Reactions of Peroxynitrite with Biomolecules
Reactions of Peroxynitrite with Biomolecules

Dissertation

zur Erlangung des Grades eines Doktors der Naturwissenschaften der Fakultät für Biologie der Universität Konstanz

vorgelegt von

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Diese Arbeit ist meiner Frau Tanja

und meinem Sohn Erik gewidmet

für ihre Liebe, ihre Geduld

und für das Glück

mit dem sie mich beschenken.
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**Relevant Publications**


Relevant Presentations

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- Poster at the "2nd International Conference on the Biology and Chemistry of Peroxynitrite” (1999) in Heraklion, Crete, Greece.


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<td>α-NT</td>
<td>anti-nitro-tyrosine</td>
</tr>
<tr>
<td>ADH</td>
<td>alcohol dehydrogenase</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulfate</td>
</tr>
<tr>
<td>BQ</td>
<td>p-benzoquinone</td>
</tr>
<tr>
<td>BM3</td>
<td>P450\textsubscript{BM-3}-monooxygenase</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CAM</td>
<td>P450\textsubscript{CAM}-monooxygenase</td>
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<tr>
<td>cGMP</td>
<td>cyclic guanosine-monophosphate</td>
</tr>
<tr>
<td>CIDNP</td>
<td>chemically induced dynamic nuclear polarization</td>
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<tr>
<td>CM</td>
<td>calmodulin</td>
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<td>CPA</td>
<td>m-chloro-perbenzoic acid</td>
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<tr>
<td>CPO</td>
<td>chloroperoxidase</td>
</tr>
<tr>
<td>DENO</td>
<td>diethyl NONOate</td>
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<tr>
<td>DMUA</td>
<td>dimethyl-uric acid</td>
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<tr>
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<td>dimethylsulfoxide</td>
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<td>DNA</td>
<td>desoxyribo-nucleic acid</td>
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<td>DNP</td>
<td>2,4-dinitro-phenol</td>
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<tr>
<td>DT</td>
<td>dityrosine</td>
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<td>DTBP</td>
<td>2,2′-dithio-bipyridine</td>
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<td>DTNB</td>
<td>Ellmans reagent, dithio-bis(nitro-benzoic acid)</td>
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<td>DTPA</td>
<td>diethylene-triamine-pentaacetic acid</td>
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</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase (NOS-II)</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MBS</td>
<td>2-mercapto-benzo-thiazole</td>
</tr>
<tr>
<td>MBT</td>
<td>2-mercapto-benz-selenazole</td>
</tr>
<tr>
<td>MET</td>
<td>metyrapone</td>
</tr>
<tr>
<td>MP-11</td>
<td>microperoxidase (11 amino acids)</td>
</tr>
<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NA</td>
<td>nitro-anisol</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>nNOS</td>
<td>neuronal or brain nitric oxide synthase (NOS-I)</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOR</td>
<td>nitric oxide reductase</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>NP</td>
<td>nitro-phenol</td>
</tr>
<tr>
<td>NT</td>
<td>nitro-tyrosine</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered solution</td>
</tr>
<tr>
<td>PCS=PGIS</td>
<td>prostacyclin (PGI₂) synthase</td>
</tr>
<tr>
<td>PEP</td>
<td>phospho-enolpyruvate</td>
</tr>
<tr>
<td>PN</td>
<td>peroxynitrite (oxoperoxonitrate(-I))</td>
</tr>
<tr>
<td>pNC</td>
<td>12-(p-nitro-phenoxy)-dodecanoic acid</td>
</tr>
<tr>
<td>RNS</td>
<td>reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RP</td>
<td>reversed phase</td>
</tr>
<tr>
<td>RSNO</td>
<td>nitroso-thiol</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl-sulfate</td>
</tr>
<tr>
<td>SIN-1</td>
<td>3-morpholino-sydnonimine</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>TBA</td>
<td>2-thio-barbituric acid</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethyl-ethylene-diamine</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoro-acetic acid</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)-amino-methane</td>
</tr>
<tr>
<td>XO</td>
<td>xanthine oxidase</td>
</tr>
</tbody>
</table>
1 Introduction

Radicals and oxidants play a major role in pathophysiological processes. In the course of this century, due to improved analytical methods, it was possible to identify several of these reactive species and even to understand the basic principles of oxidative damage. The mechanisms of many reactions of oxidants with biomolecules are known and also the strategies of cellular defense, mainly provided by antioxidants such as glutathione, ascorbate (vitamin C) and tocopherols (vitamin E) [1]. But protection against oxidative damage is also provided by enzymes such as SOD, catalase, GPx and others [2].

These oxidants have been divided into two major groups [3]:

- Reactive oxygen species (ROS), among them superoxide (O$_{2}^−$), hydrogen-peroxide (H$_2$O$_2$), singlet oxygen (¹O$_2$), hypochlorite (OCl$^−$), alkylperoxides (ROO·, ROOH) and hydroxyl radicals (-OH).

- Reactive nitrogen species (RNS), referring to nitric oxide (·NO), nitrogen dioxide (·NO$_2$), dinitrogen trioxide (N$_2$O$_3$), nitrylchloride (ONOCl) and nitrosylchloride (ONCl), but also the nitronium (NO$_2^+$) and nitrosonium (NO$^+$) cation.

These reactive species are of great biological and chemical interest, because they have been characterized by a high reactivity towards many target molecules. Especially for their characterization in biological systems techniques have been developed and optimized to investigate reactions of ROS and RNS with biomolecules. Due to their short lifetime, these species often have to be detected by indirect methods, such as the employment of spin traps, which form stable radical adducts with the initial radicals or stable 1e-oxidation products. These adducts can be detected by ESR [4]. Another possibility is the identification of ROS and RNS by their footprints, this means stable endproducts of oxidatively modified biomolecules (e.g. malondialdehyde during fatty acid peroxidation or 3-NT in PN-mediated nitration) [1, 5]. At last CIDNP (chemically induced dynamic nuclear polarization) is a very powerful method to get insight in the mechanism by which radical reactions proceed [6].

This work will focus on two of these reactive species: ·NO and O$_{2}^−$. Both radicals are rather longlived, but can react with each other in a nearly diffusion controlled reaction (4-10 × 10$^9$ M$^{-1}$s$^{-1}$) [7, 8, 9]. The resulting peroxynitrite
(PN) is the species on which we will concentrate in this work. Studies of Zou and Ullrich revealed that prostacyclin synthase, a P450 enzyme which forms the vessel-relaxing PG\textsubscript{I2}, is inactivated by PN at submicromolar concentrations [10]. This inactivation went hand in hand with a nitration [11] and since PGIS contains no other oxidizable cysteine except the heme-ligating one, the nitration remains as a reason for the inactivation [11, 12]. Therefore it was desirable to investigate the mechanism of PN-mediated nitration of phenolic compounds in presence and absence of iron catalysts.

### 1.1 Nitric Oxide

#### 1.1.1 Biosynthesis of Nitric Oxide

Tannenbaum and coworkers observed in 1981 that animals excreted more nitrate as they had taken up with the food and concluded on an enzyme which was able to synthesize NO\textsubscript{X} species [13, 14]. In the following years a corresponding NO-synthase, a P450 enzyme, was discovered, which exists in three isoforms: eNOS (NOS-III), n or bNOS (NOS-I) and iNOS (NOS-II) [15]:

- n or bNOS, type-I (160 kDa): Ca\textsuperscript{2+}/CM-dependent, constitutive and bound to specific membrane proteins in neuronal cells, skeletal muscle, kidney.
- iNOS, type-II (130 kDa): Ca\textsuperscript{2+}/CM-independent, induced by cytokines and LPS and cytosolic in macrophages, hepatocytes, astrocytes and smooth muscle.
- eNOS, type-III (134 kDa): Ca\textsuperscript{2+}/CM-dependent, constitutive and bound to fatty acids in Golgi and Caveolae membranes of endothelial cells, epithelial cells, cardiomyocytes and neurons.

Fig. 1 shows the hypothetical reaction mechanism for the formation of ·NO from oxygen and L-arginine by NOS [16]. N\textsuperscript{\textgamma}-hydroxy-arginine, which is formed within the first step could be identified as an intermediate. The other reaction steps are estimated from spectroscopical investigations. It is not yet clarified, which pathway of 4-6 or 7-8 is correct. L-arginine is converted to L-citrulline in a 5ε-oxidation. The reaction requires 2 equivalents of oxygen and 1.5 of NADPH.

This complex reaction requires several cofactors, which are shown in Fig. 2. FMN and FAD are responsible for the electron transport within the enzyme and function as shuttles for the electrons from NADPH to the heme iron (see also (9) in Fig. 1). BH\textsubscript{4} is essential for a completely activated enzyme. (E-I)
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Figure 1: Hypothetical reaction mechanism for the formation of ·NO from oxygen, arginine and NOS.

In Fig.2 proposes a mechanism by which BH$_4$ could participate in the ·NO formation. BH$_4$ may play a role during oxygen activation [16], but could also have structural effects by stabilizing the active form of NOS [15]. In another publication a pterin radical has been observed, suggesting the formation of a ·BH$_3$ radical during catalysis of NO formation [17].

Inhibitors of NOS are structural analogues of L-arginine, such as N$^G$-methyl-L-arginine (L-NMA) and N$^G$-nitro-L-arginine (L-NNA). Other often employed inhibitors are mercapto-ethyl-guanidine (MEG), guanidine, amino-guanidine and thio-urea [16].

1.1.2 Chemical Sources and Determination of Nitric Oxide

NO-donors are compounds which release ·NO in dependence of time, temperature, pH and redox reactions. Since 1879 nitro-glycerol (propanetriol-trinitric acid-ester) is used for the prophylaxis of heart attacks. The beneficial action of nitro-glycerol is based on the release of ·NO in the organism. Also other alkyl-nitrates and nitrites release ·NO [18]. NONOates and sodium nitro-prusside (SNP) also release ·NO and are used for its continuous production [18]. Spermine
1 INTRODUCTION

Figure 2: Cofactors, which are necessary for the formation of \( \cdot \text{NO} \) by NOS, in the reduced and oxidized form. (A) NADPH, (B) NADP\(^+\), (C) oxidized FMN, (D) reduced FMN, (E) tetrahydro-biopterin (BH\(_4\)), (F) 4a-hydroperoxy-biopterin, (G) 4a-hydroxy-biopterin, (H) dihydro-biopterin-quinone and (I) dihydro-biopterin.

and diethylamine NONOate (see Fig.104) are the most common NO-donors in biological systems, because they release \( \cdot \text{NO} \) over a longer time period and can thus serve as models for \( \cdot \text{NO} \) production from NOS [22]. NONOates are rather stable at alkaline pH and decay faster with decreasing pH.

\( \cdot \text{NO} \) concentrations can be easily determined by chemoluminescence: \( \cdot \text{NO} \) can diffuse through a semipermeable membrane and is transported by helium as carrier to the chemoluminometer [19]. Other possibilities are \( \cdot \text{NO} \) sensitive dyes (e.g. 2,3-diamino-naphthalene), which show a change in fluorescence after addition of \( \cdot \text{NO} \) [20]. The third possibility is the oxidation of oxyHb to metHb. The absorbance change is measured at 401 nm and 411 nm (the isosbestic point) as a wavelength pair [21].

1.1.3 Chemistry of Nitric Oxide

Nitric oxide is a colorless, paramagnetic gas. Its half-life in an oxygenated saturated 2 mM solution at 25 \(^\circ\)C is less than one second [19], but it strongly depends on the concentration of \( \cdot \text{NO} \). One of the major decomposition pathways of \( \cdot \text{NO} \).
is its reaction with oxygen:

\[ 2 \cdot \text{NO} + \text{O}_2 \longrightarrow 2 \cdot \text{NO}_2 \]  \hspace{1cm}(1)

Since under physiological conditions \( \cdot \text{NO} \) concentrations are normally in the nanomolar range, reaction (1) is very slow because it depends bimolecular on \([\cdot \text{NO}]\) (see reaction (2)):

\[ \frac{d[\cdot \text{NO}]}{dt} = 4k[\text{O}_2][\cdot \text{NO}]^2 \]  \hspace{1cm}(2)

with \( k=2.63 \cdot 10^6 \text{M}^{-2} \cdot \text{s}^{-1} \) at 25°C [23]. In an air-saturated solution containing 225 \( \mu \text{M} \text{O}_2 \) the halflife of 1 mM \( \cdot \text{NO} \) about 0.56 s, at 10 nM it is 15.5 h [19]. Under pathophysiological conditions (e.g. Ischemia/reperfusion) \( \cdot \text{NO} \) concentrations can reach the micromolar range [5] and its halflife is more than 10 s. With its diffusion coefficient, which is a factor of 1.4 higher compared to that of \( \text{O}_2 \), \( \cdot \text{NO} \) has a high radius of action and can diffuse over long distances. Furthermore, due to its lipophilicity [24], \( \cdot \text{NO} \) is able to diffuse through cell membranes and acts as a fast and, at nanomolar concentrations, also specific messenger. At high concentrations, \( \cdot \text{NO} \) gets less specific and may be cytotoxic towards all cell types.

The following equations will describe the major reactions of \( \cdot \text{NO} \) in biological systems. Under physiological conditions there will be always an excess of \( \cdot \text{NO} \) and a part of it will autoxidize to form nitrogen dioxide. Dimerization and dismutation of \( \cdot \text{NO}_2 \) (5,6) will be suppressed in favor of the fast reaction with \( \cdot \text{NO} \) (7) to form dinitrogen trioxide. The formation of \( \text{N}_2\text{O}_3 \) is also the reason, why \( \cdot \text{NO} \) in physiological concentrations almost quantitatively yields nitrite and only traces of nitrate (8) [15, 24]:

- The formation of \( \cdot \text{NO}_2 \) and its disproportionation to nitrite and nitrate in water [15, 16, 24]:

\[ 2\text{NO} \cdot + \text{O}_2 \longrightarrow 2\text{NO}_2 \cdot \]  \hspace{1cm}(3)

\[ 3\text{NO}_2 \cdot + \text{H}_2\text{O(l)} \longrightarrow 2\text{HNO}_3(\text{aq}) + \text{NO} \cdot \]  \hspace{1cm}(4)

\[ 2\text{NO}_2 \cdot + 2\text{OH}^- (\text{aq}) \longrightarrow \text{NO}_2^- (\text{aq}) + \text{NO}_3^- (\text{aq}) + \text{H}_2\text{O(l)}} \]  \hspace{1cm}(5)

\[ \text{N}_2\text{O}_4(\text{g}) \rightleftharpoons 2\text{NO}_2 \cdot (\text{g}) \]  \hspace{1cm}(6)
• The formation of N$_2$O$_3$, a nitrosating species [15, 25]:

\[
\text{NO} \cdot (g) + \text{NO}_2 \cdot (g) \rightleftharpoons \text{N}_2\text{O}_3(g) \tag{7}
\]

\[
\text{N}_2\text{O}_3 + \text{H}_2\text{O} \rightarrow 2\text{NO}^- + 2\text{H}^+ \tag{8}
\]

• The reaction with thiols [15, 24, 26]. Reaction (9) is thermodynamically and kinetically hindered and only can proceed, if an electron acceptor is present (e.g. iron(III)). Nitroso-thiols could be a kind of storage for nitric oxide and can nitrosylate other compounds by an either polar (12) or radical mechanism (of course the lifetime of "free" nitrosyl cation is short under physiological conditions):

\[
\text{NO} \cdot + \text{RSH} \rightarrow \text{RSNO} + \text{H}^+ + e^- \tag{9}
\]

\[
\text{RSH} + \cdot\text{NO} \rightarrow \text{RS} \cdot + \text{HNO} \tag{10}
\]

\[
\text{RS} \cdot + \cdot\text{NO} \rightarrow \text{RSNO} \tag{11}
\]

\[
\text{RSNO} + \text{H}^+ \rightleftharpoons \text{RSH} + \text{NO}^+ \tag{12}
\]

• Oxidation of \cdot\text{NO} [5, 16]:

\[
\text{NO} \cdot + \text{OH} \cdot \rightarrow \text{NO}^+ + \text{OH}^- \tag{13}
\]

\[
\text{NO} \cdot + \text{Fe(III)} \rightarrow \text{Fe(II)} \cdot\cdot\text{NO}^+ \tag{14}
\]

• Reduction of \cdot\text{NO} (e.g. by NADH-NO reductase, P450$_\text{NOR}$ [27]) [16, 24]:

\[
2\text{NO} \cdot + \text{Fe(III)}/\text{NADH} \rightarrow 2\text{NO}^- + \text{Fe(III)}/\text{NAD}^+ + \text{H}^+ \tag{15}
\]

\[
\text{NO}^- + \text{NO}^- \rightarrow \text{ONNO}^- \tag{16}
\]

\[
\text{ONNO}^- + \text{NO}^- \rightarrow \text{N}_2\text{O} + \text{NO}_2^- \tag{17}
\]

\[
\text{ONNO}^- + \text{H}^+ \rightarrow \text{N}_2\text{O} + \text{OH} \cdot \tag{18}
\]

\[
\text{NO}^- + \text{O}_2 \rightarrow \text{ONOO}^- \tag{19}
\]

• The reaction with superoxide will be discussed more detailed in 1.3.1.
1.1.4 Biology of Nitric Oxide

·NO is a second messenger and plays a key role in the regulation of the vascular system (for reviews see [15, 28, 29]). For this finding MURAD, IGNARRO and FURCHTGOTT received the NOBEL prize. The physiological actions of ·NO are severalfold:

- Relaxation of blood vessels: ·NO binds to the heme-iron subunit of guanylate cyclase, which undergoes a conformational change and becomes activated [15, 28]. Due to the activation the cGMP level is increased and this leads to a relaxation of the smooth muscle and a vasodilatation [28, 29]. Before its discovery ·NO was called EDRF (endothelial derived relaxing factor).

- Inhibition of platelet aggregation: This mechanism proceeds analogous to the smooth muscle relaxation, but is not yet clarified in detail. This biological process is important to inhibit thrombus formation [28].

- Signal transduction also proceeds via activation of guanylate cyclase. cGMP activates cGMP-dependent kinases, which then start a cascade of enzymatic reactions by phosphorylation [29].

- Immune defense: Macrophages produce high concentrations of ·NO after stimulation with IFN-γ, which participates in the unspecific defense of bacteria, fungi and tumor cells (phagocytosis) [30, 31].

But also pathophysiological processes of ·NO are known, especially in chronic inflammation and shock [32, 33]. In presence of oxygen protein modifications such as thioloxidation and also tyrosine nitration were observed and could lead to inactivation of the enzymes [31, 34]. Other damages arise from inhibition of the respiratory chain by the binding of ·NO to heme-thiolate iron or from deamination reactions at the DNA [31].

1.2 Superoxide

1.2.1 Biosynthesis of Superoxide

Biological sources of superoxide can originate from all processes, in which oxygen is somehow activated or the redox equilibrium is disturbed [35]:

- The respiratory chain in mitochondria (e.g. cytochrome c oxidase and other heme and heme-thiolate proteins) [35, 36, 37].
- Monooxygenation and oxygenation reactions [35, 38].
- Conversion of xanthine DH into xanthine oxidase (XO) [35, 39, 40, 41].
- Aggregation of certain protein fragments in membranes exhibits the NADH/NADPH oxidase activity [35, 43, 44].
- Activated macrophages, phagocytosis (NADH/NADPH oxidase activity) [35, 44].
- Autoxidation of polyphenols or fatty acids [35, 45].
- Under certain circumstances heme-thiolate enzymes can be modified and converted to superoxide-producing proteins. NOS has been reported to produce superoxide after treatment with PN [46].

For normal concentrations of superoxide the organism has two protection enzymes: Mn-SOD (only in mitochondria) and Cu,Zn-SOD in the cytosol [47]. Especially XO and NADPH/NADH oxidase seem to be important sources for high concentrations of superoxide. XDH from liver and milk is converted to XO by either thioloxidation and/or proteolysis by which the affinity for oxygen is increased dramatically [39, 41]. The active site of XO consists of two metal centers, a Mo-containing one and an iron-sulfur cluster [48, 41]. Inbetween these two metal centers the binding sites for FAD and NAD\(^+\) are localized [40, 41]. The Mo-coordinating sulfurs are part of a Mo-pterin-cofactor. XO is inhibited by tungsten, cyanide and allopurinol, which is oxidized to 2-oxo-allopurinol and reversibly inhibits the active site [39]. XO shows only low substrate specificity and converts hypoxanthine, xanthine, NADH/NADPH (XO has also NADH/NADPH oxidase activity) and even acetaldehyde [22]. **Fig.3** shows the major steps in the postulated mechanism of superoxide formation, which was combined from a talk of Prof. Dale and [42].

The phagocyte NADPH oxidase flavocytochrome b558 is a heterodimer and consists of a glycosylated subunit, gp91(phox), and a nonglycosylated one, p22(phox), which after assembling in the presence of two other components (p47phox, p67phox) in the membrane show the \(O_2^-\)-generating activity [43, 44]. This process is started after phosphorylation by phosphokinase C (PKC). NADPH oxidase contains two nonidentical heme groups that mediate the final steps of electron transfer. NADPH oxidase activity is stimulated by angiotensin-II [49]. This stimulation could not be observed in cell lines which were deficient in one of the subunits.
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Figure 3: Proposed mechanism for the formation of superoxide from XO and xanthine. The iron-sulfur cluster is also necessary for the full activity of the enzyme, but its role in this mechanism is still not clarified. During the conversion of xanthine to uric acid two molecules of superoxide are formed from oxygen. This mechanism was combined from a talk of Prof. Dale and [42].

1.2.2 Chemical Sources and Determination of Superoxide

Often a solution of KO$_2$ in DMSO is used as a source for superoxide [50] and to obtain low stationary concentrations of O$_2^-$ the DMSO solution can be added by a constant minimal flow. Another method described for the formation of superoxide is the autoxidation of pyrogallol in air-saturated solutions. The flux of O$_2^-$ formation can be controlled by the pH (in alkaline solutions the autoxidation is faster) and temperature [51]. The latter method is not suitable for spectroscopic measurements, since the oxidation products of pyrogallol (quinones, semiquinones) themselves show high absorbancies between 250 and 500 nm. The best system to produce constant fluxes of O$_2^-$ in vitro consists of the biological system XO/hypoxanthine in which hypoxanthine is oxidized to xanthine in a first step and to uric acid in a second one and oxygen is reduced to superoxide [22].

Superoxide can be determined by reduction of ferricytochrome c (Fe$^{3+}$) to ferrocytochrome c (Fe$^{2+}$) [21]. The reaction is followed at 550 nm. Another possibility for the detection of O$_2^-$ is the oxidation/reduction of fluorescence dyes or chemiluminogenic compounds (e.g. lucigenin). Both methods are not specific for superoxide, therefore one has to take care of other oxidizing and reducing species in the reaction solution.
1.2.3 Chemistry of Superoxide

At pH 7, superoxide is a shortlived radical with a rather low reactivity. Its short lifetime is due to its fast disproportion in aqueous solutions. By the reaction with metals and other reactive species superoxide can generate hydroxyl radicals, which may damage nearly all existing biomolecules. In vivo there are two enzymatic systems which keep the \( \text{O}_2^- \) concentration low, the Mn-SOD and Cu,Zn-SOD. The following equations show the most important reactions of superoxide and its major decomposition product hydrogen peroxide:

- Disproportionation of \( \text{O}_2^- \) in aqueous solution with \( k=7.3 \times 10^5 \, \text{M}^{-1}\text{s}^{-1} \) for the self-dismutation \([50]\), \( k=10^8 \, \text{M}^{-1}\text{s}^{-1} \) for the Mn-SOD catalyzed reaction \([50]\), \( k=2 \times 10^9 \, \text{M}^{-1}\text{s}^{-1} \) under catalysis of Cu,Zn-SOD \([50]\) and \( k=10^7 \, \text{M}^{-1}\text{s}^{-1} \) in presence of Mn- and Fe-porphyrins \([50]\). Hydrogen peroxide is scavenged by catalase (\( k=0.8-2 \times 10^7 \, \text{M}^{-1}\text{s}^{-1} \)) \([52]\):

\[
\begin{align*}
2\text{O}_2^- + 2\text{H}^+ & \rightarrow \text{O}_2 + \text{H}_2\text{O}_2 \quad (20) \\
2\text{O}_2^- + 2\text{H}^+ & \xrightarrow{\text{SOD}} \text{O}_2 + \text{H}_2\text{O}_2 \quad (21) \\
2\text{H}_2\text{O}_2 & \xrightarrow{\text{Cat}} \text{O}_2 + 2\text{H}_2\text{O} \quad (22)
\end{align*}
\]

- Reaction with transition metals (Fe(III), Cu(I) and (II), Ni(II) and Co(II)), e.g. Fenton reaction \((24)\) and Haber-Weiss cycle \((25)\)\([3, 53, 54]\):

\[
\begin{align*}
\text{Fe}^{3+} + \text{O}_2^- & \rightarrow \text{Fe}^{2+} + \text{O}_2 \quad (23) \\
\text{Fe}^{2+} + \text{H}_2\text{O}_2 & \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \text{OH}^- \quad (24) \\
\text{H}_2\text{O}_2 + \text{O}_2^- & \xrightarrow{\text{Fe(III)}} \text{O}_2 + \text{OH}^- + \cdot\text{OH} \quad (25)
\end{align*}
\]

- Reaction with hypohalogenites (X=Cl\(^-\), Br\(^-\), I\(^-\)) \([3, 53]\):

\[
\text{O}_2^- + \text{HOX} \rightarrow \text{O}_2 + \cdot\text{OH} + \text{X}^- \quad (26)
\]

- Reactions with thiols leads to thyl radical and disulfide formation \([1]\):

\[
\begin{align*}
\text{O}_2^- + \text{RSH} + \text{H}^+ & \rightarrow \text{H}_2\text{O}_2 + \text{RS} \cdot \overset{2x}{\rightarrow} \text{RSSR} \quad (27)
\end{align*}
\]
1 INTRODUCTION

- Oxidations by the perhydroxyl radical (pK_a = 4.5), e.g. lipid peroxidation of polyunsaturated fatty acids (28) and radical chain reaction of polyphenolic compounds (29)[1]:

\[
\begin{align*}
R - CH = CH - CH_2 - CH = CH - R + HOO\cdot & \rightarrow (28) \\
R - CH = CH - C - H - CH = CH - R + H_2O_2 & \rightarrow \\
A - OH + O_2^- & \rightarrow A - O^- + HOO\cdot & \rightarrow A - O\cdot + HOO^- (29)
\end{align*}
\]

- The reaction with nitric oxide will be discussed in more detail in 1.3.1.

Superoxide is unlikely to react directly with biomolecules, but in its protonated form, as the perhydroxyl radical it is much more reactive and additionally to unsaturated lipids and polyphenols reacts with antioxidants such as ascorbate and tocopherols [1]. Especially in the presence of transition metals superoxide shows a high oxidative potential, mainly mediated by hydroxyl radicals and hydrogen peroxide [53].

1.2.4 Biology of Superoxide

There are only few indications for physiological actions of superoxide. It is well known that O_2^- is produced in macrophages during phagocytosis [55] and this action can be interpreted as an unspecific immune defense. Furthermore superoxide could participate in signal transduction, because nearly all cell types show increased levels of O_2^-, when they are stimulated by cytokines. Therefore O_2^- could be a kind of unspecific second messenger. At least superoxide plays a major role as an intermediate during oxygen activation, but this physiological action should proceed protein-bound and caged. So controlled neither free superoxide should escape the cage nor other reactive species, which are formed during the catalytic cycle of this oxygen activation. The metal-oxo intermediates will be discussed in more detail.

1.3 Peroxynitrite (PN)

1.3.1 History and Properties of PN

PN (oxoperxonitrate (1-)) was first observed in 1901 by BAYER and VILLIGER in the reaction of nitrous acid and hydrogen peroxide, but was ill-defined as pernitric acid (HNO_4). In 1935 GLEU and HUBOLD introduced the name peroxynitrite for this compound. In 1954 HALFPENNY and ROBINSON observed
hydroxylation and nitration reactions of aromatics by PN. At the same time Anbar and Taube postulated the mechanism for PN formation by nitrosation of H$_2$O$_2$. In 1964 Papee and Petriconi proved the formation of PN in the photolysis of nitrate. In 1976 traces of PN were found in mars minerals by the NASA Viking-sonde. In the 80’s, the role of PN in smog chemistry and in the destruction of ozone in the stratosphere was discussed and first indications were found for the formation of PN \textit{in vivo}.

Today it is known, that PN can be formed \textit{in vivo} by the nearly diffusion controlled reaction of ·NO and O$_2^-$:

\[
\text{·NO} + \text{O}_2^- \rightarrow \text{ONOO}^- \tag{30}
\]

The velocity of this reaction ($k=4.3-10 \times 10^9 \text{M}^{-1}\text{s}^{-1}$ [7, 8, 9]) is a factor of 2-10 faster compared to the velocities of the reactions of Mn- and Cu,Zn-SOD with superoxide (see also equation (21) and k-values given in 1.2.3). For the suppression of PN formation \textit{in vivo} the ·NO concentration is essential. If the nitric oxide concentration gets too high, SOD’s cannot compete with it for the superoxide anion and formation of PN will be favored [5].

PN is a structural isomer of nitrate, but lies 150 KJ·mol$^{-1}$ higher in energy [56]. Kept at -20 to -80 °C in alkaline solutions PN can be stored for 4 weeks up to months without serious losses. The intense yellow color of the PN-anion can be used for its quantification as proposed by Hughes and Nicklin in 1968, by the use of $\varepsilon_{302} = 1670 \text{M}^{-1}\text{cm}^{-1}$ [57]. In alkaline solutions PN is present in its cis-conformation (as shown previously by $^{15}$N-NMR and Raman measurements [58, 59] and x-ray structure analysis [60]) and undergoes a very slow decomposition to form oxygen and nitrite [61]. The cis-form of ONOO$^-$ is 14.6 KJ·mol$^{-1}$ more stable than the trans-form of PN-anion [56]. In neutral solutions PN-anion undergoes protonation to peroxynitrous acid with a pK$_a$-value of 6.8 [56], which is much more reactive and isomerizes to nitrate by a yet not completely understood mechanism [56]. $\varepsilon_{302}$ of ONOOH is a factor of 100 lower compared to that of ONOO$^-$ (unpublished observation of Kissner and Koppelen). ONOOH is thought to be present in the trans-conformation, because trans-ONOOH is 4.18 KJ·mol$^{-1}$ more stable than cis-ONOOH [56, 62]. The half-life of ONOOH in aqueous phosphate buffer at pH 7.4 is 2.7 s at 25 °C and 0.8 s at 37 °C [61, 63]. \textbf{Fig.4} shows a compilation of the protonation and conformation equilibria and pathways for the decomposition of PN, as well as
1 INTRODUCTION

isomerization energies and pKₐ-values [56, 61, 62].

Concerning the postulated activated intermediate of PN, ONOOH⁺, there are two theories. Older studies about ONOOH-isomerization proposed a vibronical excitation in the trans-form by a combined deforming vibration along the N-O-O angle and a stretching vibration along the O-OH bond (see also Fig.5) [56]. During these vibrations the O-O bond is weakened and the endbonded O-atom approaches the N-atom. The intermediate can be regarded as a three-membered ring, which has either singlet or triplet character. In cis-ONOOH these vibrations are hindered. On an orbital basis, Fig.6 shows that in the trans-ONOOH HOMO and 2nd HOMO a bonding overlapping of the N- and O-orbitals becomes possible, whereas in the cis-form it is anti-bonding [59]. The radical cage mechanism of PN-isomerization and 1e-oxidative reactivity was discussed very controversially in literature [64, 65, 66, 67, 68, 69, 70].

The current opinion about PN-isomerization and 1e-oxidative reactivity has changed. Detailed kinetic measurements [71, 72], ESR measurements [73, 74, 75] and CIDNP experiments [6] favor the radical cage as reactive intermediate during PN-isomerization and also the observed 1e-oxidations are explainable this way. Equation (31) shows the formation of the radical pair in the solvent cage and the subsequent recombination to nitrate [71, 72]. If this cage encounters a 1e-donor (D-H), then a cage will be formed with a -NO₂ and donor radical pair and a molecule of water (equation (32)) [6]. This cage can either collapse by recombination of the donor and nitrogen dioxide radical, which would be a nitration or form nitrite and D⁺, which would be hydrolyzed by water and form a
hydroxy product. Of course it may also happen, that some of the radical species escape from the cage and react in a different way. The yield of these radical species is still under discussion and varies from 5 [76] - 40 % with respect to ONOOH concentration [71, 72].

\[
\text{ONO}_2 \overset{\cdot\cdot}{\longrightarrow} \text{NO}_2 - \text{OH} \quad (31)
\]

\[
\text{ONO} \cdot \cdot \cdot \text{OH} \overset{\cdot\cdot}{\longrightarrow} \text{NO}_3^- \quad (32)
\]

Also in the decomposition of PN at alkaline pH there are new findings. The formation of nitrite and oxygen is more complicated than shown in Fig.4. The following equations will introduce the major reactions taking place in an alkaline solution of PN [24, 61, 77].:

\[
\text{ONO}_2 \overset{\cdot\cdot\cdot}{\longrightarrow} \cdot\text{NO}_2 + \cdot\text{OH} \quad k = 1.2 - 1.3 \text{s}^{-1} \quad (33)
\]

\[
\text{ONO}_2^- \overset{\cdot\cdot\cdot}{\longrightarrow} \cdot\text{NO} + \text{O}_2^- \quad k = 0.02 \text{s}^{-1} \quad (34)
\]

\[
\cdot\text{NO} + \text{ONO}_2^- \rightarrow \cdot\text{NO}_2 + \text{NO}_2^- \quad k < 1.3 \times 10^{-3} \text{M}^{-1} \text{s}^{-1} \quad (35)
\]

\[
\cdot\text{NO}_2 + \text{ONO}_2^- \rightarrow \text{NO}_2^- + \text{ONO}_2 \cdot \quad k \approx 2 \times 10^4 \text{M}^{-1} \text{s}^{-1} \quad (36)
\]

\[
\cdot\text{OH} + \text{ONO}_2^- \rightarrow \text{OH}^- + \text{ONO}_2 \cdot \quad k \approx 5 \times 10^9 \text{M}^{-1} \text{s}^{-1} \quad (37)
\]

\[
\cdot\text{NO}_2 + \cdot\text{NO} \rightarrow \text{N}_2\text{O}_3 \quad k \approx 1.1 \times 10^9 \text{M}^{-1} \text{s}^{-1} \quad (38)
\]

\[
\text{N}_2\text{O}_3 + \text{ONO}_2^- \rightarrow \text{NO}_2^- + 2 \cdot\text{NO}_2 \quad k \approx 3 \times 10^8 \text{M}^{-1} \text{s}^{-1} \quad (39)
\]

Reaction (33) contributes most to the decay of PN in the physiological pH range (by formation of hydroxyl radicals which react with PN by equation (37)), above pH 8 reaction (34) causes PN-decomposition. Reaction (35) and (36) are
too slow to play a role, especially since the reaction of ·NO with ·NO₂ (mainly produced in the autoxidation of ·NO in oxygenated solutions) is much faster (see (38)). N₂O₃ is the intermediate that accelerates PN-decomposition at alkaline pH [77]. Some other reactions may play a role in this decomposition, so the formation of ONOO⁻ and O₂NOO⁻ and their further reactions [77].

1.3.2 Chemical Sources and Determination of PN

Fig. 7 gives an overview for the various methods of PN synthesis [63]. SIN-1 (9) and the NONOate/XO system (7) are mimicks for the physiological continuous formation of PN and are often used in biological model systems [16, 22]. The most common chemical syntheses of PN are the reactions of nitrite with acidified hydrogen peroxide (0) [78], of solid potassium superoxide with gaseous nitric oxide (1) [9] and of isoamyl nitrite with alkaline hydrogen peroxide (5) [79]. The reaction of tetramethyl-ammonium superoxide in liquid NH₃ with liquid nitric oxide is dangerous and complicated to handle, but yields highly purified PN as the solid N(CH₃)₄⁺-salt (deep yellow to orange color) with lowest contaminations of nitrite (2) [58]. (4) and (6) are methods rather used for physical experiments and are complicated in handling.

Figure 7: Different methods to synthesize PN (for a review see [63]).

PN concentrations in alkaline solutions are easily monitored by the absorption of ONOO⁻ at 302 nm (see also 1.3.1) [56, 62]. Other methods to determine
PN-concentrations are fluorometric measurements (e.g. oxidation of dihydro-rhodamine 123) [80, 81] and chromatographic or photometric measurements of other stable reaction products (e.g. nitro-phenols, nitroso-phenol...). But all these methods are not specific for PN and therefore are not suitable, if other oxidants may be present in a system.

1.3.3 Chemistry of PN

PN is the biological equivalent to chemical nitration reagents (e.g. NO$_2^+$BF$_4^-$ or HNO$_3$/H$_2$SO$_4$). The chemical nitration is mediated in organic solvents by a polar mechanism involving the nitronium cation (formation of a π-, a σ-complex and subsequent rearomatization by proton abstraction). In contrast PN nitrates in aqueous solutions by a radical mechanism involving nitrogen dioxide and phenoxy radicals [6], yielding also dimerization products as a consequence. In vitro, at concentrations ≥ 1 mM, PN reacts with almost all biomolecules [82]. ONOOH mainly causes 1e-oxidations, in which a ·NO$_2$ or ·OH radical together with the target radical are formed [6, 83]. ONOO$^-$ reacts by 2e-oxidations, in which an oxene is transfered to the target [84, 85, 86]. Tab.1 gives an overview about reactions of PN with proteins and other biomolecules, as well as with synthetic compounds.

Nitration of tyrosine residues and thiol or sulfoxidation of cysteine or methionine residues in proteins are the most common reactions of PN in vivo. Some examples for protein modifications are shown in Tab.1. Here I want to present some other important modified proteins:

- Studies by Zou and Ullrich on prostacyclin synthase (PGIS), a P450 enzyme, revealed that it is nitrated at a tyrosine residue near the active site by submicromolar concentrations of PN [11]. The localization of the nitrated Tyr near the heme-thiolate iron was concluded from the observation, that this 3-NT formation could be inhibited by blocking the active site of the enzyme [11]. Nitration went hand in hand with an inactivation of PGIS suggesting a possible role of the nitrated Tyr in the catalytic cycle, or a conformational change after nitration [12]. Regarding the high sensitivity of PN towards PGIS inactivation and nitration we concluded on a heme-thiolate autocatalyzed nitration of the Tyr [116].

- Beckman and coworkers found the Mn-SOD to be nitrated at low PN-concentrations, also suggesting an involvement of the manganese active center [118]. In another study the inactivation of Mn-SOD could be correlated
<table>
<thead>
<tr>
<th>Compound</th>
<th>Modification</th>
<th>$k(\text{ONOO}^-)/k(\text{ONO}_2\text{H})$</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Se-methionine</td>
<td>selenoxide, fragmentation</td>
<td>$2 \times 10^2 / 2 \times 10^4$</td>
<td>[87]</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>ascorbyl radical ...</td>
<td>$88 / 2.3 \times 10^2$</td>
<td>[88, 89]</td>
</tr>
<tr>
<td>Glutathione</td>
<td>thiol oxidation, GSSG</td>
<td>$5.8 \times 10^2 / 1.5 \times 10^6$</td>
<td>[15, 63]</td>
</tr>
<tr>
<td>Trolox, tocopherol</td>
<td>tocopheryl radical ...</td>
<td>$33 / 10^2 \times 10^4$</td>
<td>[91, 92, 93]</td>
</tr>
<tr>
<td>ADH</td>
<td>thiol oxidation, zinc release</td>
<td>$4 \times 10^5 / -$</td>
<td>[15, 94]</td>
</tr>
<tr>
<td>Phenol/tyrosine</td>
<td>phenoxyl radical ...</td>
<td>$- / 10^3$</td>
<td>[62]</td>
</tr>
<tr>
<td>Cysteine</td>
<td>thiol oxidation, cystine</td>
<td>$5 \times 10^3 / -$</td>
<td>[15]</td>
</tr>
<tr>
<td>Methionine</td>
<td>sulfoxidation, fragmentation</td>
<td>$1.8 \times 10^2 / -$</td>
<td>[15]</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>reaction with</td>
<td>$9.1 \times 10^{4a} , 1.3 \times 10^{-3b} / -$</td>
<td>[61, 77]</td>
</tr>
<tr>
<td>Carbon dioxide</td>
<td>reaction with</td>
<td>$3-5.8 \times 10^4 / -$</td>
<td>[95, 96]</td>
</tr>
<tr>
<td>Ebselen</td>
<td>ebselenoxide</td>
<td>$2 \times 10^6 / -$</td>
<td>[97]</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>metal center, nitration</td>
<td>$1.4 \times 10^4 / -$</td>
<td>[15]</td>
</tr>
<tr>
<td>oxyHemoglobin</td>
<td>metal center, nitration, Fe=O</td>
<td>$2 \times 10^4 / 8.8 \times 10^4$</td>
<td>[15, 98]</td>
</tr>
<tr>
<td>Aconitase</td>
<td>thiol oxidation</td>
<td>$1.4 \times 10^5 / -$</td>
<td>[99]</td>
</tr>
<tr>
<td>GPx</td>
<td>seleno-cysteine oxidation</td>
<td>$4.5 \times 10^4 , 8 \times 10^6 / -$</td>
<td>[101, 100]</td>
</tr>
<tr>
<td>Lactoperoxidase</td>
<td>metal center, Fe=O</td>
<td>$3.3 \times 10^5 / 8.4 \times 10^4$</td>
<td>[102]</td>
</tr>
<tr>
<td>HRP</td>
<td>metal center, nitration, Fe=O</td>
<td>$3.2 \times 10^6 / -$</td>
<td>[102]</td>
</tr>
<tr>
<td>Myeloperoxidase</td>
<td>metal center, Fe=O</td>
<td>$6.2 \times 10^6 / 2.5 \times 10^5$</td>
<td>[102]</td>
</tr>
<tr>
<td>Mn(II)TMPyP</td>
<td>metal center, Mn=O</td>
<td>$1.8 \times 10^6 / -$</td>
<td>[50]</td>
</tr>
<tr>
<td>Fe(III)TMPyP</td>
<td>metal center, Fe=O</td>
<td>$2.2-50 \times 10^6 / -$</td>
<td>[103]</td>
</tr>
<tr>
<td>Fe(III)TMPs</td>
<td>metal center, Fe=O</td>
<td>$6.5 \times 10^4$</td>
<td>[104]</td>
</tr>
<tr>
<td>Tryptophane</td>
<td>oxidation, nitration</td>
<td>$- / 1.3 \times 10^2$</td>
<td>[90]</td>
</tr>
<tr>
<td>Mn-SOD</td>
<td>metal center, nitration</td>
<td>???</td>
<td>[117, 118]</td>
</tr>
<tr>
<td>Cu,Zn-SOD</td>
<td>metal center</td>
<td>$10^6 / 10^5$</td>
<td>[105]</td>
</tr>
<tr>
<td>Mn$^{2+}$, Fe$^{3+}$</td>
<td>reaction with</td>
<td>1</td>
<td>[91, 106]</td>
</tr>
<tr>
<td>Cu$^{2+}$</td>
<td>reaction with</td>
<td>40</td>
<td>[91, 106]</td>
</tr>
<tr>
<td>Fe(III)edta</td>
<td>reaction with</td>
<td>$- / 5.5 \times 10^3$</td>
<td>[62]</td>
</tr>
<tr>
<td>Tempone</td>
<td>oxidation</td>
<td>$- / 4.5 \times 10^9$</td>
<td>[73]</td>
</tr>
<tr>
<td>β-ketoester</td>
<td>nitration</td>
<td>8</td>
<td>[107]</td>
</tr>
<tr>
<td>Ergothionein</td>
<td>thiol oxidation</td>
<td>$10^4$</td>
<td>[108]</td>
</tr>
<tr>
<td>β-carotene</td>
<td>radical intermediate ...</td>
<td>???</td>
<td>[109, 110]</td>
</tr>
<tr>
<td>Iodide,bromide</td>
<td>radicals, oxidation</td>
<td>$2.3 \times 10^4$</td>
<td>[90]</td>
</tr>
<tr>
<td>BSA</td>
<td>thiol oxidation, nitration</td>
<td>$2.5 \times 10^3$</td>
<td>[105]</td>
</tr>
<tr>
<td>P450BM−3</td>
<td>thiol oxidation, nitration, Fe=O</td>
<td>$2 \times 10^5$</td>
<td>[111]</td>
</tr>
<tr>
<td>Chloroperoxidase</td>
<td>metal center, Fe=O</td>
<td>$10^6$</td>
<td>[111]</td>
</tr>
<tr>
<td>GAPDH</td>
<td>thiol oxidation</td>
<td>$2.5 \times 10^5 / -$</td>
<td>[112]</td>
</tr>
<tr>
<td>Tyrosine hydroxylase</td>
<td>thiol oxidation, nitration</td>
<td>???</td>
<td>[113]</td>
</tr>
<tr>
<td>Tryptophane hydroxyl</td>
<td>thiol oxidation, nitration</td>
<td>$3.4 \times 10^4 / -$</td>
<td>[114]</td>
</tr>
<tr>
<td>PTP1B, CD45, LAR</td>
<td>thiol oxidation, nitration</td>
<td>$2.2 \times 10^7-2 \times 10^8 / -$</td>
<td>[115]</td>
</tr>
</tbody>
</table>

Table 1: Reactions of PN with biomolecules and synthetic compounds and velocities for these reactions (for reviews see [15, 124]). "..." in the field modification means that the first modification is followed by others (e.g. nitration, hydroxylation, nitrosation or fragmentation). k-values are given in M$^{-1}$s$^{-1}$, first value for the reaction with PN-anion/second one for the reaction with ONOOH. k-values for nitric oxide were measured in $^a$ oxygenated solution and $^b$ in deoxygenated solution. "-" does not mean that the compound does not react, but no k-value was determined. PTP1B, CD45 and LAR are protein tyrosine phosphatases.
with the exclusive nitration of Y$_{34}$ [117], although there is new evidence that inactivation may be due to dityrosine formation [119]. Cu,Zn-SOD is not inactivated by PN, but catalyzes the nitration of other proteins, such as neurofilament-L [120, 121].

- This thesis work revealed an autocatalyzed nitration of P450$_{BM-3}$ and P450$_{CAM}$ by PN at concentrations <50 µM, inactivation of P450$_{BM-3}$ by PN was due to thiol oxidation [111].
- Ca-ATPase in skeletal muscle sarcoplasmic reticulum undergoes also sensitive Tyr nitration and thiol oxidation by PN and is inactivated [122]. For P450$_{2B1}$ a nitration and inactivation by 39 µM PN has been reported [123].
- BSA contains no metal center, but is also nitrated at PN concentrations >100 µM. Thiol oxidation takes place at lower PN concentrations [124].
- NOS is inactivated by PN, probably by thiol oxidation [125]. Phosphatidylinositol 3-kinase is a target for protein nitration [126].

PN-mediated nitrations of Tyr-residues in proteins without metal-catalysis normally require PN-concentrations >100 µM. Exceptions may be cases, where Tyr is present as a tyrosinate or as a tyrosyl radical. Thiol oxidations of Cys-residues in proteins normally can be observed at PN concentrations of 5-50 µM and can get more sensitive, if the SH-group is present as a thiolate, or if it is located near to a metal center in a protein. The following list will give an overview about reactions of PN with low molecular weight biomolecules:

- Depletion of antioxidants, such as ascorbate[88], tocopherols[93], uric acid [4] and glutathione [127].
- Nitration, hydroxylation, nitrosation and dimerization of phenolic compounds (e.g. phenol, tyrosine, salicylate, L-DOPA and polyphenols) [62, 128, 65, 129, 130]. The mechanism of these reactions will be discussed later in more detail.
- Oxidation of aromatics and heterocyclic compounds (e.g. tryptophan, phenylalanine, indole, histidine and related compounds, purins and related compounds) [90, 64, 131, 132]. Especially purines play a major role in the cytotoxicity of PN, by formation of nitro- and oxo-nucleotides or radical purine intermediates and subsequent strand breaks in DNA [133, 134].
- Oxidation and nitration of polyunsaturated fatty acids or β-keto-esters (e.g. pyruvate and lipidperoxidation) [135, 136].
• Thiol oxidation (e.g. cysteine and glutathione) [138, 137]. The reaction of thiols with PN-anion or peroxynitrous acid yields a variety of products, such as dithiol (RSSR), sulfenic acid (RS-OH), sulfoxides (sulfinic acid RS(O)OH, sulfonic acid RS(O)₂OH, sulfate ROS(O)₃OH depending on the PN/RSH ratio), nitroso-thiols (RS-NO), nitrito-thiols (RS-ONO), thiol-nitrates (RS-ONO₂) or nitro-thiols (RS-NO₂) [83]. These products can be formed in either 1e- or 2e-oxidations, either involving thyl radicals or direct oxene transfer.

• Seleno- or sulfoxidation of thio- or seleno-ethers (e.g. ebselen, methionine and Se-methionine) [86, 139]. A very suitable model to distinguish ONOO⁻ from ONOOH reactivity was the reaction of PN with thianthrene-5-oxide (see also Fig.8 (1')) [84]. The sulfone (4') is the exclusive product at pH 14 (ONOO⁻) and PN reacts as a nucleophilic oxidant. Electrophilic oxidants, such as hydrogen peroxide in perchloric acid would form the disulf oxide (3'). PN in the protonated form, at pH 6.4, leads to nitration of the aromatic ring (2'). The sulfone-sulfoxide (5') is not formed.

![Figure 8: Different reactivity of ONOO⁻ and ONOOH, as monitored by using thianthrene-5-oxide as a model.](image)

• Reaction of ONOO⁻ and ONOOH with carbon dioxide [95, 140]. Nitration and dimerization of phenolic compounds, but also fragmentation of methionine by PN are increased in presence of carbon dioxide [85, 96]. Nowadays it is excepted, that PN and CO₂ form an adduct, the nitroso-peroxo-carbonate (ONOOCO₂⁻) [140, 141, 142]. This adduct has three major pathways to form reactive nitrating species: The first and dominant one, the adduct can undergo homolytic cleavage to form a radical cage-pair of nitrogen dioxide...
and carbonate radical (42). These radicals can escape from the cage (30-33 \%) and lead to the reactions (44 and 45) [141, 143]. 2. The adduct could undergo heterolytic cleavage to form a nitronium and a carbonate ion cage-pair (41). But the product pattern (high yields in radical derived dimerization and fragmentation products), as well as the extremely short lifetime of NO\textsuperscript{+}_2 in aqueous solution stand against this theory. 3. Isomerization of ONOO\textsuperscript{−} to O\textsubscript{2}NO\textsuperscript{−} (nitro-carbonate), but also this pathway does not play an essential role [141, 142]:

\[
\text{HCO}_3\textsuperscript{−} + \text{H}^+ \rightleftharpoons \text{H}_2\text{CO}_3 \rightleftharpoons \text{CO}_2 \uparrow + \text{H}_2\text{O} \quad (40)
\]

\[
\text{ONOO}^− + \text{CO}_2 \rightarrow \text{ONOOCO}_2^− \rightarrow [\text{NO}_2^+ \cdot \text{CO}_3^−]\text{cage} \quad (41)
\]

\[
\text{ONOO}^− + \text{CO}_2 \rightarrow \text{ONOOCO}_2^− \rightarrow [\text{CO}_3^− \cdot \text{NO}_2]\text{cage} \quad (42)
\]

\[
\text{CO}_3^− \cdot + \text{Tyr} \rightarrow \text{HCO}_3^− + \text{Tyr} \cdot \quad (43)
\]

\[
\text{Tyr} \cdot + \cdot \text{NO}_2 \rightarrow \text{O}_2\text{N} - \text{Tyr} \quad (44)
\]

\[
\text{Tyr} \cdot + \text{Tyr} \cdot \rightarrow \text{Dityr} \quad (45)
\]

The reaction of carbon dioxide with PN is fast (k=3\times10^4 \text{ M}^{-1}\text{s}^{-1}) and concentrations of CO\textsubscript{2} are high under physiological conditions [144]. Therefore the formation of ONOO\textsuperscript{−} could be one of the major pathways for PN-reactivity \textit{in vivo}. Nitration of Tyr by PN is increased by a factor of 3-10 in the presence of CO\textsubscript{2} [95, 145, 146], depending on pH.

### 1.3.4 Biology of PN

The biology of PN is rather characterized by its participation in pathophysiological processes. These actions can be summarized by modifications of biomolecules as shown in 1.3.3, especially nitration of Tyr-residues and oxidation of Cys- and Met-residues in proteins [148, 149, 150, 151]. All these protein modifications are not specific for PN, thiols are also oxidized by hydrogen peroxide, superoxide, hydroxyl radicals and hypochlorite [137] and tyrosines are nitrated by \cdot\text{NO}_2 or by the MPO-catalyzed reaction of nitrite with hydrogen peroxide [15, 152]. 3-NT is often taken as a marker for PN presence \textit{in vivo} [5, 153], but is also criticized for its unspecificity [155, 154, 156] and some reports even question the relevance of PN-mediated nitration under physiological conditions [22]. 3-NT can be also formed by nitrogen dioxide, the autoxidation product of nitric oxide [15], by the myeloperoxidase- or HRP-catalyzed reaction of hydrogen peroxide with nitrite [152], by the CPO-catalyzed reaction of hypochlorite with nitrite [53] and by the addition of nitric oxide to a once formed tyrosyl radical with subsequent oxidation [34, 157, 158]. Protein-bound 3-NT is popular as a footprint of PN
Table 2: Velocities for PN decay in presence of physiological concentrations of biomolecules (effective rate constant). Second column: k-value for ONOO\(^-\) / k-value for ONOOH. Fourth column: k\(_{\text{scav}}\) for ONOO\(^-\) / k\(_{\text{scav}}\) for ONOOH. Values are taken from reviews [144, 162] and corrected with values from Tab.1.

<table>
<thead>
<tr>
<th>Biomolecule</th>
<th>k(_{\text{sec}}) [M(^{-1})s(^{-1})]</th>
<th>[Biomol.][M]</th>
<th>k(_{\text{scav}}) [s(^{-1})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Self-decompos.</td>
<td>-</td>
<td>-</td>
<td>0.4-0.6</td>
</tr>
<tr>
<td>Carbon dioxide</td>
<td>3-4.6x10(^4) / -</td>
<td>10(^{-3})</td>
<td>30-46 / -</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>50-90 / 230</td>
<td>10(^{-2})</td>
<td>0.5-0.9 / 2.3</td>
</tr>
<tr>
<td>Glutathione</td>
<td>580 / 1.5x10(^6)</td>
<td>10(^{-2})</td>
<td>5.8 / 15000</td>
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<td>Myeloperoxidase</td>
<td>2.5x10(^5) / 6.2x10(^6)</td>
<td>2x10(^{-7})-5x10(^{-4})</td>
<td>0.05-125 / 0.12-3100</td>
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<tr>
<td>oxyHemoglobin</td>
<td>- / 2.5x10(^4)</td>
<td>2.3-5x10(^{-3})</td>
<td>- / 58-125</td>
</tr>
<tr>
<td>GPx</td>
<td>2x10(^4)-8x10(^6) / -</td>
<td>1.5-2x10(^{-6})</td>
<td>0.03-16 / -</td>
</tr>
<tr>
<td>Albumin</td>
<td>3-6x10(^8)</td>
<td>6x10(^{-4})</td>
<td>1.8-3.6</td>
</tr>
</tbody>
</table>

in vivo, because antibodies are available for its sensitive detection and the spectroscopic and electronic properties of 3-NT for UV/Vis and electrochemical HPLC detection are suitable for a sensitive monitoring [154, 159]. During the last year another issue was brought up, speaking against 3-NT as a biomarker for PN: Murad and coworkers have observed that 3-NT is not stable under physiological conditions and that there may be enzymatic and non-enzymatic pathways for reduction of PN [160, 161]. These findings also revitalized the discussion, that PN could be an unspecific messenger, on the basis that PN-mediated nitrations are reversible.

Depletion of antioxidants or modification of low molecular weight biomolecules requires extremely high concentrations of PN, to be harmful to cells or the whole organism. The half-life of PN in vivo is short regarding the cellular defense, which is able to intercept PN. Tab.2 shows possible pathways for PN consumption in vivo [144, 162]. Important for the PN scavenging ability are the effective velocities, that means the second order rate constant of PN with an antioxidative system multiplied with its physiological concentration. Most important is the decay velocity (k\(_{\text{scav}}\)) for the PN-anion. The reactivity of ONOOH is almost completely suppressed by glutathione (k\(_{\text{scav}}=15000\) s\(^{-1}\)). The difference of the values is due to the different second order rate constants and physiological concentration that were used for calculation of the effective velocities.

The reaction of nitric oxide and superoxide yields PN in the anionic form and although protonation is in a fast equilibrium, it is mainly present as ONOO\(^-\) at
physiological pH due to its pKₐ of 6.8 [56], therefore scavengers for the PN-anion are more important than for ONOOH. Most of ONOOH is trapped by GSH, MPO or oxyHb (see Tab.2). Uric acid also reacts very fast with ONOOH and is present in cytosol and plasma at concentrations of 100-300 µM [4]. Regarding the fact that heme- and heme-thiolate proteins increase the nitrination potential of PN [163, 164], these compounds cannot be considered as real scavengers for PN. On the other hand it had been shown, that such proteins coupled with antioxidants represent possibly the most effective scavenging systems for PN [50, 165]. Biomolecules that react with ONOO⁻ are more likely to be oxidized by PN, since only GPx and GSH have a certain protective potential. Since many metal-containing proteins and also carbon dioxide react with PN-anion and increase its oxidative properties, these two reactions could be the major activation pathways for PN in vivo and contribute the major part to PN-toxicity [163, 116, 146].

Tab.3 shows a list of human diseases, in which 3-NT could be detected and an involvement of PN was suggested or proved [166]. Tab.4 shows the same for animal models of disease [166] and Tab.5 for cellular models of disease [166].

| Atherosclerotic plaques of coronary vessels | Chronic renal failure in septic patients |
| LDL isolated from atherosclerotic lesions | Inflammatory bowel disease |
| Lungs with sepsis and/or respiratory disease | Helicobacter pylori gastritis |
| Idiopathic pulmonary fibrosis | Necrotizing enterocolitis |
| Lung transplants with obliterative bronchiolitis | Coliace disease |
| Plasma of infants with BPD | Fluid of patients with arthritis |
| Amyotrophic lateral sclerosis | Early prosthesis failure |
| Multiple sclerosis plaques | Inclusion body myositis |
| Alzheimer’s lesions | Placenta of preeclamptic pregnancies |
| Rejected renal allografts | Skin lesion with anaphylactoid purpura |

Table 3: Detection of 3-NT in human disease [166].

These tables show that PN may have a major impact on the pathophysiology of disease and how important the development of scavengers for PN is. The impact of PN on the vascular system could be established in our group by ZOU et al [167, 168, 170] and this finding is supported by other groups [171, 172]. PN not only eliminates the vasorelaxating PGI₂, but also consumes ·NO, another potent vasorelaxating factor (EDRF), for its formation. Therefore PN could contribute seriously to vasodysfunction. Nitration and inactivation of PGIS by PN was found to be involved in hypoxia-reoxygenation triggered vasospasm [169],
1 INTRODUCTION

Table 4: Detection of 3-NT in animal models of disease [166].

<table>
<thead>
<tr>
<th>Animal Model</th>
<th>3-NT Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aorta of septic rats</td>
<td></td>
</tr>
<tr>
<td>Cardiac alloraft rejection</td>
<td></td>
</tr>
<tr>
<td>Ischemia-reperfusion-injured rat heart</td>
<td></td>
</tr>
<tr>
<td>Cholesterol-induced atherosclerosis</td>
<td></td>
</tr>
<tr>
<td>Autoimmune myocarditis</td>
<td></td>
</tr>
<tr>
<td>Myocardial inflammation</td>
<td></td>
</tr>
<tr>
<td>Rabbit lungs following exposure to hyperoxia</td>
<td></td>
</tr>
<tr>
<td>Lungs of endotoxin-treated rats</td>
<td></td>
</tr>
<tr>
<td>Ischemia-reperfusion-injured rat lungs</td>
<td></td>
</tr>
<tr>
<td>Influenza-induced pneumonia</td>
<td></td>
</tr>
<tr>
<td>Pulmonary granulomatous inflammation</td>
<td></td>
</tr>
<tr>
<td>HSV-1-induced pneumonia</td>
<td></td>
</tr>
<tr>
<td>Malonate- and MPTP-induced neurotoxicity</td>
<td></td>
</tr>
<tr>
<td>Excitotoxicity model of neuronal injury</td>
<td></td>
</tr>
<tr>
<td>Transgenic model for ALS</td>
<td></td>
</tr>
<tr>
<td>ApoE-deficient mice</td>
<td></td>
</tr>
<tr>
<td>Brain vasculature of CO-poisoned rats</td>
<td></td>
</tr>
<tr>
<td>Chronic cerebral vasospasm</td>
<td></td>
</tr>
<tr>
<td>Allergic encephalomyelitis</td>
<td></td>
</tr>
<tr>
<td>Central nervous system inflammation</td>
<td></td>
</tr>
<tr>
<td>Liver transplantation</td>
<td></td>
</tr>
<tr>
<td>Dimercaptosuccinic acid nephrosclerosis</td>
<td></td>
</tr>
<tr>
<td>Guinea pig ileitis</td>
<td></td>
</tr>
<tr>
<td>Placenta of LPS-treated rats</td>
<td></td>
</tr>
<tr>
<td>Carrageenan paw edema</td>
<td></td>
</tr>
<tr>
<td>Murine leishmaniasis</td>
<td></td>
</tr>
<tr>
<td>Zymosan peritonitis</td>
<td></td>
</tr>
<tr>
<td>Autoimmune diabetes in NOD mice</td>
<td></td>
</tr>
<tr>
<td>Autoimmune uveitis</td>
<td></td>
</tr>
<tr>
<td>Dermal tumor promotion</td>
<td></td>
</tr>
<tr>
<td>Mice bearing ResX tumor</td>
<td></td>
</tr>
<tr>
<td>Aged rat skeletal muscle SERc2a isoform</td>
<td></td>
</tr>
</tbody>
</table>

Table 5: Detection of 3-NT in cellular models of disease [166].

<table>
<thead>
<tr>
<th>Cellular Model</th>
<th>3-NT Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingested bacteria in human PMN</td>
<td></td>
</tr>
<tr>
<td>IL-1β-stimulated VSMC</td>
<td></td>
</tr>
<tr>
<td>Arginine-depleted neuronal cells</td>
<td></td>
</tr>
<tr>
<td>Native LDL-treated endothelium</td>
<td></td>
</tr>
<tr>
<td>H₂O₂-exposed kidney epithelium</td>
<td></td>
</tr>
<tr>
<td>Cytokine-stimulated fetal glial cultures</td>
<td></td>
</tr>
<tr>
<td>TNF-α-treated endothelium</td>
<td></td>
</tr>
<tr>
<td>CO-exposed endothelium</td>
<td></td>
</tr>
</tbody>
</table>

In atherosclerosis [173] and in endotoxic shock [manuscripts submitted] and was found to be stimulated by IL-1β in rat mesangial cells [12].

1.4 Compound I and II

Since the nitration mediated by "free" PN is much less efficient compared to the metal-catalyzed nitration by PN [163, 174], it may be assumed, that biologically occurring nitations require either metal-catalysis [120, 168] or the presence of carbon dioxide [95, 149] to compete with the antioxidant defense of the organism. Metal-catalysis could even be a more important factor, when a tyrosine is located nearby the metal center. In this case reactive intermediates (e.g. nitrogen dioxide) have a short distance to diffuse and the shortlived species have the best chances to react with this Tyr. Therefore it is obligatory to introduce some basic information about the mechanism by which PN reacts with transition metals.

Oxidized metal species such as ferryl (Compound II, Fe⁴⁺⁻O), perferryl
(Compound I, formally Fe\textsuperscript{V}=0), Mn\textsuperscript{IV}=O and Mn\textsuperscript{V}=O play a central role in reactions of PN with these metals [163, 175]. Therefore this section will give a short introduction on the basic chemistry and properties of these high valence metal compounds.

Compound I has been described for peroxidases and P450 enzymes [176]. Theoretically all heme- and heme-thiolate proteins can form a Compound I intermediate, depending on the generating species and the environment of the active site [177]. Formation of Compound I requires heterolytic cleavage of the oxidizing species (e.g. hydrogen peroxide, periodate, chlorite, N-oxides and hypochlorite) or a direct oxene transfer (e.g. iodoso-benzene) [176, 178]. The following equations show the steps of perferryl formation and its reduction to Compound II:

\[
\begin{align*}
\text{(X)Fe}^{\text{III}} & + \text{ROOH} + \text{B}^- \rightleftharpoons \text{(X)Fe}^{\text{III}} \cdots \text{OOR}^- + \text{BH} \\
\text{(X)Fe}^{\text{III}} \cdots \text{OOR}^- & \rightarrow \text{(X)Fe}^{\text{V}} = \text{O} + \text{RO}^- \\
\text{(X)Fe}^{\text{III}} & + \text{O} = \text{R} \rightarrow \text{(X)Fe}^{\text{V}} = \text{O} + \text{R} \\
\text{(X)Fe}^{\text{V}} = \text{O} & \leftrightarrow \text{(X)Fe}^{\text{IV}} - \text{O}^- \leftrightarrow \text{(X)Fe}^{\text{IV}} = \text{O} \\
\text{(-X)Fe}^{\text{VI}} = \text{O} & + \text{e}^- + \text{H}^+ \rightarrow \text{(X)Fe}^{\text{VI}} - \text{OH}
\end{align*}
\]

Heterolytic cleavage of the oxidizing species (e.g. peroxides) requires the formation of Compound O, a coordinative complex of the oxidizing species (46), which is subsequently cleaved (47) [176]. Iodosobenzene can directly transfer an oxene to the ferric iron (48) [176]. The leaving groups, RO\textsuperscript{-} and R, must be redox stable or else they may be oxidized by the produced Compound I (this was observed for Mn\textsuperscript{V}=O and Fe\textsuperscript{V}=O and nitrite in reactions of PN with Mn(III) and Fe(III) porphyrins [50, 103, 164]). Concerning the mesomeric structures in (49) one has to consider, that iron(V) in porphyrins is not stable and was only described for some peculiar complexes [179]. Iron(V) will immediately abstract an electron or H-atom from the coordinating porphyrin (HRP) or a nearby amino acid (cytochrome c peroxidase (Trp), PGH\textsubscript{2} synthase (Tyr), cytochrome P450 mono-oxygenases and lactoperoxidase (Cys)) and form an iron(IV)-AA or -porphyrin radical or cation radical [176, 180]. The mesomer structure with an oxygen-centered radical could contribute to the 1e-oxidative potential of Compound I [178]. All mesomers are in equilibrium with their protonated form. By uptake of one electron, Compound I is reduced to Compound II (50).

Compound I is able to form epoxides from olefins [38, 181, 178, 182], to oxidize
thio-ethers (see Fig.9) and amines [176, 178], dealkylate alkyl-amines [178, 183], O-dealkylation [184], N,S-hydroxylation [178], and especially in P450 enzymes is also able to hydroxylate alkanes (mono-oxygenations) [176, 178]. Fig.10 shows the proposed mechanism for the hydroxylation of an alkane by P450 Compound I. In the first step Compound II is formed by H-atom abstraction from the alkane, followed by either "oxygen rebound" directly yielding ROH and the ferric heme [185, 186, 187], or electron transfer yielding a carbenium ion which can be further hydrolyzed by water [178]. This special ability of P450 enzymes to insert oxygen in C-H bonds leaves open room for speculations on the role of the thiolate-ligand in these reactions. Older MO-calculations revealed no radical character of the thiolate-ligand in P450 Compound I, but a stabilizing effect by its d-orbitals [188, 189]. By using new calculation methods Green assumed sulfur-based radicals in heme-thiolate Compound I [190, 191], that would explain the special reactivity in mono-oxygenations.

Compound II is formed during reduction of Compound I (see (50)), but it can also be generated by homolysis of suitable peroxides (e.g. m-chloro-perbenzoic acid (CPA), peracetic acid or PN) [176, 181]. The following equations show the formation and properties of ferryl:

\[
\begin{align*}
(X)\text{Fe}^{\text{III}} & + \text{ROOH} + \text{B}^- & \rightarrow & (X)\text{Fe}^{\text{III}}\cdots\text{OOR}^- + \text{BH} & \text{(51)} \\
(X)\text{Fe}^{\text{III}}\cdots\text{OOR}^- & \rightarrow & (X)\text{Fe}^{\text{IV}} = \text{O} + \text{RO}^- & \text{(52)} \\
(X)\text{Fe}^{\text{IV}} = \text{O} & \leftrightarrow & (X)\text{Fe}^{\text{III}} - \text{O}^- & \leftrightarrow (X)\text{Fe}^{\text{IV}} - \text{O}^- & \text{(53)} \\
(X)\text{Fe}^{\text{VI}} = \text{O} & + & \text{e}^- & + & \text{H}^+ & \rightarrow & (X)\text{Fe}^{\text{III}}\cdots\text{OH}^- & \text{(54)}
\end{align*}
\]

For the first step in Compound II generation, the formation of Compound O is assumed again (51), but in this case the peroxide is splitted homolytically
Figure 10: Proposed mechanism for the monooxygenation of an alkane by a P450 enzyme. Iodoso-benzene generates P450 Compound I, which is reduced to Compound II by abstraction of a hydrogen atom from the substrate [178, 185, 187].

yielding the ferryl and a leaving radical \((\text{52})\). \(\text{Fe}^{IV}=\text{O}\) formation with hydrogen peroxide is thermodynamically not favorable, because of the high energy of the hydroxyl radicals formed. CPA yields mesomerically stabilized CPA radicals and PN the more or less stabilized nitrogen dioxide radicals [163, 164]. The contribution of the mesomeric structure of the oxygen-centered radical \((\text{53})\) to the character of Compound II is small, because an O-radical has a higher oxidative potential compared to iron(IV), whereas the negatively charged oxygen structure contributes a lot, since the \(\text{pK}_a\) of ferryl should be low (personal communication Neese) and often is written as \(\text{Fe}^{IV-}\).
1.5 Oxidative Stress

PN formation \textit{in vivo} depends on the levels of superoxide and nitric oxide. Only under pathophysiological conditions, where the oxygen equilibrium is disturbed, PN levels are increased dramatically and may be harmful to the organism. These malfunctions can be summarized under the term ”oxidative stress”. The following list shows conditions, that fit in this topic (for reviews see [192, 193]) and Fig. 11 gives an overview about sources and defenses for reactive oxygen species in the aerobic cell [SIES, 1989]:

- Hyperbaric oxygen, \textit{Hyperoxia}
  - Retrolental \textit{Fibroplasy}
  - Pulmonary respiration dysfunction
  - \textit{Ischemia} reperfusion damage

- Hypobaric oxygen, \textit{Hypoxia}
  - Systemic hypoxia
  - Shock syndrome

- Poisons, chemicals
  - Paraquat and other redoxcyclers
  - Tetrachloro-methane and other solvents
  - Chemotherapy (adriamycin...)
  - Nitro-compounds (nitro-phenols, nitrofurantoin)
  - Cancerogens, xenobiotica (benzpyren, PCBs)

- Drug-induced hemolytic \textit{Anemia}

- Vitamin (A, C, E) deficiency

- Aging (cataract, lipofuscine)

- Acute inflammation
  - Heat shock, burn damage
  - Infections
  - Pulmonary edema after hemodialysis
  - Cerebral edema
• Chronic inflammation
  – Rheumatoid Arthritis
  – Emphysema (inactivation of α-1-antiproteinase)
  – Behcets disease
  – Cancer
  – Diabetes
  – Cataracts

• Radiation damage
  – Sun burn (UV)
  – Radiotherapy

• Air pollution
  – Photooxidants (NO$_X$, O$_3$, PAN)
  – Sulfur dioxide, hydrogen sulfite
  – Asbestos, soot
  – Cigarette smoke
Figure 11: Sources and defenses for reactive oxygen species in the aerobic cell (Sies, 1989).
2 Aims of the Study

With the nitration of prostacyclin synthase as a basis, the present study should contribute to the following issues:

- It should give a detailed picture of the mechanism by which PN reacts with phenolic compounds and especially clarify the question, whether this reaction has a radical or a polar character.

- It should give insights in the mechanism by which metal-complexes influence the reaction of PN with phenolic compounds. Reactive intermediates during the metal-catalyzed reaction of phenol with PN should be characterized.

- Other enzymes should be found, which are nitrated in a similar PN concentration sensitive manner as PGIS and therefore could be considered as suitable models for PGIS nitration by PN.

- Scavengers for PN or inhibitors for PN-mediated nitration should be identified, which could be employed to protect PGIS from inactivation by PN.
3 Materials and Methods

3.1 Reactions of Phenolic Compounds with Peroxynitrite

3.1.1 Chemicals

Chemicals were purchased from Merck, Fluka, Lancaster, Aldrich or Sigma if not indicated differently and were from the highest purity available. All buffers were made from water with Milli-Q quality, using salts and corresponding acids for the desired pH. All buffers were stored not longer than one week at +4 °C.

3.1.2 Peroxynitrite Synthesis

Peroxynitrite (PN) was synthesized according to KOPPENOL and KISNNER [9] by addition of gaseous ·NO to solid potassium superoxide. 0.3 g KO₂ were added to 5 g SiO₂ sand which was already kept under argon. Next 50 ml ·NO gas were added (2 ml/min) from a syringe to the solid mixture which was stirred on ice. The solid PN was dissolved in 5 ml 0.1 M sodium hydroxide solution, separated from the sand, freed from residual hydrogen peroxide with 1-3 g manganese(IV)oxide and then filtered through a syringe filter. Stock solutions were diluted to a final concentration of 50-100 mM, determined spectroscopically using ε₃₀₂ = 1670 M⁻¹cm⁻¹ [57]. Stock solutions were kept at 193 K for one month or at 253 K for at least one week without serious losses of PN. The ratio of PN:nitrite was determined as about 1:1 with the GRIESS assay [194].

3.1.3 Oxygen Measurements

Oxygen was determined with a modified CLARK-type electrode (Eschweiler-Kiel), using E=+0.8 V for detection of oxygen. The volume of the electrode chamber was 500 µl. Oxygen release from neutral and alkaline PN solutions was determined from the decomposition of 800 µM PN in 0.2 M K-phosphate buffer at different pH-values at 310 K and quantified using a saturated stock solution of oxygen in water (2.181 mM at 273 K) [195]. Measurements were stopped when the maximum yield was reached.

3.1.4 Nitric Oxide Measurements

For NO-detection an electrode-potential of -0.8 V was used. Nitric oxide release from acidic nitrite solutions was measured in the reaction of 5 mM sodium nitrite in 0.2 M buffer at 273 K (pH 1: KCl/HCl, pH 2 and 3: Na-citrate/citric acid, pH 4 and 5: Na-acetate/acetic acid, pH 6 and 7: K-phosphate, pH 8 Tris
(tris(hydroxymethyl)-amino-methan)) and quantified with a saturated stock solution of nitric oxide in water (3.275 mM at 273 K) [195].

3.1.5 Determination of Nitrate and Nitrite

Nitrite and Nitrate were quantified by ion chromatography. The column was an Anion-II 250x4.6 Nucleosil strong anion exchanger (trimethyl-ammonium) from Macherey & Nagel, the mobile phase consisted of 200 mM potassium phosphate buffer pH 6.5, the flow was 0.35-0.5 ml/min. The anions were detected UV-spectroscopically at 225 nm.

3.1.6 Products of the Phenol-PN-reaction

All products from the reaction of phenol with PN were identified and quantified by HPLC from mixtures containing 5 mM phenol and 800 µM PN in 0.2 M K-phosphate buffer at 310 K with HPLC using internal and external standards. Since the buffer was heated and degassed before use a possible contamination by bicarbonate was low. PN was rapidly added under Vortex-mixing. Standard incubation times were 5 min. The required minimal incubation times were determined by spectroscopically recording the absorbance at 350 nm or 400 nm in the pH-range from 7-12 after addition of PN to the phenolic solution, the endpoints were determined after 30 sec at pH 8, 120 sec at pH 9, 6 min at pH 10, 30 min at pH 11, 50 min at pH 12. Some products required other incubation times: for detection of 2-nitroso-phenol the incubation time was only 1 min, also biphenols were very sensitive to incubation times. Products were detected on different HPLC-systems, to avoid overlapping of peaks for identification. Tab.6 shows a list of all HPLC-systems used and the corresponding retention times of references. The flow in all HPLC-systems was 1 ml/min. The pH dependent quantitation of nitrophenols (NP) was done on HPLC-system (VI), of hydroxyphenols (HP), p-benzoquinone (BQ) and 4-nitrosophenol (p-NO) on system (I) and of biphenols (BP) on system (V).

\[^{1}\text{HP for hydroxy-phenol, BQ for benzoquinone, Phe for phenol, NO for nitroso-phenol, NC for nitro-catechol, NH for nitro-hydroquinone, NP for nitro-phenol and BP for biphenol. The composition of HPLC-systems was: LKB Bromma 2150 pumps for I-VI, a Spectra Physics SP8490 variable wavelength detector, SP4290 integrator and an ODS II C\text{18} 250x4.6 \text{mm column (Bischoff) for I-III. A forward optical scanning detector spectra focus from Spectra Physics, a Merck-Hitachi D-2000 integrator and a Nucleosil C\text{4} 250x4.6 \text{mm column (Bischoff) for IV-VI. The mobile phase of all systems consisted of 0.1 M citric acid/citrate buffer pH 2 and acetonitrile. The percentage of acetonitrile is listed beside the system numbers. Hydroxyproducts were}}\]
### 3 MATERIALS AND METHODS

#### Table 6: HPLC retention times for products of the phenol-PN reaction.

<table>
<thead>
<tr>
<th></th>
<th>HPLC-systems</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I,5%</td>
</tr>
<tr>
<td>Reference</td>
<td>Retentiontime [min]</td>
</tr>
<tr>
<td>-------------</td>
<td>------------------</td>
</tr>
<tr>
<td>3-HP</td>
<td>7.2</td>
</tr>
<tr>
<td>2-HP</td>
<td>9.2</td>
</tr>
<tr>
<td>p-BQ</td>
<td>10.2</td>
</tr>
<tr>
<td>α-BQ</td>
<td>7.7</td>
</tr>
<tr>
<td>Phe</td>
<td>19.2</td>
</tr>
<tr>
<td>4-NO</td>
<td>15.5</td>
</tr>
<tr>
<td>2-NO</td>
<td>-</td>
</tr>
<tr>
<td>4-NC</td>
<td>-</td>
</tr>
<tr>
<td>2-NH</td>
<td>-</td>
</tr>
<tr>
<td>4-NP</td>
<td>50</td>
</tr>
<tr>
<td>3-NP</td>
<td>-</td>
</tr>
<tr>
<td>2-NP</td>
<td>78</td>
</tr>
<tr>
<td>2,2′-BP</td>
<td>-</td>
</tr>
<tr>
<td>4,4′-BP</td>
<td>-</td>
</tr>
<tr>
<td>2,4′-BP</td>
<td>-</td>
</tr>
</tbody>
</table>

For 2,4′-biphenol (2,4′-dihydroxy-biphenyl) an authentic sample was prepared according to [196, 197], the UV-spectra of both, the isolated product from the phenol-PN-reaction as well as the authentic sample showed two shoulders at 245 and 285 nm. For 4-nitroso-phenol we purified a sample from the phenol-PN-reaction and characterized it by UV/Vis-spectra ($\lambda_{\text{max}}=300$ nm in acidic milieu and $\lambda_{\text{max}}=256$ and 396 nm in alkaline milieu) [198], NMR-spectra (a para-substituted aromatic with two doublet-signals with $\delta=7.61$ and 6.58 ppm and $J_{\text{vic}}=8.78$ and 9.18 Hz at 333K) [199] and EI-MS-spectra (M$^+$ at m/z=123). For 2-nitroso-phenol an authentic sample was prepared, according to [200, 201], which in two HPLC-systems showed the same retention time as the product from the phenol-PN-reaction see (Tab.6). A UV/Vis-spectrum of 2-nitroso-phenol could not be obtained. Another product was characterized as 4-phenoxy-phenol ($t_R=38.3$ min on system (VI) and $t_R=14.6$ min on system (VI) with 30 % acetonitrile and showed symmetrical peaks after spiking). An authentic sample of o-benzoquinone was prepared by the oxidation of catechol with ferric chloride. We also synthesized a sample of phenyl-nitrite and phenyl-nitrate according to [202, 203], but both detected at 265 nm, nitro- and nitrosophenols at 287 nm.
were unstable in aqueous solutions and were hydrolyzed to phenol and nitrite or nitrate, respectively.

### 3.1.7 Products of the Anisol-PN-reaction

Products of the anisol-PN reaction were identified and quantified on system (V), using 2-, 3-, 4-nitro-anisol (NA) and 2-, 3-, 4-hydroxy-anisol (HA) as internal and external standards, with UV-detection at 270 nm. 5 mM anisol was incubated with 800 µM PN for 5 min at 310 K. The anisol stock solution was 100 mM in acetonitrile, allowing 25 µl acetonitrile to be added to 475 µl buffer. We ensured, that this concentration of acetonitrile had no effect on product formation by adding 25 and 50 µl acetonitrile to the phenol-PN reaction. Retention times: 4-HA (5.8 min), 2-, 3-HA (6.6 and 6.9 min), 4-NA (13.8 min), anisol (15.2 min), 2-NA (17.7 min) and 3-NA (20.4 min). Reactions with NO were done by using a saturated 3.275 mM standard solution of NO at 273 K [195]. Nitrogen dioxide was added by a gas-syringe (100 µl ≈ 4.54 µmol) to 1 ml reaction mixtures, containing 5 mM of the aromatic compound in 0.2 M K-phosphate buffer pH 6 in a sealed microflask with septicum.

### 3.1.8 Reactions of Tyrosine with Peroxynitrite, Nitric Oxide and PN-generating Systems

1 mM tyrosine were incubated with different concentrations of PN, Spermine NONOate (1 or 2 mM) (≥98 %, Cayman Chemical, Ann Arbor, USA) or xanthine (0.5 or 1 mM)/ xanthine oxidase (28 or 56 mU/ml) (grade III, Sigma, Germany) and NONOate. Decomposition of Spermine NONOate was monitored at 250 nm (ε<sub>250</sub>=8000 M<sup>-1</sup>cm<sup>-1</sup>) and was found to be ≈16 nmol/min at 37 °C in 0.1 M KP<sub>i</sub> buffer pH 7.4. The stock solution was kept on ice in 0.1 M NaOH. Tyrosine nitration was measured in dependence on PN-concentration and time-dependent in the case of NO-donors and PN-generating systems by the use of HPLC. A C<sub>18</sub> Nucleosil (100-5) 250x4.6 column was used and the mobile phase consisted of 7.5 % acetonitrile in 50 mM citrate buffer pH 2.4, the flow was 1.25 ml/min. Products were monitored at 275 and 360 nm. Furthermore hydroxylation in these systems was monitored on the same HPLC system, but the flow was 1 ml/min and a gradient was used: 0-5 min 0-10 % acetonitrile, 5-15 min 10 % acetonitrile in 50 mM citrate buffer pH 2.5. An authentic sample of 3,3'-dityrosine was synthesized according to [204].
3 MATERIALS AND METHODS

3.2 Scavengers for Peroxynitrite

3.2.1 Materials

- Photometer: An Aminco DW-2 dual beam spectral photometer equipped with a magnetic stirrer and connected to a computer.

- HPLC-systems:
  
  I) Jasco components: PU-980 pump, UV-975 fast scanning detector, LG-980-02 low pressure mixer. Solvent degaser unit SDU 2003 from Labsource and helium pressure. This system was connected to a computer with the Borwin-HPLC-software.


- Chemicals were purchased from Merck, Sigma, Lancaster or Aldrich and were from the highest purity available. The dimethyl-uric acid derivatives and dithio-purine and -pyrimidine were purchased from Sigma, Germany. Ebselen was a kind gift from Prof. Wendel (Universität Konstanz). TEMPO and TEMPO were purchased from Alexis.

3.2.2 Inhibitors of Phenol Nitration

5 mM phenol and 800 μM PN were incubated in 0.5 M K-phosphate buffer pH 7 at 25 °C. The formation of 2- and 4-nitro-phenol was followed photometrically at 405 nm (after addition of 20 μl saturated NaOH to 980 μl reaction solution) or by separation of 2- (t_R=28 min) and 4-nitrophenol (t_R=16 min) by HPLC (system II, 1 ml/min, 30 % acetonitril and 75 % 0.1 M citrate buffer pH 2, Bischoff C_{18}-Nucleosil-100-5 250x4.6, detection at 280 nm). For the determination of the IC_{50}-values, the scavengers were added before addition of PN by vortex-mixing and IC_{50}-values were the concentrations of scavengers, where the 405 nm absorbance or nitro-phenol peak area reached the half amount compared to the reaction without scavengers.

In another experiment 5 mM phenol were incubated either with 655 μM PN in 0.2 M K-phosphate buffer pH 6 (1 min) or with 400 μM PN at pH 9 (10 min) and 37 °C. At pH 6 the formation of 2- (t_R=15.6 min) and 4-nitro-phenol (t_R=12.8 min) was monitored by HPLC (system I, 1 ml/min, 10 % acetonitril and 90 % 0.05 M citrate buffer pH 2, Macherey Nagel C_{4}-Nucleosil-300-5 250x4.6,
detection at 280 nm). At pH 9 the formation of 4-nitroso-phenol ($t_R=11$ min) was either followed photometrically at 395 nm or by HPLC (system I, 1 ml/min, 10 % acetonitril and 90 % 0.05 M citrate buffer pH 2, Macherey Nagel C_{18}-Nucleosil-100-5 250x4.6, detection at 300 nm). Once more the IC$_{50}$-values were estimated as described above.

### 3.2.3 Inhibitors of Microperoxidase-catalyzed Phenol Nitration

The same method as in the phenol system at pH 6 was used, but with 5 µM microperoxidase (MP-11) (a porphyrin with 11 amino acids originating from the degradation of cytochrome c). MP-11 like other heme containing enzymes and iron-porphyrins leads to a strong increase in nitration of phenol. No IC$_{50}$-values were estimated, but the effect of 100 µM scavenger on nitration was monitored using HPLC.

### 3.2.4 Inhibitors for the Inactivation of ADH by PN

The activity of 26 nM ADH with 300 µM NAD$^+$ for the conversion of 172 mM ethanol to acetaldehyde in 0.1 M K-phosphate buffer pH 7.6 was followed spectrosopically at 37 °C. The formation of NADH at 340 nm was measured time dependently and the basic specific activity was calculated from the slope of the linear area (first 25 sec) and was found to be $0.044\pm0.007$ U/mg. In a next step the amount of PN was estimated that led to a complete inhibition of the enzyme. In this case the ethanol and NAD$^+$ were added after complete reaction of ADH with PN (5 min) and the activity was measured. 20 µM PN quantitatively inhibited the enzyme, therefore this value was used for the following experiments. In a following step we determined the IC$_{50}$-values of several PN-scavengers in this systems, by addition of different concentrations to the enzyme, prior to addition of PN by vortexing carefully. Subsequently the activity was determined. The IC$_{50}$-value given for the scavenger corresponded to the concentration, that inhibited 50 % of the ADH-activity.

### 3.2.5 Kinetics of PN-decomposition

Decay of PN was followed by UV/Vis spectroscopy at 302 nm and the reference wavelength at 400 nm. The samples were measured in a special cuvette which was equipped with a magnetic stirrer. Because of inadequate mixing during the first 2-3 sec the decomposition curves were only compared qualitatively and no kinetic constants could be obtained from these measurements. 400-800 µM PN were injected into a stirred pH 7, 8 or 9 buffer solution which contained the
scavenger in different concentrations. The velocity of decay was then compared with the one in controls.

### 3.2.6 Investigation of the Reaction of Uric Acid with PN

In a first attempt we chose dimethyl analogues of UA, because they were easier to handle on HPLC and 13C-NMR-data should be easier to interpret, due to the methyl signals. 2 mM 1,3-dimethyl-uric acid were incubated with 8 mM PN in 1 M K-phosphate buffer pH 7. The reaction solution (1 ml) was injected on system II equipped with a semipreparative column (4 ml/min, A: 0.2 M KCl/HCl buffer B: 2-propanol, gradient: 0-10 min 100 % A, 10-23 min linear to 35 % B, 23-27 min linear to 0 % B, Merck C18-LiChrosorb-7 250x10, detection at 210 nm). The products were isolated from 20 runs, each of them concentrated and purified once more with the same HPLC-system, but water as the mobile phase. This time the isolated peaks were evaporated to dryness and dissolved in D6-DMSO. From this solutions 1H- and 13C-NMR (also one DEPT) spectra were made on a Bruker NMR DRX 600, the frequency for 1H was 600MHz, for 13C 150 MHz. Also EI-MS and FAB-MS spectra were made on a Varian MAT 312 respectively Varian MAT 312/amd 5000 (EI: 3 kV / 70 eV; FAB: primary ions (133Cs) 10 kV, secondary ions 6 kV).

In a second attempt 0.5-1 mM uric acid were incubated with 250-2000 µM PN in 0.2 M K-phosphate buffer pH 6-9 (1-10 min). 20 µl of the reaction solutions were injected on system I (0.7 ml/min, 0.2 M K-phosphate buffer pH 4.5, two Macherey Nagel C18-Nucleosil-100-5 250x4.6 columns connected in series, detection at 220 nm). The HPLC system was modified from the description in [205]. From each product a UV spectrum was taken and the already described oxidation products of uric acid (allantoin, allantoic acid, alloxanthin, oxonic acid, cyanuric acid and alloxan) were used as external and internal standards [206, 207]. Some reaction solutions were additionally incubated with uricase, to see whether some peaks dissapeared, which could mean that the basic structure was recognized by uricase. Allantoin and allantoic acid were estimated by hydrolysis (0.12 M NaOH) of allantoin to allantoate, further hydrolysis to glyoxylic acid and its coupling to 2,4-dinitro-phenyl-hydrazine and identification of the coupling product on HPLC as described [208]. Urea was quantified by the method of urea degradation to ammonia by urease and the colorimetric measurement of the ammonia-pH-shift with phthalein purple (o-cresol-phthalein complexone) [209] or by the o-phthalaldialdehyde method [210]. Reagent A consisted of 30 mM Tris, 5 mM
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complexone, 10 mM edta, 30.8 mM sodium azide and the pH was adjusted to 7.4. Reagent B consisted of 150 mM sodium chloride and 20 U/ml urease (Sigma, U1500) in water. For assays 250 µl sample were mixed with 125 µl reagent A and/or 125 µl reagent B respectively in assays without urease, the amount of reagent B was replaced by equal amounts of 150 mM NaCl-solution.

3.2.7 Reaction of Ebselen with Peroxynitrite

3.2.7.1 Materials Two HPLC systems were used: I) Jasco PU-980 pump, UV-975 variable-wavelength detector and LG-980-02 low-pressure gradient mixer. II) LKB 2150 pump, Spectra Physics spectra focus detector and SP4290 integrator. In both systems solvents were gassed with helium. Kinetics were recorded on an Aminco DW-2 UV/Vis spectrophotometer in the dual-wavelength mode, equipped with a magnetic stirrer.

3.2.7.2 Separation of ebselen, GSH-ebselen and ebselenoxide System I was used with a C4-Nucleosil-300-5 column 250x4.6 (Macherey-Nagel, Düren, Germany), the mobile phase consisted of 80 % 0.1 M K-phosphate buffer pH 4.5 and 20 % acetonitrile. The flow was 1.2 ml/min and the products were detected at 270 nm. Spectra were taken from each peak. Solutions of 500 µM ebselen (from a 10 mM stock solution of ebselen in acetonitrile) in 0.1 M K-phosphate buffer at pH 7 and 9 were treated with different concentrations of GSH (0.05-5 mM) and were then reacted with 500 µM PN. 50 µl of each sample were injected on the HPLC.

3.2.7.3 Determination of free ebselen concentrations in bovine aortic microsomes and tissue-containing solutions System II was used with a C4-Nucleosil-300-5 column 250x4.6 (Macherey-Nagel, Düren, Germany), the mobile phase consisted of 80 % 0.1 M K-phosphate buffer pH 4.5 and 20 % acetonitril for the tissue-samples (1) and 75 % water and 25 % acetonitril with 0.1 % added trifluoroacetic acid in the microsomal experiments (2). The flow was 1.2 ml/min and the products were detected at 270 nm. (1) 4.3 µM ebselen were incubated with 0.134 g dry weight (first sample) and 0.077 g (second sample) of bovine coronary tissue in 10 ml PBS-buffer and aliquots of 50 µl were taken after 1, 15, 30 and 35 min and injected on HPLC. Ebselen and GSH-ebselen were identified by external standards. (2) 500 µl bovine aortic microsomes (1 mg/ml protein) were incubated with 500 µl 1 mM DTNB for 10 min in 0.2 M K-phosphate buffer pH 7.5. The solution turned yellow, indicating the reaction of DTNB with free thiol-groups. After centrifugation the supernatant was removed and 1 ml
fresh K-phosphate buffer was added. The sample was vortexed and then 20 or 50 µM ebselen were added and incubated for 5 min. 50 µl of this solution were injected on HPLC. The same procedure was performed for the non-DTNB-treated microsomes. Concentrations of ebselen were quantified by external standards.

### 3.2.7.4 Protective effects of ebselen, GSH and ebselen-GSH on phenol- and BSA-nitration

Solutions of 5 mM phenol in 0.1 M K-phosphate buffer at pH 7 or 9 were supplemented with ebselen, GSH or both and were reacted with 1 mM PN. The yield of nitro-phenols (pH 7) or 4-nitroso-phenol (pH 9) was determined from the absorbance at 400 nm (after addition of NaOH(aq)). PN- and ebselen decomposition kinetics were recorded at 320 against 370 nm. At this wavelength PN as well as ebselen absorb, but not ebselenoxide and the ebselen-GSH-adduct has a very low absorbance. PN was always added last to the stirred solution in the cuvette by a syringe through a septum. The decomposition of 100 µM PN and 50 µM ebselen with 0, 20 or 40 µM BSA in 0.2M K-phosphate buffer at pH 10 was recorded and also the decomposition of 250 or 500 µM PN and 200 µM ebselen with 0, 200 and 400 µM GSH in 0.2 M K-phosphate buffer pH 8, 9 and 10.

### 3.3 Metal-catalyzed Reactions of PN

#### 3.3.1 Decomposition Kinetics of PN

The decay of PN was followed by the absorbance difference at 302 and 350 nm. The metal-complex was preincubated in 0.1 M K-phosphate buffer at pH 8 or 9 and 10 °C. 200-600 µM PN were injected into the stirred solution with a syringe. The spectrophotometer was equipped with a magnetic stirrer and the conditions were analogous to those described in 3.2.5. Dioxygen formation in solutions of PN and heme or heme-thiolate enzymes were measured polarografically as described in 3.1.3 [130]. Nitrite and nitrate were measured according to 3.1.5 and NO$_2^-$ additionally by the GRIESS test [194]. The latter was also tried for the determination of nitrate, which was first reduced by nitrate reductase [194], but this method failed in our experiments.

#### 3.3.2 Metal-catalyzed Nitration of Phenol

5 mM phenol were preincubated with the 0.5-20 µM of the metal complexes or enzymes in 0.1 M KP$_i$ pH 6-8 at 37 °C. Then 400-800 µM PN were added in 0.1 M NaOH (not more than 5 vv% of the reaction solution to avoid a pH-shift) by Vortex-mixing. The yield of nitrated products was either measured
qualitatively by their absorbance at 350 nm at neutral and 420 nm at alkaline pH or quantitatively together with hydroxylated and dimerized products by using HPLC as described in 3.1.6 [130, 163]. To see whether an effect was really due to enzyme-catalysis controls were done with heat-denatured enzymes. In these controls the enzymes were preincubated in buffer, then heated for 10 min at 95 °C, allowed to cool down. Next phenol was added and at last mixed with PN. In some cases also 10 vv% of acetonitril were added to enhance the denaturation of the enzymes.

### 3.3.3 Spectral Intermediates During Reaction of PN with Transition-metals

Stopped-flow measurements were done in collaboration with Dr. Herold at ETH Zürich according to [111, 163]. Measurements were carried out with an On-Line Instrument Systems, Inc. stopped-flow instrument equipped with an OLIS RSM 1000 rapid scanning monochromator (cell-width 2 cm). The temperature was maintained at 12 °C and the pH was measured at the end of the reactions for control. Data were taken every millisecond and the spectra which are shown were averaged (62/s). NOR solutions at 4-20 μM in 0.1 M phosphate buffer pH 5-8, were reacted with an excess of PN, 66-800 μM in an equal volume of 10 mM NaOH. The final concentrations were 2-10 μM NOR and 33-400 μM PN in 0.05 M KP, at pH 5.9-8.5. Further stopped-flow measurements were done together with Prof. Dr. Ghisla at Universität Konstanz. This instrument consisted of an J&M diode array detector and the setup for the other parts was developed in the group of Prof. Dr. Ghisla [211]. The stopped-flow data was interpreted by using the Spectacle software of Labcontrol.

All other spectra were recorded on an Aminco photometer. The HRP-ferryl and -NO spectra were obtained by difference absorbance spectroscopy. 5 μM HRP were incubated with either 400 μM SIN-1 or 25 μM DENO in 0.1 M KP, pH 7.4 at 37 °C. After some minutes of incubation 200 μM ascorbate were added to both solutions.

### 3.4 Reactions of PN with P450\textsubscript{BM−3}

#### 3.4.1 Materials

P450\textsubscript{BM−3} (CYP 102) from Bacillus megaterium [182, 212] (this enzyme was a gift of J.A. Peterson, USA) and P450\textsubscript{NOR} (CYP 55AI) from Fusarium oxysporum [213] (this enzyme was a gift of H. Shoun, Japan) were purified as described.
Chloroperoxidase (EC 1.11.1.10) from *Calderomyces fumago*, horseradish peroxidase (EC 1.11.1.7) (lyophilized powder) xanthine oxidase (XO) (EC 1.13.22) (grade II) from buttermilk, Cu,Zn-SOD (EC 1.15.1.1) from bovine erythrocytes, Mn-SOD (EC 1.15.1.1) from E.coli, Uricase (type V) from porcine liver and the protease inhibitor cocktail (P8465 and P2714) were purchased from Sigma (Steinheim, Germany). Spermine NONOate (N-[4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl]) and diethylamine NONOate (Ethanamine, N-ethyl, compound with 1,1-diethyl-2-hydroxy-2-nitrosohydrazine (1 : 1)) were purchased from Cayman Chemical Company (Ann Arbor, USA). SIN-1 hydrochloride (3-morpholino sydnonimine) was from Calbiochem-Novabiochem Corporation (La Jolla, CA, USA) 12-(p-nitrophenoxy)-dodecanoic acid was a kind gift from Dr. U. Schwaneberg (University of Stuttgart) [214]. All other chemicals were purchased in the highest purity available. Peroxynitrite was synthesized according to the method described by Kissner and Koppenol [9] from ·NO and potassium superoxide and purified from residual H2O2 with MnO2. 3,3'-dityrosine (3,3'-DT) was synthesized by incubation of tyrosine with HRP and H2O2 as previously described [204].

3.4.2 Nitration of Free Tyrosine by Bolus Addition and Simultaneously Generated PN

1 mM tyrosine and 10-100 µM PN in 0.1 M K-phosphate buffer pH 7.4 were vortexed at 37 °C, incubated for 5 min and assayed for the product pattern. For the following experiments the same conditions as described previously [22] were employed: 1 mM tyrosine was incubated with 1 or 2 mM spermine NONOate for 16 h at pH 7.4 and aliquotes were taken after 20, 40, 60, 120, 240, 480 and 960 min and directly injected in the HPLC. The same procedure was used for 1 mM tyrosine incubated with 1 mM spermine NONOate and 28 or 56 mU XO in buffer pH 7.4, containing 1 mM hypoxanthine. Products were identified and quantified by using a HPLC system from Jasco (PU-980, UV-1575 and LG-980-02) and a Nucleosil 100-5 C18-column (250 x 4.6 mm) from Macherey & Nagel (Düren, Germany). The mobile phase consisted of 7.5 % acetonitrile and 92.5 % 50 mM citrate buffer pH 2.4 (1 ml/min) and UV/Vis detection at 275 as well as 360/428 nm. The detection limit for a 3-NT standard was 40 nM for 50 µl injection volume. Typical retention times were 3.6 min for tyrosine, 6.7 min for 3-NT and 7.5 min for 3,3'-DT.
3.4.3 Nitration of P450 \(_{\text{BM}−3}\) WT and F87Y Variant by Bolus Addition and Simultaneously Generated PN

The nitration of P450\(_{\text{BM}−3}\) WT, F87Y and CPO was monitored by Western blot analysis, using a rabbit polyclonal nitrotyrosine antibody (K552) made in our laboratory according to the method of Beckman et al [215]. Proteins were separated by SDS-PAGE on a 10 % acrylamide gel, 1 % SDS, 1 % APS and 0.05 % TEMED in 375 mM Tris pH 8.8. The accumulation gel consisted of 4 % acrylamide, 1 % SDS, 0.5 % APS and 0.1 % TEMED in 125 mM Tris pH 6.8. 5 \(\mu\)l of each sample in Laemmli buffer were put on the gel, the electrolyte consisted of 25 mM Tris, 192 mM glycine and 5 mM SDS at a current of 35 mA for 1 h. Proteins were transferred by a semi-dry blot procedure to a nitrocellulose membrane with a current of 0.8 mA/cm\(^2\) for 90 min in 48 mM Tris, 39 mM glycine, 0.07 % SDS and 20 % methanol. Proteins were controlled by Ponceau S staining on the membrane. The membrane was blocked with 5 % milkpowder in PBS/T (with 1 % Tween 20) for 2 h at RT. Then the membrane was incubated for 90 min at RT in 15 ml \(\alpha\)-NT 1:2000 in PBS/T with 0.05 % milkpowder, washed three times in PBS, three times in PBS/T and another three times in PBS. In the next step the membrane was incubated for 45 min at RT in 15 ml goat-a-rabbit (1:10000 dil.) in PBS/T with 0.05 % milkpowder. Then the 3 washing steps were repeated and the blot was developed by ECL. In standard reactions 5 \(\mu\)M of the enzyme were prepared in 0.1 M phosphate buffer pH 7.4 and rapidly mixed with 2.5-5 \(\mu\)l of PN in 0.1 M NaOH giving a final volume of 100 \(\mu\)l and the desired final concentration of PN. Other enzymes such as HRP or SOD (150-200 U/100 \(\mu\)l reaction solution) (1-2 \(\mu\)l of an aqueous stock solution) were added to the buffer together with P450\(_{\text{BM}−3}\), the starting volume of the buffer was adjusted to give 100 \(\mu\)l in all reactions. The buffer for XO assays contained 500 \(\mu\)M hypoxanthine. To avoid the accumulation of the known PN-scavenger uric acid, uricase was added in some experiments, but it turned out that contaminations in the employed uricase decreased the yield of nitration. 2.8, 5.6 or 28 mU/ml XO (1-2 \(\mu\)l of a stock solution in reaction buffer), 0.1 or 1 mM spermine NONOate (1-2 \(\mu\)l of a stock solution in 0.1 M NaOH) and 0.1 or 1 mM SIN-1 (1-2 \(\mu\)l of a freshly prepared stock solution in water) were added directly to the reaction buffer and incubated for at least 10 h. PN scavengers (500 \(\mu\)M) were added together with P450\(_{\text{BM}−3}\) before mixing with 50 \(\mu\)M PN. Protease inhibitors were prepared as described in the product information from Sigma and were solved in 1 ml DMSO / 4 ml water (for bacterial cell extracts) and 100 ml water (for general use). 2.5 \(\mu\)l of these stock solutions were added
where indicated (100 µl final volume).

Another PN-generating system consisted of pyrogallol, which was described to generate superoxide during its autooxidation [51] and diethyl NONOate, but the nitration results were not reproducible probably due to the PN-scavenging effect of pyrogallol [216].

### 3.4.4 Inhibition of PN-mediated Nitration of Phenol and P450BM−3 by Palmitate

Palmitate was prepared as a 20 mM stock solution in acetonitril, this allowed additions of small volumes, since acetonitril has a slightly increasing effect on nitration by PN [130]. The palmitate only was dissolved after sonication and fell out, when the solution cooled down. Over all experiments the volume of acetonitril was kept the same. 1-1.5 µM BM3-WT and F87Y variant were preincubated with 10-200 µM palmitate in 0.1 M K-phosphate buffer pH 7.4 at RT. In cases were the effect on phenol nitration was measured, phenol was present at 500 µM. PN was added by Vortex-mixing from a stock solution in 0.1 M NaOH. Nitration of phenol was monitored by UV/Vis spectroscopy (Aminco photometer) at 400 nm after addition of 25 µl 1 M NaOH to 250 µl reaction solution. Nitration of BM3-WT and F87Y variant was measured by the already described Western blot technique, but this time an anti-NT was used from Upstate Biotechnology Incorporated (Hamburg, Germany). A secondary antibody (goat anti-mouse IgG) was obtained from Pierce (Rockford, Illinois, USA).

### 3.4.5 Localisation of Nitrated Tyrosines in P450BM−3 WT and F87Y

From each sample of the Western blots aliquots were directly taken for HPLC analysis: 20 % acetonitrile were added to the sample, then heated for 10 min at 95 °C. After denaturation the pH was adjusted to 8, the pH-optimum for trypsin, which was added 1:10 based on the concentration of P450BM−3 and incubated for 5-10 h. The degradation was checked by SDS-gel electrophoresis and Coomassie staining. 50 µl of the degraded sample were injected on the Jasco HPLC system, by using a Nucleosil 100-5 C18-column (250 x 4.6) from Macherey & Nagel (Düren, Germany) and a gradient of 0-75 % B in 40 min (A: 10 % acetonitrile and 0.1 % TFA in water, B: 80 % acetonitrile and 0.08 % TFA in water) (1 ml/min). After it had passed the first detector the mobile phase was made alkaline by mixing with 0.5 ml/min 50 mM borate buffer pH 10 from a second pump. This step allowed simultaneous detection of nitrotyrosinate at 428 nm by a second detector. A list
of the theoretically obtained peptide fragments and the corresponding masses were calculated by using the computer program ExPASy-PeptideMass.

### 3.4.6 Electrospray Ionization (ESI) Mass Spectrometry

Mass spectra were obtained with an AUTOSPEC-Q tandem hybrid mass spectrometer (VG Analytical, Manchester, England), equipped with the Mark IV ESI source (Micromass, Manchester, England) and an OPUS data system. Samples were diluted with acetic acid to a final concentration of 50 % acetic acid and injected onto a column (10 x 1 mm) of polymeric beads with 4000 Å pores (Michrom BioResources, Auburn, CA) equilibrated with 0.1 % formic acid. The trapped peptides were desalted by elution with 0.1 % formic acid. Subsequently, the peptides were eluted directly into the ESI source with 0.1 % formic acid/90 % methanol (v/v) at a flow of 8 µl/min through a 130-µm ID stainless steel needle and nebulized with a coaxial gas flow of nitrogen at 10 l/h.

### 3.4.7 Measurements of P450BM−3 Reductase Activity and Determination of Thiol-groups within the Enzyme

The reductase activity of 0.01 µM P450BM−3 was measured by the known NADPH-dependent reduction of 10 µM cytochrome c [217] in 0.1 M potassium phosphate buffer pH 7.4 at 37 °C. Reduced cytochrome c was followed by time-dependent measurement of the absorbance difference between 550 and 560 nm. The inactivation of the reductase-domain of 0.01 µM P450BM−3 by PN was determined by addition of 0-20 µM PN to the enzyme and, after 5 min, addition of 10 µM cytochrome c and 25 µM NADPH to start the reduction.

Thiol-groups were determined spectroscopically by addition of 2,2’-dithiobipyridine (DTBP) (Sigma, Germany), which forms mixed disulfides with free SH-groups within the enzyme. For each SH-group one equivalent of free mercaptopyridine is formed, which has a high absorption at 343 nm (ε343=7600 M\(^{-1}\)cm\(^{-1}\) [218]). The mechanism is similar to that one of Ellmans reagent (dithio-bis-(nitro-benzoic acid), DTNB) and although the extinction coefficient of DTBP is half as high compared to DTNB, it has other advantages. DTBP shows a high pH stability and does not easily undergo self hydrolysis. 5 µM enzyme in 0.1 M K-phosphate buffer pH 7.4 were either treated or not treated with 50 µM PN. Next 50 µM DTBP (2 µl in acetonitril) were added from a 5 mM stock solution in acetonitril. Kinetics were measured at 343 and 400 nm as a wavelength pair for 5 min. The DTBP dimer only showed marginal absorption when employed at
this concentration.

### 3.4.8 Monooxygenase Activity Assay for P450\textsubscript{BM−3} WT

The method described by Schwaneberg et al [214], was used with 12-(p-nitrophenoxy)-dodecanoic acid as a substrate. This test is based on the release of p-nitro-phenolate from an unstable hemiacetal formed by ω-hydroxylation of the fatty acid. 0.1 µM P450\textsubscript{BM−3} WT were incubated with 90 µM substrate in 0.1 M potassium phosphate buffer pH 8.2 at 37 °C. The reaction was started by addition of 200 µM NADPH. The release of p-nitro-phenolate was monitored at 410 nm on an Aminco DW-2 UV/Vis spectrophotometer equipped with a magnetic stirrer. For inhibition experiments 0.1 µM enzyme were mixed with different amounts of PN (2.5-5 µl in 0.1 M NaOH) to give a final volume of 500 µl. Substrate was added after this first step. Scavengers were added first followed by PN.

### 3.4.9 Stopped-flow Measurements

Measurements were carried out with an On-Line Instrument Systems, Inc. stopped-flow instrument equipped with an OLIS RSM 1000 rapid scanning monochromator (cell-width 2 cm). The temperature was maintained at 12 °C and the pH was measured at the end of the reactions for control. Enzyme solutions, 1-10 µM in 0.1 M phosphate buffer, were reacted with an excess of PN, 33-800 µM in 10 mM NaOH. Alternatively, m-chloro-peroxy-benzoic acid, H\textsubscript{2}O\textsubscript{2} and persulfate in H\textsubscript{2}O/phosphate buffer were used as oxidizing agents.

### 3.4.10 Structure of P450\textsubscript{BM−3}

The structure shown in Fig. 8 was obtained from the corresponding protein database file (1bu7.pdb) published by Sevrioukova et al (1998) and was rendered with the computer programs Raster 3D [219] and Molscript [220]. The calculations of the distances between heme-iron and OH-groups of tyrosines or 4’-carbon of F87 were done by using Rasmol [221] and Molmol [222].

### 3.5 Reactions of P450\textsubscript{CAM} with PN

#### 3.5.1 Materials

P450\textsubscript{CAM} from *Pseudomonas putida* (E.C. 1.14.14.1) was expressed in *Escherichia coli* strain TB1 and purified as described [223, 224] (this enzyme was a gift from Dr. C. Jung, Max-Delbrück-Centrum, Berlin, Germany). Ethanolic camphor was added to a 50 µM solution of P450\textsubscript{CAM}, resulting in final
concentrations of 500 µM camphor and 132 mM ethanol (0.8 v%). Metyrapone (1,2-di-(3-pyridyl)-2-methyl-1-propanon) was purchased from Roth, Germany. PN was synthesized as described in 3.1.2.

A monoclonal antibody against 3-nitrotyrosine (anti-NT, clone 1A6) was procured from Upstate Biotechnology Incorporated (Hamburg, Germany). The antibody concentration in the stock solution was 2 µg / ml. Secondary antibody (goat anti-mouse IgG) was obtained from Pierce (Rockford, Illinois, USA). The stock solution was 0.8 mg / ml. The enhanced chemiluminescence (ECL) kit and nitrocellulose transfer membrane (Hybond C, pore size 0.5 µm) were purchased from Amersham (Braunschweig, Germany).

3.5.2 P450CAM-catalyzed Nitration of Phenol and Tyrosine Residues by PN

5 mM phenol were reacted with 800 µM PN in 0.1 M KP, at pH 7 and 7.5 in the presence of different heme and heme-thiolate enzymes (1-2 µM). The products were identified and quantified as described in 3.1.6.

2.5 µM CAM was treated with 0-500 µM PN in 0.1 M KP, pH 7 or 7.5 in the presence or absence of 0.5 mM camphor or 50 µM metyrapone. 10 % acetonitrile were added to the ”nitrated” enzyme solution, which was then kept for 5 min at 95 °C. After denaturation 250 nM trypsin were added and the mixture was incubated for 10-18 h. 50 µl of the peptide mixture were analyzed by using HPLC. All conditions were the same as described in 3.5.4 except the gradient which was used here: 0-40 min 0-75 % B (A: 5 % acetonitrile and 0.1 % TFA in water, B: 80 % acetonitrile and 0.1 % TFA in water). Identification and localization of the nitrated tyrosine residues was done according to 3.5.5. 10 µl of the HPLC samples were used for Western blot analysis. α-NT Western blots were made according to 3.5.3.

Protein samples were treated with Laemmli sample buffer, kept at 95 °C for 5 min and then separated by 7.5 % (v/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis with an amount of 2 µg protein per well, with a constant current of 30 mA for 50 min. The size of the separation gel was 8.2 x 7.5 cm². Next proteins were transferred from the gel to a nitrocellulose membrane in a semi-dry blot procedure with a constant current of 50 mA for 90 minutes. The blot buffer consisted of 48 mM Tris / 39 mM glycine / 20 % (v/v) methanol / 0.07
% (w/v) SDS. Proteins were visualized with 0.1 % Ponceau S in 5 % (v/v) acetic acid to check efficiency of the transfer. After destaining with phosphate-buffered saline (PBS), the membrane was blocked with 5 % (w/v) milk powder in PBS (pH 7.4) for 2 hours at room temperature and after several washing steps with PBS and PBS containing 0.1 % Tween-20 it was incubated with a monoclonal antibody against nitrotyrosine at a dilution of 1:1000 overnight at 4 °C. After further washing, the membrane was incubated with a goat anti-mouse antibody for 45 minutes at a dilution of 1:7500. Finally antibody binding was detected by the ECL technique according to the instructions of the merchant.

3.5.3 Camphor Binding Spectra of P450 CAM after Treatment with PN

2.5 µM camphor-free CAM were titrated with an ethanolic aqueous solution of camphor in 0.1 M KP, pH 7.5 at 10 °C and the absorbance shift from 417 nm (camphor-free form) to 393 nm (camphor-bound form) was followed by UV/Vis spectroscopy. After each addition of camphor (1, 2, 3, 13, 113 and 1113 µM total concentration) a spectrum was recorded. This procedure was repeated for CAM which was treated with 50 and 250 µM PN.

3.5.4 CO-binding Spectra of Reduced P450 Enzymes after Treatment with PN

2.1 µM CAM (substrate-free, 250 µl) were mixed with 250 µM CO (50 µl of a 1.56 mM, saturated CO solution in water at 0 °C [195]), then 5 µl of a saturated sodium dithionite solution were added to the total volume of 300 µl. The same procedure was done for CAM after treatment with 50 and 250 µM PN. Also BM3 and NOR were examined by this method.

3.5.5 Decomposition Kinetics of PN in Presence of P450 CAM, P450 NOR and Microperoxidase and Stopped-flow Analysis of the Reaction of P450 CAM with PN

PN-decomposition kinetics were followed according to 3.4.1. Stopped-flow experiments were done together with Prof. GHISLA at Universität Konstanz as described in 3.4.3.
4 Results

4.1 Reactions of Peroxynitrite with Phenolic Compounds

4.1.1 Short Introduction

Our group became involved in the chemistry of PN by the observation that the heme-thiolate enzyme prostacyclin synthase became inactivated by submicromolar concentrations of PN and a nitration of a tyrosine residue at the active site was likely to be involved [10, 11]. Current work could establish that this reaction has a major impact on cardiovascular pathophysiology (Zou et al [167, 169]) and therefore more detailed studies were undertaken to elucidate the mechanism of tyrosine nitration. Phenol can be used as a convenient model and several reports have been dealing with this compound in order to characterize the reaction pathways of PN [62, 128]. As a result from a number of investigations a complex behavior of PN emerged which was dominated by the pH-value employed [62, 56, 61]. PN is quite stable as its anion and only few compounds can react directly by oxene transfer or a so-called two-electron oxidation pathway. Seleno compounds [85, 225], thioethers [84, 139] or transition metal-complexes [104, 174] are among such acceptors. At neutral pH with a pKₐ of 6.8 PN is rapidly protonated and undergoes a transition from the cis-form to the trans-form of peroxynitrous acid [56, 59]. This species at room temperature is supposed to reach a vibronically excited state or a triplet state with properties of a hydroxylradical-nitrogen dioxide radical complex [HO⋅-NO₂] [56, 66]. This highly reactive intermediate eventually would isomerize to nitrate, but during its lifetime can react with organic molecules by one-electron oxidations [75, 82, 89] by hydroxylation [66, 62], nitration or by generation of hydroxy-nitrito adducts [226]. It also was reported that above pH 7 dioxygen is formed together with two molecules of nitrite [9, 72, 61] which can be envisaged as a disproportionation at high concentrations of PN. In the present study we use phenol as a model for tyrosine nitration and follow the product pattern over the whole pH range. By comparison with anisol as a substrate we show that for nitration by PN but not for hydroxylation the phenoxylradical must be considered an obligatory intermediate.

4.1.2 The Contribution of Nitrite

PN in aqueous solutions undergoes a series of unusual transitions accompanied by the generation of reactive intermediates which interact with each other so that a complex pattern of strongly pH-dependent reactions will be a consequence. Care has to be taken that nitrite as a contaminant and a secondary product
at higher pH-values does not lead to PN unrelated products. We therefore first determined the content of nitrite in our preparation and established its contribution to reactions with phenol over the whole pH-range [227]. As shown in Fig.13 there is a significant formation of 4-nitroso-phenol starting at about pH 5 towards the acidic range. The same pH-dependence but with lower yields one sees for 2- and 4-nitro-phenol formation and a similar one could be observed for the NO-release from nitrite solutions (see Fig.12).

4.1.3 The Disproportionation of Peroxynitrite

It has been reported that PN can undergo isomerization to nitrate at acidic pH-values [56] but above pH 7 the release of dioxygen with a stoichiometric formation of two moles of nitrite can be observed [61]. Since this reaction may depend on buffer composition and on PN concentration the generation of dioxygen was also followed polarographically under our defined incubation conditions with 800 µM PN as a function of pH (Fig.14).

Being low at pH 7 the release of dioxygen increased until a maximum of about 120 µM at pH 9 in 0.2 M K-phosphate buffer (Fig.14). The kinetics of the release are relatively fast at pH 8 and below and at pH 11 become too slow to reach completion in an observable time scale (Fig.15). Below pH 7...
the disproportionation pathway was virtually absent and was replaced by the isomerization as measured by an increasing amount of nitrate (determined by ion chromatography with UV/Vis detection. See Fig. 16).

4.1.4 The Reaction of Phenol with Peroxynitrite

In the presence of 5 mM phenol at pH 5 hydroxylated, nitrated and nitrosylated metabolites were detected as illustrated by the chromatograms shown in Fig. 17, 18.

As could be expected for such oxidizing conditions the primary hydroxylation products catechol and hydroquinone were partly oxidized to their quinones, from which p-benzoquinone was clearly identified. Another unstable compound

Figure 14: pH-dependent oxygen-release of 800 µM peroxynitrite in 0.2 M K-phosphate buffer at 310 K. Measured with a Clark-type-electrode (Eschweiler, Kiel) and \( E = +0.8 \) V for oxygen detection. At pH 10 the reaction was completed after 20 min. Representative values from a series of measurements with different PN concentrations.

Figure 15: Time-dependent oxygen-release from 800 µM PN in 0.2 M K-phosphate at 310 K and different pH. (line) pH 8, (bold line) pH 9, (broken line) pH 10, (bold broken line) pH 11. Typical experiment out of five.

Figure 16: Formation of nitrite and nitrate from 655 µM PN in dependency of pH. Solid squares for total amount of nitrite and nitrate, open squares for the ratio, solid circles for nitrite and open circles for nitrate. Determined by ion chromatography (UV-detection, Anion-II-column).
Figure 17: Chromatogram of the reaction mixture of 800 µM PN and 5 mM phenol at pH 5 and 310 K (incubated for 5 min) on system I, detected at 265 nm.

Figure 18: Chromatogram of the reaction mixture of 800 µM PN and 5 mM phenol at pH 2 and 310 K (incubated for 5 min) on system VI, detected at 287 nm.

(1) 4-nitroso-phenol, p-benzoquinone and 2-hydroxy-phenol; (2) 4-nitro-catechol; (3) 2-nitroso-phenol; (4) 4-nitro-phenol; (5) 2-nitro-phenol; (6) 2,4'-biphenol.

could be identical with o-benzoquinone since the peak was also formed as the main product from catechol in the presence of ferric chloride. At acidic pH 2- and 4-nitro-phenol as well as 2- and 4-nitroso-phenol were present, together with 4-nitro-catechol and a minor peak (Fig.18) which co-chromatographed with 2,4'-biphenol in two solvent systems (verified by spiking the peak with an authentic sample) and showing identical UV-spectra. Yields of known products were in good agreement with previous studies [5, 62, 128], except 2-hydroxyphenol in the first one.

A quantitation of hydroxyphenols (Fig.19) as a function of pH resulted in an optimum at 4.5 and low activities above 8 or below 3. From 800 µM PN about 85 µM were converted to hydroxylated phenols under optimum conditions.

The formation of nitrated phenols, 2- and 4-nitro-phenol, showed two pH-optima at 6-7 and at 2 (Fig.20). Since 4-nitroso-phenol was also formed at pH-values below 4 it can be conceived that the acidic pH-optimum is due to nitrite contamination as shown in Fig.13. Unexpected was the high amount of 4-nitroso-phenol, contrary to previous observations [5, 128], formed between pH 7 and 12 with a maximum at pH 9-10 (Fig.20). Since in this range the disproportionation takes place it could be postulated that nitrosating species must be involved in this reaction. Although hardly visible at the scale of Fig.20 distinct amounts of 2- and 4-nitrophenol are also formed under alkaline conditions inferring nitrating
4 RESULTS

Figure 19: Product yield of 5 mM phenol and 800 µM PN in 0.2 M K-phosphate buffer at 310 K after 5 min (pH 1-8) and 10 min (pH 9-12). Longer incubation times at higher pH led to loss of nitroproducts. The maximum of 4-nitroso-phenol was at pH 10, when incubating for 1 hour at pH 9-12. Symbols mean: (A) (open circles) 4-hydroxy-phenol, (open triangles) p-benzoquinone, (solid circles) 2-hydroxy-phenol.

Figure 20: Yield of nitration and nitrosation products: (solid squares) 4-nitroso-phenol, (open squares) 4-nitro-phenol and (solid diamonds) 2-nitro-phenol. Nitroproducts were quantified on HPLC-system VI, hydroxy-phenols, 4-nitroso-phenol and benzoquinone on HPLC-system I according to the conditions mentioned in the method section. Arrow bars indicate mean values from three measurements.

species in the course of PN decomposition.

4.1.5 Dimerization of Phenolic Intermediates

We could confirm earlier results [128] that 4,4’- and 2,2’-biphenols are formed and additionally 2,4’-biphenol was identified. Their maximum formation occurred between pH 6 and 8 and between 10 and 12 with a distinct second maximum (Fig.21).

Figure 21: Formation of biphenols from 5 mM phenol and 800 µM PN in 0.2 M K-phosphate buffer at 310 K after 1 min (pH 1-6), 5 min (pH 7 and 8), 10 min (pH 9), 15 min (pH 10), 20 min (pH 11) and 35 min (pH 12). The incubation times shown are optimized for biphenol formation. Symbols mean: (open circles) 2,2’-biphenol [µM], (open squares) 2,4’-biphenol [relative area], (open triangles) 4,4’-biphenol [µM]. Quantitation and identification on HPLC-system V. Arrow bars indicate mean values from four measurements.

When investigating the dependency of phenol nitration on substrate con-
4 RESULTS

It was noticed that a steep increase occurred up to 1.25 mM phenol but then rather a decrease was seen at higher phenol concentrations. In the chromatograms the dimerization products increased as depicted for 2,2'- and 2,4'-biphenol in Fig. 23 using the relative area of the product peaks. The same tendency could be observed when working in alkaline solutions, where the 4-nitrosophenol decreased with increasing phenol concentration in favor of dimerization products (see Fig. 22).

![Figure 22: Formation of nitrophenols and 2,4'-biphenol from 800 µM PN and phenol after 5 min in 0.2 M K-phosphate buffer pH 7 in dependency of phenol concentration. (open squares) 2-nitro-phenol, (open circles) 2-nitro-phenol, (open triangles) 2,4'-biphenol and (solid circles) 2,2'-biphenol. Representative values out of two experiments.](image1)

![Figure 23: Formation of biphenols and 4-nitroso-phenol from 655 µM PN in dependence of phenol concentration at pH 9.](image2)

Although the quantitation of the sum of all biphenols could not be performed exactly, because of lack of an authentic sample, it appeared that at the given pH of 7 the biphenols compensated for the decrease in nitrophenols. In view of the nature of possible precursors it was of interest to look also for diphenylethers as potential dimerization products. Indeed, a compound which co-chromatographed with 4-hydroxy-diphenylether was noticed and also co-chromatographed with the authentic sample in a second solvent system. Another peak could be observed, which would fit to the retention time of 2-hydroxy-diphenylether, for which however no authentic reference compound was available.

4.1.6 The Reaction of Anisol with PN

The formation of dimerization products from phenolic compounds would be compatible with phenoxy radicals as intermediates which also would be in
accordance with the known one-electron oxidizing properties of PN [75, 82, 89]. Blocking of the phenolic hydroxyl group by methylation would prevent this one-electron oxidation but still would allow hydroxyl radical addition reactions to the aromatic ring. We therefore set up an analytical system for anisol nitration and hydroxylation products. 10 % acetonitrile as a solvent provided comparable conditions for phenol and anisol reactions with PN. In a test system with nitronium tetrafluoroborate as a known nitrating agent similar nitration yields were observed (44 and 45 µM 4- and 2-nitro-phenol and 12 and 36 µM 4- and 2-nitro-anisol). When using 800 µM PN at pH 4 anisol was converted to about 40 µM 2-hydroxy-anisol and 17 µM 4-hydroxy-anisol (see Fig.24). However, the corresponding nitration products were only about 1 % of the hydroxylated products (0.27 µM 2-nitro- and 0.11 µM 4-nitro-anisol). Further comparisons between phenol and anisol were performed with ·NO and ·NO$_2$ as reactive species. With ·NO only phenol gave the 4-nitroso-phenol derivative (0.67 µM) and smaller amounts of nitro-phenols (0.2 µM). Also with ·NO$_2$ only phenol formed nitro-phenols (7 µM) but no nitration of anisol could be detected (see Fig.25). However, it seemed that a strong 1e-oxidant such as Ce(IV) was reactive enough to abstract an electron from the anisol-ring. This species then could be nitrated by nitrogen dioxide.)

![Figure 24: Formation of hydroxy- and nitro-anisols from 800 µM PN and 5 mM anisol.](image1)

![Figure 25: Nitroproducts from reactions of 5 mM phenol or anisol with 163 µM nitric oxide or 4.5 mM nitrogen dioxide. Effect of 1 mM Ce(IV) on anisol nitration by 800 µM PN at pH 6.](image2)

4.1.7 Nitrosylation of Phenol and Anisol

As shown in Fig.20 the yield of 4-nitroso-phenol in the reaction of phenol with PN is quite high in the alkaline pH-range. 2-nitroso-phenol is known to be
unstable under these conditions and probably undergoes rearrangement of the NO-group, forming an O-nitroso-phenol. This nitrito-aryl is a nitrous acid ester and therefore should easily hydrolyze yielding nitrite and phenol. The maximum of nitrosylation (pH 9-10) went hand in hand with maxima of oxygen release and nitrite formation at this pH (see Fig. 14, 16). The nitrosylation itself showed a linear dependence on PN-concentration as shown in Fig. 27. Azide, a well known \( \text{N}_2\text{O}_3 \) scavenger decreased the yield of 4-nitroso-phenol (Fig. 26).

**Figure 26:** Inhibition of 4-nitroso-phenol formation from 5 mM phenol and 310 µM PN by 5 mM azide. Measured by the absorbance of 4-nitroso-phenol at 395 nm.

**Figure 27:** Dependence of 4-nitroso-phenol yield on PN-concentration.

The nitrosylation of anisol could not be investigated because no nitroso-standards were available. But we could observe several products in reaction mixtures of anisol with nitrosonium tetrafluoroborate and also with PN. These products were no nitro-products, but had similar absorbancies in the corresponding wavelength region (not shown). So it is not possible to exclude the formation of nitroso-products from reactions of anisol with PN.

### 4.1.8 Tyrosine Nitration by Bolus-added and Simultaneously Generated PN

In this chapter we reinvestigated the findings of Pfeiffer et al [22]. In this publication it had been reported that simultaneously generated PN was not able to nitrate tyrosine. **Fig. 28** shows the PN-concentration-dependent formation of 3-nitro-tyrosine from 1 mM tyrosine. We see a linear relationship between 3-NT formation and PN-concentration. Indeed at low fluxes of PN 3-NT was not detectable anymore, but the 3,3'-dityrosine peak increased, see **Fig. 29**. Neither 2 mM spermine NONOate nor 2 mM spermine NONOate coupled with 28 or 56 mU/ml XO and 1 mM xanthine formed more than 500 nM
3-NT. Most of the PN reacted by dimerization as shown in Fig.29,30. The products in these systems were released time-dependent. Only by monitoring 3-hydroxy-tyrosine we could distinguish the PN-generating system from the NO-source (see Fig.32,33). Bicarbonate and metallo-enzymes which are known as good PN nitration catalysts did not affect the nitration in these systems. This was good evidence, that nitration was rather mediated by NO-derived species and not by PN. This theory was also supported by the fact that deoxygenation of the reaction solution decreased the 3,3'-DT and 3-NT formation (see Fig.31).

Figure 28: 3-NT yield from 1 mM tyrosine at pH 7.4 in dependence of PN concentration.

Figure 29: Chromatograms of the reaction mixture of 1 mM tyrosine with either 100 µM PN or 2 mM spermine NONOate in 0.1 M KPi pH 7.4 after 260 min. Products were detected simultaneously at 275 and 360 nm.

Figure 30: Time-dependent formation of 3-NT and 3,3'-DT from Spermine NONOate and tyrosine. (○) for 3-NT and (●) for 3,3'-DT from 1 mM tyrosine and 1 mM NONOate. (△) for 3-NT and (solid triangles) for 3,3'-DT from 1 mM tyrosine and 2 mM NONOate.

Figure 31: Solutions which contained 1 mM tyrosine and 5 mM nitrite at pH 3 were bubbled with either nitrogen or oxygen during incubation for 15 or 60 min. 3-NT was measured by HPLC.
4.2 Scavengers for Peroxynitrite

4.2.1 Short Introduction

4.2.1.1 Searching for scavengers of PN requires understanding the nature of PN-reactions

In the PN literature all reactions involving homolytic OO-bond cleavage have summarized as "1e-oxidations" in contrast to a few reactions that transfer one oxygen atom from PN and therefore have been termed "2e-oxidations" [74, 75, 75, 86]. The formation of ebselen-oxide from ebselen or the sulfoxide from methionine are examples for this type which can originate from the PN anion as evidenced from the pH-dependency of such oxidations. To complicate the reaction pattern of PN further the PN anion can behave as a nucleophile and form adducts e.g. with carbonyls. The peroxy compound arising from carbon dioxide is a well-studied example [6, 140].

In view of the plethora of oxidations by PN it is evident that no typical inhibitor or scavenger will be identified but only mechanism-based subtypes can be found. In the present study phenol will be used as substrate since our previous investigations have revealed that the "1e-oxidation" pathway leads to a phenoxy-radical to which an -NO_2 radical can add to form 2- and 4-nitro-phenol [6, 130]. By measuring this parameter, which also closely mimicks the nitration of tyrosine in PGIS, we attempted to define a basis for PN scavengers.

The aim of the present study was to reinvestigate a series of organic compounds as inhibitors of PN with special emphasis on biologically occurring substances as lead structures. Among them would be compounds such as ascorbate...
or tocopherols, but also oxo derivatives since carbon dioxide is known to form an addition product with the PN anion [142]. Our main interest, however, was focused on uric acid which had been described as an especially good scavenger for PN [4, 131, 228, 230] and hydroxyl radicals [229].

4.2.1.2 The test systems  As a test system we employed the hydroxylation and nitration of phenol [62, 85], which not only allowed to monitor the formation of the radical pair but also could serve as a model for tyrosine nitration in proteins. The analytics and the mechanisms involved were recently published by us [130] and had been extended to the involvement of heme catalysis [163]. Another aim of this study was to distinguish between scavengers that mainly react with the protonated form of PN and those that show high reactivity for the PN-anion. For this reason we used as a second test system an enzymatic one based on ADH-activity. For ADH Beckman and coworkers have reported a k-value of $4 \times 10^5 \text{M}^{-1}\text{s}^{-1}$ for the reaction with PN-anion [94]. Therefore this enzyme seemed to be a perfect model to monitor scavenging ability and protective effects of the employed antioxidants. Furthermore decomposition kinetics of PN at alkaline pH should show if a compound was able to react directly with PN-anion or if it required the protonated form or one of the decomposition intermediates for the reaction. We here present data on reactivity of PN-scavengers in different systems.

4.2.2 Inhibitors for the Nitration of Phenol by Peroxynitrite

Nitration of phenol by PN has been reported to involve phenoxy radicals [6, 130] and therefore can be considered as two one-electron oxidation steps mediated by peroxynitrous acid (ONOOH), or more precisely the radical cage form of decaying PN [72, 71]. Fig.34 shows the formation of nitro-phenols in the PN-phenol system at pH 6 in dependence of the concentration of some representative PN-scavengers. It is obvious, that two major groups of inhibitors for phenol nitration exist: The first one shows an exponential dependence (uric acids, dithio-purine and -pyrimidine), the second one a more linear dependence (thiols, ebselen, methionine, tyrosine and ascorbate) from scavenger concentration. This behavior is directly linked to the reactivity towards ONOOH or ONOO$^-$ as will be shown later.

Tab.7 shows the IC$_{50}$-values of some natural antioxidants and synthetic compounds. It turned out, that among the best inhibitors of phenol nitration at pH 6 or scavengers for the radical cage form of PN are uric acid and its
1.3- and 3.7-dimethyl analogues (DMUA), as well as 2-TBA, 2.6-DTPy and 2.6-DTPu, followed by cysteine, ascorbate, ebselen, 3.9-DMUA and GSH (left values). Se-met and methionine itself showed very poor protection in this system, whereas xanthine, allopurinol, caffeine, allantoin and alloxan, which are all structurally related to uric acid showed only poor protection (see Tab.8). Exactly the same observations were made during phenol nitration at pH 7 (not shown). Very low IC$_{50}$-values have been reported for TEMPO/TEMPONE in phenol nitration reactions [106]. Therefore we also used this compound in our system and found IC$_{50}$-values of less than 2 $\mu$M for the nitration of 5 mM phenol by 800 $\mu$M PN (not shown). Additionally we found increased levels of 4-nitroso-phenol, when using TEMPO/TEMPONE, supporting the mechanism previously postulated for this reaction [106]. The results in the phenol system could be reproduced in a test system, where the same scavengers were employed to prevent BSA from nitration by PN (see Tab.9)[231].

![Figure 34: Effect of scavengers on phenol nitration by PN in dependence of their concentration. The IC$_{50}$-values were determined from these curves. Furthermore this graphic allowed to separate the scavengers into two major groups (linear and exponential dependence on concentration).](image)

<table>
<thead>
<tr>
<th>Scavenger</th>
<th>IC$_{50}$ [µM] pH 6</th>
<th>IC$_{50}$ [µM] pH 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteine</td>
<td>64±14 / 425±125</td>
<td>Glutathione</td>
</tr>
<tr>
<td>Methionine</td>
<td>450±34 / 690±75</td>
<td>Se-methionine</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>88±18 / 133±18</td>
<td>Uric acid</td>
</tr>
<tr>
<td>2-TBA</td>
<td>37±6 / 26±4</td>
<td>2.6-DTPu</td>
</tr>
<tr>
<td>2.6-DTPy</td>
<td>32±4 / 43.5±9.5</td>
<td>1.3-DMUA</td>
</tr>
<tr>
<td>3.7-DMUA</td>
<td>19±3 / -</td>
<td>3.9-DMUA</td>
</tr>
<tr>
<td>Ebselen</td>
<td>127.5±4.5 / 190±11</td>
<td>Xanthine</td>
</tr>
<tr>
<td>Allopurinol</td>
<td>&gt; 1 mM / &gt; 1 mM</td>
<td>Caffeine</td>
</tr>
<tr>
<td>Allantoin</td>
<td>&gt; 1 mM / -</td>
<td>Alloxan</td>
</tr>
</tbody>
</table>

Table 7: IC$_{50}$-values [µM] of scavengers for phenol nitration at pH 6 or nitrosylation at pH 9 by peroxynitrite. First value for nitration (pH6), second value for nitrosylation (pH9). Mean-values of two independent series with at least four concentrations. 5mM phenol were reacted with 655 $\mu$M PN at pH 6 and 400 $\mu$M PN at pH 9.

At pH 9 the nitrosylation of phenol by PN was monitored and the effects of
### Table 8:
Compounds with poor reactivity towards PN. Effect of 1 mM compound on nitration of 5 mM phenol by 655 µM PN at pH 6 and on nitrosation of 5 mM phenol by 400 µM PN. Values are the remaining percentage of nitration or nitrosation compared to the control without added compound.

<table>
<thead>
<tr>
<th>Inhibitor [1 mM]</th>
<th>% Nitration (pH6)</th>
<th>% Nitrosation (pH9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine</td>
<td>100</td>
<td>80</td>
</tr>
<tr>
<td>Caffeine</td>
<td>68</td>
<td>85</td>
</tr>
<tr>
<td>Guanosine-5’-P</td>
<td>90</td>
<td>82</td>
</tr>
<tr>
<td>Alloxan</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>Xanthine</td>
<td>76</td>
<td>97</td>
</tr>
<tr>
<td>Allantoine</td>
<td>100</td>
<td>78</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>100</td>
<td>88</td>
</tr>
<tr>
<td>GSSG</td>
<td>89</td>
<td>52</td>
</tr>
<tr>
<td>Allopurinol</td>
<td>98</td>
<td>91</td>
</tr>
<tr>
<td>D-mannitol</td>
<td>100</td>
<td>88</td>
</tr>
</tbody>
</table>

Table 9: IC$_{50}$-values [µM] for the inhibition of BSA nitration at pH 7 by peroxynitrite. Mean values of two independent series with at least four concentrations. Reaction of 15 µM BSA with 1 mM PN. TBA, thiobarbituric acid; DMUA, dimethyluric acid.

<table>
<thead>
<tr>
<th>Glutathione</th>
<th>150±26</th>
<th>Methionine</th>
<th>200±38</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbate</td>
<td>170±17</td>
<td>Uric acid</td>
<td>75±13</td>
</tr>
<tr>
<td>2-TBA</td>
<td>45±12</td>
<td>1.3-DMUA</td>
<td>60±14</td>
</tr>
<tr>
<td>3.7-DMUA</td>
<td>185±37</td>
<td>3.9-DMUA</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>Ebselen</td>
<td>180±12</td>
<td>Xanthine</td>
<td>&gt; 500</td>
</tr>
<tr>
<td>Allopurinol</td>
<td>&gt; 500</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

the same scavengers as used above. These results are also shown in Tab.7 (right values) and once more the uric acids and the thio-purines and -pyrimidines were best in preventing phenol from nitrosylation, suggesting that also this process involves radical species (also in the alkaline pH range high amounts of biphenols are formed, implicating the involvement of phenoxy radicals, see herefore Fig.23), for which the same group of scavengers is most effective.

In another experiment we used constant concentrations of PN, phenol and scavengers, but additionally measured the hydroxylation of phenol by PN (see Fig.35) and it turned out, that UA was most effective in suppressing hydroxy-products and benzoquinone, followed by 2-mercapto-benz-selenazol, 2-thio-barbituric acid and 2-mercapto-benz-thiazol. Ascorbate, GSH, cysteine,
ebselein and methionine showed only small effects on hydroxylation. In the case of 2,6-DTPu and -DTPpy hydroxy-products and benzoquinone could not be determined because of interfering scavenger peaks (marked with *). Additional experiments in this system showed no significant effect on neither PN-mediated hydroxylation nor nitration by PEP, oxalate, acetone, a-ketoglutarate, but a small decrease of nitration by acetaldehyde and a trialkyl-phosphine. Pyruvate showed a small increase of nitration (see Fig. 37).

Figure 35: Effect of 200 µM scavenger on nitration (pH 7) and hydroxylation (pH 5) of 5 mM phenol by 1 mM PN at RT. Products were determined by HPLC and error bars represent mean values out of 3 measurements.

Figure 36: Effect of 100 µM scavenger on nitration of 5 mM phenol by 655 µM PN at pH 6 and RT, catalyzed by 5 µM MP-11. o- and p-nitro-phenol were quantified by HPLC, error bars indicate mean values out of 3 measurements.

Additionally a system containing phenol and MP-11 was used as a model for the metal-catalyzed tyrosine nitration in proteins. Nitration of 5 mM phenol

Figure 37: Effect of 200 µM scavenger on nitration of 5 mM phenol by 800 µM PN at pH 6 and RT. Products were quantified by HPLC, error bars indicate mean values out of 3 measurements.
by 655 µM PN at pH 6 is increased by a factor of 4-5 by 5 µM MP-11. The same scavengers as tested above were employed in this system and it turned out that also in the metal-catalyzed nitration UA, 1,3-DMUA, 2,6-DTPu and -DTPy were much more effective in preventing phenol from nitration than were GSH, ascorbate, ebselen, methionine and Se-met (see Fig.36). 2-TBA showed an unusually small effect in this system.

4.2.3 Inhibitors for the Oxidation and Inactivation of ADH by PN

Our second test system used the oxidation of ADH as a probe for PN-anion reactivity. The protecting effect of the employed scavengers on ADH-activity could be directly correlated with the reactivity of the scavenger towards the PN-anion. Beckman et al [94] reported about a sensitive thiol-oxidation and ADH-inactivation by low concentrations of PN. We could confirm these findings in our system (see Fig.38). The IC$_{50}$-value for PN was at 1-2 µM, for ·NO alone (from diethyl NONOate) in air-saturated solution it was five magnitudes higher, in deoxygenated solutions by a factor of 10 (see Fig.39). Fig.10 shows the effects of PN-scavengers in this system and it turned out, that uric acids, tyrosine and tryptophan were un effective in protecting 26 nM ADH from inactivation by 20 µM PN, whereas GSH, cysteine, Se-met followed by methionine were highly efficient. We made also controls to ensure that the scavengers employed could not reactivate already inactivated ADH. 2,6-DTPy and -DTPu showed also a protecting activity in this system, but led themselves to an inhibition of the enzyme, when employed at higher concentrations. Therefore no complete conservation of ADH-activity could be achieved. Ebselen, which is known to react with thiols by forming adducts [232, 233] inhibited ADH itself at nanomolar concentrations (not shown). Ascorbate seemed to form intermediates with PN, that were also inhibiting the enzyme, therefore only a maximum of 60 % conserved ADH-activity could be achieved.

4.2.4 Kinetics of Reactions of Scavengers with PN

To determine whether a scavenger reacts with the PN-anion or not we followed the decay of PN at 302 nm at pH 8 and 9 in absence and presence of various scavengers. In some special cases the wavelength for monitoring was optimized, e.g. for ebselen the formation of ebselenoxide was followed at 275 nm and for UA the decrease of UA itself together with PN was observed at 280 nm. Ebselen accelerated the decay of PN equally over the pH-range from 8-12, giving clear evidence
Figure 38: IC₅₀-values for the oxidation and inactivation of 26 nM ADH by PN at pH 7.6. Error bars are the mean value of two measurements.

Figure 39: IC₅₀-values for the oxidation and inactivation of 26 nM ADH by nitric oxide at pH 7.6, in presence and absence of oxygen. Error bars are the mean value of two measurements.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ [µM]</th>
<th>Compound</th>
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<tr>
<td>Cysteine</td>
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<td>Glutathione</td>
<td>31±2.5</td>
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<td>Methionine</td>
<td>185±7</td>
<td>Se-methionine</td>
<td>15±1</td>
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<tr>
<td>Tyrosine</td>
<td>420±30</td>
<td>Tryptophane</td>
<td>400 / 35±3*</td>
</tr>
<tr>
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<td>400 / 42±1.5*</td>
<td>Uric acid</td>
<td>400 / 26±2*</td>
</tr>
<tr>
<td>2.6-DTPu</td>
<td>40±5**</td>
<td>2.6-DTPy</td>
<td>45±8**</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>***</td>
<td>Ebselen</td>
<td>***</td>
</tr>
</tbody>
</table>

Table 10: IC₅₀-values [µM] for the protection of 26 nM ADH by different antioxidants from inhibition by 20 µM peroxynitrite. *highest employed concentration of scavenger and conserved percentage of ADH-activity at this concentration. **scavenger itself reduced ADH-activity by 20 % when employed at 50 µM. ***strong inhibition of ADH by ebselen and probably a product from the PN-ascorbate reaction.

that this compound reacts with the PN-anion as previously reported [97]. Other compounds that clearly reacted with the anionic form of PN were Se-methionine [85], methionine [139], GSH [15], cysteine [15]. Pyruvate was even able to react several times with PN, tri-carboxy-ethyl-phosphine (TCEP), α-ketoglutarat, dimercapto-propyl-sulfonat and nitric oxide also accelerated the decomposition of PN at alkaline pH (for the latter it remained unclear, if ·NO is the reactive form or ·NO₂). Phenol, tyrosine, tryptophane, TEMPO and TEMPONE, oxalate, PEP, ascorbate (showed complicated kinetics, no simple decomposition of PN, maybe this observation is correlated to the behavior of ascorbate in the ADH-system), UA, acetone, acetaldehyde and sulfite showed less acceleration with increasing pH, clearly indicating that these compounds require ONOOH or its reactive cage form for reaction. Based on these experiments we can propose the following order of scavenger-reactivity towards PN-anion (the last 10 compounds are hard to
distinguish by the measured kinetics):

\[
\text{Ebselen} > \text{phosphine} > \text{GSH} \geq \text{cysteine} \geq \text{Se-methionine} > \\
\text{DTT} \geq \text{Pyruvate} \geq \text{Methionine} \geq \text{DMPS} > \alpha\text{-ketoglutarate} > \\
\text{ascorbate} > \text{acetaldehyde} > \text{PEP} \equiv \text{acetone} \equiv \text{oxalate} > \\
\text{phenol} \equiv \text{tyrosine} \geq \text{tryptophane} \equiv \text{TEMPO/TEMPONE} \equiv \\
\text{Uric acid} \geq \text{sulfite}
\]

Figure 40: Kinetics of PN-decomposition in dependence of pH and scavengers. (A) Decomposition of 1 mM PN and 250 µM UA in 0.2 M KP\textsubscript{i} at pH 7 (1), 8 (2), 9 (3) and 10 (4). (B) Formation of ebselenoxide from 400 µM ebselen and 500 µM PN in 0.2 M KP\textsubscript{i} at pH 8 (1), 10 (2) and 12 (3). (C) Decay of 500 µM PN in presence of keto- and aldehyde-compounds (500 µM, except pyruvate which was 750 µM). (D) Decay of 250 µM PN in presence of sulfur compounds (500 µM, except DTT and TEMPONE which were 250 µM and sulfite was 2.5 mM).

4.2.5 Reaction of Uric Acid with PN

For better understanding, why UA was much more efficient as a PN-scavenger than for example xanthine, caffeine or allopurinol, we tried to identify all stable products of the reaction of UA with PN in the pH-range from 6-9. Fig.41 shows the representative chromatograms of the reaction of UA with PN at pH 6, 7, 8 and 9. At pH 6 the reaction yielded three peaks (t\textsubscript{R}=8.6, 9.5 and 11 min) eluting before UA (t\textsubscript{R}=15-16.5 min). At pH 9 the peak at 11 min increased and
additional peaks at $t_R=12$ and 17 min appeared, supporting the different action of PN at neutral and alkaline pH. The slight shifts in retention time are due to the pH of the injected reaction solutions into the mobile phase. The UV/Vis spectra of the peaks showed no significant absorbance above 300 nm as reported elsewhere for a product from the UA-PN reaction [131], even no significant band above 250 nm except uric acid itself. The reaction of 1.3-DMUA and PN showed a comparable product pattern in a different HPLC-system (see also Fig. 42).

Figure 41: HPLC-chromatograms of the reaction of 0.5 mM uric acid with 1.3 mM PN in 0.2 M K-phosphate buffer (A) pH 6 (1 min), (B) pH 7 (1 min), (C) pH 8 (5 min) and (D) pH 9 (10 min). The broken lines were incubated 1 h longer. Conditions as mentioned in materials and methods.

Peaks from the reaction of 1.3-DMUA and PN were collected for further identification by $^1$H- and $^{13}$C-NMR and EI-MS ([234] contained reference signals). These investigations only yielded poor results due to the instability of the products. 1.3-DMUA was chosen for better monitoring of ring opening and abstraction of urea from the UA-ring system. Indeed some of the product peaks had lost one or even two methyl groups, but it could not be determined if this was already mediated by PN or was due to a slower degradation process. UA has been also reported to undergo oxidation to allantoin and oxonic acid in alkaline solutions [208]. $^{13}$C-NMR of freshly prepared UA-PN reaction solutions indicated a lack of allantoin, oxonic acid, cyanuric acid, diimine, parabanic acid, alloxanthin and alloxan in the product pattern of this reaction, although these products have been reported for other oxidation reactions of UA.
RESULTS

[206, 207, 208, 235]. Allantoin would have been also visible as a shoulder in the salt peak of the chromatograms. Furthermore we have tested the content of allantoin, as well as allantoic acid by the method described elsewhere [208]. What we found were distinct amounts of urea, determined as previously described [209]. From 1 mM PN and 0.5 mM uric acid 345±78 µM urea are formed at pH 7.4 (determined colorimetrically by the NH3-pH-shift after enzymatic degradation of urea by urease). Uricase had no effect on the peaks above except UA itself, suggesting that none of these products has a structure similar to UA. We cannot present any structural proposal for the UA-PN products, but only the product pattern on HPLC and the finding, that the products formed from reactions of PN with UA undergo further degradation. All possible oxidation products were commercially available and also tested on the HPLC-system from Fig. 41 and could be excluded: Allantoin, allantoic acid, oxonic acid, alloxan, alloxanthine and cyanuric acid.

![Figure 42: HPLC-chromatogram of the reaction of 2 mM 1,3-dimethyl-uric acid and 8 mM PN at pH 7. Peak 1, 2, 3, 4 were isolated and investigated by NMR and MS. Conditions as described in materials and methods.](image)

4.2.6 Reactions of Ebselen with Peroxynitrite

4.2.6.1 Short introduction Ebselen is known for its very fast reaction with PN-anion [97]. Therefore it seemed to be one of the best candidates to protect PGIS from inactivation by PN, but no protective effect could be observed when bovine aortic microsomes or bovine aortic rings were preincubated with ebselen in a 10-50 fold excess with respect to PN concentration. Since ebselen already reacts with ONOO− with a second order rate constant of 2x10^6 M^{-1}s^{-1} [97], the enzyme would have to react with 10^7-10^8 M^{-1}s^{-1}. This requires an almost diffusion controled reaction, regarding the low concentration of PGIS in bovine aortic microsomes. However evidence for a a very fast reaction of PN with PGIS was based on competitive kinetics with several PN-scavengers (e.g. GSH, ascorbate and trolox) [12]. For this reason we developed a test to see what was happening to ebselen in tissue or at membranes.
4.2.6.2 **The fate of ebselen in tissues** It is known that ebselen forms adducts with thiols, especially glutathione (GSH) [232, 233] which could have been responsible for the lack of the expected ebselen effect. Indeed, ebselen disappeared time-dependently from a tissue-containing bath solution and two metabolite peaks were formed concomitantly as seen in Fig. 43. According to the optical spectra the band with a retention time of 7 min proved to be identical with the spectra of the ebselen-GSH adduct as seen from a titration of ebselen with GSH (see Fig. 44). The second metabolite was not identified but it had a similar retention time as the ebselen-cysteine adduct and disappeared after excess addition of GSH to the bath solution.

![Figure 43](image1.png)  
**Figure 43:** Time-dependent loss of "free" ebselen in a solution containing 0.134 g (dry weight) bovine coronary tissue and parallel formation of the ebselen-GSH-adduct and an unknown product (similar retention time as the ebselen-cysteine adduct). After addition of 5 µM GSH after 30 min all ebselen and the unknown product are converted to the ebselen-GSH-adduct. Separation and quantification by HPLC.

![Figure 44](image2.png)  
**Figure 44:** UV/Vis-spectra of 100 µM ebselen with 0 (2), 50 (3), 100 (4), 200 (5) 400 µM (6) GSH in 0.2 M K-phosphate buffer pH 7. (1) buffer without ebselen and GSH.

Not only GSH but also reactive thiols in proteins can bind ebselen as had been shown for albumin [233] and the nonenzymatic compound lipoate [232]. Using a microsomal fraction from aortic rings it was once more possible to observe a decrease in "free" ebselen after addition of this membrane fraction by HPLC analysis. When the protein-bound thiols were pre-oxidized with Ellman’s reagent (DTNB, 5,5’-dithio-bis-(2-nitro-benzoic acid)), 38 µM of the added 50 µM (respectively 4 µM of 20 µM) ebselen remained unbound in solution and could be detected by HPLC. Using non-treated microsomes these values dropped to 13 from 50 µM and 0 from 20 µM (see chromatogram in Fig. 45).
Since this microsomal fraction also contained PGI₂ synthase it was possible to conduct an experiment with PN and ebselen with and without previous treatment with DTNB. Additions of 1 and 10 µM PN caused the expected inhibitions of 6-keto PGF₁α formation in the presence of ¹⁴C-PGH₂ with DTNB having no significant modulatory effect. However, when 10 µM ebselen were added before PN the inhibition was largely prevented (not shown) [236].

A qualitative confirmation of this result was obtained by immunoprecipitation experiments with a monoclonal anti-nitrotyrosine antibody [236]. Without DTNB pretreatment of aortic microsomes 1 and 10 µM PN caused a massive nitration not influenced by ebselen (Western blots not shown) whereas the DTNB mediated modification of microsomes allowed ebselen to partially trap PN leading to a diminished nitration of PGI₂ synthase (not shown) [236]. It is interesting to note that control microsomes already contain a certain amount of nitrated enzyme which was consistently found also in previous experiments [10, 11].

4.2.6.3 The reaction of PN with ebselen in the absence and presence of thiols  Ebselen and the PN anion rapidly react to give the corresponding selenoxide and nitrite [144]. When this reaction was carried out at pH 9 the stability of PN is high enough to lead to an almost quantitative conversion of ebselen to its selenoxide after equimolar mixing with PN. Only a minor amount of ebselen remained due to some isomerization of PN to nitrate [19] or its dismutation to nitrite and dioxygen [61] (Fig.46). At pH 7 only about 50 % of ebselen were converted to the oxide. If the same experiment was carried out when GSH in a slight molar excess over ebselen had been added 1 min before PN only a small amount of ebselen oxide appeared at pH 7 and a larger amount...
at pH 9 (Fig. 46).

The peak of the ebselen-GSH adduct formed under these conditions suggested that this adduct does not undergo a rapid reaction with PN to its corresponding selenoxide. At pH 9 a small peak with a lower retention time as the ebselen-GSH adduct could be consistent with such a selenoxide confirming the better stability of PN at pH 9 which allowed to form this product at pH 9 but not at pH 7.

Such results point to a slower reaction of PN with the ebselen-GSH adduct than with ebselen itself. Direct kinetic measurements are difficult to perform since the absorbancies of PN and that of the adduct are in the same region between 290 and 340 nm. However, when choosing 320/370 nm as a wavelength pair and pH 10 when the stability of PN is high enough one can observe kinetics of PN decomposition with ebselen (Fig. 47 and 48). At the same wavelength pair a preincubated equimolar mixture of ebselen and GSH decomposes PN with slower kinetics although the maximum absorbance change is somewhat larger (Fig. 47).

This is at least a qualitative confirmation of the slower reaction of the adduct. As a control a secondary addition of PN resulted only in a minor change due to the absorbance of PN at 302 nm [57]. A corresponding experiment could be performed with the ebselen-albumin adduct (Fig. 48). Additions of 20 and 40 µM albumin to 100 µM ebselen at pH 10 slowed down the kinetics of the ebselen reaction. Again a second addition of PN did not change the absorbance difference significantly proving that in the initial reaction the disappearance of the adduct through oxidation by PN had been monitored.

A weaker scavenging effect of the ebselen-GSH adduct than with GSH alone could also be observed during the nitration of 5 mM phenol by 650 µM PN. The
Figure 47: Kinetics of the decomposition of peroxynitrite and disappearance of ebselen. The system consisted of (1) 400 µM ebselen and 500 µM PN in 0.2 M K-phosphate buffer at pH 10, (2) second addition of 500 µM PN to the reaction solution of (1), (3) 400 µM ebselen and 200 µM GSH and 500 µM PN and (4) second addition of 500 µM PN to the reaction solution of (3).

Figure 48: Kinetics of the decomposition of peroxynitrite and disappearance of ebselen. The system consisted of (1) 100 µM ebselen and 100 µM PN in 0.2 M K-phosphate buffer at pH 10, (2) second addition of 100 µM PN to the reaction solution of (1), (3) 100 µM ebselen and 20 µM BSA and 100 µM PN and (4) 100 µM ebselen and 40 µM BSA and 100 µM PN.

The corresponding hydroxylated and nitrated phenol metabolites were separated by HPLC as described [130]. In this system 100 µM ebselen caused 21±2 % inhibition, 100 µM GSH gave 88±1 %, and the combination of 100 µM ebselen/100 µM GSH yielded only 83±3 % inhibition (mean values of five measurements). With tyrosine the corresponding differences were even more distinct (see Fig.49).

Figure 49: Effect of ebselen, GSH and ebselen-GSH-adduct on tyrosine nitration by PN.

4.2.6.4 Why is ebselen a good scavenger for PN-anion? Ebselen shows an outstanding reactivity towards the PN-anion with a $k_{sec}=2\times10^6 \text{ M}^{-1}\text{s}^{-1}$ [97].
Also Se-methionine reacts with PN-anion, but some magnitudes slower with a $k_{sec} = 2 \times 10^2 \text{ M}^{-1}\text{s}^{-1}$ [85]. Regarding this difference, the high reactivity of ebselen towards ONOO$^-$ cannot be only due to the selenium atom. Another reason could be a special ring-tension in the selenazolon ring of ebselen, which is decreased by formation of the ebselenoxide. To test this we used two related compounds: 2-mercapto-benz-selenazole (2-MBS) and 2-mercapto-benzo-thiazole (2-MBT), which were also tested as scavengers in the phenol system (see Fig.35), where the selenium compound provided better protection. Reaction of 2-MBS with PN at pH 7.6 could be confirmed by a change of the absorbance spectrum after titration with PN (see Fig.50). So far 2-MBS showed a similar behavior as ebselen, which also changes its absorbance when the ebselenoxide is formed.

![Figure 50: Titration spectra of 100 µM 2-MBS in 0.1 M KP, pH 7.6, every trace was recorded after addition of 10 µM PN.](image)

![Figure 51: Decomposition-kinetics of 100 µM PN in presence of 100 µM 2-MBS at pH 7 and 9 (black lines) and absence of 2-MBS (grey lines).](image)

The kinetics in Fig.51 proved, that 2-MBS reacts rather with the protonated form of PN or its activated form than with the anionic form. For the kinetics the decrease of 2-MBS absorbance at 320 nm was measured, all starting absorbancies are relative. PN itself absorbs only weak at this wavelength as shown by the controls. Additional results to prove the exceptional ebselen reactivity were obtained, when 2-MBS was treated with GSH. Contrary to the adduct formation in the case of ebselen and GSH, no such adduct could be observed for 2-MBS. Even after addition of a 4-fold excess of GSH the absorbance spectrum of 2-MBS remained unchanged (not shown). Also when the absorbance at 320 nm disappeared after treatment with PN (see Fig.50), it could not be restored by GSH as it was the case for ebselen (not shown). Ebselenoxide, the product from ebselen and PN can be reduced by GSH at equimolar concentrations [144]. So the basis for high reactivity of ebselen towards PN-anion seems to be connected to the
special conditions in the selenazolon ring, but these circumstances seem also to be responsible for the high reactivity of ebselen towards thiols. With thiols, as we and others have shown, ebselen forms adducts by ring-opening [232, 236].

4.3 Reactions of Peroxynitrite with Transition-metals

In the first part of this work we have investigated the self-decomposition of PN and the influence of different antioxidants on this decomposition and on the PN-mediated nitration of phenol. Here we want to present the effect of transition-metals on the nitration of phenol by PN and its decomposition [116, 163]. Especially manganese, iron and copper-containing complexes and enzymes have been reported to modify PN-chemistry [164, 174, 237]. Furthermore cobalt and nickel seem to interact with PN [76]. All these metals probably form high-valence species with PN as reported for heme-enzymes and porphyrin complexes (Compound I and II) [50, 102, 103].

4.3.1 Effect of Metal-containing Enzymes on PN-decomposition Kinetics and the Nitrite:Nitrate Ratio

A simple test to measure the efficacy with which metal-complexes and -enzymes can react with PN is based on the observation of PN-decomposition at 302 nm. Fig.52,53 show the kinetics of PN-decomposition in presence of different metal-enzymes and complexes.

In Fig.52 P450NOR (NOR) was the best catalyst for PN-decomposition,
followed by coupled systems of hemoglobin (HG) or horseradish peroxidase (HRP) with ascorbate (asc). Next was $\text{P450}_{\text{BM-3}}$ wildtype followed by ascorbate in a 100-fold excess and HG and HRP alone showed the weakest effect on PN-decay. In Fig. 53 chloroperoxidase (CPO) was more efficient than $\text{P450}_{\text{BM-3}}$ wildtype (BM3-WT), followed by microperoxidase (MP-11) for which it should be noted that it was used at a 5-fold higher concentration compared to the other enzymes. $\text{P450}_{\text{CAM}}$ (CAM) showed approximately the same reactivity as MP-11 and the $\text{P450}_{\text{BM-3}}$ F87Y (BM3-Mut) variant showed the weakest effect on PN-decomposition. The reasons for the difference between BM3-WT and -Mut will be discussed later. Summarizing the results of the kinetic measurements we can conclude that heme-thiolate enzymes were more efficient in catalyzing the decomposition of PN than heme-enzymes. Hemin (ferric protoporphyrin IX) and other manganese- and iron-containing porphyrins were less efficient compared to the enzymes. Simple metal complexes, such as EDTA showed only effects when employed at high concentrations (not shown).

It should be noted that the decay curves were obtained with a 30 - 150 fold excess of PN and therefore must include a turnover of the heme proteins implying that the supposed ferryl intermediates had been rereduced to the ferric state by PN during the turnover [50, 163]. In agreement with this assumption it had been reported that in the HRP-phenol system or with a manganese-porphyrin complex the absorbance decrease at 302 was enhanced in the presence of ascorbate as a suitable reductant for the intermediate higher oxidation states of the metals. This was also found to be true for HRP and HG in our system Fig. 54. The subsequent events in the reaction cycle could be analyzed by the products deriving from PN during its decomposition by the heme compounds. Such products are dioxygen, water and $\text{N}_2\text{O}_3$, but also nitrate from the isomerization of PN. Since dioxygen formation can be followed polarographically with an oxygen electrode the effects of MP-11 and $\text{P450}_{\text{NOR}}$ on PN decomposition were investigated with this method. Fig. 55 shows kinetics of dioxygen release in presence of MP-11 and NOR. It clearly turned out that the heme proteins enhanced the initial rates of dioxygen formation. The total release of $\text{O}_2$ was lower with NOR than with MP-11 or CPO at pH 9 and 10, but higher at pH 7.5. Catalase and myeloperoxidase (MPO) showed only minor effects (not shown), although for the latter a fast on-reaction with PN had been reported [102].

The parameters nitrite and nitrate are more difficult to measure since the
PN solution already contains nitrite and nitrate and tests for nitrate are rather insensitive. We therefore analyzed only the ratio of nitrite/nitrate at the end of the reaction. Time dependent kinetic measurements are not meaningful by the methods available since any remaining PN will eventually decompose to nitrite and nitrate during the work-up procedures. As a result of an end point determination no significant changes in the nitrite/nitrate ratio could be observed when control assays were compared to the ones with MP-11 and NOR with PN concentrations much larger than enzyme concentrations, but more nitrate was found at a 1:5 ratio of enzyme to PN (Fig. 56). With large amounts of MP-11 the nitrite:nitrate ratio decreased in agreement with reports in literature, where even 1 μM concentrations of iron-porphyrins caused a shift of the nitrite:nitrate ratio by a factor of 7 [104, 238]. This strong shift could neither be observed with 1 μM concentrations of MP-11, nor with NOR.

Figure 54: Kinetics of PN-decomposition in presence of different heme- and heme-thiolate enzymes (part III). Reaction conditions as indicated in the legend box and at 10 °C. These traces are representative for at least three independent measurements.

Figure 55: Time-dependent oxygen release from mixtures of 800 μM PN in 0.2 M KP4, at pH 9 with no enzyme, 1 μM MP-11, 1 μM NOR and 1 μM CPO. Typical experiment out of five.

Figure 56: Nitrite:nitrate ratio from decayed PN (400 μM) in dependence of KP4 buffer and MP-11 concentration.
4.3.2 Metal-catalyzed Nitration of Phenol by Peroxynitrite

Another tool to investigate the reactivity of metal-enzymes and -complexes towards PN is the nitration of phenol in presence of these compounds [116, 163, 174]. The intermediates which are formed during reactions of PN with metal-containing complexes and enzymes were found to increase the nitration of phenol by PN in a more or less efficient way. This increase can be measured qualitatively by the absorbance of the entity of products which absorb at 350-430 nm (e.g. nitro- and nitroso-products) (see Fig. 57). The formation of nitroso-products at pH 7 is low, therefore the absorbance at 400 nm will be due to nitration products. The nitration of 5 mM phenol by 655 µM PN is increased 6-fold in presence of 5 µM MP-11. Also the initial velocity of nitration seems to be increased. Anisol nitration was not increased by heme or heme-thiolate enzymes suggesting that also the metal-catalyzed nitration by PN requires phenoxy radicals as intermediate-species (not shown). The phenolic OH-group in anisol is protected by the methyl group, therefore the abstraction of an electron or ·H is hindered.

![Figure 57: Kinetics of phenol (5 mM) nitration by 655 µM PN at pH 7 in presence (broken line) and absence (solid line) of 5 µM MP-11. The traces were measured at 400 and 450 nm as a wavelength pair.](image)

A more powerful tool is the quantitation of all products by HPLC. Fig. 58 shows the nitration and dimerization of phenol by PN with increasing concentration of some representative metal-complexes and -enzymes. It is evident, that the simple complex Fe(III)edta requires high concentrations (> 600µM) until a plateau-value for the nitration is reached. In the case of hemin the plateau is reached at 10 µM and with MP-11 it is also reached at around 10 µM. But with CPO the plateau is already reached at 1 µM. When even higher concentrations of the enzymes were used after the plateau had been reached, then the yield of nitro-products started to decrease. This might be due to increases in dimerization or other products [163]. Also in catalyzing the phenol nitration a P450 enzyme was most efficient, supporting the idea of
a special role of the thiolate in the heme-thiolate moiety during reactions with PN.

Figure 58: Nitrination of 5mM phenol by 800 μM PN at pH 7 in dependence of iron-containing complexes and enzymes: o- and p-nitro-phenol [μM] and o,p'-biphenol [rel.area]. (A) Fe(III)edta, (B) hemin, (C) MP-11 and (D) CPO. For all figures: o-NP (circles), p-NP (triangles) and o,p'-BP (squares).

Valuable information could be obtained by adding phenol as a probe for hydroxylating or nitrating intermediates to the decomposing PN solutions with and without metal complexes. In the absence of metals we and others have described catechol and hydroquinone as well as 2- and 4-nitro-phenols as the main reaction products [56, 145, 62]. In addition we have noticed an appreciable amount of 4-nitroso-phenol as a consequence of a high contribution of the dismutation pathway generating N₂O₃ as the likely nitrosylating agent. Also the whole spectrum of dimerization products could be identified with the conclusion of phenoxyradicals as intermediates. In the following experiments we also have included Fe(III)edta as a nonpeptide iron complex (see Tab.11). The data clearly point to a decrease in hydroxylated metabolites and an increase in nitrated phenols when ferric complexes are present. An extrapolation to the pure contribution of iron indicates a complete suppression of the hydroxylation pathway. This would rule out hydroxyl radicals as intermediates of the heme-catalyzed pathways. Also a high yield of 4-nitroso-phenol was determined from which a major participation of the dismutation pathway can be concluded.
### RESULTS

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<table>
<thead>
<tr>
<th>7.5</th>
<th>2-hydroxyphenol [μM]</th>
<th>Nitrophenols [μM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>without iron*</td>
<td>13.5±1.5</td>
<td>22.2±1.6</td>
</tr>
<tr>
<td>with 1 μM NOR</td>
<td>&lt; 0.5</td>
<td>31±4.3</td>
</tr>
<tr>
<td>with 1 μM MP-11</td>
<td>10.1±3.8</td>
<td>34.9±2.6</td>
</tr>
<tr>
<td>with 2.5 μM MP-11</td>
<td>&lt; 0.5</td>
<td>42.7±5.4</td>
</tr>
<tr>
<td>with 1 μM HRP**</td>
<td>&lt; 0.5</td>
<td>31.3±2.7</td>
</tr>
<tr>
<td>with 2.5 μM HRP***</td>
<td>&lt; 0.5</td>
<td>28.8±1.9</td>
</tr>
<tr>
<td>with 20 μM Fe(III)edta</td>
<td>1.1±0.2</td>
<td>27.3±0.7</td>
</tr>
<tr>
<td>with 100 μM Fe(III)edta</td>
<td>&lt; 0.5</td>
<td>32.6±3.1</td>
</tr>
</tbody>
</table>

Table 11: Effect of iron-proteins and iron-edta on PN-mediated nitration and hydroxylation. (+) 4-hydroxy-phenol is only present in trace amounts at this pH (*) 800 μM PN and 5 mM phenol in 0.1 M KP_i-buffer pH 5 or 7.5 at 37 °C for 1 min (**) formed high amounts of biphenols (***) formed highest amounts of biphenols.

Whereas the mechanisms of nitration with free PN via its acid form is still ill-defined, the presence of ferric complexes very convincingly has been shown to occur via initial formation of a ferryl ion and the ·NO₂ radical [111, 164, 163]. The data with NOR were in agreement with such homolytic cleavage of PN although with cytochrome P450 monooxygenases hydroperoxides usually are forming the perferryl state (Compound I) in which the oxygen atom possesses oxene transfer properties and provides the basis for monooxygenase reactions [38]. Therefore, by this mechanism phenol could be converted to 2- and 4-hydroxy-phenols which should then increase the yield of these products in addition to the pattern of hydroxy- and nitro-phenols derived from the decomposition of free PN. However, catalysis by NOR rather decreased the yields of hydroxy-phenols (see Tab.11) [163]. It was interesting therefore to test the product pattern with a real P450-monooxygenase like BM3 as shown Tab.12. When tested under the same
Nitro-phenols [µM]

<table>
<thead>
<tr>
<th>Conditions</th>
<th>pH 6</th>
<th>pH 7</th>
<th>pH 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>without iron*</td>
<td>35.5±5.2</td>
<td>38±4.1</td>
<td>3.3±1.6</td>
</tr>
<tr>
<td>with 2 µM CPO**</td>
<td>97.4±10.8</td>
<td>100±18.2</td>
<td>50.4±6.8</td>
</tr>
<tr>
<td>with 2 µM NOR**</td>
<td>95.7±12.2</td>
<td>77±9.3</td>
<td>13.5±4.7</td>
</tr>
<tr>
<td>with 2 µM BM3**</td>
<td>89.5±6.3</td>
<td>56±4.5</td>
<td>20±7.5</td>
</tr>
<tr>
<td>with 4 µM MP-11</td>
<td>124.4±22.3</td>
<td>62±11</td>
<td>18±5.2</td>
</tr>
<tr>
<td>with 4 µM catalase</td>
<td>32.4±3.7</td>
<td>28.6±6.3</td>
<td>5.3±1.8</td>
</tr>
<tr>
<td>with 2 µM HRP</td>
<td>51.4±5.7</td>
<td>48±8.2</td>
<td>9.2±3.5</td>
</tr>
</tbody>
</table>

Table 12: Effect of heme-thiolate and heme-proteins on PN-mediated nitration of phenol. (*) 5 mM phenol and 800 µM PN in 0.1 M K-phosphate buffer for 5 min at 37 °C. (**) In controls with heat-denatured enzymes the nitration decreased to the non-catalyzed level.

We could show, that heme-proteins are efficient catalysts for phenol nitration and heme-thiolate enzymes are even more efficient in nitration increase and hydroxylation decrease. These findings are the bases for explanation of selective and autocatalyzed nitration of tyrosine residues in some of the heme and heme-thiolate proteins, which were investigated during this thesis work.

4.3.3 Formation of High-valence Metal-species during Reactions of Transition-metals with Peroxynitrite

Chelated transition-metals are known to form high-valent intermediates with peroxides. Also PN behaves alike and it has been previously shown that PN forms ferryl intermediates (Compound II, Fe$^{IV}=$O) during reactions with iron (III) porphyrins [103] and Mn$^{IV/V}=$O with manganese (II) and (III) porphyrins [174, 50]. Also peroxidases such as HRP and myeloperoxidase (MPO) have been reported to perform similar reactions [102].

Our first observations with Fe(III)edta and PN. Fig.59 showed similar intermediate bands in the reaction of Fe(III)edta with either hydrogen peroxide (left) or PN (right). These bands disappeared faster if an antioxidant such as ascorbate was added. Hence Fe(III)edta also increased the yield of phenolic...
Nitrlation by PN, this intermediate must represent the reactive species, during Fe(III)edta-catalyzed nitration.

Figure 59: Reaction of 1 mM Fe(III)edta with either 2 mM H$_2$O$_2$ (left side) or 800 µM PN (right side) at pH 7. Both spectra show the absorbance at around 500 nm before addition of peroxide and 0, 5 and 10 min after the addition.

HRP was the only heme-enzyme which could form a more or less stable ferryl-intermediate during reaction with PN. Bolus-added as well as simultaneously-generated PN caused a shift of the Soret band of HRP at 398 nm to a new intermediate band at 424 nm, which was the ferryl (Compound II) intermediate as previously described [102]. Fig. 60A shows the time-dependent accumulation of the ferryl-species during decomposition of SIN-1, which is known to form simultaneously ·NO and O$_2^-$ by autoxidation and serves as a source of continuously-generated PN [22]. This ferryl-species could be converted back to the ferric-heme by treatment with ascorbate, indicating the reactivity of this intermediate towards e-donors. This observation was in agreement with the increased nitration of phenol and the accelerated decomposition of PN in presence of HRP. Fig. 60B shows the Fe-NO-complex of HRP, which is hard to distinguish from the ferryl-species, but shows no reactivity towards ascorbate. Bolus addition of either PN or H$_2$O$_2$ resulted in similar spectra, suggesting that also in these cases the ferryl-species was formed (not shown). Fig. 61 shows the visible region of the ferryl species with the characteristic double bands at 525 and 557 nm. Once more the Fe-NO-complex of HRP showed similar absorbance maxima (not shown). P450's, hemin and MP-11 formed no stable ferryl-species with PN which could be observed by conventional spectroscopy. Stopped-flow spectra of hemin and MP-11 revealed only small spectral changes and a decomposition of the porphyrin-ring.

After the finding that P450's could catalyze the nitration of phenol by PN, we
Figure 60: Difference absorbance spectra, measured against 5 \( \mu M \) native HRP: (A) incubated with 400 \( \mu M \) SIN-1 at pH 7.4 for 1-13 min (black lines) and 0.5 or 2 min after addition of 200 \( \mu M \) ascorbate (broken grey lines). (B) incubated with 25 \( \mu M \) DENO at pH 7.4 for 0.2, 1 and 2 min (black lines) and 0.5 or 2 min after addition of 200 \( \mu M \) ascorbate (broken grey lines).

Figure 61: Visible region of the HRP ferryl-species generated during the reaction of 5 \( \mu M \) HRP with 650 \( \mu M \) PN at pH 7.5.

concentrated on the mechanism of metal-catalyzed nitration of phenol. Since we found dimerization products also in metal-containing reactions, the mechanism was likely to proceed via radical species and to involve a ferryl intermediate, which we postulated for NOR and PN [163]. As shown in Fig.62 we observed an intermediate during reaction of NOR with PN which could be assigned to the ferryl-species in agreement with the Compound II spectrum of CPO [239]. This ferryl-band increased until most of the PN had decayed (first 100 ms in the kinetic trace in Fig.62) and then started to decompose and formed back the ferric form of NOR (more than 2000 ms). The bimolecular rate constant \( k_{sec} \) for the formation of the ferryl-species was \( 8 \times 10^4 \) to \( 2 \times 10^5 \) M\(^{-1}\)s\(^{-1}\), \( k_{obs} \) was 20 s\(^{-1}\) at pH 7.2 and 43 s\(^{-1}\) at pH 5.9 (see Fig.64). The monomolecular rate constant \( k_{obs} \) for the decomposition was 1.4-1.6 s\(^{-1}\). In the pH range shown the rate constant decreases, but at pH 8.8 it increases again (not shown). From the short life-time we could conclude that Compound II of P450\(_{NOR}\) (and also other P450’s as will be shown later) is much more reactive compared to that one of HRP, which is stable for at least minutes. This also explains the high efficiency of P450’s in catalyzing PN-decomposition and phenol nitration by PN.
4.4 Autocatalytic Tyrosine Nitration of Cytochrome P450BM−3 and its F87Y Mutant by Peroxynitrite

4.4.1 Short Introduction

In order to explain the high selectivity of the enzyme for nitration, we have used chemical models [130] which suggest that a tyrosine at the active site is nitrated after a homolytic cleavage of PN and a ferryl species as well as nitrogen dioxide are generated [163]. Interestingly, we have also shown that the P450 protein NADH-NO-reductase (P450NOR) can catalyze the nitration of added phenol but does not show autocatalytic protein nitration at low PN concentrations [163]. In contrast, the monooxygenase P450BM−3 [240] gave a positive reaction with NT-Ab in Western blots after treatment with low PN concentrations [116]. This finding prompted the present study on the location of nitrated tyrosines in P450BM−3 and in a mutant in which a phenylalanine located close to the active site was exchanged against a tyrosine (F87Y) [182]. This protein displays similarities to
PGI₂-synthase and can thus be used to study mechanistic aspects on PN mediated nitrations, in particular the role of the heme in this process. In addition, since Pfeiffer and Mayer [22] had reported the lack of tyrosine nitration by simultaneously generated ·NO and O₂⁻ under physiological conditions, we in this work emphasize that this is not valid when tyrosines are in a protein close to a metal-containing active site. In this case reaction with the metal centers can compete effectively with other chemical processes of PN such as isomerization to nitrate or dismutation to dioxygen and dinitrogen trioxide. Uncatalyzed nitration reactions are rather inefficient and may require 100 µM or more PN and are thus less likely to occur in cellular systems.

4.4.2 Nitration of P450BM₃

Previous work with heme-thiolate enzymes had shown that these enzymes can catalyze the decomposition of 100 µM PN. When P450BM₃ is reacted with PN it becomes nitrated [116] and thus displays an analogy to PGI₂-synthase nitration. Nitration of P450BM₃ was monitored by Western blotting with a polyclonal NT-antiserum which gave only a very weak background staining with the untreated native enzyme. Fig.65 A and B shows the effect of incubation of 5 µM wild-type P450µ with 100 µM PN, resulting in a clearly stained band at 116 kDa. Addition of 500 µM PN intensified this band but also gave a strong background of contaminating protein, which was not visible on Ponceau S stained gels for these blots. This suggested that this aggregated material was only present at low concentrations (Fig.67). With 50 µM PN, the staining of the holo enzyme (NADPH reductase domain plus the heme-thiolate protein) became weaker but appeared rather selective compared to other bands. Also 5 µM PN still resulted in a distinct and selective staining which became faint but still visible after addition of 0.5 µM (not shown). The F87Y variant behaved qualitatively similarly but displayed significant nitration even when 5 µM enzyme were incubated with 0.5 µM PN. Other heme-thiolate enzymes such as P450NOR [163] and CPO were nitrated at PN concentrations above 100 µM (data not shown). Both enzymes showed a strong NT-positive staining at concentrations of 250-500 µM of PN.

To demonstrate that tyrosine nitration was effective when the enzyme was exposed to simultaneously generated ·NO and O₂⁻, we incubated 5 µM BM3 aerobically with 100 µM SIN-1 and obtained a strong nitration of the 116 kDa holo enzyme, but also of proteins of somewhat lower size and of an obviously
Figure 65: α-NT Western blot of BM3 Wildtype and F87Y Variant (major band at 116 kDa) after treatment with different amounts of PN at pH 7.4.

Figure 66: α-NT Western blot of BM3 WT after treatment with PN-generating systems or nitric oxide. A comparable blot was made for BM3 F87Y.

aggregated material (Fig.66, lane 2). A control in which O$_2^-$ generated by SIN-1 autoxidation was scavenged by Cu,Zn-SOD (lane 3) showed negligible nitration and so did a sample which was only exposed to ·NO, generated through the thermal decomposition of 100 µM spermine NONOate (lane 4). As expected, the exposure of BM3 to hypoxanthine/xanthine oxidase resulted in no nitration (lane 1) unless the reaction system was supplemented with 0.1 mM NONOate (lane 5) which then caused strong nitration of the 116 kDa protein but also of a second band at 54 kDa which corresponds to the size of the heme domain alone. Weak staining was seen also in a fragment at 66 kDa which could represent the reductase domain. Both the addition of Cu,Zn-SOD or the omission of XO blunted the nitration (Fig.66 lanes 6 and 7, respectively).

Figure 67: Ponceau S stained gel for Western blot in Fig.65.

Figure 68: Ponceau S stained gel for Western blot in Fig.69.

These blots suggest that the incubation of the enzyme with xanthine oxidase resulted in a splitting of the 116 kDa holoenzyme into the 66 and 56 kDa
domains of the reductase and the heme domain, respectively (this was also observed on Ponceau S stained gels for these blots, see Fig.68). As xanthine oxidase preparations are known to be contaminated with proteases [241] we performed similar assays in the presence of a protease inhibitor cocktail from Sigma (for bacterial and general use) and could indeed block the cleavage with no effect on the nitration yields (data not shown). Similar experiments with similar results were performed with the F87Y mutant (see Fig.69). Massive 3-NT immunoreactivity of the holoenzyme was obtained by simultaneous generation of \( \cdot \text{NO} \) and \( \text{O}_2^- \) with significant or negligible cleavage in the absence or presence of the protease inhibitor cocktail (Fig.69 lanes 3 and 7, respectively).

Figure 69: \( \alpha \)-NT Western blot of BM3 F87Y Variant after treatment with XO/NONOate in presence of protease inhibitors at pH 7.4. All samples contained 0.5 mM hypoxanthine. A comparable blot was made for BM3 WT.

Interestingly, Cu,Zn-SOD could not completely suppress the nitration of the BM3 F87Y mutant and also NONOate alone had a weak nitrating effect. We noticed, however, that the 3-NT immunoreactivity after incubation with NONOate required dioxygen (data not shown) suggesting the involvement of nitrogen dioxide (\( \cdot \text{NO}_2 \)). The latter can nitrate tyrosine through hydrogen abstraction followed by radical-radical reaction between the tyrosyl radical and a second molecule of \( \cdot \text{NO}_2 \) [242, 243]. To underline the significance of heme catalysis in the nitration of BM3, it was important to prove that the reaction with \( \cdot \text{NO} \) is an artefact which would be unlikely to occur under physiological dioxygen concentrations. With tyrosine as a model compound, we found that the nitration by NONOate strongly depended on the dioxygen concentration in solution and did not proceed under anaerobic conditions (data not shown). Moreover, we detected high relative yields of 3,3'-dityrosine as an indicator
of a radical pathway different from the PN product pattern. Moreover, other products appeared which could not be detected with PN as the nitrating agent.

Potential scavengers for PN were employed in order to study their efficiency to compete with protein nitration. When glutathione, ascorbate and tyrosine were added in a tenfold excess over PN only 30 - 40 % inhibition could be observed whereas 500 µM ebselen and TEMPONE were partially (50 - 70 %) inhibitory and uric acid showed nearly a complete inhibition. However, complete inhibition of nitration could be observed when ascorbate, glutathione or phenol were supplemented with 5 µM horseradish peroxidase. HRP alone had no effect but was also nitrated (see Fig.70 A and B).

For pyrogallol it had been described, that it releases superoxide during autooxidation [51]. Therefore pyrogallol could be another chemical source of superoxide and thus when coupled with a NO-donor, such as diethyl NONOate would form PN. We optimized the pH and temperature, so that fluxes of superoxide from pyrogallol and ·NO from diethyl NONOate were nearly equal (determined by the reduction of cytochrome c by $O_2^-$ and by the ·NO-derived fluorescence of dihydrorhodamine). Employed in our system it turned out, that the behavior of BM3-WT and F87Y was different (see Fig.71): For the wildtype enzyme the PN-scavenging ability of pyrogallol seemed to be more effective than the nitration increase through PN formation and as a consequence the nitration rather decreased in presence of pyrogallol. Thus BM3-WT was nitrated more efficiently by NONOate alone. The F87Y variant gave more intensive signals, when pyrogallol and NONOate were present. Y$_{87}$ and Y$_{334}$ showed a completely different sensitivity for nitration in this system.

To prove the autocatalytic mechanism of the nitration of BM3, two additional experiments were made. The BM3-catalyzed nitration of phenol as well as the
autocatalyzed nitration of BM3 by PN have to be influenced if the active site of
the enzyme was blocked by a substrate. Palmitate was chosen, because saturated
fatty acids are known to react slowly with PN and therefore it was expected that
palmitate shows no effect on the nitration. Phenol nitration was monitored by
the absorption of NP at 400 nm in alkaline solution. Fig.72 shows the decrease
of BM3-WT-catalyzed nitration in dependence of palmitate concentration.
Although no clear concentration dependency was found, the nitration decreased
overall experiments which contained palmitate. This result got more obvious in
the case of the F87Y variant, when higher concentrations of palmitate were used
(see Fig.73). Thus palmitate can block the active site of BM3 and inhibit the
enzyme-catalyzed nitration of phenol.

Figure 72: Palmitate inhibits the BM3-WT-catalyzed nitration of phenol by PN. Reaction
of 5 mM phenol with 800 µM PN in 0.1 M K-phosphate buffer pH 7.4 at RT.

Figure 73: Palmitate inhibits the BM3 F87Y-catalyzed nitration of phe-
nol by PN. Reaction of 5 mM phenol
with 800 µM PN at pH 7.4.

A comparable experiment was done for the autocatalyzed nitration of BM3-
WT and the F87Y variant. Fig.75 shows the decrease of nitration of both,
BM3-WT and F87Y, in dependence of palmitate concentration. Unfortunately
Fig.74 shows different results for BM3-F87Y, which is less nitrated by 10 µM
PN in presence of palmitate. BM3-WT showed no difference in staining in either
presence or absence of palmitate. For both enzymes no difference in nitration by
100 µM PN could be observed between palmitate-containing and -free samples.
However, palmitate is not completely soluble at these concentrations and is prob-
ably present as micells. Therefore it may be that palmitate concentrations varied
from the indicated concentrations and caused the differences.
4.4.3 Localisation of Nitrated Tyrosine Residues

Nitration was quantitatively monitored by HPLC, and nitrotyrosine/nitrotyrosinate was detected at 360/428 nm, respectively. Simultaneous detection at these two wavelengths was possible by postcolumn pH-change of the mobile phase. The PN-treated enzyme was proteolyzed by trypsin and the nitrotyrosine-positive peptides were detected by HPLC (see Fig. 76), collected, and subjected to mass spectrometrical analysis. The area of NT-positive peptide peaks increased linearly with the PN-concentration up to 250 µM and reached a plateau at 500 µM (see insert in Fig. 76). The chromatograms show that in the WT BM3 only one NT-positive peptide could be detected ($t_R = 21$ min). At PN concentrations above 500 µM also other NT-containing peaks accumulated. MS-analysis of the peak with $t_R = 21$ min showed that the WT was nitrated at $Y_{334}$ in the fragment 324-336 ([M + 1] 1464.7 + 45 Da). Mass spectrometric analysis of a complete mixture of proteolytic peptides, not separated by HPLC, indicated the incorporation of a single oxygen atom in the fragments 640 - 657 ([M + 1] 1893.9 + 16 Da), 16-31 ([M + 1] 1795.2 + 16 Da) and 350-362 ([M + 1] 1521.1 + 15.6 Da). As these sequences do not contain F, Y or W, the single incorporation of oxygen might be due to sulfoxidation of $M_{653}$, $M_{30}$ and $M_{354}$, respectively. For F87Y, the HPLC and MS analyses revealed nitration of $Y_{87}$ in the fragment 80 - 94 ([M + 1] 1771.8 + 45 Da) ($t_R = 16$ min) in addition to $Y_{334}$ ($t_R = 21$ min). Another signal ([M + 1] 1968 Da) could be correlated to the mass of the nitrated fragment 420-434 ($Y_{429}$), but since this signal was also observed in samples without PN-treatment, we considered it as an artefact or contamination. More evidence for an artificial signal came from HPLC-analysis, where this nitrated "peptide" could not be detected. Tab. 13 shows that the mass spectrometric analysis of the complete
RESULTS

peptide mixtures, not separated by HPLC, served to monitor ca. 35-40 % of the peptide fragments including several tyrosine-containing fragments which were not nitrated. However, most importantly the nitrated peaks identified by HPLC have been characterized. The sequence of BM3 is shown in Tab.18-20.

Figure 76: Chromatograms of the tryptic digestion of PN-treated WT and F87Y. Enzymes were treated with 250 µM PN at pH 7.4 and digested with 1 µM trypsin. The NT-positive peptides were detected at 360 and 428 nm. (A) 10 µM BM3 WT, the insert shows the area of the tR=21 min peak at 360 nm (solid circles) and 428 nm (open circles) in dependence of PN-concentration. (B) 10 µM BM3 F87Y, the insert shows the area of the tR=16 min peak at 360 (open circles) and 428 nm (solid circles), as well as the area of the tR=21 min peak at 360 (open squares) and 428 nm (solid squares).

4.4.4 Spectral Intermediates During Reactions of P450BM−3 and Chloroperoxidase with Peroxynitrite

The reaction of an excess PN (128 µM) with BM3 WT (1 µM) was studied by rapid-scan UV/vis spectroscopy between 300 and 450 nm at pH 6.8 and 12 °C. As shown in Fig.77, in the first 300 ms of the reaction the characteristic band for native BM3 WT at 418 nm rapidly decreased (trace 2) and concomitantly, a new product with an absorbance maximum around 430 nm was partly formed (trace 5). The spectrum of this species remained unchanged up to about 4 s (trace 6) while the absorbance band of PN at 302 nm decreased (see insert in Fig.77). Finally, when about two thirds of PN had been consumed, the intermediate species decayed back to the iron(III)-form of native BM3 WT, as indicated from the increase in absorbance at 418 nm over the last 20 s (traces 7 and 8). Two exponential equations were needed to fit the first 3 s of the kinetic trace at 435 nm and the following kobs values were obtained: 6.5 ± 0.6 s⁻¹ and 1.29 ± 0.04 s⁻¹. An observed rate constant of 0.084 ± 0.001 s⁻¹ was obtained from the fit of the trace at 435 nm between 10 and 50 s; this
corresponds to the decay rate of the intermediate species back to native BM3 WT.

Two analogous experiments were carried out with different PN and enzyme concentrations, that is 1 μM BM3 WT with 30 μM PN (A) and 2 μM BM3 WT with 128 μM PN (B). Surprisingly, the observed rate constants obtained from the fits of the first 3 s of the traces at 435 nm (7 ± 2 s⁻¹; 1.0 ± 0.1 s⁻¹ (A) and 7.7 ± 0.6 s⁻¹; 1.44 ± 0.02 s⁻¹ (B)) appeared to be independent from the PN as well as the enzyme concentration, and they were almost identical to the values obtained when 1 μM BM3 WT was reacted with 128 μM PN. Nevertheless, because of the relatively large signal to noise ratio of the extracted traces, the uncertainty of the observed rate constants is significant and, thus, small changes in the rates cannot be detected by these data. As expected, the decay rate of the intermediate species back to native BM3 WT, obtained from the fits of the traces at 435 nm between 10 and 50 s (0.077 ± 0.001 s⁻¹ (A) and 0.072 ± 0.01 s⁻¹ (B)), was as well independent from the concentrations of the two reagents.
Figure 77: Rapid-scan UV/vis spectra of the reaction of 1 μM BM3 WT with 128 μM PN in 0.05 M phosphate buffer at pH 6.8, 12 °C. Scans are shown at the following measuring times. 2: 320 ms; 3: 640 ms; 4: 960 ms; 5: 1.60 s; 6: 3.84 s; 7: 15.84 s; 8: 27.84 s. In the inset kinetic traces at 302, 418 and 435 nm. For clarity, 0.015 absorbance units were subtracted to the trace at 302 nm and the traces 1-8 were smoothed.

Figure 78: Rapid-scan UV/vis spectra of the reaction of 2 μM CPO with 30 μM PN in 0.05 M phosphate buffer at pH 6.8, 12 °C. Scans 1-5 represent the formation of CPO Compound II whereas scans 6-9 (bold) show its decay back to native CPO after consumption of PN. Scans are shown at the following measuring times. 2: 16 ms; 3: 32 ms; 4: 48 ms; 5: 128 ms; 6: 1.41 s; 7: 3.65 s; 8: 6.89 s; 9: 27.01 s. In the inset kinetic traces at 302, 397 and 434 nm.

The yield of the intermediate species with the absorbance maximum around 430 nm depended on the PN-concentration: when only 30 μM PN were used, the spectrum of the intermediate showed a significant shoulder at 418 nm which indicated that a large amount of enzyme was still present in its native form. The kinetic traces extracted at 302 nm could not be fitted well to a single exponential equation probably because the spectra of the different enzyme species involved in the reaction also contribute significantly to the absorbance changes at this wavelength. However, qualitatively no significant change was observed for the decay rate of PN in the presence or in the absence of the enzyme.

Rapid-scan kinetic studies of the reaction of the F87Y mutant of BM3 (1 μM) with an excess of PN (30 μM) showed that the yield of this reaction is significantly lower than that of the analogous reaction with the native enzyme (data not shown). Nevertheless, analysis of the kinetic trace at 435 nm gave a result similar to that observed with the WT enzyme. The first 6 s of the trace could be fitted to a two exponential equation that gave the following k_{obs}-values: 8 ± 1 s^{-1} and 0.58 ± 0.01 s^{-1}. The decay rate of the intermediate species, obtained by fitting the trace at 435 nm between 10 and 50 s, was 0.035 ± 0.001 s^{-1}. 

In a similar stopped-flow experiment a solution of 2 \( \mu \)M CPO was reacted with an excess PN at pH 6.8 and 12 °C. As shown in Fig.78, by addition of 30 \( \mu \)M PN the broad absorbance band around 397 nm, arising from the iron(III)-form of native CPO, rapidly disappeared within the first 100 ms of the reaction (traces 1-5). Simultaneously, a new species appeared with two absorbance maxima around 434 and 372 nm, characteristic for the ferryl-form of CPO Compound II [239]. As shown from the inset in Fig.77 as long as significant amounts of PN were present (about 1 s), the spectrum of Compound II remained unchanged whereas a significant decrease in the absorbance was observed at 302 nm. Finally, when most of the PN had been consumed, CPO Compound II slowly decayed back (over 30 s) to the native iron(III)-form, as indicated from the reappearance of the absorbance band at 397 nm (traces 6-9). The first 300 ms of the traces extracted at 397 and 434 nm were fitted to a single exponential expression and \( k_{\text{obs}} \)-values of 30 ± 2 s\(^{-1}\) and 32 ± 3 s\(^{-1}\) were obtained, respectively. From these data, a second order rate constant of about 1 \times 10^6 M\(^{-1}\)s\(^{-1}\) was calculated for the formation of CPO Compound II. Single exponential fits of the two traces between 3 and 30 s, which correspond to the decay of CPO Compound II to the native iron(III)-form, gave values of 0.284 ± 0.001 s\(^{-1}\) and 0.301 ± 0.002 s\(^{-1}\), respectively. As in the reactions with BM3, the kinetic trace extracted at 302 nm could not be fitted well to single exponential equation but qualitatively it could be observed clearly that in the presence of 2 \( \mu \)M CPO PN decayed at a significantly faster rate than under the same conditions in the absence of the enzyme.

4.4.5 Effect of Peroxynitrite on Monooxygenase Activity

BM3 is composed of a single polypeptide chain which includes the FAD-FMN containing reductase domain and the heme-thiolate domain embedded in its active site which accommodates fatty acid substrates that undergo monooxygenations in the presence of NADPH and dioxygen [244]. A convenient assay uses dodecanoic acid p-nitro-phenol ether for the assessment of enzyme activity [214]. BM3 WT activity was lowered by PN in a concentration-dependent way with an IC\(_{50}\)-value of 2.3 ± 1 \( \mu \)M (Fig.79 and 80). Compared to the non-treated control preincubation of 0.1 \( \mu \)M BM3 with 10 \( \mu \)M PN lowered the monooxygenase activity to 16 ± 5 %.

The inactivation of the enzyme could be partially blocked with a tenfold excess relative to the PN-concentration of GSH (92 ± 3 % relative to non-treated
control) and ascorbate (88 ± 2 % relative to non-treated control), but uric acid showed no protection (activity was even less than the non-treated control) (see Fig. 81). In agreement with earlier reports on the BM3 F87Y variant no enzymatic activity was measurable for this enzyme [182]. As the IC\textsubscript{50}-value of 2 - 3 μM obtained for the inactivation was lower than the estimated PN concentration needed for 50 % nitration we considered also other targets for modification besides tyrosine especially since the reductase domain is known to contain essential thiol groups [245]. Indeed, the inactivation could be partly restored by incubation with 1 mM DTT (30-40 % of the not PN-treated control) (see Fig. 81). Also 10 μM ebselen, which is known to form adducts with thiols [232, 233, 236] and to react with an essential thiol group of the NADPH reductase [245], was found to block the enzyme activity completely. It is therefore likely that oxidation of an essential thiol group and not nitration of a tyrosine residue is responsible for enzyme inactivation.
measured by the NADPH-dependent reduction of cytochrome c, the reductase activity was lowered by PN in a concentration-dependent way (see Fig.82). The IC$_{50}$-value for the inactivation of 0.01 µM BM3 by PN was about 1-2 µM and for ebselen it was < 1 µM (Fig.83). Unfortunately, m-chloroperbenzoic acid as an artificial oxene donor did not support enzyme activity so that this hypothesis could not be tested by use of the heme-thiolate domain alone.

![Figure 82](image1.png)  
**Figure 82:** Reductase activity of the BM3 reductase domain in dependence of PN-concentration. Measured by the reduction of 10 µM cytochrome c by 0.01 µM PN-treated BM3 in the presence of 25 µM NADPH at pH 7.4.

![Figure 83](image2.png)  
**Figure 83:** Measurement of the time-dependent reduction of cytochrome c by 0.01 µM BM3 WT, treated with different concentrations of PN, in presence of 200 µM NADPH at pH 7.4.

Furthermore we could prove, that two thiol groups in BM3-WT are oxidized very quickly by PN, in a system were PN was added in a 10-fold excess with respect to the enzyme concentration. We used 2,2’-dithio-bipyridine, since it had some advantages concerning its pH stability and also its reactivity towards SH-groups located at the surface of the enzyme and buried ones [218, 246]. Fig.84 shows the kinetic trace for the reaction of DTBP with BM3-WT, which was either treated or not treated with PN. It is obvious, that the not treated enzyme had fast (thus easily accessible) reacting thiol groups, which were absent in the PN-treated enzyme. By using the extinction coefficient of DTBP we calculated an absorption increase of 0.038 for each free thiol group per 5 µM enzyme. The absorption difference for the traces in Fig.84 was ≈0.07, thus about two thiols per enzyme molecule are oxidized by a ten-fold excess of PN.
4.5 Reaction of P450\textsubscript{CAM} with Peroxynitrite

4.5.1 P450\textsubscript{CAM}-catalyzed Nitration of Phenol by Peroxynitrite

P450\textsubscript{CAM} is probably the most thoroughly investigated P450 enzyme at all. Lots of kinetic, substrate specific, mechanistic and mutagenesis-related studies have been done with this enzyme (for reviews see [247, 248]). According to the known crystal structure and sequence of P450\textsubscript{CAM} some tyrosine residues are located close to the active site [249, 250].

We previously reported about a significant increase of phenol nitration by PN, in the presence of iron complexes, especially of heme- and heme-thiolate-enzymes [116, 163]. Therefore also a nitration increase in reactions of phenol with PN was expected, when CAM was present at catalytic concentrations. Since not only the camphor-containing but also the -free enzyme was available, we expected a significant effect of an empty or occupied active site on the nitration activity.

Tab.14 shows the yields of nitration, hydroxylation and dimerization products from the reaction of phenol with PN in presence and absence of different heme and heme-thiolate proteins. These values were determined for the reaction of 5 mM phenol with 800 \(\mu\)M PN at pH 7 and 7.5, using RP-HPLC. The metallo proteins were present at concentrations of 1-2 \(\mu\)M. As previously shown [116, 163], the nitration increase by 1 \(\mu\)M NOR is much higher compared to that of 2 \(\mu\)M HRP. Whereas 1 \(\mu\)M camphor-free CAM could increase the nitration at pH 7 and 7.5 by 80-90 %, 1 \(\mu\)M camphor-containing CAM did not and yielded values comparable to the protein-free control. The hydroxylation at pH 7 was best suppressed by NOR, followed by camphor-free CAM and HRP. Once more camphor-containing CAM showed the smallest effect. More surprising were the results concerning the dimerization: Normally the increase in phenol nitration is accompanied by an increase in dimerization as well, because phenoxyradicals are the common intermediary species in both reactions, the iron-catalyzed
and proton-assisted pathway. As can be seen in the table, dimerization in the HRP-catalyzed reaction is increased by a factor of about 18, in the case of NOR by a factor of 4, with camphor containing CAM it reaches the value of the control and with the camphor-free form it is even decreased by 30%. It should be noticed that 2,4′-BP is the major dimerization product [130] and was not available as a standard, therefore it was quantified by its relative peak area in HPLC chromatograms. The simultaneous increase in nitration and decrease in dimerization was first observed with this enzyme. Camphor-free CAM seems to be able to increase the nitration by a highly specific mechanism, by which less free radicals are formed or where formed radicals are somehow caged in a more efficient way.

<table>
<thead>
<tr>
<th>Reaction at pH 7</th>
<th>p-NP [μM]</th>
<th>o-NP [μM]</th>
<th>o-OH [μM]</th>
<th>o,p′-BP [A]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16.6±0.1 (2)</td>
<td>24±0.4 (2)</td>
<td>21.2±0.2 (2)</td>
<td>30441</td>
</tr>
<tr>
<td>+ 2 μM HRP</td>
<td>39</td>
<td>57</td>
<td>9.5</td>
<td>543360</td>
</tr>
<tr>
<td>+ 1 μM NOR</td>
<td>52</td>
<td>75</td>
<td>4</td>
<td>114914</td>
</tr>
<tr>
<td>+ 1 μM CAM\text{free}</td>
<td>29.6±2.9 (3)</td>
<td>43.9±4.6 (3)</td>
<td>6.0±0.7 (3)</td>
<td>21625±75 (2)</td>
</tr>
<tr>
<td>+ 1 μM CAM\text{cont}</td>
<td>17.9±2.1 (3)</td>
<td>26.0±2.8 (3)</td>
<td>12.1±2.1 (3)</td>
<td>27650±1300 (2)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reaction at pH 7.5</th>
<th>p-NP [μM]</th>
<th>o-NP [μM]</th>
<th>o-OH [μM]</th>
<th>o,p′-BP [A]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.7±0.2 (4)</td>
<td>13.2±0.6 (4)</td>
<td>8.9±1.4 (4)</td>
<td>23000±400 (2)</td>
</tr>
<tr>
<td>+ 1 μM CAM\text{free}</td>
<td>16.4±5 (2)</td>
<td>24.9±7.1 (2)</td>
<td>8.5±1.3 (2)</td>
<td>20000±3400 (2)</td>
</tr>
<tr>
<td>+ 1 μM CAM\text{cont}</td>
<td>9.3±3.2 (2)</td>
<td>14.0±4.7 (2)</td>
<td>8.3±1.9 (2)</td>
<td>25000±1000 (2)</td>
</tr>
</tbody>
</table>

Table 14: CAM catalysis of phenol nitration by PN. 5 mM phenol were reacted with 800 μM peroxynitrite in 0.1 M potassium phosphate buffer pH 7 and 7.5 at RT.
4.5.2 Autocatalyzed Nitration of P450\textsubscript{CAM} by Peroxynitrite

Since nitration of added phenol is increased, it would be also feasible if the nitration of tyrosyl-residues within the enzyme requires low concentrations of PN. To prove an autocatalytic nitration of CAM we used the Western blot technique and stained with anti-NT as already described for other nitrated P450-enzymes, such as PGI\textsubscript{2} synthase, BM3 and NOR [10, 163, 116]. Fig.85 shows an anti-NT Western blot of camphor-containing and -free CAM samples after treatment with different concentrations of PN. Obviously the free-form shows a clear signal at 50 \( \mu \text{M} \), whereas the camphor-containing-form shows an intense signal only at 250 \( \mu \text{M} \).

![Figure 85: \( \alpha \)-NT Western blot of CAM in the camphor-free and -containing (25 \( \mu \text{M} \) camphor) form after treatment with different concentrations of PN.](image)

Figure 85: \( \alpha \)-NT Western blot of CAM in the camphor-free and -containing (25 \( \mu \text{M} \) camphor) form after treatment with different concentrations of PN.

In another experiment the same samples as taken for these Western blots in Fig.85 were injected to a HPLC-system, optimized for the detection of NT-positive peptides at 428 nm and alkaline pH. Before injection, the enzyme was digested by trypsin. Fig.87 shows the HPLC chromatograms of camphor-free (A) and -containing (B) trypsin-digested CAM after treatment with 50 or 500 \( \mu \text{M} \) PN. The same pattern of NT-positive peaks was found in both cases, except a strong signal at 12.3 min, which only appeared in the camphor-free enzyme. The areas of all peaks (except that one of the porphyrin) increased with increasing PN-concentration and reached a plateau at around 500 \( \mu \text{M} \) PN (see inserts in Fig.87). It should be noted that the chromatograms are shown at different scales and that the areas of all peptide-containing NT-positive peaks were decreased by a factor of 2-3 in the camphor-containing experiments (see inserts). Thus autocatalytic nitration of CAM was hindered by camphor.

![Figure 86: \( \alpha \)-NT Western blot of CAM, nitrated in the presence and absence of 12.5 \( \mu \text{M} \) metyrapone.](image)

Figure 86: \( \alpha \)-NT Western blot of CAM, nitrated in the presence and absence of 12.5 \( \mu \text{M} \) metyrapone.
The peaks were isolated and send for MS-analysis. The following Tab.15 shows the identified tyrosine-containing peptide fragments and if present, the mass-shift due to nitration.

<table>
<thead>
<tr>
<th>R.time [min]</th>
<th>$t_R$=12.3</th>
<th>$t_R$=15.5</th>
<th>$t_R$=18</th>
<th>$t_R$=19.5</th>
<th>$t_R$=22</th>
<th>$t_R$=28</th>
<th>$t_R$=37</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fragment</td>
<td>no peptide</td>
<td>366-372</td>
<td>213-239</td>
<td>179-197</td>
<td>91-109, 300-313</td>
<td>198-211</td>
<td>porphyrin</td>
</tr>
<tr>
<td>Nitrated Y</td>
<td>no peptide</td>
<td>366-372</td>
<td>213-239</td>
<td>179</td>
<td>96 and 305</td>
<td>201 or 203</td>
<td>-</td>
</tr>
<tr>
<td>m/z, q</td>
<td>663.5, +</td>
<td>815.49, +</td>
<td>923.16, 3+</td>
<td>796.83, 3+</td>
<td>1099.5, 2+</td>
<td>565.62, 3+</td>
<td>890.97, 2+</td>
</tr>
</tbody>
</table>

Table 15: Identified NT-positive fragments from trypsin-digested CAM, after treatment with 250 µM PN (R.time=retention time).

For better support of the autocatalytic nitration of CAM we additionally used metyrapone to block the active site. Metyrapone is well known for its tight binding to the heme-iron and an active site located tyrosine in CAM [250, 251]. This inhibitor was already efficient at low concentrations. Fig.86 shows an anti-NT Western blot of CAM after treatment with different concentrations of PN in presence and absence of 12.5 µM metyrapone. Without metyrapone, the nitration of CAM starts at PN concentrations of around 25 µM, whereas in presence of this inhibitor the nitration shows only a faint signal at 50 µM. Also for the higher concentrations of PN the metyrapone-free samples always show a stronger staining compared to the metyrapone-containing one and it clearly turned out, that nitration of the enzyme was decreased by a factor of 2-3 when the active site was blocked. The same effect was observed for the peak area of NT-positive peptides in chromatograms, where the samples used for the Western blot in Fig.86 were injected after digestion with trypsin (see Fig.88). Tab.16
shows the areas of the NT-positive peptides in absence or presence of 50 µM metyrapone. The areas of the Y$_{179}$- and Y$_{201/203}$-containing fragments only are half as high in the metyrapone-containing samples and the peak of the Y$_{96}$- and Y$_{305}$-containing fragments disappeared completely. To ensure that metyrapone at the employed concentrations had no effect on the PN-mediated nitration a comparable Western blot was made for BM3 and no difference could be observed between the nitration of metyrapone-containing and -free samples (see Fig. 89). Furthermore metyrapone had no effect on the PN-mediated nitration of phenol, when employed at these concentrations (not shown).

![Figure 88](chromatograms.png)

**Figure 88:** Chromatograms of trypsin-digested CAM (2.5 µM) after treatment with 250 µM PN in presence and absence of 50 µM metyrapone at pH 7.

![Figure 89](western blot.png)

**Figure 89:** α-NT Western blot of BM3, nitrated in the presence and absence of 12.5 µM metyrapone.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Y179</th>
<th>Y96+305</th>
<th>Y201/203</th>
</tr>
</thead>
<tbody>
<tr>
<td>without metyrap.</td>
<td>25168</td>
<td>72237</td>
<td>10607</td>
</tr>
<tr>
<td>+ 50 µM metyrap.</td>
<td>12895</td>
<td>-</td>
<td>5927</td>
</tr>
</tbody>
</table>

**Table 16:** Area of NT-positive peaks of digested CAM, nitrated in absence and presence of 50 µM metyrapone.

4.5.3 Camphor-binding and CO-binding of Native and PN-treated P450$_{\text{CAM}}$

It had been reported that PN-mediated nitration of a tyrosyl-residue or oxidation of essential thiols, or even sulfoxidation of methionine could inactivate enzymes [11, 147]. Camphor has no characteristic absorbance, therefore it is hardly possible to develop photometric activity assays for CAM. But since we found
a PN-mediated nitration of Y$_{96}$, which is known to be also binding site for camphor [249], we expected also a modulation of the camphor binding capacity. Therefore we investigated the binding of camphor to the enzyme. The camphor-free enzyme has an absorption maximum at 418 nm, the camphor-containing enzyme-substrate complex at around 390 nm [247]. **Fig.90**A, B and C shows the spectra of titration of camphor-free CAM with camphor, after treatment with 0, 50 and 250 µM PN. It is obvious that the PN-treated enzyme could interact with camphor less efficiently than the non-treated one. 250 µM PN completely abolished the substrate binding spectrum (C).

![Figure 90](image)

**Figure 90**: Camphor-binding spectra of 2.5 µM CAM after treatment with 0 (A), 50 (B) or 250 (C) µM PN at pH 7.5. Camphor was added stepwise to give final concentrations of 1-1000 µM.

CO-binding spectra of dithionite-reduced PN-treated and non-treated enzyme showed a significant decrease of the 450 nm band in dependence of the PN concentration (see **Fig.91**). The characteristic band of reduced CO-bound P450 enzymes at 450 nm was completely formed in the non-treated enzyme and decreased after preincubation with 50 and 250 µM PN. Therefore a loss in activity could have been assigned neither exclusively to the nitration of Y$_{96}$ and a resulting decreased camphor affinity nor to the oxidation of the heme-thiolate moiety and the resulting altered ability to form the necessary Compound I and II species.

![Figure 91](image)

**Figure 91**: CO-binding spectra of 2.1 µM CAM (substrate-free) after treatment with 0, 50 and 250 µM PN at pH 7.5. Enzyme was first treated with PN, then 250 µM CO were added followed by dithionite to reduce the ferric heme. After these steps the characteristic CO-binding spectrum at around 450 nm appeared.
4.5.4 Kinetic Measurements of the Reaction of P450$_{\text{CAM}}$ with PN

For NOR [163], BM3 and CPO [111] we observed a red-shifted Soret band during reactions with PN and in the case of CPO this species could directly be assigned to the heme-thiolate ferryl band. Stopped-flow measurements of camphor-free CAM revealed the existence of a similar band as in the cases of NOR, BM3 and CPO, suggesting a common mechanism of all P450 enzymes in reactions with PN. This would also explain the low required concentrations for autocatalyzed nitration in these enzymes if a tyrosine residue is nearby the heme-iron [11, 111, 252], the high efficiency in catalyzing the nitration of added phenol [116, 163, 252] and the high turnover in PN decomposition kinetics [116, 163, 252].

Fig. 92 and 93 show the stopped-flow spectra of the camphor-free and -containing CAM. In absence of camphor it seems at a first sight as there would be no reaction taking place, but in the insert, which shows the traces at 417 (Soret band of native or P420 enzyme), 422 (isosbestic point) and 435 nm (intermediate band), one can see a very fast increase of an intermediate at around 433-436 nm followed by a slower decrease of this species (Fig. 92). The formation of the intermediate reaches a maximum after 1000 ms, whereas its decrease was completed after 15000 ms (see insert). The kinetic features and the absorbance maximum of this intermediate seem to be comparable to that ones which were found for the other P450 enzymes. Also the low yield of this intermediate seems feasible regarding the nearby tyrosine which probably trapps the ferryl species as it was postulated for the F87Y variant of BM3 [111].

In presence of camphor no such species could be observed. Also a red-shifted band increased, as invisisaged by the formation of the shoulder at 415-418 nm (Fig. 93). But since this species was stable for at least 20 s (see insert of kinetic traces) we interpreted it as the oxidized P420-form of the enzyme. It might be that also in this case a ferryl intermediate is formed, but is too unstable to be observed. Similar to other P450 enzymes camphor-free CAM was highly efficient in catalyzing the decomposition of PN at alkaline pH, but showed no effect in the presence of camphor (Fig. 94).
Figure 92: Stopped-flow spectra of 1 μM CAM (substrate-free) with 300 μM PN in 0.1 M KPi, pH 6.5 at 20 °C. Spectra were taken after 10, 100, 500, 1000, 5000 and 15000 ms. The insert shows the traces at 417, 422 and 435 nm.

Figure 93: Stopped-flow spectra of 2 μM CAM (substrate-bound) with 200 μM PN at pH 6.5 and 20 °C. Spectra were taken after 10, 100, 1000, 5000 and 15000 ms. The insert shows the traces at 390, 403 and 416 nm.

Figure 94: Decomposition of 150 μM PN in 0.1 M KPi at pH 8 and 10 °C in the presence of 1 μM NOR, 1 μM CAM (free), 1 μM CAM (cont.) or 4 μM HRP.
5 Discussion

5.1 The Role of Phenoxy Radicals in Reactions of Peroxynitrite with Phenolic Compounds

The results of our investigation clarify the sequence of events starting from the protonation of PN and leading to the nitrosylation, nitration and hydroxylation of aromatic compounds. Especially the nitration mechanism of PGIS with PN could be characterized by using phenol as a model. Our preparation of PN contained nitrite but its contribution to the nitration and nitrosylation reactions could be well separated from those mediated by PN. Nitration and nitrosylation of phenol by nitrite in the acidic range occurred parallel to the protonation to nitrous acid (pK$_a$=3.5) and therefore could involve a polar mechanism via [H$_2$ON=O]$^+$ or "NO"$^+$. Since also ·NO$_2$ must be formed (or N$_2$O$_3$ as the reaction product with ·NO [77]) the system is able to perform nitrosylations as well as nitratations [128, 145]. Since such reactions were not observed with anisol it is likely that phenoxy radicals are first formed by ·NO and/or ·NO$_2$ and that subsequently an addition of these radicals to the ring system followed [243, 254].

The nitrosylation with PN in the alkaline pH-range could not be due to nitrite formation but rather to the reaction of the protonated PN (peroxynitrous acid) with a second molecule of PN [9, 61]. This reaction yields dioxygen and two moles of nitrite but since nitrite at higher pH-values can neither nitrate nor nitrosylate phenol, the reactive intermediates must be ·NO and ·NO$_2$ or N$_2$O$_3$ as already postulated [61] or could even originate directly from a postulated HOON=O/-OO-N=O dimer [9]. However, the mechanism of phenol nitrosylation was not a central part of our study.

![Figure 95: The major reactions taking place in a system that consists of phenol and PN.](image-url)
At pH-values below 7 the protonation equilibrium keeps PN in its acid form and the disproportionation will be suppressed in favor of the isomerization to nitrate. Indeed nitrate formation is the only reaction occurring under acidic conditions [61] which could be a consequence of having no PN available to form an HOONO/−OON=O adduct. If one describes the postulated electronically activated form of the trans-peroxynitrous acid as a triplet state then it becomes feasible that before spin conversion the excited state has time to react with organic molecules as observed repeatedly [62, 65, 90]. In the case of phenol the hydroxylated and nitrated derivatives are formed, whereas with anisol the nitro derivatives were virtually absent (1% as compared to phenol). If one takes into account a high amount of dimerization products which only can be explained via phenoxyradical formation, it is evident that nitration would occur as a consequence of addition of ·NO₂ to the previously formed phenoxyradical. From the fact that phenol can be nitrated with ·NO₂ alone it is evident that from the excited state intermediate not only the OH-radical part but also the remaining NO₂-radical can convert phenol to its phenoxyradical. This would explain that with higher phenol concentrations a dramatic shift to the dimerization products was observed. The dimerization products shown in Fig.96 may be formed via a sandwich intermediate of two phenoxyradicals as postulated elsewhere [196, 255]. This intermediate leads to a characteristic dimerization product pattern with low yields of 4,4′-biphenol and high yields of 2,4′-biphenol [196, 256]. Products in brackets could not be identified, but probably are formed as observed in other reactions involving phenoxyradicals [256]. We also detected additional peaks with retention times that would fit to those expected for 2-phenoxy-phenol and diphenyl-peroxide. Other hypothetical products potentially could be formed from the addition of ·NO, ·NO₂ or ·OH to an oxygen-centered phenoxyradical leading to the highly unstable benzene-nitrite, benzene-nitrate and benzene-hydroperoxide. The major products 2-,4-hydroxy- and nitro-phenols [62, 145] are formed as discussed below. p-benzoquinone probably is an oxidation product of hydroquinone and 4-nitro-catechol a nitration product of catechol as postulated for the reaction of salicylate with PN [65].

This sequence of reactions with ·NO₂ and ·OH radicals forming phenoxyradicals also explains why an excess of phenol leads to lower yields of nitro-phenols and to higher amounts of dimerization products. It is worth mentioning that the nitration of phenol has a second maximum between pH 9 and 12 with only low activities at pH 8. We suggest that beyond the pKₐ of phenol at ≈10 increasing phenolate anion formation must occur and this would lead to a facilitated
abstraction of an electron and hence to higher yields of phenoxyradicals. Alternatively, the ·NO₂ radical could add directly to the highly activated phenolate ring. It should be noted that at pH 11 PN is stable for longer than 5 min, allowing incubations for a longer time period in order to have all PN reacted.

The most striking support for a phenoxyradical intermediate is the lack of nitration with anisol. The methoxy group has similar electronic effects as the hydroxy group and if nitration would occur as an addition of ·NO₂ to the aromatic ring followed by subsequent one-electron oxidation it should also take place with anisol. In contrast, the hydroxylation of anisol was comparable to phenol hydroxylation by PN suggesting that the hydroxyl radical is strong enough for an addition to the aromatic ring (see Fig.97). Whether the ·NO₂ radical is able to cause the rearomatization or whether an excited state of peroxynitrous acid is required cannot be answered from our present experiments.

Physiological concentrations of PN are normally in the nanomolar range, only under pathophysiological conditions it can reach the micromolar range [5, 63].
Therefore systems have been developed to simulate these physiological concentrations. Low fluxes of PN can be produced from SIN-1 or by coupling superoxide sources, e.g. xanthine oxidase/xanthine with NO-donors such as NONOates [22, 243]. But at physiological fluxes of PN we have the same situation as shown in Fig. 22. Decreasing PN concentration has the same effect as increasing phenol or tyrosine concentration. Due to the large excess of tyrosine (1 mM) the probability for PN (10-20 nmol/min) to react two times with one tyrosine molecule or for a ·NO_2 radical to meet a tyrosyl radical is small. Instead the reaction of two tyrosyl radicals to form 3,3'-dityrosine is preferred as shown in Fig. 29,30 [242]. Hence protein-bound tyrosine is not likely to dimerize, 3,3'-DT containing proteins are rare in vivo, nitration is more likely [11, 118, 257].

5.2 The Nature of Peroxynitrite-mediated Reactions and the Requirements for Potent Inhibitors

Our different test systems were examples for the double sided nature of PN-mediated reactions. As already pointed out in the short introduction for this topic, PN can react either by 1e-oxidations (e.g. nitration and hydroxylation of phenolic compounds) or by 2e-oxidations (e.g. sulfoxidation). 1e-oxidations are rather mediated by the protonated form of PN (ONOOH) or its activated form ONOOH^*, which seems to possess similar reactivity as the radical cage of [ONO· ·OH], in which each of the radicals can abstract an electron from a target molecule [75]. 2e-oxidations are mostly mediated by the anionic form of PN (ONOO^-) via oxene transfer, but also require more specific targets, e.g. thiols, thioethers, selenium compounds or metals which can stabilize oxo-complexes (e.g. Mn^{III} to Mn=O^V) [84, 86, 50]. The 2e-oxidation formally follows a heterolytic cleavage of the OO-bond in PN and this would yield nitrite as a byproduct in such reactions. Indeed, increased levels of nitrite could be found in reactions of ebselen with PN (result was not shown). But it is known that there is no clear cut reaction mechanism, neither for the protonated nor for the anionic form. This means, if the reaction of PN-anion with the target is too slow, then dimerization of PN will yield intermediates which are also able to
perform 1e-oxidations. On the other hand ONOOH has been shown to yield same products with thiols as ONOO$^-$, maybe via two 1e-oxidation steps.

Since ADH reacts with ONOO$^-$ in a very fast reaction with $10^5$ M$^{-1}$s$^{-1}$ [94], we could be certain that it was the anion which reacted at pH 7.6 in our system. Therefore it could be expected that only PN-anion scavengers would be protective. In the phenol or BSA test system it had to be the protonated form that mediated nitration and hydroxylation of phenolic compounds, since we also could find dimerization products and other indications for involvement of radicals in these reactions. The pattern of scavengers for each of the test systems allowed a clear structure-activity relationship: Thiols (cysteine, GSH, DTT,...) react with both, ONOOH and ONOO$^-$ and therefore provided protection from PN-mediated oxidations in all systems. Therefore thiols also could be the best PN-scavengers in biological systems. Of course, there were more specific inhibitors for either 1e- or 2e-oxidation, but no other compounds were as much effective in both systems. Other known antioxidants such as ascorbate, tocopherol were highly efficient in the 1e-oxidation, but not in ADH-oxidation. This went hand in hand with the results from the kinetic measurements. Ebselen as the best candidate for the protection of ADH and scavenging of PN-anion, failed in both systems. As we could show, ebselen forms easily adducts with thiols and in this form has lost most of its reactivity towards the PN-anion. In addition, ebselen has been reported to inhibit several thiol-dependent enzymes [258, 259, 260] and we observed this effect also for ADH. For this reason we could not test ebselen in the ADH system. In the BSA system we had the same problems, since it is known that ebselen binds to thiols in BSA [233]. In the phenol system ebselen showed also weak protection, but this time it was probably due to its fast reaction with PN. This means, that after addition of 400 µM PN to a reaction solution containing 200 µM ebselen half of the amount will immediately react with ebselen to form ebselenoxide, but the remaining 200 µM PN will perform oxidations. The results from our studies can be summarized by the following scheme (Fig.98).

Uric acid was one of the most surprising test compounds. Except TEMPO and TEMPONE it was the only scavenger which was highly effective in the phenol and BSA system and showed no protection in the ADH system. The low IC$_{50}$-values for phenol and BSA nitration implied a high specificity for the active form of PN in these systems. When 800 µM PN react with 5 mM phenol the yield of nitration, hydroxylation and dimerization products is about
10-20 % with respect to PN concentration. This indicates that only 10-20 % of the PN reach the active state and perform these reactions. The IC$_{50}$ values of UA and its dimethyl derivatives were about 4-6 % with respect to PN concentration. Such low half-inhibitory concentrations require UA to react several times with ONOOH*, suggesting that UA forms radical intermediates in a first reaction step which react further with ONOOH*. Other compounds which were structurally related to UA provided only poor protection in this system (adenosine, guanosine, xanthine, hypoxanthine, caffeine, alloxan and allantoin), leading to the conclusion that the aromaticity of the imidazole ring had to be interrupted as it is the case in UA by the amide-function formed by N$^7$, N$^9$ and the carbonyl-group at position 8. There is no other difference between xanthine and UA except the 8-oxo-group. However the lack of reactivity of xanthine towards PN could be eliminated by exchange of the oxo-groups at position 2 and 6 against thio-groups. 2,6-dithio-purine as well as 2,6-dithio-pyrimidine were highly effective in all systems, suggesting that the introduced sulfur performed similar reactions as a thioether. TEMPO and TEMPONE showed the highest activity of all compounds in the phenol system. But they only suppressed the formation of nitro- and hydroxy-products by catalyzing the nitrosation of phenol and therefore are no useful scavengers for phenol oxidation, but only modified the product pattern, as already reported [106].

We could separate five major products from the reaction of phenol with PN. Three of them were formed at pH 6 and 7, two of them decreased and one in-
creased at alkaline pH. At pH 8 and 9 two new products appeared, one of them was not stable and formed another new product when standing for longer times. These products were isolated from HPLC and investigated by MS- and NMR-analysis, but no results could be obtained from these measurements. These observations were in agreement with detected radical intermediates during reactions of UA with PN and poor stability of oxidized products [4, 230].

5.2.1 Pitfalls when Using Ebselen as a Scavenger in Biological Systems

There are several aspects of the results presented. First, the unexpected lack of protection by ebselen of PG12 synthase nitration and inhibition has found an explanation which is consistent with the high reactivity of ebselen with thiol compounds [236, 232, 233]. Given to in vitro preparations of aortic rings ebselen is present almost exclusively as its adducts to thiols. Due to its high concentration of about 5 mM in cells GSH is a favorite reactant but some protein-bound thiols/thiolates can even be more nucleophilic and therefore could also form adducts.

![Chemistry of the system ebselen, glutathione and peroxynitrite.](image)

Our results can be summarized by Fig.99, which also includes the known complex chemistry, ebselen can perform under physiological conditions. These numerous reactions and equilibria, that take place in this system are also the reason why we could not determine a kinetic constant for the reaction of PN with ebselen-GSH adduct. There are several species absorbing in the same wavelength region and thus complicating such kinetic measurements, but at least one can qualitatively see a much slower reaction compared with ebselen itself (see Fig.47 and 48). As shown in Fig.99 GSH also reacts with PN (6), as already described [144]. So it is impossible to distinguish in the kinetics between effects which originate from ebselen, GSH or ebselen-GSH adduct. We could not
determine, whether the reactivity towards PN in a ebselen and GSH containing system comes from the small amount of "free" ebselen (1) which is formed by a slow equilibrium from the adduct (3) (if so it must be very slow, or else one would not get a peak for the adduct in HPLC), or whether it comes directly from the adduct. The latter would mean that a reaction from the adduct (3) to the ebselenoxide (2) is possible, but then the question is what happens to the ebselen-bound GSH? Ebselenoxide (2) can be converted back to ebselen (1) by two molecules of GSH [144]. Another possibility is the conversion of the adduct (3) to the corresponding selenol (4) by one molecule of GSH followed by the reaction with another molecule of ebselen to give the diselenide (5) [232]. Of course also (4) and (5) may react with PN.

So the mechanism of ebselen inactivation towards PN is complex, but this reaction scheme fits to the observations made in this study: ebselen binds to thiols and forms isolatable adducts. These systems show a decreased reactivity towards PN-anion, at least comparable to that of other tested seleno-compounds such as Se-methionine [85, 86]. Therefore the loss of reactivity of ebselen towards PN in biological systems seems to be due to this thiol-binding, hence in the absence of any thiols as shown after preincubation with Ellman’s reagent ebselen can well compete with microsomal PGI<sub>2</sub> synthase for PN. Although the ebselen adducts reacted too slow to protect PGI<sub>2</sub> synthase they could interact with PN and therefore at least could still function as antioxidants in cells. Therefore the free radical nitration of phenol could be inhibited. In the context of PN nitration of PGI<sub>2</sub> synthase the results lend support to our mechanistic proposal that PN reacts as its anion directly at the ferric heme and that the nitrated tyrosine must be at the active site as judged from the protection against nitration by quasi-substrates blocking the substrate binding site [12]. Thus, by kinetic and steric reasons none of the known antioxidants was able to interfere with this process. In case of an initially assumed inhibitory action of ebselen it might have been possible to decide whether the nitration of PGI<sub>2</sub> synthase, which now has been identified to occur during atherosclerosis [173] and endotoxin exposure of aortic rings [167] is only a pathological and damaging process or whether the simultaneous trapping of ·NO and PGI<sub>2</sub> by superoxide generation is a physiological reaction required for host defense and cellular activation.
5.3 Spectral Intermediates During Reaction of PN with Transition-metals and Their Effects on PN-decomposition and PN-mediated Nitration

The experimental design of our study allows to conclude on a catalytic role of ferric complexes in the decomposition of PN. Not only the decrease of the absorbance of PN at 302 nm was enhanced but also the kinetics of dioxygen formation as a consequence of PN disproportionation were increased to a similar extent [163]. Since a molar ratio of Fe(III) complex to PN in the range of 1 : 100 was used a catalytic cycle must have been involved. Repetitive additions of PN to NOR resulted in the same kinetics and indicated that true catalysis took place. The catalytic cycle starts with a reaction of the ferric complex with the PN anions since the pH-dependence indicates a higher efficiency of iron catalysis compared to the metal-free decomposition at pH-values above the pK of PN. MP-11, NOR and other heme thiolate proteins and a HRP-ascorbate coupled system were the best candidates to promote the catalytic decomposition of PN with NOR and CPO exhibiting even higher turnover numbers than MP-11. Catalase and MPO were inactive [116, 163].

The resulting species from the interaction of NOR with PN absorbs at 435 nm and is formed in a rapid reaction with a fully developed spectrum already 80 ms after mixing [163]. Its subsequent decomposition back to the ferric NOR in presence of an excess of PN occurs with a half-life time of 2.9 s which is in the same range as the kinetics of the decrease of PN measured at 302 nm or the increase in dioxygen formation (it has to be mentioned that there are minor differences in pH and temperature of these different assays) [163]. From this one can conclude that the second reaction step involving the decay of the primary product represents the rate-limiting step in the cycle. Concerning the nature of the primary intermediate one can assume a higher oxidation state of the heme iron which could be either the ferryl (Fe^{IV}=O) or perferryl (Fe^{V}=O). For NOR or other P450 enzymes the exact absorbance spectra of such species is not known, but for HRP the observed Soret absorbance (shift from 398 to 418 nm in a difference spectrum) has been reported to correspond to a ferryl complex [102]. Thus the first reaction would result in a ferryl species in addition to the ·NO₂ radical [175]. In the subsequent and rate-limiting reaction the ferryl complex can oxidize a second PN molecule resulting in dioxygen and ·NO formation the latter combining with the remaining ·NO₂ to N₂O₃. For HRP this is a slow reaction. For MP-11 it becomes faster and for NOR and
CPO it proved to be a very fast process determining the efficacy to decompose PN.

Alternatively to this 1e-oxidation of PN an electronic rearrangement between the ferryl species and the ·NO₂ radical could give nitrate and the ferric complex as described for hemoglobin [98] and metal porphyrins [104, 238] at nearly equal concentrations of PN to metal porphyrin. Both pathways seem to be functional since no significant change in the nitrite/nitrate ratio by NOR or MP-11 with PN ratios of 1 : 100 was observed. This, however, must be a function of PN concentration since at a low PN : enzyme ratio the disproportionation becomes less likely.

The postulate of a ferryl complex was fully compatible with the observed reactivity of phenol in the system and earlier reports about increased nitrination in presence of metal-catalysts [174, 152]. The increase of nitro- and dimerization-products allow to conclude on phenoxy radicals as intermediates. It was important that unlike the metal-free decomposition of PN no hydroxylated phenols were formed in the presence of ferric complexes which agrees with the formation of a ferryl species instead of a quasi-OH radical species derived from the protonated PN (see Fig.100). If the ferryl-nitrogen dioxide pair could react with phenol as well as with PN then a competition between both reaction pathways could be expected and indeed was found. Hence the following reaction cycle meets all experimental data (Fig.101). By comparison with the mechanism of the proton-catalyzed pathway of PN action (see Fig.100) it is evident that the ferryl ion substitutes for the OH-radical-like species which explains the loss of hydroxylating capability but the retention of the nitrating power.

This would explain that the yield of nitration parallels the decay kinetics since during the lifetime of PN more can be diverted to the ferryl-nitrogen dioxide pathway instead to the proton-catalyzed pathway. A reason for this higher reactivity of the P450’s towards PN may be related to the nature of the iron-thiolate structure which by having a partial covalent character could enhance the reactivity of the oxo-species by preventing back-bonding as postulated for the iron-oxene complex Fe⁵=0 ↔ Fe⁴-O· [191]. Thus, the thiolate ligand not only influences the reactivity of the oxene complex (Compound I) in monooxygenases, but also of the ferryl complex (Compound II) (see equations 55 and 56) which so far has not yet been clearly identified with P450 enzymes, but UV/Vis and resonance Raman spectras exist for CPO Compound II [239, 253].
Figure 100: Reaction pathways of PN in metal-free systems.

\[
\begin{align*}
S^-\text{Fe}^{V} = O & \leftrightarrow S - \text{Fe}^{V} - O^- \leftrightarrow S \cdot \text{Fe}^{IV} = O \leftrightarrow S - \text{Fe}^{IV} - O^- \\
S^-\text{Fe}^{IV} = O & \leftrightarrow S - \text{Fe}^{IV} - O^- \leftrightarrow S \cdot \text{Fe}^{III} = O \leftrightarrow S - \text{Fe}^{III} - O^- 
\end{align*}
\] (55) (56)

The implications of this mechanism for the biological significance of PN reactivity are severalfold. Similar metal-catalyzed reactions may be responsible for the Mn-SOD nitration in vivo [118] which now might be explained in an analogous way by assuming a higher valence state of manganese (Mn\textsuperscript{V}=O). This complex might abstract a hydrogen atom from the phenolic group, and a NO\textsubscript{2}-radical for the subsequent nitration reaction. Manganese complexes have been investigated

Figure 101: Reaction pathways of PN in metal-containing systems.
which also show the catalysis of PN conversion to nitrate [50]. After completion of this work Lee et al. [103, 164] have also obtained evidence for the formation of a ferryl nitrogen dioxide complex with the iron porphyrin Fe(III)TMPyP.

5.4 Reactions of the Monooxygenase P450BM−3 with Peroxynitrite

5.4.1 Metal-catalyzed Nitration of Protein-bound Tyrosines and Inactivation of P450BM−3

The results presented in this work extend our previous study of a the heme-catalyzed nitration of tyrosine residues by PN [116]. Unlike the heme-thiolate protein NOR which can catalyze the nitration of added phenol, but not of its own tyrosine residues [163], BM3 is able to catalyze nitration of Y334 at the entry of the substrate channel, 19.8 Å distant from the heme-iron center as shown in the crystal structure displayed in Fig.102 [111]. Since this autcatalytic nitration proceeds with micromolar concentrations of PN, it nicely mimics the autocatalytic nitration of the heme-thiolate enzyme PGI2-synthase [11]. The proposed sequence of events is initiated by the formation of a ferryl intermediate and the rather stable ·NO2 radical although the semantic problem regarding the detailed mechanism of reactions of PN with P450 enzymes could not yet be completely established and shall be addressed later in more detail. Overall, a one-electron transfer or H-atom abstraction from a tyrosine residue to PN generates ·OH- and a tyrosyl radical which adds ·NO2 to give the nitrated tyrosine in position 334 of the WT enzyme. The F87Y variant contains an additional tyrosine residue located close to the active site (6.5 Å distant from the heme-iron center, see Fig.102). The additional potentially competitive nitration at this tyrosine residue confirmed the requirement that the nitration target is located adjacent to the active site. However there are some other tyrosines within the range of 20 Å of the active site, which were not found to be nitrated. This means, that the nitrated tyrosines either are activated for nitration by PN by neighboured amino acids (e.g. aspartate or glutamate which could create a partial tyrosinate), or are easily accessible for reactive species which are formed at the iron through tunnels which cannot be seen in the X-ray structure.

Fig.103 describes the underlying mechanism of the monooxygenase activity test we used in this study [214]. Surprisingly, the nitration of tyrosine is of little consequence for the activity of BM3, quite in contrast to PGI2-synthase where
tyrosine nitration is the predominant enzyme-inactivating modification. However, as judged from the peak areas in chromatograms of trypsin-degraded BM3 WT incubated with different concentrations of PN, the observed $IC_{50}$-value for inactivation of 2-3 $\mu$M PN was too low to yield significant nitration. We therefore consider the possibility of additional oxidation of an essential sulfhydryl group. Indeed, the reductase domain contains a thiol important for the activity, in agreement with the observed inhibition by ebselen [245]. PN-dependent thiol modification is a well-known phenomenon [83, 137], and the fact that enzyme activity could be partly restored by incubation with DTT at concentrations which do not reduce 3-NT suggests PN-dependent thiol modification of BM3. Furthermore the activity of the reductase in the NADPH-dependent reduction of cytochrome c is inhibited by PN in a concentration-dependent way similar to what was observed for the inactivation of the monooxygenase activity. Furthermore, we could show by using the thiol-group determination reagent DTBP [218], that 2 thiol-groups per enzyme molecule were oxidized at 10-fold molar excess of PN with respect to
the enzyme. This result supports the hypothesis that oxidation of a thiol group rather than tyrosine nitration is responsible for enzyme inactivation.

Figure 103: Underlying mechanism for the monooxygenase activity assay of BM3.

5.4.2 Nitration of Tyrosines under Biological Conditions

Apart from the special significance for heme-thiolate proteins our results allow to address the controversial problem of PN-mediated tyrosine nitration in vivo. Pfeiffer and Mayer [22] questioned the biological relevance of PN-mediated tyrosine nitration, since in their experiments a simultaneous generation of superoxide and ·NO did not lead to a higher nitration of free tyrosine as ·NO alone. This was probably due to high yields of 3,3'-DT, which was described to be the major product in reactions of Tyr with ·NO₂ [242]. Here, we clearly show that ·NO and O₂⁻, simultaneously generated by SIN-1 or a XO/NONOate system, do nitrate tyrosine (Fig.104 explains the mechanism by which SIN-1 forms PN and NONOate forms ·NO). This nitration could be suppressed by Cu,Zn-SOD to the levels obtained with ·NO alone from spermine NONOate. The nitration by ·NO was significantly lower and depended on the oxygen concentration. Meanwhile the experiments of Pfeiffer and Mayer have been extended and the results were corrected with respect to the high yields of 3,3'-DT and to errors in their experimental setup [243].

Two arguments can be brought up to explain the reasons for the discrepancy between the results with free tyrosine and those obtained in our enzymatic systems. First, if an excess of tyrosine reacts with PN, the intermediate ·NO₂ radical can competitively react with tyrosine to form another tyrosyl radical fol-
Figure 104: Mechanism for the formation of PN by SIN-1 and of \( \cdot \text{NO} \) by NONOate.

ollowed by dimerization to 3,3’-dityrosine [242]. Second, the presence of the ferric heme acts as an efficient catalyst for the nitration according to mechanisms established previously [163]. The same applies for other metallo-enzymes like P450<sub>2B1</sub> [123], Mn-SOD [118] and the copper ions in tyrosine hydroxylase and Cu,Zn-SOD [113, 120]. Thus, autocatalysis by enzyme-bound transition metals results in a higher specificity of PN under physiological conditions. However, other non-transition metal containing proteins such as the SR Ca-ATPase have also been nitrated with high yields in vitro by both systems, PN and various concentrations of SIN-1 [122, 149].

### 5.4.3 Spectral Intermediates During Reactions of P450 Enzymes and PN

Stopped-flow studies of the reaction between an excess PN and BM3 at neutral pH indicate that this enzyme is not an efficient catalyst for the decomposition of peroxynitrous acid (ONOOH) as there is no significant difference in the PN decay rate in the presence or in the absence of the enzyme. In contrast, when less than 1 % CPO is reacted with PN at neutral pH the half-time for the disappearance of the characteristic absorbance at 302 nm is about one third of that measured under the same conditions but in the absence of CPO. Although both enzymes increased the velocity of PN-decomposition at pH 8 and 9 [116, 163]. The rapid reaction of CPO with PN yields the ferryl, iron(IV)-form of CPO (Compound II) [239]. This reaction is likely to proceed via a one-electron oxidation of native CPO by PN to Compound II and nitrogen dioxide, as already proposed for the reaction of PN with NOR [163]. However, it cannot be excluded that, in a first step, PN reacts with native CPO to the two-electron oxidized species Compound I and nitrite, which then react very rapidly to the observed Compound II and \( \cdot \text{NO}_2 \). CPO Compound I is partly formed when the enzyme is reacted with an excess of \( \text{H}_2\text{O}_2 \) and decays back to native CPO with an observed rate constant of about 0.3 s<sup>−1</sup> [261].
The mechanism of the reaction of PN with BM3 is more complex as two exponential equations are needed to fit the partial build-up of an intermediate species with an absorbance maximum around 430 nm. In analogy to the reaction of PN with CPO as well as with NOR and as the 430 nm absorbance band is comparable to that of ferryl forms of other heme proteins, this species is assigned as Compound II of BM3. The two observed reactions could represent the formation of Compound I and \( \cdot \text{NO}_2 \) from the two-electron oxidation of native iron(III) BM3 and the following fast reaction to yield the observed Compound II and \( \cdot \text{NO}_2 \). Surprisingly, the observed rate constants for both reactions do not seem to depend on the PN-concentration. However, because of the small changes in the absorbance and the relatively large signal to noise ratio small changes can probably not be detected in the measured data.

In both systems, as long as large amounts of PN are present the spectrum of Compound II remains unchanged: This observation suggests a catalytic reaction which involves PN and has the reaction of the ferryl ion with PN as the rate-limiting step. The different amounts of Compound II formed in the two systems might indicate two different reaction mechanisms. With CPO, in analogy to the iron(III)-porphyrin complexes [103], Compound II may catalyze the isomerization of PN to nitrate. This would explain the quantitative yield and the persistence of the characteristic absorbance of Compound II in the presence of PN. With BM3 and in particular with the F87Y mutant the ferryl-species might rapidly react with a close tyrosine residue via hydrogen atom abstraction to yield the tyrosyl radical and the iron(III)-form of the proteins. This reaction is likely to be faster with the F87Y mutant as the inserted tyrosine residue is closer to the heme. The tyrosyl radical then reacts rapidly with \( \cdot \text{NO}_2 \) to nitrotyrosine. In agreement with the nitration results presented above, the stopped-flow data suggest that BM3 and its F87Y mutant do not accelerate the isomerization of PN at neutral pH but catalyze the nitration of a tyrosine residue. As the active species in this catalytic cycle is the iron(III)-form, this mechanism would explain the low yields of Compound II, in particular with the F87Y mutant.

In summary, the reaction of BM3 with PN not only resulted in a valid model for PGI\(_2\) synthase nitration, but also clarified other open questions in the biology of PN. Future projects will include a further characterization of the ferryl intermediate by Resonance Raman spectroscopy, as well as the identification of tyrosyl radicals by ESR as intermediates during ferryl formation in P450 enzymes.
5.5 Reactions of Peroxynitrite and Cytochrome P450\textsubscript{CAM}

All P450 enzymes (including P450\textsubscript{CAM}) tested in our studies increased the yield of o, and p-nitro-phenol in the PN-mediated phenol nitration (see Tab.14). In the presence of substrate-free CAM the yields of nitrated phenols almost doubled compared to the control experiments at both pH values. The yield of hydroxylation to catechol decreased sharply at pH 7 but remained unaffected at pH 7.5. The total amount of o,p'-BP formed is significantly lower, compared to the experiments with HRP and NOR. The reason is not yet clear. It is conceivable that HRP essentially behaves as a peroxidase and effectively catalyzes the one-electron oxidation of phenol in agreement with its high rate constant in the reaction with PN [102, 165]. NOR was more efficient in the nitration with very little hydroxylation and moderate dimerization, as already shown [163]. The results obtained here are in agreement with the known chemistry of HRP involving one-electron transfer (either in the form of a H-atom abstraction from a phenol or an electron transfer from a phenolate). The open active site of NOR [262, 263, 264] probably enables phenol to bind to the protein and allows the ferryl species and ·NO\textsubscript{2} to act in a concerted way.

The picture of a required access to the active site for an increased phenol nitration is well supported by the finding that in the presence of camphor CAM looses its nitrating function almost completely since the tight binding of substrate obviously hinders the reaction of phenol with PN to take place. In this case the yields are close to those in control experiments except for the decreased catechol formation. Since this hydroxylation is believed to proceed by OH-radicals it is conceivable that in the presence of the protein and camphor the concentration of such highly reactive species are reduced in contrast to the ferryl species responsible for the one-electron oxidation of phenols which was not affected. This effect is more evident at pH 7.0 than at 7.5 consistent with the mechanism of OH-radical formation being dependent on the protonation of PN [72]. The increase of PN-mediated nitration of phenol by CAM could also be suppressed by methyrapone, which had no effect on the uncatalyzed nitration (not shown).

According to the known crystal structure and sequence of CAM some tyrosine residues are located close to the active site (see Fig.105 and 106) [250]. Indeed a quite sensitive nitration of the substrate-free enzyme was found, which started at 25-50 µM PN (see Fig.85 and 86). An involvement of
iron-catalysis in the self-nitration of PN could also be proved by addition of camphor and metyrapone to the reaction solutions. In both cases the nitration was decreased by a factor of 2-3 in presence of these active site blockers, as observed on Western blots and in chromatograms (see Fig.85, 86, 87 and 88). Metyrapone had no direct effect on PN-mediated nitrations as shown in Fig.89 for the autocatalyzed nitration of BM3. Thus, the metyrapone effect can be attributed to blocking the active site of CAM as a substrate analogue.

In a second experiment the same samples as used for Western blots were digested with trypsin and the resulting peptides were separated by HPLC. The isolated NT-positive peptides as well as trypsin degraded enzyme mixtures were investigated by MS. Y_{179}, Y_{96}, Y_{305} and Y_{201,203} were found to be nitrated. Fig.105 and 106 show all tyrosine residues in CAM labeled according to the sequence Tab.17. All nitrated Tyr residues are located nearby the heme-iron, except Y_{306}, which is a bit more distant. The crystal structure also explains, why nitration of Y_{96} is affected much more by metyrapone, compared to the other Tyr residues. Y_{96} is directly located at the active site and is known to interact with camphor. Since metyrapone also binds to Y_{96}, CAM cannot be nitrated at low concentrations of PN in the presence of metyrapone as shown in the chromatogram in Fig.88.

The substrate-binding spectra of CAM (at 390 nm) was decreased by addition of PN (see Fig.90). At a first view this observation seemed to be due to nitration of Y_{96} by PN, which would decrease the binding affinity of camphor to the
enzyme, but CO-binding spectra of PN-treated CAM revealed (see Fig.91), that additionally a conversion of the P450 to the P420 form occurred. It is known, that the P420 form of CAM is less efficient in binding the camphor molecule.

In decomposition kinetics of PN CAM showed a similar acceleration as the other investigated P450 enzymes. In the presence of camphor this acceleration decreased to the value of the uncatalyzed decomposition (see Fig.94). Contrary to our previous observations neither camphor-free nor -containing CAM showed the accumulation of a ferryl band (see Fig.92 and 93). The only observation which could be made, was the conversion of the camphor-containing enzyme (390 nm) into the P420 form (416 nm) (see Fig.93). The substrate-free enzyme (417 nm) showed a similar kinetic behavior as NOR, BM3-WT and CPO, but the yield of the ferryl intermediate (435 nm) was less (see Fig.92). If the absence of a ferryl species is due to the easy oxidation of the thiolate ligand, to a slower formation of the intermediate compared to its decomposition or to an effect at the neighbouring Y96 will be examined as soon as another protein sample is available.

At present we favor the latter one, since BM3-F87Y also showed no ferryl band and has a Tyr close to the active site. From these observations we suggest that the ferryl intermediate can be trapped by a nearby Tyr, resulting in the ferric iron complex together with a tyrosyl radical, which may react with the remaining nitrogen dioxide from the first step. Whether a nitrated Tyr located at the active site then sterically hinders another PN to reach the heme-iron or is able to react a second time with a ferryl intermediate is not clear yet.
6 Outlook

Here are some topics that remained to clarify or for which we already have preliminary data:

- The ferryl species, which is formed during reactions of P450 enzymes and PN have to be further characterized. Raman resonance measurements seem to be suitable for this intention, since this technique has already been used for the characterization of the ferryl species in CPO [253].

- CAM and the F87Y variant of BM3 showed no ferryl spectrum during their reaction with PN. Since both have a tyrosine nearby the iron at the active site, it is feasible that the ferryl is immediately trapped by these tyrosines. In this case there should be a tyrosyl radical formed, which can be detected by ESR spectrometry.

- By collaboration with Dr. Hager we will receive a sample of native CPO and a mutant in which the heme-thiolate-containing cysteine is exchanged by an alanine. We hope to find significant differences between these two enzymes, which could give new insights in the importance of the thiolate ligand during reactions with peroxides.

- We also plan to do experiments with non-heme proteins such as dopamine β-hydroxylase, which contains a sulfur coordinated copper ion (Cu$^{2+}$). Since we and others have shown that thiol oxidation could be a major pathway by which PN inactivates enzymes (ADH, BM3, aconitase, GAPDH,...) potentially all metal-sulfur containing proteins could be inactivated by PN. β-hydroxylase could be a key to widespread PN-mediated damage during neurodegenerative diseases (e.g. Parkinson).
7 Summary

- The reaction mechanism of phenol with peroxynitrite was characterized. This reaction was used as a model for PN-mediated nitration of tyrosine-residues in proteins, especially in prostacyclin synthase. 2- and 4-nitrophenol as well as 2- and 4-hydroxy-phenol had already been identified as major products of this reaction. In this work we identified 2,4'-biphenol and 4-phenoxy-phenol as additional dimerization products, furthermore 2-nitroso-phenol, 4-nitro-catechol and o-benzoquinone as additional products. High yields of 4-nitroso-phenol at alkaline pH were found and a complete picture of the dimerization product pattern was given, allowing to conclude on phenoxy radicals as obligatory intermediates in this reaction. The latter finding was also supported by the results obtained from reactions of anisol with PN. Other new results concerned a maximum of nitrosation and oxygen release at pH 9-10 and an involvement of radical species also at this pH.

- New scavengers for PN were identified and compared with known antioxidants in different test systems. The choice of these test systems allowed a detailed insight in the mechanism by which scavengers interact with PN. Alcohol dehydrogenase reacts with the PN-anion and the enzyme is inactivated by thioloxidation. Scavengers which are efficient in this system also have to react with the PN-anion. We identified GSH, cysteine, Se-methionine, dithio-purine and -pyrimidine as highly efficient in this system. The scavenger concentration for the half-maximal protection were in the range of 15-45 µM, in a system where 26 nM ADH were inactivated by 20 µM PN. Uric acid and its dimethyl analogues, ebselen and ascorbate were inefficient in this system.

Phenol nitration and hydroxylation as well as nitration of BSA requires peroxynitrous acid or its activated intermediate as the reactive species. In these test systems uric acid and its 1,3- and 3,7- dimethyl analogues, 2-thio-barbituric acid, 2,6-dithio-purine and -pyrimidine were highly efficient in the inhibition the nitration and hydroxylation of phenol, followed by cysteine and ascorbate, which were especially less efficient in the suppression of hydroxylation. Methionine, Se-methionine and GSH were less efficient in both. The trend in the BSA system was similar. Scavengers which could accelerate the decomposition of PN could also have beneficial effects on PN-mediated oxidations. Phosphine and pyruvate showed high effects on PN-decay, pyruvate even seemed to react in a catalytic manner, but also increased the nitration of phenol.
Ebselen is known to react very fast with the PN-anion. Nevertheless it was inefficient in our systems. Ebselen could also not protect PGIS from inactivation by PN, due to adduct formation with thiols. The ebselen-GSH adduct was identified and isolated by HPLC. The reaction of this adduct with PN was very slow, but ebselen could protect PGIS from inactivation, when thiol groups in microsomes were blocked with Ellmans reagent.

- Heme- and heme-thiolate proteins had a high catalytic effect on this nitration. P450\textsubscript{NOR}, P450\textsubscript{BM-3}, chloroperoxidase and microperoxidase not only showed high effects on the nitration and dimerization increase, but also on the decrease of hydroxylation. Furthermore P450\textsubscript{NOR} and CPO were most efficient in catalyzing the decomposition of PN. We identified a ferryl intermediate in the reactions of P450\textsubscript{NOR}, P450\textsubscript{BM-3} and CPO with PN. Our results point towards a special role of the thiolate-ligand for the reactivity of the ferryl intermediate. This high reactivity could be related to a partial radical character of the sulfur in Compound II of P450 enzymes (S-Fe-O· ↔ S·Fe=O).

- P450\textsubscript{BM-3} started to be nitrated at 0.5 μM PN and was inactivated with an IC\textsubscript{50}-value of 2-3 μM. Y\textsubscript{334} was found to be nitrated in the wildtype enzyme, Y\textsubscript{87} and Y\textsubscript{334} were nitrated in the F87Y variant. Our studies revealed, that the inactivation was not due to nitration, but due to thioloxidation in the reductase domain. Stopped-flow measurements revealed the formation of a ferryl species during reactions of P450\textsubscript{BM-3} and CPO with PN and second order rate constants of k(BM3)=2x10\textsuperscript{5} M\textsuperscript{-1}s\textsuperscript{-1} and of k(CPO)=1x10\textsuperscript{6} M\textsuperscript{-1}s\textsuperscript{-1} for this process.

- P450\textsubscript{CAM} started to be nitrated at 25 μM PN. Y\textsubscript{179}, Y\textsubscript{96}, Y\textsubscript{305} and Y\textsubscript{201,203} were found to be nitrated after treatment with 250 μM PN. When metyrapone was added, which blocks the active site, the nitration was decreased by a factor of 2-3. Treatment with PN decreased the camphor-binding spectrum of P450\textsubscript{CAM} and resulted in the oxidized P420-species. P450\textsubscript{CAM} could catalyze the decomposition of PN. In stopped-flow spectra no ferryl species could be observed, a similar behavior was observed as for the F87Y variant of P450\textsubscript{BM-3}.

### 7.1 Zusammenfassung

- Es wurde ein wesentlicher Beitrag zur Aufklärung des Reaktionsmechanismus von Peroxynitrit (PN) mit Phenol geleistet. Diese Reaktion wurde...
als Modell für die Nitrierung von Tyrosinresten in Proteinen durch PN benutzt, speziell in der Prostacyclin Synthase. Sowohl 2- und 4-Nitrophenol, als auch 2- und 4-Hydroxyphenol wurden schon in der Vergangenheit als Hauptprodukte dieser Reaktion beschrieben. In dieser Arbeit wurden zusätzlich 2,4'-Biphenol und 4-Phenoxyphenol als Dimerisierungsprodukte identifiziert, als auch 2-Nitrosophenol, 4-Nitrocatechol und o-Benzochinon als neue Oxidationsprodukte. Hohe Ausbeuten an 4-Nitrosophenol im alkalischen pH Bereich wurden nachgewiesen und ein komplettes Bild des Dimerisierungsprodukt-Schemas konnte erstellt werden, was den Rückschluss auf eine obligatorische Beteiligung von Phenoxyradikalen in dieser Reaktion zuließ. Diese Schlussfolgerung wurde weiterhin durch die Ergebnisse unterstützt, die aus der Reaktion von Anisol mit PN erhalten wurden. Weitere neue Erkenntnisse aus dieser Arbeit betrafen das Maximum der Nitrosylierung und Sauerstofffreisetzung zwischen pH 9 und 10.


Sowohl die Phenol-Nitrierung und Hydroxylierung als auch die Nitrierung von BSA benötigen die protonierte Form von PN oder deren angeregten Zustand. In diesen Testsystemen waren Harnsäure und seine 1,3- und 3,7-Dimethylanalogen, 2-Thiobarbitursäure, 2,6-Dithiopurin und -Pyrimidin hoch wirksam bei der Unterdrückung der Nitrierung und Hydroxylierung von Phenol, gefolgt von Cystein und Ascorbat, die vorallem auf die Hydroxylierung einen kleineren Einfluss hatten. Methionin, Se-Methionin und GSH waren in beiden Reaktionen weniger aktiv. Im BSA System waren ähnliche Trends erkennbar. Hemmstoffe, die den Zerfall von PN beschleunigen, könnten ebenso positive Effekte auf die von PN verursachten Oxidationen haben. Phosphin und Pyruvat zeigten starke Beschleunigung des
Zerfalls, Pyruvat schien sogar katalytisch zu wirken, allerdings erhöhte es
die Nitrierung von Phenol.

Ebselen ist bekannt für seine schnelle Reaktion mit PN. Trotzdem zeigte
es in unseren Testsystemen kaum Effekte und konnte PGIS nicht vor der
Inaktivierung durch PN schützen. Ebselen ist bekannt, Addukte mit Thiolgruppen zu bilden. Das Ebselen-GSH-Addukt wurde isoliert und identi-
fiziert, sowie seine Reaktion mit PN als langsam erkannt. In mit Ellmans
Reagenz behandelten Mikrosomen, in denen alle Thiolgruppen blockiert
waren, konnte Ebselen die PGIS vor der Inaktivierung durch PN schützen.

• Häm- und Häm-Thiolat-Proteine haben eine starke katalytische Wirkung
auf diese Nitrierung. P450_{NOR}, P450_{BM−3}, CPO und Mikroperoxidase
erhöhten nicht nur die Nitrierung und Dimerisierung, sondern erniedrigten
zugleich die Hydroxylierung. Weiterhin waren P450_{NOR} und Chloroperox-
idase am effizientesten in der Katalyse des PN-Zerfalls. Wir konnten eine
Ferryl-Zwischenstufe in der Reaktion von P450_{NOR}, P450_{BM−3} und CPO
mit PN identifizieren. Unsere Ergebnisse lassen den Schluss zu, dass der
Thiolat-Ligand eventuell eine Schlüsselrolle für die Reaktivität der Fer-
ryl Spezies in P450 Enzymen einnimmt. Die könnte an einem partiellen
Radikalcharakter des Schwefels liegen (S-Fe-O· ↔ S·Fe=O).

• Die Nitrierung von P450_{BM−3} begann bei 0,5 µM PN und das Enzym wurde
mit einem IC_{50}-Wert von 2-3 µM inaktiviert. Y_{334} konnte im Wildtyp-
Enzym als nitriert identifiziert werden, Y_{87} zusätzlich in der F87Y Mutante.
Unsere Studien zeigten, dass die Inaktivierung nicht von der Nitrierung
herrührte, sondern vielmehr von einer Thioloxidation in der Reduktase
Domäne. Stopped-flow Messungen zeigten die Bildung von Compound II
und eine Geschwindigkeitskonstante zweiter Ordnung von k(BM3)=2x10^5
M^{−1}s^{−1} und k(CPO)=1x10^6 M^{−1}s^{−1} für diesen Prozess.

• Die Nitrierung von P450_{CAM} begann bei 25 µM PN. Y_{179}, Y_{96}, Y_{305}
and Y_{201,203} konnten als nitriert identifiziert werden nach Behandlung mit
250 µM PN. Nach Zugabe von Metyrapon, welches das aktive Zentrum
blockiert, war die Nitrierung um einen Faktor 2-3 verringert. Behand-
lung mit PN verringerte das Campher-Bindungsspektrum und resultierte
in der oxidierten P420-Spezies. P450_{CAM} konnte den Zerfall von PN nicht
katalysieren. Auch in Stopped-flow Spektren zeigte P450_{CAM} nicht das für
andere P450-Enzyme beobachtete Ferryl-Spektrum. Genau dasselbe Ver-
halten war bei der F87Y Mutante von P450_{BM−3} beobachtet worden.
References


REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


This thesis was written with \LaTeX an intelligent freeware text editor [265].
A Appendix

A.1 Chemical Structures

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Figure 107: Structures of PN scavengers.
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Figure 108: Structures of PN scavengers.
## A.2 Sequences of P450<sub>Cam</sub> and P450<sub>BM3</sub>

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Table 17: Peptide-fragments from trypsin-digested P450<sub>Cam</sub> calculated by using “Peptide mass” (www.expasy.ch).
Table 18: Peptide-fragments from trypsin-digested P450BM-3 (part I) calculated by using "Peptide mass" (www.expasy.ch).

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Table 19: Peptide-fragments from trypsin-digested P450BM−3 (part II) calculated by using "Peptide mass" (www.expasy.ch).
Table 20: Peptide-fragments from trypsin-digested P450<sub>BM-3</sub> (part III) calculated by using "Peptide mass" (www.expasy.ch).

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