reaction is $34 \pm 2 \text{ M}^{-1}\text{s}^{-1}$ (data not shown), a value that is in the same order of magnitude than that of the reaction between ferryl myoglobin and nitrite. A third source for tyrosyl radical is the oxidation of tyrosine by the ferryl myoglobin protein radical (equation 15), a reaction that is likely to proceed faster than the corresponding oxidation by ferryl myoglobin. For comparison, the reaction between tyrosine and Compound I of myeloperoxidase ($2.93 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ at pH 7.4) is about one order of magnitude faster than the reaction between tyrosine and Compound II of myeloperoxidase ($2.46 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ at pH 7.4) [63].

\[
\begin{align*}
\text{MbFe}^{IV}=O + \text{Tyr} & \rightarrow \text{MbFe}^{IV}=O + \text{Tyr}^* \quad (15) \\
\text{MbFe}^{IV}=O + \text{Tyr} & \xrightarrow{\text{H}_2\text{O}} \text{Mb[Fe}^{III}\text{OH}_2]^3+ + \text{Tyr}^* \quad (16) \\
\text{NO}_2^* + \text{Tyr} & \rightarrow \text{NO}_2^- + \text{Tyr}^* \quad (17) \\
\text{NO}_2^* + \text{Tyr}^* & \rightarrow \text{NO}_2^-\text{-Tyr} \quad (18)
\end{align*}
\]

As the nitrite- and the tyrosine-induced reductions of ferryl myoglobin are very slow, the concentrations of tyrosyl radicals and nitrogen dioxide in solution are rather low. Therefore, the yields of 3-nitrotyrosine are low in the presence of ferryl myoglobin. On the other side, the concentrations of tyrosyl radical and nitrogen dioxide should be higher in the reactions with the ferryl myoglobin protein radical and this explains the higher 3-nitrotyrosine yields.

With increasing concentrations of nitrite, large amounts of nitrite react with the ferryl myoglobin protein radical and limit the yield of tyrosyl radical (equation 11) which is needed for formation of 3-nitrotyrosine (equation 18).
We also studied the nitration of free tyrosine by \textit{in situ} reaction of methemoglobin, tyrosine, hydrogen peroxide, and variable concentrations of nitrite. Here we found a maximum yield of about 12\% 3-nitrotyrosine relative to protein concentration (Figure 15).

When the \textit{in situ} reaction between metmyoglobin, tyrosine, hydrogen peroxide (one equivalent relative to protein concentration) and variable concentrations of nitrite is carried out at pH 6.0, maximum yield of 3-nitrotyrosine is reached at lower concentrations of nitrite with a maximum level of about 11\% yield (relative to protein) (Figure 16). At lower pH, the reactions involved in the formation of 3-nitrotyrosine (equations 11–13, 15–17) may have higher reaction rates. Therefore, concentration levels of both, tyrosyl radical and nitrogen dioxide should reach sufficient levels for effective generation of 3-nitrotyrosine already in the presence of lower concentrations of nitrite. However, as rates of yield limiting reactions (equation 9, 10) may also increase, maximum yield of 3-nitrotyrosine is not changed significantly. Therefore, under conditions of low tissue pH observed after ischemia/reperfusion, nitration of tyrosine may be of particular physiological relevance.

When the \textit{in situ} reaction between myoglobin, hydrogen peroxide, and nitrite is carried out in the presence of variable concentrations of tyrosine, yield of 3-nitrotyrosine decreases with increasing concentrations of tyrosine (Table 9). In addition, when the \textit{in situ} reaction between nitrite and tyrosine is carried out in the presence of variable concentrations of myoglobin/hydrogen peroxide, yield of 3-nitrotyrosine (relative to the protein) also decreases with increasing concentrations of myoglobin (Table 10).

As shown in Table 11, the relative ratios of metmyoglobin/hydrogen peroxide, nitrite, and tyrosine seem to determine the 3-nitrotyrosine yield. In the presence of equal concentrations of nitrite and tyrosine, the yield of 3-nitrotyrosine (relative to the protein) increases with increasing concentrations of
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nitrite and tyrosine. In the presence of the same ratios metmyoglobin/hydrogen peroxide to nitrite/tyrosine, however, also yields of 3-nitrotyrosine are identical.

Table 11  % Yield of 3-nitrotyrosine (relative to protein) shown for relative ratios of the concentrations of myoglobin, nitrite and tyrosine.

<table>
<thead>
<tr>
<th>metmyoglobin</th>
<th>nitrite</th>
<th>Tyr</th>
<th>Yield of 3-nitrotyrosine (% relative to protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (250 μM)</td>
<td>2</td>
<td>2</td>
<td>2.3</td>
</tr>
<tr>
<td>1 (500 μM)</td>
<td>2</td>
<td>2</td>
<td>2.5</td>
</tr>
<tr>
<td>1 (250 μM)</td>
<td>4</td>
<td>4</td>
<td>3.1</td>
</tr>
<tr>
<td>1 (250 μM)</td>
<td>9</td>
<td>9</td>
<td>4.2</td>
</tr>
</tbody>
</table>

In contrast to myeloperoxidase-catalyzed formation of dityrosine in the presence of tyrosine and hydrogen peroxide [63], we could never detect any dityrosine, even with very high concentrations of free tyrosine and in the absence of oxygen. Most likely, concentrations of tyrosyl radical generated in the course of the reaction are too small to lead to detectable amounts of dityrosine or tyrosine radicals may react with the protein.

3.5 Conclusions

In the present work we have determined the rate constant for the nitrogen monoxide-mediated reduction of ferryl hemoglobin to be $$(24 \pm 2) \times 10^6 \text{ M}^{-1}\text{s}^{-1}$$ (at pH 7.0 and 20 °C). Therefore, under pathological conditions when the ferryl hemoglobin concentration can be significant, this reaction may contribute to detoxify this highly oxidizing species. In contrast, the rate constant for the nitrite-mediated reduction of ferryl hemoglobin, $$(7.5 \pm 0.4) \times 10^2 \text{ M}^{-1}\text{s}^{-1}$$ (at pH
7.0 and 20 °C) is too slow to play a significant role under physiological conditions. Although the reaction is about one order of magnitude faster than the corresponding reaction of ferryl myoglobin, this reaction still only may be important if no other effective antioxidant species like nitrogen monoxide or oxoperoxonitrate(1-) is present. As the rate increases at lower pH, it may be of higher relevance under conditions of ischemia/reperfusion, which are characterized by acidic tissue pH.

Finally, we have shown that in the presence of nitrite the in situ reactions of metmyoglobin or methemoglobin with hydrogen peroxide can yield significant amounts of 3-nitrotyrosine. Under patophysiological conditions in which hydrogen peroxide and nitrite levels are elevated, these reactions may thus contribute to nitration of tyrosine, free or incorporated in proteins.

3.6 References

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Reactions of nitrogen monoxide and oxoperoxonitrate(1-) with iron porphyrin model complexes

4.1 Introduction

It has been shown that the reaction between nitrogen monoxide and oxyhemoglobin (Hb[FeO_2]^{2+}) is very fast (89 \times 10^6 \text{ M}^{-1}\text{s}^{-1} at pH 7.0, 20 °C) [1] and most likely proceeds via formation of the oxoperoxonitrato methemoglobin complex (Hb[Fe^{III}OONO]^{2+}) which subsequently decays to methemoglobin (HbFe^{III}) and nitrate [1]. Given the high reaction rate between nitrogen monoxide and oxyhemoglobin, the reaction is expected to be the main route for depletion of endothelium nitric oxide synthase-derived nitrogen monoxide in the bloodstream [2]. In order to synthesize a model for the oxoperoxonitrato methemoglobin complex, there are two possible approaches how to obtain a oxoperoxonitrato complex. When a dioxygen iron porphyrin complex reacts with nitrogen monoxide, a oxoperoxonitrato complex should be obtained as an intermediate. It has been shown that in the reaction between pentacyanosuperoxocobaltate(III) and nitrogen monoxide a quite stable oxoperoxonitrato complex is generated [3, 4]. The other strategy to form a oxoperoxonitrato complex is to mix an iron(III) porphyrin complex with
oxoperoxonitrate(1-) which also has been proposed to form a oxoperoxonitrato complex as an intermediate [5].

Different types of porphyrin model complexes of hemoglobin and myoglobin have been developed to study reversible binding of dioxygen. Simple iron porphyrin complexes such as [Fe(TPP)] (TPP: mesotetraphenylporphyrin\(^1\)) are easy to prepare by insertion of an iron ion into the porphyrin system. In order to mimic the active site of hemoglobin, the model complexes need a coordinated nitrogen base ligand (imidazole, pyridine etc.) [6]. The dioxygen complex [Fe(TPP)(1-MeIm)(O\(_2\))] with an 1-methylimidazole (1-MeIm) ligand coordinated to the iron has been shown to reversibly bind dioxygen [7, 8]. However, it is only stable at very low temperature and in diluted solution. At room temperature, [Fe(TPP)(1-MeIm)(O\(_2\))] irreversibly forms the \(\mu\)-oxo iron(III) dimer [(TPP)Fe\(^{III}\)-O-Fe\(^{III}\)(TPP)] [9]. Therefore, the active centre needs to be sterically protected to prevent irreversible oxidation. The first iron model complex which binds dioxygen reversibly at room temperature has been the “picket fence” iron porphyrin complex [Fe(TpivPP)] [TpivPP: meso-tetra-(\(\alpha,\alpha,\alpha,\alpha\)-o-pivalamidophenyl)-porphyrin\(^2\)] [10]. The aprotic cavity formed by the four pivalamidophenyl substituents prevents autoxidation. [Fe(TpivPP)(1-MeIm)(O\(_2\))] is very stable at room temperature and reversibly binds dioxygen [10].

In order to mimic the relaxed- and tense-state of hemoglobin, it has been shown that variation of the ligand determines the oxygen affinity of the porphyrin complex [6]. The steric hindrance caused by the 2-methylimidazole and 1,2-dimethylimidazole ligands decreases the dioxygen affinity of the deoxy iron porphyrin complex analogously to the reduced affinity of dioxygen in the tense-state of hemoglobin [11]. Oxygenation experiments in the crystal showed that when 1-methylimidazole is coordinated to the iron-TpivPP complex, no

\(^1\) TPP: meso-tetraphenylporphyrin

\(^2\) TpivPP: meso-tetra-(\(\alpha,\alpha,\alpha,\alpha\)-o-pivalamidophenyl)-porphyrin
cooperativity effect is observed. However, complexes with the more bulky imidazoles 2-methylimidazole and 1,2-dimethylimidazole show significant cooperativity during binding of dioxygen [12]. It has been proposed that coordination of dioxygen to the iron may cause a conformational change within the crystal which enhances the dioxygen affinity of the remaining deoxy sites [12].

One of the problems with the iron porphyrin complexes such as [Fe(TpivPP)(1-MeIm)(O_2)] is that an excess of the free proximal ligand has to be present in solution as the coordinated ligand is in an equilibrium with the free ligand. This problem has been solved by covalently attaching the ligand to the porphyrin structure [6]. In complexes such as iron-TpivPP, the ligand can be attached by amide linkage to one of the phenyl substituents which is directed to the other side of the porphyrin plane [13]. The base can also be attached via binding to a β-pyrrole position of the tetraphenylporphyrin ring system [14]. Another approach to connect the ligand with the porphyrin is to attach a histidine-containing peptide fragment via a thioether linkage [15]. In both-face-hindered porphyrins, the nitrogenous base is covalently bound at two positions of the porphyrin. Therefore, the ligand is fixed close to the iron ion and a high degree of iron-coordinated base is ensured [6]. In addition, the double face hindering should inhibit the dimerization which is observed when a simple attached base of one complex coordinates to the iron ion of another porphyrin complex at low temperature [6].

In this chapter we present kinetic and mechanistic studies of reactions of nitrogen monoxide and oxoperoxonitrate(1-) with the water-insoluble porphyrin model complex [Fe(TpivPP)(1-MeIm)(O_2)] and with iron- tetraphenylporphyrin complexes in different oxidation states. We attempted to obtain a model for the oxoperoxonitrato methemoglobin complex which is formed during nitrogen monoxide-mediated oxidation of oxyhemoglobin. During our studies, others
Chapter 4 showed that water-soluble iron(III) porphyrin complexes have significant reactivity towards oxoperoxonitrate(-1) [5, 16, 17]. Therefore, it was interesting to compare these results with our results which we obtained by performing analogous reactions in organic solvents.

4.2 Materials and methods

Reagents

[Fe$^{III}$TPP(Cl)] was obtained from Midcentury Chemicals. Concentrations of [Fe$^{III}$TPP(Cl)] solutions were determined by UV-visible spectroscopy ($\varepsilon_{418} = 99.1$ mM$^{-1}$cm$^{-1}$; $\varepsilon_{507} = 12$ mM$^{-1}$cm$^{-1}$ in dichloromethane) [18]. [Na(18-crown-6)]$_2$S$_2$O$_4$ (18-crown-6: 1,4,7,10,13,16-hexaoxacyclooctadecane) was prepared according to the literature [19]. Dichloromethane, toluene (Baker, analyzed grade) and 1-methylimidazole (1-MeIm) (Fluka, puriss.) were used without further purification.

Nitrogen monoxide was obtained from Linde and passed through a sodium hydroxide solution as well as a column of sodium hydroxide pellets to remove higher nitrogen oxides before use. Nitrogen monoxide stock solutions (dichloromethane or toluene) were prepared by degassing the solvents for 45 minutes with nitrogen, followed by saturation with nitrogen monoxide for 3 hours. The concentrations of the saturated solutions are 11.0 mM (dichloromethane) and 11.4 mM (toluene).

The complex [Fe(TpivPP)(1-MeIm)(O$_2$)] was prepared according to the literature [10], but by using sodium dithionite as the reducing agent instead of [Cr(acetylacetonate)$_2$]. In brief, 200 mg (200 $\mu$mol) meso-tetra-(a,a,a,a-o-pivalamidophenyl)-porphyrin, 200 mg (930 $\mu$mol) iron(II)bromide, 73 mL pyridine, and 13.3 mL 1,2-dimethoxyethane were heated under reflux for 2 hours under nitrogen. Then the solution was brought to dryness on a rotary
evaporator on air. The solid residue was redissolved in trichloromethane, purified chromatographically over alumina (activity I) with trichloromethane as eluent and dried again on a rotary evaporater to yield \([\text{Fe}^{III}(\text{TpivPP})(1-\text{MeIm})(\text{Br})]_{1/2}\text{CHCl}_3\). To obtain \([\text{Fe}(\text{TpivPP})(1-\text{MeIm})(\text{O}_2)]\), \([\text{Fe}^{III}(\text{TpivPP})(1-\text{MeIm})(\text{Br})]_{1/2}\text{CHCl}_3\) (20 mg, 17 \(\mu\)mol) and sodium dithionite (55 mg, 316 \(\mu\)mol) were placed in a reaction flask under nitrogen. 10 mL of degassed dichloromethane, 0.25 mL of a degassed dichloromethane solution of 1-methylimidazole (1 M, 250 \(\mu\)mol) and 1 mL of degassed water were added into the flask. The resulting solution was allowed to react for 2 hours at room temperature. The reaction mixture was washed with water (3 \(\times\) 2 mL) to remove sodium sulfate and excess sodium dithionite in the presence of air. Finally, the solution was brought to dryness on a rotary evaporator. The concentration of the \([\text{Fe}(\text{TpivPP})(1-\text{MeIm})(\text{O}_2)]\) solutions was determined spectroscopically \((\varepsilon_{426} = 159 \text{ mM}^{-1}\text{cm}^{-1}; \varepsilon_{548} = 15 \text{ mM}^{-1}\text{cm}^{-1}\) in dichloromethane) [13]. To increase the stability of the complex in dichloromethane as solvent, 1-methylimidazole was added in excess (about 10 equivalents).

**Stopped-flow kinetic analysis**

Kinetic studies were carried out with an On-Line Instrument System stopped flow instrument equipped with an OLIS RSM 1000 rapid scanning monochromator and with an Applied Photophysics SX17MV single wavelength stopped-flow instrument. The pathlengths of the cells in the two spectrophotometers were 1 cm. With the Applied Photophysics apparatus, kinetic traces were taken at different wavelengths between 300 and 430 nm and the data were analyzed with the SX17MV operating software or with Kaleidagraph, Version 3.0.5. The results of the fits of the traces (averages of at least 10 single traces) from at least four experiments were averaged to obtain each observed rate constant, given as the mean \(\pm 2 \times\) standard deviation.
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Solutions of \([\text{Fe(TpivPP)(1-MeIm)(O}_2\text{)}]\) were prepared in dichloromethane in the presence of an excess of 1-methylimidazole (about 10 equivalents) to increase stability of the solution. The used solutions were in the concentration range 5-12 \(\mu\text{M}\).

Nitrogen monoxide solutions were prepared by diluting the stock solutions with degassed solvent in gas-tight SampleLock Hamilton syringes. The final nitrogen monoxide concentrations were controlled with an ANTEK Instruments nitrogen monoxide analyzer, equipped with a chemiluminescence detector. The organic solutions were directly injected into the water vessel of the analyzer apparatus to determine nitrogen monoxide concentrations.

Solutions of \([\text{Fe}^{III}(\text{TPP})(\text{Cl})]\) for the stopped-flow measurements were prepared by first dissolving \([\text{Fe}^{III}(\text{TPP})(\text{Cl})]\) in dimethyl sulfoxide and then adding acetonitrile (final ratio 15 : 85). The used solutions were in the concentration range 12-18 \(\mu\text{M}\).

Oxoperoxonitrate(1-) was prepared from potassium superoxide and nitrogen monoxide according to the method described in the literature [20] and stored in a polyethylene bottle at \(-20 ^\circ\text{C}\). The concentration of the oxoperoxonitrate(1-) solutions was determined spectrophotometrically by measuring the absorbance at 302 nm \((\varepsilon = 1.70 \text{ mM}^{-1}\text{cm}^{-1})\) [21].

Oxoperoxonitrate(1-) solutions for measurements were prepared by diluting an oxoperoxonitrate(1-) solution of a specific concentration with acetonitrile and aqueous sodium hydroxide (0.01 M) in a ratio of 0.5 : 10 : 1.5 and stored on ice. Under these conditions, oxoperoxonitrate(1-) was shown to be stable for about 30 minutes. The final oxoperoxonitrate(1-) concentration was in the range 125-580 \(\mu\text{M}\).

**UV-visible spectra**

UV-visible spectra were collected on a UVIKON 820 spectrophotometer with 1 cm quartz cuvettes.
Ion chromatographic product analysis of the reaction of $[\text{Fe}^{III}(\text{TPP})(1-\text{MeIm})(\text{O}_2)]$ with nitrogen monoxide

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 as described previously [22]. A phthalic acid solution (2.5 mM phthalic acid, 5 % acetonitrile, pH 4.2 Tris) was used as eluent. Calibration curves were obtained by measuring 5-10 standard aqueous sodium nitrite and sodium nitrate solutions. 50 µL of a dichloromethane solution of 1-methylimidazole (250 mM, 12.5 µmol) were added to 10 mL of a dichloromethane solution of $[\text{Fe}^{III}(\text{TPP})(\text{Cl})]$ (100 µM, 1.0 µmol). The resulting solution was degassed with nitrogen for 30 minutes and then 40 µL of a suspension of $[\text{Na}(18\text{-crown-6})]_2\text{S}_2\text{O}_4$ (68 µmol suspended in 1 mL water, 2.7 µmol, 2.7 equivalents) was added. The reaction mixture was allowed to react for 1 hour. During the reaction, the colour of the mixture changed from green-brown to red. The resulting mixture was cooled to −78 °C and transferred under argon into a new Schlenk flask to remove water and excess $[\text{Na}(18\text{-crown-6})]_2\text{S}_2\text{O}_4$ from the reaction solution. The solution was saturated with dioxygen for 20 minutes and 180 µL of a dichloromethane solution of nitrogen monoxide (11 mM, 2 µmol) was added into the sealed Schlenk flask. The resulting solution was allowed to react for 30 minutes. Finally, the solution was allowed to warm up to room temperature and nitrite and nitrate were extracted with $2 \times 10$ mL of water. The aqueous solution was analyzed by ion chromatography.

The amount of contaminating nitrite and nitrate present in the nitrogen monoxide solution was determined by injecting with a gas-tight syringe 1 mL of the nitrogen monoxide saturated solution into a Schlenk flask filled with nitrogen and sealed with a rubber septum. Nitrogen monoxide was then removed with the pump-freeze-and-thaw method. The resulting solution was extracted with water and analyzed by ion chromatography.
4.3 Results

4.3.1 Nitrogen monoxide-mediated oxidation of

\[ \text{[Fe(TpivPP)(1-MeIm)(O_2)]} \]

The reaction between nitrogen monoxide and \([\text{Fe(TpivPP)(1-MeIm)(O_2)}] \) was first studied by UV-visible spectroscopy. Stepwise addition of nitrogen monoxide to a not degassed dichloromethane solution of \([\text{Fe(TpivPP)(1-MeIm)(O_2)}] \) in a sealed cuvette resulted in the changes of the absorbance spectra shown in Figure 1. After addition of 10 equivalents of nitrogen monoxide, the maximum of the Soret band shifted from 426 (spectrum 1) to 420 nm (spectrum 5) with an isosbestic point at 422 nm. Further addition of nitrogen monoxide resulted in an additional shift of the maximum of the Soret band to 418 nm (spectrum 13), accompanied by a significant decrease in the absorbance of the band and an isosbestic point at 409 nm. In the visible part of the spectrum (Figure 1B), the band at 540 nm first shifted to 547 nm (spectrum 5 in Figure 1B) and then the absorption of the band decreased. At 504 and 643 nm, two bands were formed. The absorbance of these two bands continuously increased during addition of nitrogen monoxide. There is an isosbestic point observed at 522 nm for the spectra 1-5 and three isosbestic points at 480, 528, and 581 nm for the spectra 5-13.
**Figure 1** UV-visible spectra (A Soret band and B, visible part) of a [Fe(TpivPP)(1-MeIm)(O₂)] solution (9.3 μM in dichloromethane) after subsequent additions of a nitrogen monoxide solution in dichloromethane. Spectrum 1: [Fe(TpivPP)(1-MeIm)(O₂)], spectra 2-8: addition of 1-7 equivalents of nitrogen monoxide, spectrum 9: after addition of 10 equivalents of nitrogen monoxide, spectra 10-12 after addition of 19, 28, 37 equivalents nitrogen monoxide, and spectrum 13 after addition of a total of 54 equivalents of nitrogen monoxide.
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As nitrogen monoxide solutions are always contaminated with nitrite, we also determined the absorbance changes arising from stepwise addition of a dichloromethane solution of [Na(18-crown-6)]NO₂ to a solution of [Fe(TpivPP)(1-MeIm)(O₂)] (Figure 2). By addition of nitrite, the maximum of the Soret band continuously shifted from 426 nm (spectrum 1 in Figure 2A) to 420 nm (spectrum 11), maintaining approximately the same intensity, with an isosbestic point for the spectra 1-3 at 406 nm. In the visible part of the spectrum, addition of 0.78 equivalents of nitrite induced a shift of the absorption band from 536 (spectrum 1 in Figure 2B) to 538 nm (spectrum 3), with an isosbestic point at 572 nm. Further addition of nitrite caused a continuous shift of its maximum to 549 nm (spectrum 11), with an isosbestic point for the spectra 4-10 at 548 nm.

Figure 2 UV-Vis spectra (A Soret band and B, visible part) of a [Fe(TpivPP)(1-MeIm)(O₂)] solution (9.3 μM in dichloromethane) after subsequent additions of [Na(18-crown-6)]NO₂ (in dichloromethane). Spectrum 1: [Fe(TpivPP)(1-MeIm)(O₂)], spectra 2-4: after addition of 0.26, 0.52, and 0.78 equivalents of nitrite, spectra 5-10: after addition of 1.3, 3.9, 10.4, 16.9, 23.4 and 29.9 equivalents of nitrite and spectrum 11 after addition of a total of 56 equivalents of nitrite.
The reaction between nitrogen monoxide and \([Fe(TpivPP)(1-MeIm)(O_2)]\) in dichloromethane was also studied by single-wavelength stopped-flow spectroscopy at 20 °C. In order to avoid problems related with the correct determination of the concentration of the nitrogen monoxide solution, kinetic measurements were performed with an 8- to 10-fold excess of \([Fe(TpivPP)(1-MeIm)(O_2)]\). As shown in Figure 3, when nitrogen monoxide (1.5 µM) was mixed with an excess of \([Fe(TpivPP)(1-MeIm)(O_2)]\) (11.5 µM), the trace measured at 418 nm showed first a perturbation over 150 ms which is always present and independent of porphyrin concentrations. It may be caused by the compression of the organic solvent used. Over a longer time-scale, 1-5 s, the measurement showed an increase of the absorption. Analysis of this part of the trace showed that a two-exponential expression was needed to fit the data. The rate of the first step depended linearly on the iron-porphyrin complex concentration. The second-order rate constant for this reaction, estimated from the linear fit of the plot of \(k_{observed}\) versus \([Fe(TpivPP)(1-MeIm)(O_2)]\)-concentration (Figure 4), is \(k_1 = (1.0\pm0.2) \times 10^6 \text{ M}^{-1}\text{s}^{-1}\). The observed rate constant for the second step varied between 0.89 and 2.32 s\(^{-1}\) (Figure 4), with an
average of $k_2 = 2 \pm 1 \text{s}^{-1}$, without showing a clear dependence on the complex concentration.

**Figure 3** Time course measured at 418 nm for the reaction of [Fe(TpivPP)(1-MeIm)(O$_2$)] (11.5 µM) with nitrogen monoxide (1.5 µM) in dichloromethane at 20 °C. The solid line corresponds to the best fit, which resulted in the observed rate constants of 10 s$^{-1}$ and 1.1 s$^{-1}$.

**Figure 4** (▲) Plot of $k_{1,\text{observed}}$ versus [Fe(TpivPP)(1-MeIm)(O$_2$)] concentration for the reaction between [Fe(TpivPP)(1-MeIm)(O$_2$)] and nitrogen monoxide in dichloromethane at 20 °C. The second-order rate constant obtained from the linear plot is $(1.0 \pm 0.2) \times 10^6 \text{M}^{-1}\text{s}^{-1}$. (●) Data obtained for $k_{2,\text{observed}}$. 

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Finally, the reaction between nitrogen monoxide and \([\text{Fe(TpivPP)(1-MeIm)(O}_2)]\) in dichloromethane was studied by rapid-scan UV-visible spectroscopy between 300 and 450 nm at 8 °C. Upon mixing \([\text{Fe(TpivPP)(1-MeIm)(O}_2)]\) (5.9 μM) with nitrogen monoxide (17 μM), the Soret band shifted from 426 (\([\text{Fe(TpivPP)(1-MeIm)(O}_2)]\)) to 420 nm, accompanied by a continuous decrease of the absorption (Figure 5).

![Absorbance vs Wavelength](image)

**Figure 5** Rapid-scan UV-visible spectra of the reaction of \([\text{Fe(TpivPP)(1-MeIm)(O}_2)]\) (5.9 μM) with nitrogen monoxide (17 μM) in dichloromethane at 8 °C. Time intervals of the shown spectra are: traces 1-8, every 200 ms, and trace 9 after a total of 2.0 s.

### 4.3.2 Analysis of the nitrogen-containing products from the reaction of \([\text{Fe(TPP)(1-MeIm)(O}_2)]\) with nitrogen monoxide

In order to determine the final nitrogen-containing products of the reaction between nitrogen monoxide and \([\text{Fe(TPP)(1-MeIm)(O}_2)]\), we performed ion chromatographic analysis of nitrite and nitrate. As \([\text{Fe(TPP)(1-MeIm)(O}_2)]\) (in dichloromethane) is stable only at very low temperatures in dioxygen containing solutions [8], reactions had to be performed at −78 °C. \([\text{Fe(TPP)(1-MeIm)(O}_2)]\)
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was obtained by reduction of $[\text{Fe}^{III}(\text{TPP})(\text{Cl})]$ with sodium dithionite and subsequent oxygenation in the presence of an excess of 1-methylimidazole. After reaction with nitrogen monoxide, nitrite and nitrate formed in the course of the reaction were extracted with water from the organic solvent. Addition of two equivalents nitrogen monoxide to a solution of $[\text{Fe(TPP})(1\text{-MeIm})(\text{O}_2)]$ yielded less than one equivalent nitrate relative to the complex concentration. Furthermore, results were not reproducible and also the sum of formed nitrite and nitrate were always less than expected (Table 1). For control, nitrogen monoxide was allowed to react with oxygen in dichloromethane in the absence of the iron porphyrin complex. Also under this conditions, the amount of formed nitrate and nitrite were much less than expected (Table 1).

<table>
<thead>
<tr>
<th>[TPP(1-MeIm)Fe(O_2)] (µM)</th>
<th>NO* added (µM)</th>
<th>Contamination deriving from the NO* stock solution</th>
<th>Total concentrations found after reaction a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[NO₂⁻] (µM)</td>
<td>[NO₃⁻] (µM)</td>
<td>[NO₂⁻] (µM) [NO₃⁻] (µM)</td>
</tr>
<tr>
<td>125</td>
<td>250</td>
<td>26±8</td>
<td>110±16 [NO₂⁻] 98±12 [NO₃⁻]</td>
</tr>
<tr>
<td>135</td>
<td>270</td>
<td>9±1</td>
<td>12±4 [NO₂⁻] 147±8 [NO₃⁻]</td>
</tr>
<tr>
<td>124</td>
<td>248</td>
<td>23±4</td>
<td>0 [NO₂⁻] 112±12 [NO₃⁻]</td>
</tr>
<tr>
<td>0</td>
<td>270</td>
<td>9±1</td>
<td>25±8 [NO₂⁻] 104±4 [NO₃⁻]</td>
</tr>
</tbody>
</table>

a The amount of contaminating nitrite and nitrate present in the nitrogen monoxide solution were not substracted.
4.3.3 \([\text{Fe}^{\text{III}}(\text{TPP})(\text{Cl})]\)-accelerated decay of oxoperoxonitate(1-)

The reaction of \([\text{Fe}^{\text{III}}(\text{TPP})(\text{Cl})]\) with oxoperoxonitate(1-) was studied by single-wavelength stopped-flow spectroscopy as well as by rapid-scan UV-visible spectroscopy at 20 °C. As \([\text{Fe}^{\text{III}}(\text{TPP})(\text{Cl})]\) is not water-soluble, the iron-porphyrin solution was prepared by first dissolving \([\text{Fe}^{\text{III}}(\text{TPP})(\text{Cl})]\) in dimethyl sulfoxide and then adding acetonitrile to get a final volumetric ratio of 15 : 85. Under these conditions, a mixture of iron(III)-tetraphenylporphyrin-solvents complexes with iron-coordinated dimethyl sulfoxide and acetonitrile ligands is formed. The spectrum shows a maximum of the Soret band at 394 nm and a shoulder at 408 nm (spectrum A in Figure 6, Table 2). Addition of aqueous sodium hydroxyde (0.01 M) to this solution yields the \([\text{Fe}^{\text{III}}(\text{TPP})]_2\text{O}\) complex with a maximum of the Soret band at 414 nm (spectrum B in Figure 6, Table 2). The oxoperoxonitate(1-) solutions were prepared by diluting a stock solution (in 0.01 M aqueous sodium hydroxyde) with aqueous sodium hydroxyde (0.01 M) to a specific concentration. This solution was further diluted with aqueous sodium hydroxyde (0.01 M) and acetonitrile at a volumetric ratio of 0.5:1.5:10 and stored on ice. Under these conditions, oxoperoxonitate(1-) was shown to be stable for about 30 minutes.
Figure 6 Soret band and visible spectra of iron(III)-tetraphenylporphyrin complexes in acetonitrile/dimethyl sulfoxide (85:15). A Solvents complexes derived from dissolving [Fe\textsuperscript{III}(TPP)(Cl)] (11.3 μM) in acetonitrile/dimethyl sulfoxide (85:15) and B, [Fe\textsuperscript{III}(TPP)]\textsubscript{2}O (11.3 μM) formed by addition of an aqueous sodium hydroxide solution (0.01 M) to the acetonitrile/dimethyl sulfoxide solution of [Fe\textsuperscript{III}(TPP)(Cl)].

Table 2 Spectroscopic data for some iron-tetraphenylporphyrin complexes.

<table>
<thead>
<tr>
<th>Complex</th>
<th>Soret</th>
<th>Visible</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\lambda_{\text{max}}) ((\varepsilon))</td>
<td>(\lambda_{\text{max}}) ((\varepsilon))</td>
</tr>
<tr>
<td>[Fe\textsuperscript{III}(TPP)(Cl)] (CHCl\textsubscript{3})</td>
<td>417 (99.1)</td>
<td>510 (12)</td>
</tr>
<tr>
<td>[Fe\textsuperscript{III}(TPP)]NO\textsubscript{3} (dichloromethane)</td>
<td>412 (113)</td>
<td>513 (11.5)</td>
</tr>
<tr>
<td>[Fe\textsuperscript{III}(TPP)]\textsubscript{2}O (benzene)</td>
<td>408 (106)</td>
<td>571 (10.7)</td>
</tr>
<tr>
<td>[Fe\textsuperscript{III}(TPP)(DMSO)\textsubscript{x}]\textsuperscript{+} (DMSO)</td>
<td>490 (10.2)</td>
<td>531 (13.2)</td>
</tr>
<tr>
<td>[Fe\textsuperscript{III}(TPP)(1-MeIm)\textsubscript{2}]Cl (CHCl\textsubscript{3})</td>
<td>417 (156)</td>
<td>462 (16.9)</td>
</tr>
<tr>
<td>[Fe\textsuperscript{III}(TPP)(Cl)] (DMSO/MeCN)\textsuperscript{b}</td>
<td>394 (101)</td>
<td>498 (10.0)</td>
</tr>
<tr>
<td>this work</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ref.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
When $[\text{Fe}^{III}(\text{TPP})(\text{Cl})]$ was mixed with an excess of oxoperonitrate(1-), we first observed a shift of the maximum of the Soret band from 416 nm to 422 nm (spectrum 5 in Figure 7). The spectrum of this species remained unchanged for about 15 s, while the absorbance band of oxoperonitrate(1-) at 302 nm decreased. Finally, when most oxoperonitrate(1-) had been consumed, the intermediate started to decay (spectrum 6 in Figure 7). During decay, the absorbance maximum of the Soret band shifted to 418 nm (spectrum 11 in Figure 7B), with isosbestic points at 417 and 439 nm.
Figure 7 Rapid-scan UV-visible spectra of the reaction of [Fe$^{III}$(TPP)(Cl)] (4.5 μM) with oxoperoxonitrate(1-) (170 μM) in acetonitrile/dimethyl sulfoxide at 20 °C. The formation of an intermediate (trace 5) from [Fe$^{III}$(TPP)(Cl)] (traces 1-4) and its decay (traces 6-11) are presented. Traces 1-6 recorded 0, 384, 768, 1152, and 1536 ms after mixing. Traces 6-11 recorded 22.5, 30.0, 37.5, 45.0, 52.5, and 60 s after mixing.

As shown in Figure 8A, when [Fe$^{III}$(TPP)(Cl)] (6.2 μM) was mixed with an excess of oxoperoxonitrate(1-) (480 μM), the trace measured at 426 nm showed an increase of the absorption. Analysis of the trace showed that a two-exponential expression was needed to fit the formation of the intermediate. The
observed rate constants obtained for the first process depended linearly on the oxoperoxonitrate(1-) concentration. The second-order rate constant, obtained from the linear fit of the plot of the observed rate constants versus oxoperoxonitrate(1-) concentration (Figure 9), is \( k_3 = (1.1 \pm 0.4) \times 10^5 \text{ M}^{-1}\text{s}^{-1} \). In contrast, the observed rate constants of the second step varied between 2.5 and 9.7 s\(^{-1}\) (Figure 9), with an average of \( k_4 = 5 \pm 2 \text{ s}^{-1} \), without showing a clear oxoperoxonitrate(1-) concentration dependence. When all oxoperoxonitrate(1-) had been consumed, the intermediate decayed at a rate of \( k_5 = 0.37 \pm 0.08 \text{ s}^{-1} \), independent of the oxoperoxonitrate(1-) concentration (Figure 8B).

![Graph](image)

**Figure 8** Time courses measured at 426 nm for the reaction of [Fe\(^{III}\)(TPP)(Cl)] (6.2 \, \mu\text{M}) with oxoperoxonitrate(1-) (480 \, \mu\text{M}) in acetonitrile/dimethyl sulfoxide at 20 °C. **A** the solid line corresponds to the best fit, which results in the observed rate constants of \( k_3 = 79 \text{ s}^{-1} \) and \( k_4 = 4.9 \text{ s}^{-1} \); **B** the solid line corresponds to the best fit, which results in the observed rate constant of \( k_5 = 0.36 \text{ s}^{-1} \).
Figure 8 (continued)

Figure 9  (▲) Plot of $k_{3,\text{observed}}$ versus oxoperoxonitrate(1-) concentration for the reaction between [Fe$^\text{III}$(TPP)(Cl)] and oxoperoxonitrate(1-) in acetonitrile/dimethyl sulfoxide at 20 °C. The second-order rate constant obtained from the linear plot is $k_3 = (1.1 \pm 0.4) \times 10^5 \text{M}^{-1}\text{s}^{-1}$. (●) Data obtained for $k_{4,\text{observed}}$.

As shown in Figure 10A and 10B, presence of [Fe$^\text{III}$(TPP)(Cl)] led to a significant acceleration of the decomposition rate of oxoperoxonitrate(1-). Oxoperoxonitrate(1-) alone has not decayed completely after 100 s (Figure 10B), whereas in the presence of [Fe$^\text{III}$(TPP)(Cl)] [(about 1.3 % molar ratio relative to oxoperoxonitrate(1-)], oxoperoxonitrate(1-) disappeared completely.
within about 1 s (Fig 10A). The trace measured at 302 nm in the presence of [Fe$^{III}$(TPP)(Cl)] had to be fitted to a two-exponential expression. In the presence of [Fe$^{III}$(TPP)(Cl)] (6.2 μM) and oxoperoxonitrate(1-) (480 μM), the observed rate constants for the decay of oxoperoxonitrate(1-) are 31 s$^{-1}$ and 3.8 s$^{-1}$ (Figure 10A). However, due to the difference in the rate constants, it is not clear whether the reactions observed at 302 and 426 nm ($k_{3,observed} = 79$ s$^{-1}$ and $k_{4,observed} = 4.9$ s$^{-1}$, Figure 8A) are identical. From the form of the trace in Figure 10B it may be assumed that also decay of pure oxoperoxonitrate(1-) does not follow a first-order mechanism under our conditions.

![Graph](image)

**Figure 10** Time courses measured at 302 nm for the decay of oxoperoxonitrate(1-) (480 μM) in acetonitrile/dimethyl sulfoxide at 20 °C. **A** in the presence of [Fe$^{III}$(TPP)(Cl)] (6.2 μM), the *solid line* corresponds to the best fit, which results in the observed rate constants of 31 s$^{-1}$ and 3.8 s$^{-1}$ and **B**, self-decay of oxoperoxonitrate(1-).
The reaction of the iron(II)-tetraphenylporphyrin complex with an excess of oxoperoxonitrate(1-) was studied under analogous conditions. Reduction of [Fe$^{III}$(TPP)(Cl)] with sodium dithionite generates the iron(II)-tetraphenylporphyrin complex with an absorption maximum of the Soret band at 428 nm (Figure 11, Table 2). As the complex is very air sensitive, reduction had to be carried out in situ in the presence of an excess of sodium dithionite. Rapid-scan UV-visible spectra show that when the iron(II)-tetraphenylporphyrin complex is mixed with an excess of oxoperoxonitrate(1-), the maximum of the Soret band shifted from 422 nm to 423 nm (spectrum 2 in Figure 12). The difference between the maximum of the Soret band found in the iron(II)-tetraphenylporphyrin complex (428 nm) and the maximum of the Soret band found in spectrum 1 in Figure 11 may derive from reaction of the iron(II)-tetraphenylporphyrin complex with oxoperoxonitrate(1-) already during mixing time. Finally, the intermediate decayed to a species with an absorption maximum at 417 nm (spectrum 8 in Figure 12), with an isosbestic point at 416 nm. As dithionite absorbs in the same spectral region as oxoperoxonitrate(1-) and dithionite slowly decays, the exact time when all oxoperoxonitrate(1-) has been consumed cannot be determined. Therefore and in contrast to the trace
found for oxoperoxonitrate(I-) decay in the presence of [Fe$^{III}$(TPP)(Cl)], the trace measured at 302 cannot be fitted to a two-exponential expression. In addition, a possible reaction between oxoperoxonitrate(I-) and dithionite cannot be excluded.

Figure 11 UV-visible spectra of iron(II)tetraphenylporphyrin (4.5 μM) in acetonitrile/dimethyl sulfoxide formed by reduction of [Fe$^{III}$(TPP)(Cl)] with sodium dithionite.
Figure 12 Rapid-scan UV-visible spectra of the reaction of iron(II)-
tetraphenylporphyrin (4.5 μM) with oxoperoxonitrate(1-) (170 μM) in
acetonitrile/dimethyl sulfoxide at 20 °C. The formation of an
intermediate (trace 2) from iron(II)tetraphenylporphyrin (dashed trace
1) and its decay (traces 3-8) are presented. Traces 1-2 recorded 0 and
767 ms after mixing. Traces 3-8 recorded 37.3, 55.9, 74.5, 93.1, 111.7
s, and 149 s after mixing.

4.4 Discussion

4.4.1 Nitrogen monoxide-mediated oxidation

of [Fe(TpivPP)(1-MeIm)(O$_2$)]

The nitrogen monoxide-induced oxidation of oxyhemoglobin to methemoglobin
is considered to be the major route for depletion of nitrogen monoxide in the
blood stream [2]. We attempted to obtain a model for the oxoperoxonitrato
methemoglobin complex which has been proposed to be formed as the
intermediate in the course of the reaction between nitrogen monoxide and
oxyhemoglobin [1]. We chose the “picket fence” complex [Fe(TpivPP)(N-
Melm) because [Fe(TpivPP)(N-MeIm)] is the only commercially available porphyrin complex that can bind reversibly dioxygen at room temperature.

When nitrogen monoxide is allowed to react with [Fe(TpivPP)(1-MeIm)(O₂)], a shift of the maximum of the Soret band from 420 to 418 nm with an intermediate at 420 nm is observed (Figure A). In the visible region, formation of two bands at 504 nm and 643 nm is observed with an intermediate with a band at 547 nm (Figure 1B). The bands at 504 and 643 nm are typical features for high-spin iron(III)porphyrin systems. When all [Fe(TpivPP)(1-MeIm)(O₂)] has reacted with nitrogen monoxide, further addition of nitrogen monoxide may lead to formation of [Fe^{III}(TpivPP)(1-MeIm)(NO)]⁺. However, the maxima of the bands are quite different from the maxima found in the nitrosyl complex [Fe^{III}(OEP)(1-MeIm)(NO)]⁺ (OEP = octaethylporphyrin) with maxima at 410, 525, and 558 nm (in dichloromethane) [26]. Differences may be due to the different porphyrin ligands. The spectral changes during rapid-scan UV-visible spectroscopy of the reaction (Figure 5) are very different from the absorbance changes observed by stepwise addition of nitrogen monoxide. Therefore, it is not very likely that the intermediate with a maximum of the Soret band at 420 nm is the expected product of a complete reaction between [Fe(TpivPP)(1-MeIm)(O₂)] and nitrogen monoxide. As the nitrogen monoxide solution is always contaminated with nitrite, it is very likely that [Fe(TpivPP)(1-MeIm)(O₂)] also reacts with nitrite, a reaction which is expected to be slower than the reaction between nitrogen monoxide and the dioxygen complex as it is observed in the reaction between nitrite and oxymyoglobin [27]. However, as between additions of nitrogen monoxide some time is needed to record the spectra, this reaction may be of relevance.

When nitrite was allowed to react with a [Fe(TpivPP)(1-MeIm)(O₂)] solution, formation of a species with a maximum for the Soret band at 420 nm and a band in the visible region with a maximum at 549 nm were observed. These values are quite similar to the absorption maxima found for
[Fe^{III}(TpipPP)(HIm)(NO_2)] at 422, 459 (sh) and 550 nm (in chlorobenzene) [28]. Therefore, it might be assumed that [Fe^{III}(TpipPP)(1-MeIm)(NO_2)] is generated during addition of nitrite.

The reaction between nitrogen monoxide and an excess of [Fe(TpipPP)(1-MeIm)(O_2)] proceeds in two reaction steps. In the first step, nitrogen monoxide reacts in a porphyrin-concentration dependent reaction with [Fe(TpipPP)(1-MeIm)(O_2)] with a second-order rate constant of $k_1 = (1.0 \pm 0.2) \times 10^6$ M$^{-1}$s$^{-1}$ at 20 °C (Figure 4). The first reaction is followed by a porphyrin-concentration independent reaction with a rate constant of $k_2 = 2 \pm 1$ s$^{-1}$. In the course of the reaction between nitrogen monoxide and oxyhemoglobin, the oxoperoxonitrato iron(III) complex is generated which subsequently decays to methemoglobin and nitrate [1]. Therefore, it might be assumed that also in the reaction between [Fe(TpipPP)(1-MeIm)(O_2)] and nitrogen monoxide an oxoperoxonitrato complex might be formed as an intermediate which subsequently decays to an iron(III) species and nitrate. It has been proposed that the rate determining step of the reaction of nitrogen monoxide with oxyhemoglobin is the diffusion of nitrogen monoxide into the distal protein pocket [29]. Since the iron center of [Fe(TpipPP)(1-MeIm)(O_2)] should be more accessible to nitrogen monoxide than the active center of the protein, we expected the reaction rate for the model complex to be faster than that of the corresponding reaction with hemoglobin. In contrast, the reaction is about two orders of magnitude slower. The reaction rate between nitrogen monoxide and oxyhemoglobin is $89 \times 10^6$ M$^{-1}$s$^{-1}$ at pH 7.0 and 20 °C [1].

The rapid-scan UV-visible spectroscopy of the reaction between nitrogen monoxide and [Fe^{III}(TpipPP)(1-MeIm)(O_2)] showed a continuous decrease in absorption with shift of the maxima of the Soret band from 426 to 422 nm (Figure 5). Therefore, we could not assign a spectrum to an intermediate. During nitrogen monoxide-mediated oxidation of oxyhemoglobin, the maximum of the Soret band first shifted from 417 nm (oxyhemoglobin) to 407 nm.
(oxoperoxonitrato methemoglobin) and then to 405 nm (methemoglobin) [1].
These results are quite different from the absorbance changes which we
observed in the reaction between $[\text{Fe}^{III}(\text{TpivPP})(1\text{-MeIm})(\text{O}_2)]$ and nitrogen
monoxide. The differences might derive from the different protein and
porphyrin environment and the different solvents.

There are several conceivable mechanisms by which the expected
$[\text{Fe}^{III}(\text{TpivPP})(1\text{-MeIm})(\text{OONO})]$ complex may decay to an iron(III) species and
nitrate as observed for the decay of oxoperoxonitrato methemoglobin to
methemoglobin and nitrate [1]. The oxygen-oxygen-bond in $[\text{Fe}^{III}(\text{TpivPP})(1\text{-MeIm})(\text{OONO})]$ may be cleaved homolytically to form the ferryl species
$[\text{Fe}^{IV}(=\text{O})(\text{TpivPP})(1\text{-MeIm})]$ and nitrogen dioxide as it is the case in the
reaction of $[\text{Fe}^{III}(\text{TMPS})]$ [TMPS: $5,10,15,20$-tetrakis(2,4,6-trimethyl-3,5-
sulfonatophenyl)-porphyrin$^3$] with oxoperoxonitrate(l-) [17]. Nitrogen dioxide
would recombine with the ferryl group to form the nitrato complex
$[\text{Fe}^{III}(\text{TpivPP})(1\text{-MeIm})(\text{NO}_3)]$. Nitrate is not a good ligand for iron and
therefore it would dissociate to form $[\text{Fe}^{III}(\text{TpivPP})(1\text{-MeIm})]^+$ and nitrate. The
oxygen-oxygen-bond also could be cleaved heterolytically to yield
$[\text{Fe}^{III}(-\text{O})(\text{TpivPP})(1\text{-MeIm})]$ and nitryl cation, which would again lead to
formation of $[\text{Fe}^{III}(\text{TpivPP})(1\text{-MeIm})]^+$ and nitrate. Data obtained for the decay
of the oxoperoxonitrato methemoglobin complex suggest that the
oxoperoxonitrato ligand can rearrange to nitrate in a concerted mechanism [1].

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$^3$ TMPS: $5,10,15,20$-tetrakis(2,4,6-trimethyl-3,5-sulfonatophenyl)-porphyrin
4.4.2 Analysis of the nitrogen-containing products from the reaction of [Fe(TPP)(1-MeIm)(O₂)] with nitrogen monoxide

We attempted to carry out ion chromatographic analysis of the nitrogen-containing products formed in the course of the reaction between [Fe(TPP)(1-MeIm)(O₂)] and nitrogen monoxide. If the reaction were stoichiometric, one equivalent nitrate relative to the porphyrin concentration would be expected. However, the yield of nitrate was always less (Table 1). In addition, the sum of obtained nitrite and nitrate were always less than calculated. These problems may be caused by the instability of the dioxygen porphyrin complex under our experimental conditions. If [Fe(TPP)(1-MeIm)(O₂)] had not formed completely in the course of the reduction/oxygenation of [Fe^{III}(TPP)(Cl)], addition of nitrogen monoxide would have generated less than one equivalent nitrate relative to the porphyrin concentration. We prepared the [Fe(TPP)(1-MeIm)(O₂)] complex according to the literature [8], but we could not control the concentration and the stability of the [Fe(TPP)(1-MeIm)(O₂)] solutions, as we do not have the equipment to record UV-visible spectra at low temperature. In addition, as [Fe(TPP)(1-MeIm)(O₂)] is stable only in the presence of an excess of dioxygen [8], we could not degass the solution. Therefore, under our experimental conditions, nitrogen monoxide can rapidly react with oxygen to yield nitrogen dioxide and dinitrogen trioxide which can react with the organic solvent. This is supported by the colour change, from colourless to yellow, seen when dichloromethane is used as solvent. It has been shown that nitrogen dioxide reacts with dichloromethane and leads to nitrometylation of porphyrins [30]. Therefore, formation of organic nitrogen oxide products would reduce the amount of nitrite and nitrate.
4.4.3 $[\text{Fe}^{\text{III}}(\text{TPP})(\text{Cl})]$-accelerated decay of oxoperoxonitrate(1-)

We attempted to generate an oxoperoxonitrato iron porphyrin model complex from the reaction of oxoperoxonitrate(1-) with the water-insoluble $[\text{Fe}^{\text{III}}(\text{TPP})(\text{Cl})]$ complex.

While this work was in progress, Groves et al. investigated the decomposition of oxoperoxonitrate(1-) catalyzed by the water-soluble porphyrin complexes $[\text{Fe}^{\text{III}}(\text{TMPyP})]$ [TMPyP: 5,10,15,20-tetrakis(N-methyl-4’pyridyl)porphyrin$^4$] and $[\text{Fe}^{\text{III}}(\text{TMPS})]$ [5, 17]. In the reaction of oxoperoxonitrate(1-) with $[\text{Fe}^{\text{III}}(\text{TMPyP})]$, formation of an oxoperoxonitrato complex has been proposed with a second-order rate constant of $k^6 > 5 \times 10^7$ M$^{-1}$s$^{-1}$ (at pH 7.4 and 25 °C) (equation 1) [5]. Subsequently, cleavage of the oxygen-oxygen-bond of the oxoperoxonitrate(1-) ligand yields nitrogen dioxide and the ferryl species $[\text{Fe}^{\text{IV}}(=\text{O})(\text{TMPyP})]$ with a rate constant of $k_7 = 62.2$ s$^{-1}$ (equation 2). Excess oxoperoxonitrate(1-) is catalytically isomerized to nitrate in the presence of the ferryl complex $[\text{Fe}^{\text{IV}}(=\text{O})(\text{TMPS})]$ (equation 4). In order to explain the mechanism of the isomerization, it has been postulated that oxoperoxonitrate(1-) reacts with $[\text{Fe}^{\text{IV}}(=\text{O})(\text{TMPyP})]$ to form an adduct “$[\text{Fe}^{\text{IV}}(=\text{O})(\text{TMPyP})(\text{OONO})]$" (equation 3). In this adduct, oxoperoxonitrate(1-) may either coordinate to the iron ion on the opposite side of the porphyrin plane, relative to the oxo-ligand, or form an adduct by cyclic addition to the iron-oxo group to yield a Fe-OON(0)O-five-membered ring [5]. As soon as all oxoperoxonitrate(1-) has been consumed, the $[\text{Fe}^{\text{IV}}(=\text{O})(\text{TMPyP})]$ complex decays back to the iron(III) species (equation 5) [5].

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$^4$ TMPyP: 5,10,15,20-tetrakis(N-methyl-4’pyridyl)-porphyrin
Because of solubility properties of \([\text{Fe}^{III}(\text{TPP})(\text{Cl})]\), the reaction between oxoperoxonitrate(1-) and \([\text{Fe}^{III}(\text{TPP})(\text{Cl})]\) had to be carried out in organic solvents. We performed the measurements in the presence of an excess of oxoperoxonitrate(1-). In the course of the reaction, we observed three reaction steps. As the first step is oxoperoxonitrate(1-) concentration-dependent, it can be assumed that in analogy to the reaction with the iron-TMPyP complex (equation 1), the oxoperoxonitrato iron(III) complex \([\text{Fe}^{III}(\text{TPP})(\text{OONO})]\) is formed with a second-order rate constant of \(k_3 = (1.1\pm0.4) \times 10^5 \text{ M}^{-1}\text{s}^{-1}\). In addition, the second reaction step, which is oxoperoxonitrate(1-) concentration-independent may, be due to the formation of the ferryl species \([\text{Fe}^{IV}(=\text{O})(\text{TPP})]\) with \(k_4 = 5\pm2 \text{ s}^{-1}\) (analogous to equation 2). The reaction rates of the two steps in the reaction between \([\text{Fe}^{III}(\text{TPP})(\text{Cl})]\) and oxoperoxonitrate(1-) are about two and one order of magnitude lower than those obtained with \([\text{Fe}^{III}(\text{TMPyP})]\). The decay of oxoperoxonitrate(1-) is significantly increased in the presence of \([\text{Fe}^{III}(\text{TPP})(\text{Cl})]\). Therefore, it can be assumed that also \([\text{Fe}^{IV}(=\text{O})(\text{TPP})]\) may catalytically isomerize oxoperoxonitrate(1-) to nitrate as it has been found for \([\text{Fe}^{IV}(=\text{O})(\text{TMPyP})]\) (equation 4). Over a longer time scale, we observed a much slower reaction. This may be due to the decay of the ferryl species to an iron(III) species analogous to equation 5.

In the course of the reaction, the absorption maximum of the Soret band first shifted from 416 nm to 422 nm (Figure 7). The absorption maximum for
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[Fe$$^{IV}(=O)(TPP)$$] at 422 nm is slightly different from that found for [Fe$$^{IV}(=O)(TMPyP)$$] at 427 nm which might be due to the different solvents and porphyrins. When all oxoperoxonitrate(1-) is consumed, the ferryl species decays with a shift of the maximum of the Soret band to 418 nm. Under alkaline conditions such as used in our system, the final product is most likely [Fe$$^{III}(TPP)_2$$O]. Spectral differences between the final product found after reaction with excess oxoperoxonitrate(1-) (spectrum 11 in Figure 7, maximum of the Soret band at 418 nm) and [Fe$$^{III}(TPP)_2$$O formed from the direct reaction of [Fe$$^{III}(TPP)(Cl)$$] with aqueous sodium hydroxide (spectrum B in Figure 6, maximum of the Soret band at 414 nm) may derive from subsequent binding of nitrite to iron.

4.5 Conclusions

We stopped our research on this project as during our work it has been shown that the mechanism of nitrogen monoxide-induced oxidation of oxyhemoglobin gives strong evidence for a oxoperoxonitrato methemoglobin intermediate [1]. Groves et al [5, 17] showed that the mechanism of the accelerated decay of oxoperoxonitrate(1-) in the presence of more relevant water soluble porphyrin-complexes is also very complex.

4.6 References

Chapter 4


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