Nitrite as a Substrate and Inhibitor of Myeloperoxidase

IMPLICATIONS FOR NITRATION AND HYPOCHLOROUS ACID PRODUCTION AT SITES OF INFLAMMATION*

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Christine J. van Dalen‡, Christine C. Winterbourn, Revathy Senthilmohan, and Anthony J. Kettle

From the Free Radical Research Group, Biomedical Research Unit, Department of Pathology, Christchurch School of Medicine, P. O. Box 4345, Christchurch, New Zealand

Myeloperoxidase is a heme enzyme of neutrophils that uses hydrogen peroxide to oxidize chloride to hypochlorous acid. Recently, it has been shown to catalyze nitration of tyrosine. In this study we have investigated the mechanism by which it oxidizes nitrite and promotes nitration of tyrosyl residues. Nitrite was found to be a poor substrate for myeloperoxidase but an excellent inhibitor of its chlorination activity. Nitrite slowed chlorination by univalently reducing the enzyme to an inactive form and as a consequence was oxidized to nitrogen dioxide. In the presence of physiological concentrations of nitrite and chloride, myeloperoxidase catalyzed little nitration of tyrosyl residues in a heptapeptide. However, the efficiency of nitration was enhanced at least 4-fold by free tyrosine. Our data are consistent with a mechanism in which myeloperoxidase oxidizes free tyrosine to tyrosyl radicals that exchange with tyrosyl residues in peptides. These peptide radicals then couple with nitrogen dioxide to form 3-nitrotyrosyl residues. With neutrophils, myeloperoxidase-dependent nitration required a high concentration of nitrite (1 mM), was doubled by tyrosine, and increased 4-fold by superoxide dismutase. Superoxide is likely to inhibit nitration by reacting with nitrogen dioxide and/or tyrosyl radicals. We propose that at sites of inflammation myeloperoxidase will nitrate proteins, even though nitrite is a poor substrate, because the co-substrate tyrosine will be available to facilitate the reaction. Also, production of 3-nitrotyrosine will be most favorable when the concentration of superoxide is low.

The presence of 3-nitrotyrosine at sites of inflammation has been used to implicate peroxynitrite in inflammatory tissue damage (1, 2). Peroxynitrite is formed from the rapid reaction of nitric oxide with superoxide (k ≈ 5 × 10^9 M⁻¹ s⁻¹) (3–5). It nitrates free tyrosine and tyrosyl residues in proteins (6). However, it was recently found that peroxynitrite is extremely inefficient at nitrating tyrosine when it is formed at physiological fluxes of superoxide and nitric oxide (7). This result suggests that other mechanisms are likely to contribute to production of 3-nitrotyrosine in vivo. One route may involve the neutrophil protein myeloperoxidase. This heme enzyme has been shown to catalyze nitration of tyrosine and tyrosyl residues in peptides and proteins (8). To assess the potential of myeloperoxidase to contribute to formation of 3-nitrotyrosine at sites of inflammation, it is essential to appreciate how this enzyme catalyzes nitration of proteins.

Myeloperoxidase is the most abundant protein in neutrophils and is also present in monocytes (9, 10). Ferric myeloperoxidase reacts with hydrogen peroxide, which is produced by stimulated neutrophils, to form the redox intermediate compound I (Reaction 1). Compound I is strongly oxidizing and reacts with a variety of substrates. Its main physiological substrate is assumed to be chloride, which undergoes a two-electron oxidation to form hypochlorous acid (Reaction 2). This is the most powerful oxidant produced by neutrophils in appreciable amounts. Under physiological conditions, thiocyanate is an equally preferred substrate and is oxidized to hypothiocyanite (11). In addition to this halogenation activity, myeloperoxidase acts as a classical peroxidase. Compound I is reduced by organic substrates (RH) in a one-electron reaction to form compound II and a free radical product (Reaction 3). Compound II then reacts with a second molecule of RH to regenerate the native enzyme (Reaction 4).

\[ \text{MP}^{+1} + \text{H}_2\text{O}_2 \rightarrow \text{compound I} \]  
\[ \text{REACTION 1} \]

\[ \text{Compound I} + \text{Cl}^- \rightarrow \text{MP}^{+1} + \text{HOCl} \]  
\[ \text{REACTION 2} \]

\[ \text{Compound I} + \text{RH} \rightarrow \text{compound II} + \text{R} \]  
\[ \text{REACTION 3} \]

\[ \text{Compound II} + \text{RH} \rightarrow \text{MP}^{+1} + \text{R} \]  
\[ \text{REACTION 4} \]

\[ \text{NO}_2^- + \text{HOCl} \rightarrow \text{NO}_2\text{Cl} + \text{HO}^\cdot \]  
\[ \text{REACTION 5} \]

Eiserich and co-workers (12) have proposed that myeloperoxidase catalyzes nitration by two distinct mechanisms. They showed that hypochlorous acid reacts with nitrite to form nitryl chloride (Reaction 5) (12). This species both nitrates and chlorinates tyrosine to give 3-nitrotyrosine and 3-chlorotyrosine. Given that Reaction 5 is relatively slow compared with the reaction of hypochlorous acid with thiols and amines (13, 14), it is unlikely that nitryl chloride is a significant nitrating agent in vivo. They also demonstrated that nitrite is oxidized directly by myeloperoxidase to a species that is capable of nitrating tyrosine (15). A two-electron oxidation would result in the production of the nitronium cation, whereas removal of one electron would give nitrogen dioxide.

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‡ To whom correspondence should be addressed. Tel.: 64-3-3640-588; Fax: 64-3-3641-083; E-mail: cvdalen@chmeds.ac.nz.
Nitrite Oxidation by Myeloperoxidase

The rate of oxidation of substrates by peroxidases is strongly dependent on their one-electron potentials. Although substrates with reduction potentials of 1.05–1.2 V are readily oxidized by compound I of myeloperoxidase, they are poor peroxidase substrates because they are unable to reduce compound II (16). Nitrite has a one-electron reduction potential of 0.99 V (17). Thus, on thermodynamic grounds nitrite should be a poor substrate for myeloperoxidase and inhibit its chlorination activity. To reconcile this argument with the experimental findings that myeloperoxidase catalyzes nitration of tyrosine and proteins, we have investigated the mechanism of oxidation of nitrite by this enzyme. In previous studies, tyrosine or small peptides containing tyrosyl residues have been used to investigate myeloperoxidase-catalyzed nitration. These compounds are substrates for myeloperoxidase and will be oxidized by the enzyme to produce tyrosyl radicals (18, 19), which may influence nitration. To more realistically mimic protein nitration, we have used peptides containing tyrosyl residues that are not oxidized directly by myeloperoxidase. We show that nitrite is indeed a poor substrate for myeloperoxidase but free tyrosine facilitates nitration of tyrosyl residues by acting as a co-substrate in the reaction.

EXPERIMENTAL PROCEDURES

Materials

Myeloperoxidase was purified from human leukocytes as described previously (20). Its purity index (A$_{405}$/A$_{280}$) was at least 0.80, and its concentration was determined using A$_{405}$ 91,000 μm$^{-1}$·cm$^{-1}$ (21). Solutions of sodium nitrite (BDH Chemicals) were prepared daily and kept on ice. 5,5′-Dithiobis-2-nitrobenzoic acid, acetyl-Ser-Gln-Asn-Tyr-Pro-Val-Val-amide (peptide I), Ala-Pro-Arg-Leu-Arg-Phe-Ser (peptide II), acetyl-Asp-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (peptide III), diethylthiomercuri-pentaacetic acid (DTPA), 1′-tyrosine, superoxide dismutase, and 4-phosphoribitol-12-myristate 13-acetate (PMA) were purchased from Sigma. 2-Nitro-tyrosine was from Fluka Chemicals (Buchs, Switzerland). S-Methionine was from BDH Chemicals. Complete™ peptide inhibitor mixture tablets, which are inhibitory to a large spectrum of serine proteases, were from Roche Molecular Biochemicals. 5-Thio-2-nitrobenzoic acid was prepared from 5,5′-dithiobis-2-nitrobenzoic acid as described previously (22). Hydrogen peroxide solutions were prepared daily by diluting a 30% stock, and the concentration was calculated by measuring its absorbance at 240 nm (ε$_{240}$ 43.6 μm$^{-1}$·cm$^{-1}$) (23). Inactive superoxide dismutase was prepared by treating 4.5 mg/ml enzyme with 88 mM hydrogen peroxide in 10 mM carbonate buffer, pH 10.9, for 1 h. It was then dialyzed against 10 mM phosphate buffer, pH 7.4, containing 140 mM sodium chloride and 10 μM/mL catalase (24).

Methods

Measurement of Hydrogen Peroxide Utilization by Myeloperoxidase—The activity of purified myeloperoxidase was measured by continuously monitoring hydrogen peroxide concentration with a YSI 2510 oxidase probe fitted to a YSI model 25 oxidase meter (Yellow Springs Instrument Co., Yellow Springs, OH). The electrode was covered with a single layer of dialysis tubing, except when using tyrosine. In these experiments an exclusion membrane (22) was placed directly on the electrode and then covered with dialysis tubing. The electrode was calibrated against known concentrations of hydrogen peroxide. All reactions were started with the addition of myeloperoxidase. For experiments containing nitrite, the electrode was equilibrated at each concentration of nitrite.

Spectral Analysis of Myeloperoxidase—The visible absorption spectrum of myeloperoxidase was recorded during the oxidation of chloride with and without nitrite using a Beckman 7500 diode array spectrophotometer. Each spectrum was recorded over 2 s and is an average of 20 spectra.

Measurement of Hypochlorous Acid Formation by Myeloperoxidase and Neutrophils—Hypochlorous acid production by purified myeloperoxidase was determined by measuring accumulation of taurine chloramine (22). Myeloperoxidase (10 μM) was incubated with 10 mM taurine in 10 mM sodium phosphate buffer, pH 7.4, plus 140 mM sodium chloride (PBS) at 21 °C for 5 min. The reaction was started by addition of 30 μM hydrogen peroxide and stopped with 20 μM/mL catalase. The amount of taurine chloramine was assayed using 5-thio-2-nitrobenzoic acid. Neutrophils were isolated from normal individuals according to established procedures (25). Neutrophils (1 × 10$^6$) were stimulated with PMA (100 ng/ml) and incubated at 37 °C in 1 ml of PBS containing 5 mM glucose, 1 mM CaCl$_2$, and 0.5 mM MgCl$_2$, 20 mM tyrosine, and varying concentrations of nitrite as indicated. Reactions were stopped after 30 min with catalase, and neutrophils were pelleted at 10,000 × g for 2 min. The supernatant was assayed for taurine chloramine as described previously (22).

Measurement of the NADPH-oxidase Activity of Neutrophils—The NADPH-oxidase activity of neutrophils was determined using the hydrogen peroxide electrode (22). Neutrophils were stimulated with PMA under the conditions described above for production of hypochlorous acid and the accumulation of hydrogen peroxide was monitored for 20 min in the absence or presence of 200 μM nitrite.

Nitrification of Tyrosyl Residues by Myeloperoxidase and Human Neutrophils—Nitration of the peptides by purified myeloperoxidase was carried out at 21 °C in 50 mM sodium phosphate buffer, pH 7.4, containing 100 μM nitrite, 20 μM DTPA, and 100 μM peptide. Reactions were started by the addition of 100 μM hydrogen peroxide, stopped after 1 min, and catalyzed (20 μM/mL catalase), then analyzed by HPLC (see below). Neutrophils (2 × 10$^6$/ml) were incubated at 37 °C in PBS with 100 μM peptide I, 5 mM glucose, 1 mM CaCl$_2$, 0.5 mM MgCl$_2$, 1 mM nitrite, and additions of 100 μM tyrosine, 10 μM/mL superoxide dismutase, 1 mM methionine, 100 μM azide, or 20 μM/mL catalase. The cells were stimulated with PMA (100 ng/ml). After 1 h, reactions were stopped by adding 20 μM/mL catalase and placing cells on ice. Neutrophils were pelleted by centrifugation at 10,000 × g for 5 min. COMPLETE™ protease inhibitors (0.8 mg/ml) were then added, and the supernatant fraction was stored at −20 °C until analyzed by HPLC. Nitrite concentrations were monitored using the Griess reagent (26).

High-performance Liquid Chromatography—Products of nitrification reactions were analyzed by HPLC using a 5-μm Spherisorb ODS-2 column with a gradient of 50 mM potassium hydrogen phosphate, pH 3.0/methanol (v/v) over 20 min, and identification of products was made by the comparative time of elution with standards and by spiking experiments. Analysis of products was made by comparison of the area under the curve for each peak at 274 nm relative to a standard curve for 3-nitrotyrosine.

Analysis of Nitrated Peptides—After nitration, peptide I was injected into the HPLC, and fractions were collected every minute from just before the parent peak eluted until just after the major modified peptide eluted. Fractions were dried down, dissolved in 0.1% trifluoroacetic acid, then desalted by solid-phase extraction on a reverse phase C18 column (Alltech extract-clean C18 column, 500 mg/2.8 ml) equilibrated with 0.1% trifluoroacetic acid. Bound peptides were washed off the column with 80% methanol. They were then dried down and hydrolyzed under nitrogen for at least 18 h at 110 °C using 6 n HBr supplemented with 1% phenol. The content of 3-nitrotyrosine, 3-chlorotyrosine, and tyrosine in the parent and nitrated peptides was assayed using stable isotopic dilution gas chromatography mass spectrometry essentially as described by Heinzecke and co-workers (27), except that amino acids were derivatized with trifluoroacetic anhydride. For amino acid analysis, hydrolyzed samples were derivatized with o-phthalaldehyde (28).

RESULTS

Oxidation of Nitrite by Myeloperoxidase and Hydrogen Peroxide—To determine how effective nitrite is as a substrate for myeloperoxidase, we measured enzyme activity by monitoring the loss of hydrogen peroxide (Fig. 1). Steady-state loss of hydrogen peroxide was minimal at concentrations of nitrite greater than 50 mM, decreased with increasing concentration of nitrite. The activity of myeloperoxidase could not be determined above 10 mM nitrite because the hydrogen peroxide electrode could no longer be equilibrated. Therefore, it was not possible to determine the specificity constant for nitrite. An alternative approach to...
Reactions were started by adding 10 nM myeloperoxidase to 30 \( \mu M \) \( \text{H}_2\text{O}_2 \) and varying concentrations of nitrite, in 100 \( \text{mM} \) phosphate buffer, pH 7.4, containing 20 \( \mu M \) DTPA. Steady state rates of \( \text{H}_2\text{O}_2 \) loss at 21 °C were calculated over the first minute. Data are means and ranges of at least duplicate experiments.

**Nitrite as an Inhibitor of Myeloperoxidase**—The effect of nitrite on the chlorination activity of myeloperoxidase was determined by continuously monitoring the loss of hydrogen peroxide in the presence of chloride (Fig. 2A). Under the conditions of this assay essentially all the hydrogen peroxide consumed by myeloperoxidase is converted to hypochlorous acid (22). As shown in Fig. 2A, on addition of myeloperoxidase, there was an initial rapid loss of hydrogen peroxide that slowed over time. When nitrite was present, the loss of hydrogen peroxide was markedly inhibited. There was progressive inhibition of enzyme activity with increasing concentration of nitrite up to a maximum of 80% (Fig. 2B). The concentration of nitrite that resulted in 50% maximal inhibition of myeloperoxidase activity (IC\(_{50}\)) was 1.3 \( \mu M \). Taurine chloramine formation, which is a measure of production of hypochlorous acid, was also assayed under the conditions described in Fig. 2A. With 10 \( \mu M \) nitrite, production of taurine chloramine was inhibited by 79 ± 8% (\( n = 3 \)). This confirms that nitrite was inhibiting the conversion of hydrogen peroxide to hypochlorous acid.

To check whether myeloperoxidase was reversibly or irreversibly inactivated by nitrite, we added tyrosine to the reaction system subsequent to inhibition of the enzyme. Since tyrosine is a good peroxidase substrate it should reverse inhibition that is due to accumulation of compound II. Indeed, upon addition of 100 \( \mu M \) tyrosine, enzyme activity was completely restored (Fig. 2A). We confirmed that nitrite converts the enzyme to compound II by recording the absorption spectra of myeloperoxidase during its reaction with hydrogen peroxide and chloride. In the absence of nitrite, addition of hydrogen peroxide to myeloperoxidase and chloride caused a minimal change in the absorption spectrum of the enzyme. However, when 10 \( \mu M \) nitrite was present, there was a shift in the Soret maximum to about 450 nm and a new peak appeared at 627 nm (Fig. 3). Based on the extinction coefficients for the native enzyme and its redox intermediates (32), these changes indicate that about 80% of ferric myeloperoxidase was converted to compound II. Compound II was unstable and decayed back to the native enzyme within 5 min.

**Effect of Nitrite on Hypochlorous Acid Production by Neutrophils**—We also determined the effect of nitrite on production of hypochlorous acid by human neutrophils. Hypochlorous acid was trapped with taurine, and accumulated taurine chloramine was measured (22). Under the conditions of our experiments, scavenging of hypochlorous acid by nitrite would have gauging how readily nitrite will be oxidized by myeloperoxidase *in vivo* is to compare the turnover numbers of myeloperoxidase at physiological concentrations of competing substrates. Chloride and thiocyanate concentrations in plasma range between 100–140 \( \text{mM} \) and 20–120 \( \mu M \), respectively (10). Therefore, we determined the turnover numbers of myeloperoxidase at 100 \( \mu M \) nitrite, 100 \( \mu M \) thiocyanate, or 100 \( \text{mM} \) chloride. The concentration of hydrogen peroxide was 30 \( \mu M \). The turnover number for nitrite was calculated from the steady state rate of hydrogen peroxide loss to be 0.7 s\(^{-1}\). For chloride and thiocyanate, the turnover numbers were 28.5 and 30.5 s\(^{-1}\), respectively. Thus, nitrite is a comparatively poor substrate for myeloperoxidase.
been negligible, as taurine was present in a 50-fold excess and has a rate constant for its reaction with hypochlorous acid 100 times that for nitrite (14). In the absence of superoxide dismutase, nitrite caused modest inhibition of hypochlorous acid formation (Fig. 4). Adding superoxide dismutase to cells caused an approximately 50% increase in the production of hypochlorous acid, as has been reported previously (33). This occurs because superoxide is prevented from reacting with myeloperoxidase and ensures that the enzyme is present predominantly in its active form (20). With superoxide dismutase present, nitrite was considerably more effective at inhibiting production of hypochlorous acid. The IC_{50} was 12 μM. These results demonstrate that superoxide limits the ability of nitrite to inhibit myeloperoxidase-dependent production of hypochlorous acid by neutrophils. Nitrite was not acting by inhibiting the NADPH-oxidase because, at 200 μM, it had no effect on hydrogen peroxide production by neutrophils (result not shown).

Nitration of Tyrosyl Residues by Purified Myeloperoxidase—In view of our result that nitrite is a poor substrate and inhibits chlorination activity, it is surprising that effective nitration of tyrosine, peptides, and proteins by both isolated myeloperoxidase and neutrophils has been demonstrated (8, 15, 34). To reconcile these findings, we investigated nitration of the peptide acetyl-Ser-Gln-Asn-Tyr-Pro-Val-Val-amide (peptide I) at physiological concentrations of nitrite, chloride, and tyrosine. We chose this heptapeptide because its tyrosyl residue should not be oxidized directly by myeloperoxidase (18, 19). This was confirmed using the hydrogen peroxide electrode (results not shown). Consequently, it should be a good model for investigating how myeloperoxidase nitrates proteins. Also, it should be poorly chlorinated because it does not contain an amino group (35). Therefore nitration of the tyrosyl residue should not be overwhelmed by formation of 3-chlorotyrosine.

To establish that the peptide could undergo nitration, it was incubated with a high concentration of nitrite plus tyrosine, myeloperoxidase, and hydrogen peroxide. Nitration was then monitored using HPLC with UV detection at 274 nm (Fig. 5A). With the complete reaction system, two new peaks in addition to tyrosine and peptide appeared in the chromatogram (Fig. 5A, trace a). The peak eluting immediately after tyrosine was identified as 3-nitrotyrosine based on its retention time and absorption spectrum (not shown). The late eluting peak was attributed to the peptide containing a 3-nitrotyrosyl residue based on the following results. When one of either the peptide, nitrite, myeloperoxidase, or hydrogen peroxide were omitted from the reaction system, the late eluting peak was not formed (Fig. 5A, trace b), indicating that it was derived from the peptide and an oxidation product of nitrite. This peak was also absent when the peptide was incubated with myeloperoxidase and hydrogen peroxide plus tyrosine or chloride (not shown). Thus it could not be attributed to tyrosylated or chlorinated peptide. The absorption spectrum of the peak had maxima at 280 and 350 nm, which is characteristic of a nitrated tyrosyl residue (Fig. 5B).

To further characterize the modified peptide, fractions of eluant were collected from the HPLC corresponding to the parent peptide and the late eluting peak. The fractions were then analyzed by gas chromatography with mass spectrometry after the hydrolyzed amino acids were derivatized with trifluoroacetic acid anhydride. In this assay, 3-nitrotyrosine is reduced and detected as 3-aminotyrosine. Tyrosine and 3-chlorotyrosine are also measured in this assay. In the fractions corresponding to the parent peptide only tyrosine was present. In fractions corresponding to the late eluting peak, a component had a mass spectrum with major ions having m/z of 526 and 429 mass units (Fig. 6). This mass spectrum is expected for derivatized 3-aminotyrosine. The relative amount of 3-nitrotyrosine to tyrosine was 300:1. These results demonstrate that the late eluting peak is due to a peptide that contains 3-nitrotyrosine. Hydrolyzed fractions from the HPLC were also as-

![FIG. 4. Effect of nitrite on production of hypochlorous acid by neutrophils. Human neutrophils (1 × 10⁶ cells/ml) were incubated at 37 °C in PBS containing 1 mg/ml glucose, 1 mM calcium chloride, 0.5 mM magnesium chloride, 20 mM taurine, and varying concentrations of nitrite. They were stimulated with 100 ng/ml PMA in the presence and absence of 10 μg/ml superoxide dismutase. The reactions were stopped after 30 min with 20 μg/ml catalase, and formation of taurine chloroacetate was measured. Results are representative of four individual experiments.](image)

![FIG. 5. Nitration of tyrosyl residues by myeloperoxidase. A, peptide I (100 μM) was incubated at 21 °C in 50 mM phosphate buffer, pH 7.4, with 50 mM myeloperoxidase, 1 mM nitrite, and 10 μM tyrosine. The reaction was started by adding 100 μM hydrogen peroxide and stopped after 1 h with 20 μg/ml catalase. The reaction mixture was then analyzed by HPLC as described under "Methods." HPLC traces are the complete reaction system (trace a), representative of control reactions in which one of either myeloperoxidase, nitrite, or hydrogen peroxide was omitted (trace b), and standards of 100 μM tyrosine, 100 μM 3-nitrotyrosine, and 100 μM peptide (trace c). B, UV spectrum of the nitrated peptide peak in trace a, which was recorded using a diode array detector.](image)
Nitrite Oxidation by Myeloperoxidase

**TABLE I**

<table>
<thead>
<tr>
<th>Condition</th>
<th>[Nitrate peptide]</th>
<th>µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide I alone</td>
<td>0.00 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>+ MPO, H₂O₂, NO₂</td>
<td>0.93 ± 0.18</td>
<td></td>
</tr>
<tr>
<td>+ Cl⁻</td>
<td>0.92 ± 0.26</td>
<td></td>
</tr>
<tr>
<td>+ Cl⁻, methionine</td>
<td>0.05 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>+ Tyrosine</td>
<td>2.80 ± 0.22</td>
<td></td>
</tr>
<tr>
<td>+ Tyrosine, Cl⁻</td>
<td>3.55 ± 0.48</td>
<td></td>
</tr>
<tr>
<td>+ Tyrosine, Cl⁻, methionine</td>
<td>3.87 ± 0.68</td>
<td></td>
</tr>
<tr>
<td>Peptide II alone</td>
<td>0.00 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>+ MPO, H₂O₂, NO₂</td>
<td>2.54 ± 0.18</td>
<td></td>
</tr>
<tr>
<td>+ Tyrosine</td>
<td>4.65 ± 0.54</td>
<td></td>
</tr>
<tr>
<td>Peptide III alone</td>
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<td></td>
</tr>
<tr>
<td>+ MPO, H₂O₂, NO₂</td>
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</tr>
<tr>
<td>+ Tyrosine</td>
<td>5.05 ± 0.30</td>
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Gas chromatography with mass spectrometry analysis of the reaction system containing 100 µM each of peptide I, nitrite, and hydrogen peroxide, plus 100 mM chloride and 10 µM tyrosine under the conditions given in Table I, revealed that 3% of the hydrogen peroxide was used to produce 3-chlorotyrosine. This result confirms that peptide I, which lacks a free amine group, is a poor substrate for chlorination.

**Nitrination by Stimulated Neutrophils—**When neutrophils were stimulated with FMA in the presence of 100 µM nitrite there was no detectable nitration of peptide I. There was also none when the concentration of peptide I was increased from 100 to 500 µM. The lack of nitration was not due to consumption of the nitrite because there was no appreciable loss of nitrite (not shown). With 1 mM nitrite, less than 1% of the peptide was nitrated (Fig. 8). The extent of nitrination increased dramatically in the presence of superoxide dismutase (Fig. 8), whereas inactive enzyme had no effect (not shown). These results indicate that superoxide suppresses formation of 3-nitrotyrosine on the peptide. Tyrosine almost doubled the efficiency of the reaction. Methionine, which scavenges hypochlorous acid, enhanced nitrination of the peptide, which excludes the involvement of nitryl chloride. No nitrated peptide was detected with unstimulated cells or when azide and catalase were added to the reaction system. These results confirm that nitrination was dependent on myeloperoxidase and an active respiratory burst.

![Negative ion chemical ionization mass spectrum of 3-aminotyrosine](Image)

**Fig. 6.** Negative ion chemical ionization mass spectrum of 3-aminotyrosine. Peptide I was nitrated under the same conditions as the parent peptide with nitrite and the nitrated peptide was detected by gas chromatography with mass spectrometry analysis of the GC peak. The ions at m/z 526 and 429 for derivatized 13C9 3-aminotyrosine. The structure and expected fragmentation pattern of derivatized 13C9 3-aminotyrosine.

![Effects of nitrite and tyrosine on nitration of peptide I](Image)

**Fig. 7.** Effects of nitrite and tyrosine on nitration of peptide I by myeloperoxidase. Peptide I was nitrated by myeloperoxidase in the presence (●) and absence (■) of 10 µM tyrosine under the same conditions as described in the legend to Fig. 5 except that the nitrite concentration was varied from 0 to 0.1 mM. Data are means and ranges of duplicate experiments. Inset, the range of nitrite concentration was extended to 1 mM.

Gas chromatography with mass spectrometry analysis of the reaction system containing 100 µM each of peptide I, nitrite, and hydrogen peroxide, plus 100 mM chloride and 10 µM tyrosine under the conditions given in Table I, revealed that 3% of the hydrogen peroxide was used to produce 3-chlorotyrosine. This result confirms that peptide I, which lacks a free amine group, is a poor substrate for chlorination.


Fig. 8. Nitration of tyrosyl residues by human neutrophils. Neutrophils (2 x 10^6 cells/ml) were incubated in PBS at 37 °C with 100 μM peptide I, 1 mM nitrite, and either 100 μM tyrosine, 10 μg/ml superoxide dismutase (SOD), 1 mM methionine, 100 μM azide, or 20 μg/ml catalase as indicated on the graph. Other conditions were the same as described in the legend to Fig. 5. Neutrophils were stimulated with 100 ng/ml PMA, and after an hour the reactions were stopped by pelleting the cells. Concentrations of nitrated peptide were determined by HPLC as described in the legend to Fig. 5. Results are representative of duplicates of three individual experiments.

**DISCUSSION**

In this investigation we have demonstrated that at concentrations less than 100 μM, nitrite is a poor substrate for myeloperoxidase, and nitration of tyrosyl residues is extremely inefficient. However, free tyrosine acts as a co-substrate of myeloperoxidase and enhances the nitration reaction. We obtained these results using peptides that contained tyrosyl residues that could not be oxidized directly by myeloperoxidase and were therefore good models for protein nitration. We propose that at sites of inflammation myeloperoxidase will nitrate proteins, even though nitrite is a poor substrate, because tyrosine will be available to facilitate the reaction. We found that above 250 μM nitrite, tyrosine was no longer required to promote nitration. Such high concentrations of nitrite have not been detected in vivo, but they may be feasible at localized sites where there are high fluxes of nitric oxide. In accord with our findings, high concentrations of nitrate are required for nitration of bovine serum albumin (34) and low density lipoproteins (36). Constraints on myeloperoxidase-dependent nitration identified in our study are that with neutrophils it is inefficient and largely attenuated by superoxide. Thus, the concentration of superoxide at sites of inflammation will have a significant impact on formation of 3-nitrotyrosine via this route. Within phagosomes, where the flux of superoxide is high, nitration would not be expected to occur. In contrast, when there is extracellular release of myeloperoxidase and when superoxide is trapped within phagosomes (37), nitration should proceed. Consistent with this conclusion, Jiang and Hurst (38) found that fluorescein-coated particles were readily nitrated in extracellular reactions of neutrophils, but no nitrated products were detected within their phagosomes.

Our investigation into how nitrite affects the chlorination activity of myeloperoxidase has provided insights into how it will influence production of hypochlorous acid in vivo. It has also revealed how this anion is oxidized by the enzyme and demonstrated that nitrogen dioxide must be the initial product. We found that nitrite is a potent inhibitor of the purified enzyme because it converts myeloperoxidase to compound II. However, it was a poor inhibitor of the production of hypochlorous acid by neutrophils. The discrepancies between the results with the purified enzyme and neutrophils are largely explained by the effect superoxide has on the activity of myeloperoxidase. Superoxide would prevent inhibition of chlorination by reducing compound II back to the native enzyme and thereby maintain enzyme turnover (20). In support of our findings, nitrite has been shown to have little effect on intraphagosomal chlorination until its concentration exceeds 1 mM (38). Therefore, nitrite will not attenuate the generation of hypochlorous acid at sites of inflammation.

The reduction of compound I to compound II is a one electron reaction. Therefore, when nitrite converts myeloperoxidase to compound II, it must be oxidized to nitrogen dioxide. Compound II will also oxidize nitrite to nitrogen dioxide and complete the classical peroxidase cycle (Reactions 1, 3, and 4). However, because nitrite is so effective at inhibiting the enzyme's chlorination activity, reduction of compound II by nitrite must be slow. Thus, at physiological concentrations of nitrite, its oxidation and the nitration of proteins are inefficient because the enzyme lingers at compound II and is turned over slowly. The nitrogen dioxide produced by myeloperoxidase will nitrate tyrosyl residues. It reacts with tyrosyl residues (p-TyrH) with a rate constant of 3.2 x 10^5 M^-1 s^-1 to form tyrosyl radicals (Reaction 6) (39). Tyrosyl radicals also react with nitrogen dioxide and at a rate that is almost diffusion controlled (k = 3 x 10^9 M^-1 s^-1) to give 3-nitrotyrosine (Reaction 7) (39).

\[
\text{NO}_2 + \text{p-TyrH} \rightarrow \text{NO}_2^- + \text{p-Tyr}^- + \text{H}^+
\]

**REACTION 6**

\[
\text{NO}_2 + \text{p-Tyr}^- \rightarrow \text{p-Tyr-NO}_2^-
\]

**REACTION 7**

Adding tyrosine would eliminate the need for Reaction 6 because tyrosyl radicals would be efficiently generated in Reactions 3 and 4 (40), and then rapidly exchange with tyrosyl residues in the peptide (18). As a consequence, the dominant reaction for nitrogen dioxide would be coupling with tyrosyl radicals (Reaction 7). Thus, at low nitrite concentrations, where production of nitrogen dioxide is limiting, tyrosine will enhance nitration by ensuring that most of the nitrogen dioxide formed is trapped by tyrosyl radicals. In previous studies (8, 15) the effect of tyrosine would not have been apparent because the targets for nitration were either free tyrosine or tripeptides containing tyrosyl residues that are directly oxidized by myeloperoxidase to tyrosyl radicals.

We found that neutrophils did not promote detectable nitration of the peptide I unless nitrite was present at 1 mM. It is not apparent why nitration by the cells was less efficient than with the isolated enzyme. Nitrite could not have had a substantial effect on release of myeloperoxidase from cells because its inhibition of hypochlorous acid product was only modest. Nitration was largely suppressed by superoxide. The most plausible explanation for this inhibition is that superoxide reacted with both nitrogen dioxide and tyrosyl radical. The reaction with tyrosyl radical is fast, having a second order rate constant of 1.5 x 10^9 M^-1 s^-1 (41). Reaction of superoxide with nitrogen dioxide is thermodynamically favorable and should also be fast (17).

In conclusion, we have shown that at physiological concentrations of nitrite, the propensity of myeloperoxidase to catalyze nitration of tyrosyl residues is greatly enhanced by tyrosine because it acts as a co-substrate in the reaction. Although stimulated neutrophils can promote nitration, it will be not favored due to inhibition by superoxide. Therefore, before production of 3-nitrotyrosine at sites of inflammation is attributed to myeloperoxidase, it will be necessary to show that other specific footprints of this enzyme, such as 3-chlorotyrosine (35, 42) and 3,5-dichlorotyrosine (43), are also present.

**REFERENCES**


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Nitrite Oxidation by Myeloperoxidase

Nitrite as a Substrate and Inhibitor of Myeloperoxidase: IMPLICATIONS FOR NITRATION AND HYPOCHLOROUS ACID PRODUCTION AT SITES OF INFLAMMATION
Christine J. van Dalen, Christine C. Winterbourn, Revathy Senthilmohan and Anthony J. Kettle

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