Mechanism of Reaction of Myeloperoxidase with Nitrite*

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Myeloperoxidase (MPO) is a major neutrophil protein and may be involved in the nitration of tyrosine residues observed in a wide range of inflammatory diseases that involve neutrophils and macrophage activation. In order to clarify if nitrite could be a physiological substrate of myeloperoxidase, we investigated the reactions of the ferric enzyme and its redox intermediates, compound I and compound II, with nitrite under pre-steady state conditions by using sequential mixing stopped-flow analysis in the pH range 4–8. At 15 °C the rate of formation of the low spin MPO-nitrite complex is (2.5 ± 0.2) × 10^6 M⁻¹ s⁻¹ at pH 7 and (2.2 ± 0.7) × 10^6 M⁻¹ s⁻¹ at pH 5. The dissociation constant of nitrite bound to the native enzyme is 2.3 ± 0.1 mM at pH 7 and 31.3 ± 0.5 μM at pH 5. Nitrite is oxidized by two one-electron steps in the MPO peroxidase cycle. The second-order rate constant of reduction of compound I to compound II at 15 °C is (2.0 ± 0.2) × 10^6 M⁻¹ s⁻¹ at pH 7 and (1.1 ± 0.2) × 10^6 M⁻¹ s⁻¹ at pH 5. The rate constant of reduction of compound II to the ferric native enzyme at 15 °C is (5.5 ± 0.1) × 10^7 M⁻¹ s⁻¹ at pH 7 and (8.9 ± 1.6) × 10^7 M⁻¹ s⁻¹ at pH 5. pH dependence studies suggest that both complex formation between the ferric enzyme and nitrite and nitrite oxidation by compounds I and II are controlled by a residue with a pK_a of (4.3 ± 0.3). Protonation of this group (which is most likely the distal histidine) is necessary for optimum nitrite binding and oxidation.

Nitrite is a major oxidation product derived from nitrogen monoxide that is produced by a wide variety of cell types by nitric oxide synthases (1). Induction of NO⁺ synthesis during inflammatory processes represents a defense mechanism against invading microorganisms, although excessive formation of NO⁺ has been implicated in host tissue injury (2, 3). Recently, it has been demonstrated that myeloperoxidase can oxidize nitrite to a species capable of nitrating tyrosine and tyrosyl residues in proteins (4–6). This is one of the most intriguing developments in the biochemistry of MPO, because nitration of tyrosine residues in proteins occurs in a wide range of inflammatory diseases involving activated neutrophils and macrophages. Since nitrite is observed to accumulate upon nitric oxide synthase activation in many inflammatory diseases (7) and nitrite concentrations can be locally higher in tissues than those measured in plasma (8, 9), nitration mediated by MPO could be an additional defense function.

Myeloperoxidase (MPO, EC 1.11.1.7) is a member of the homologous mammalian peroxidase family and is the most abundant protein of neutrophils (polymorphonuclear leukocytes) (10). In activated neutrophils during phagocytosis, MPO has been shown to form hypochlorous acid (11, 12) which is important for microbial killing (13, 14). MPO catalyzes both one- and two-electron oxidations. Thus principally, oxidation of nitrite (NO₂⁻) could result in the formation of either NO₂ (nitrogen dioxide) or NO₂⁻ (nitrTy). Generally, ferric or native myeloperoxidase reacts with hydrogen peroxide forming compound I (MPO-I) (Reaction 1). This redox intermediate is known to oxidize halides via a single two-electron reaction to produce the respective hypohalous acids and regenerate the native enzyme (Reaction 2). Alternatively, stepwise reduction of compound I by two donor-derived electrons produces compound II (MPO-II) and subsequently the resting ferric state (Reactions 3 and 4).

MPO + H₂O₂ ⇌ MPO-I + H₂O

REACTION 1

MPO-I + X− ⇌ MPO + HOX

REACTION 2

MPO-I + AH₂ → MPO-II + AH⁺

REACTION 3

MPO-II + AH₂ → MPO + AH⁺

REACTION 4

In the present paper we have investigated the direct reaction of the ferric enzyme and both redox intermediates with nitrite by using multimeixing sequential stopped-flow spectroscopy. We show that nitrite is an excellent substrate for compound I (MPO-I) but reacts slowly with compound II (MPO-II). Nitrite oxidation occurs during the peroxidatic cycle of MPO in two one-electron oxidation steps (Reactions 3 and 4) and yields nitrogen dioxide. Nitrite not only acts as electron donor for compounds I and II but also forms a low spin complex with the ferric enzyme (15) (Reaction 5).

MPO + NO₂⁻ → MPO-NO₂⁻

REACTION 5

For the first time the actual bimolecular rate constants for Reactions 3–5 in the pH range 4–8 are reported. The consequences for MPO-mediated nitration in vivo are discussed.

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‡ The abbreviations used are: MPO, myeloperoxidase; MPO-I, compound I; MPO-II, compound II; NO₂⁻, nitrite; NO₂⁺, nitrogen dioxide; NO₂⁻, nitrTy ion.
EXPERIMENTAL PROCEDURES

Materials—Purification of myeloperoxidase from human leukocytes to a purity index ($A_{405}/A_{280}$) of at least 0.85 and determination of its concentration and that of hydrogen peroxide was performed as reported previously (16). Hydrogen peroxide and nitrite solutions were prepared shortly before use. All chemicals were purchased from Sigma at the highest grade available.

Stopped-flow Spectroscopy—The sequential stopped-flow apparatus (model SX-181MV) and the associated computer system were from Applied Photophysics (UK). For a total of 100 μl/slot into a flow cell with 1-cm light path the fastest time for mixing two solutions and recording the first data point was about 1.5 ms. Formation of the low spin MPO-nitrite complex was followed at its Soret maximum at 447 nm. In a typical experiment one syringe contained MPO (1 μM heme) in 5 mM phosphate buffer (pH 7), and the other contained various concentrations of nitrite in 200 mM phosphate buffer (pH 5–8) or 200 mM citrate/phosphate buffer (pH 4 and 5). The bimolecular rate constant of complex formation ($k_{off}$) was derived from a plot of $k_{off}$ versus varying nitrite concentrations. The finite intercept of this plot represented $k_{on}$ and the ratio of the rate constants $k_{off}/k_{on}$ resulted in a dissociation constant of the MPO-nitrite complex. Complex formation was also studied with a diode array detector (PD1, Applied Photophysics). Typically, the heme concentration used in these experiments was 2 μM.

Because of the inherent instability of MPO compound I (16), sequential stopped-flow (multimixing) analysis was used for determination of rates of the reaction of compound I with nitrite. The kinetics of compound I formation and its stability were as described previously by Furtado et al. (16). To determine the pH dependence of the reduction of compound I, sequential stopped-flow experiments were performed at different pH values from 4 to 8. The reactions were followed at the Soret maximum of compound II (456 nm).

In a typical pH-jump experiment MPO (2 μM heme) in 5 mM buffer (pH 7) was premixed with 20 μM H$_2$O$_2$ in distilled water. After a delay time of 20 ms, compound I was allowed to react with varying concentrations of nitrite in 200 mM phosphate buffer (pH 5–8) or in 200 mM citrate/phosphate buffer (pH 4 and 5). The final pH after mixing was measured at the outlet.

Reduction of compound II by nitrite was performed as recently described by Burner et al. (17). In a typical pH-jump experiment MPO (2 μM heme) in 5 mM buffer (pH 7) was premixed with 20 μM H$_2$O$_2$ and 1.8 mM homovanillic acid in distilled water. After a delay time of 40 s, compound II was allowed to react with varying concentrations of nitrite in 200 mM phosphate buffer (pH 5–8) or 200 mM phosphate buffer (pH 4 and 5). The reactions were followed at 456 nm (disappearance of compound II), 430 nm (formation of ferric MPO), and 440 nm (isosbestic point between ferric MPO and its nitrite complex).

At least three determinations (1000–20000 data points) of $k_{off}$ were performed for each substrate concentration, and the mean value was used in a plot of $k_{off}$ versus substrate concentration. The final nitrite concentrations were at least 10 times in excess of the enzyme to ensure first-order kinetics. All reactions were performed at 15 °C.

Reduction of both compound I and compound II was also studied with the diode array detector. Typically, the heme concentration used in these experiments was 2 μM.

Measurement of Hydrogen Peroxide Utilization by Myeloperoxidase—The activity of MPO in the presence of nitrite 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.). The electrode was covered with a single layer of dialysis tubing and calibrated against known concentrations of hydrogen peroxide (18). All reactions were started by the addition of myeloperoxidase.

RESULTS AND DISCUSSION

Formation of the Nitrite Complex of Myeloperoxidase—Addition of nitrite to native myeloperoxidase induces both a reduced absorbance and a red shift of the Soret peak from 430 to 447 nm as well as formation of a new peak at 627 nm (Fig. 1A). The isosbestic points between native MPO and its nitrite complex are found at 440, 498, 594, and 650 nm. Since nitrite is a strong field ligand, a typical low spin iron complex is formed as has been confirmed by EPR spectroscopy (15). Under conditions of excess of nitrite, the pseudo first-order rate constant, $k_{on}$, was obtained from the exponential time course at 447 nm. The inset of Fig. 1B shows a typical stopped-flow trace for the complex formation displaying monophasic exponential character. The second-order rate constant ($k_{on}$) at pH 7, obtained from the slope of the plot of $k_{off}$ versus nitrite concentration is $(2.5 \pm 0.2) \times 10^6$ M$^{-1}$ s$^{-1}$ at 15 °C (Fig. 1B). The intercept of this plot, $(60 \pm 2)$ s$^{-1}$, represents the dissociation rate ($k_{off}$). The ratio of the rate constants, $k_{on}/k_{off}$, gives a value for the dissociation constant ($K_{Diss}$) of the nitrite complex to the native enzyme and nitrite of $(2.3 \pm 0.2)$ M. Previously, $K_{Diss}$ calculated from the changes in optical spectra following titration with nitrite, has been reported to be 1–2 μM at pH 7.0 (15).

The nitrite affinity was also determined between pH 4 and 8. Fig. 1C plots the apparent bimolecular rate constant for the complex formation ($k_{on}$) and the dissociation constant for the complex ($K_{Diss}$) between pH 4 and 8. At lower pH $k_{on}$ is dramatically higher ($2.2 \pm 0.7) \times 10^6$ M$^{-1}$ s$^{-1}$ at pH 5). Since the
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The pH-dependent second-order rate constant for complex formation and \( K_a \) is the dissociation constant of an ionizable group of the enzyme. The data were best fit with the values of \( p_{K_a} = 3.9 \pm 0.1 \), \( k_{on(int)} = (2.8 \pm 0.1) \times 10^6 \text{ M}^{-1} \text{s}^{-1} \). These data suggest similarities in complex formation of MPO with both nitrite and chloride, since the pH dependence of chloride binding exhibits a similar pattern with a \( pK_a \) of about 4 \((19)\). Since binding of both nitrite \((15)\) and chloride \((19)\) influences the spin state of the iron, it is reasonable to assume that the amino acid involved is the distal histidine, which, upon protonation, allows such a direct interaction between these two anions and the iron.

\[ \text{REACTION 6} \]

\[ \text{MPO-H}^+ \rightleftharpoons \text{MPO-H} \]

which leads to Equation 1,

\[ k_{on,app} = (k_{on,int} \times [\text{H}^+]) / [K_a + [\text{H}^+]] \]  

(Eq. 1)

where \( k_{on} \) is the intrinsic or pH-independent second-order rate constant for complex formation and \( K_a \) is the dissociation constant of an ionizable group of the enzyme. The use of Equation 2 in the nonlinear least square analysis yields a \( pK_a \) of \( 4.6 \pm 0.2 \). The \( k_{on} \) value of \( 4.2 \pm 0.5 \times 10^7 \text{ M}^{-1} \text{s}^{-1} \) is very similar to that published for halide oxidation by MPO compound I \((i.e., 4.6) \) \((16)\) and that of the acid-base group involved in the formation of the nitrite complex from the native enzyme. Apparently, the distal histidine is involved in both ligand binding by the ferric protein and the oxidation of
small anions by compound I. However, as Fig. 2C demonstrates, the data points could not be fitted very well by the computed curve based on Equation 2. Specifically in the neutral pH region the actual rate constants were larger than the theoretical values. One explanation for this deviation could be the strong oxidizing capacity of MPO compound I (21). The data suggest that the binding site of nitrite in compound I is the same as in the ferric enzyme and that to some extent nitrite is also oxidized by the unprotonated form of compound I.

Compound II Reduction by Nitrite—Fig. 3A demonstrates that nitrite also acts as one-electron donor for compound II. At pH 7 with 500 μM nitrite the direct conversion of compound II to the resting enzyme can be followed with an isosbestic point at 442 nm. With 5 mM nitrite the subsequent reaction of the ferric protein with nitrite was monitored (Fig. 3B). This was consistent with the determined complex dissociation constant of 2.3 mM at pH 7.

At neutral pH, the reduction of compound II by nitrite is relatively slow. The reaction was followed at 440 nm (the isosbestic point between the ferric protein and its nitrite complex). At this wavelength the time course was monophasic and exhibited a typical single exponential behavior (inset to Fig. 3C). The total increase in absorbance did not vary distinctly in the pH range investigated (about 0.010–0.012 absorbance units with 1.0 μM heme in the cuvette). The bimolecular rate constant determined at pH 7 is $(5.5 \pm 0.4) \times 10^7$ M$^{-1}$ s$^{-1}$ (Fig. 3C) and 15°C. The intercept $(k_{obt})$ at $(0.1 \pm 0.02$ s$^{-1}$) is the sum of rate constants leading from compound II to other enzyme species.

As shown in Fig. 3D, there is also a distinct effect of pH on the apparent second-order rate constants for the reaction of compound II with nitrite. Similar to compound I reduction by nitrite, compound II reduction increases with decreasing pH. The bimolecular rate constant at pH 5 is $(8.9 \pm 1.6) \times 10^7$ M$^{-1}$ s$^{-1}$ (Fig. 3D). Thus, in the acidic pH region nitrite is as a good electron donor of compound II. The reaction is best described by Reactions 10 and 11 where MPO-II-H$^+$ is the protonated form of compound II,

\[
MPO-II + H^+ \leftrightarrow MPO-II-H^+ \quad \text{REACTION 10}
\]

\[
MPO-II-H^+ + NO_2^- \rightarrow MPO + NO_2^* \quad \text{REACTION 11}
\]

By using Equation 2 in the nonlinear least square analysis (with $k_{int}$ being the pH-independent second-order rate constant of the reaction between compound II and nitrite) yields a pK$\alpha$ of 4.1 ± 0.3 and a $k_{int}$ value of $(8.2 \pm 0.3) \times 10^6$ M$^{-1}$ s$^{-1}$.

Interestingly, in all three reactions investigated (i.e. complex formation and the reduction of compounds I and II) the same amino acid appears to participate in nitrite binding and oxidation. Both formation of compound I by hydrogen peroxide as well as the binding of chloride to the native ferric enzyme and its kinetics of oxidation by compound I involve a group with a similar pK$\alpha$ value (16). When one averages data from this work one finds a pK$\alpha$ value of 4.3 ± 0.5, in agreement with the literature (16, 19). We suggest that the distal histidine is not only involved in the cleavage of hydrogen peroxide but is also the binding site of these small anionic substrates. Unprotonated distal histidine favors compound I formation, whereas its protonated form enhances the reactivity toward nitrite and halides.

Oxidation of Nitrite by Myeloperoxidase and Hydrogen Peroxide—We also measured the ability of myeloperoxidase to catalyze the oxidation of nitrite by monitoring the loss of hydrogen peroxide (Fig. 4). There is minimal activity at neutral pH but a sharp increase in activity below pH 6. These results are consistent with the stopped-flow data and indicate that nitrite will be readily oxidized by myeloperoxidase at acidic pH. Since the turnover of compound II determines the rate at which peroxidase substrates are oxidized by myeloperoxidase, nitrite can be regarded as a poor substrate for the enzyme in the neutral pH region. It is also apparent from this study that when a good substrate is available to recycle compound II, the reduction potential of the compound I/compound II couple can be exploited to oxidize nitrite to a larger extent even in the neutral region. Phenols and anilines with one-electron reduction potentials between 1.05 and 1.2 V are oxidized by compound I but not compound II and thus trap the enzyme in the compound II form. Nitrite, which has a one-electron reduction potential of 1.04 V (22), has been shown to be a poor substrate.
oxidizing nitrite by two electrons to directly yield peroxynitrite absolutely exclude that to some extent MPO compound I is kinetic investigations of the reactions of nitrite with human Reactions were started by adding recent published findings that high concentrations of nitrite compound II and is turned over slowly. This is in accord with nitrite mediated by MPO compound I is unlikely to occur (26). From a thermodynamic point of view nitryl ion formation from observed an intermediate with similar spectral features in the characteristic absorption maxima in the visible region. We never published (25). It was shown that the complex exhibited char-
troscopic data of a methemoglobin-peroxynitrite complex was compound I to compound II, and (ii) there was no spectral inclusion is based on several facts as follows: (i) the observed (23, 24).

Summary—Summing up, for the first time transient state kinetic investigations of the reactions of nitrite with human myeloperoxidase have been performed. Although we cannot absolutely exclude that to some extent MPO compound I is oxidizing nitrite by two electrons to directly yield peroxynitrite (6), our findings demonstrate that nitrite is oxidized mainly in one-electron steps by both MPO intermediates in the pH range 4–8. The data suggest that the reaction yields NO2, although we present no direct evidence for NO2 as a product. This conclusion is based on several facts as follows: (i) the observed spectral transition that is typical for a direct conversion of compound I to compound II, and (ii) there was no spectral evidence for a MPO-peroxynitrite complex. Recently, the spectroscopic data of a methemoglobin-peroxynitrite complex was published (25). It was shown that the complex exhibited characteristic absorption maxima in the visible region. We never observed an intermediate with similar spectral features in the course of reaction of compound I with nitrite. (iii) Moreover, from a thermodynamic point of view nitryl ion formation from nitrite mediated by MPO compound I is unlikely to occur (26).

The bimolecular rate constants presented here indicate that nitrite is a poor substrate for myeloperoxidase mainly because it is a bad electron donor for compound II. Thus, at physiological concentrations of nitrite, its oxidation and the nitration reactions should be inefficient because the enzyme lingers at compound II and is turned over slowly. This is in accord with recently published findings that high concentrations of nitrite are required for MPO-mediated nitration of tyrosyl residues (27), bovine serum albumin (6), and low density lipoproteins (28). However, in the presence of better electron donors for compound II (e.g. tyrosine) nitration reactions are enhanced (27). Thus, one can expect that at sites of inflammation myeloperoxidase will mediate nitration reactions because co-substrates like tyrosine will be available to facilitate the turnover of MPO and thus the formation of the powerful oxidizing species NO2 (29), which oxidizes and nitrates many cellular constituents. With increasing concentrations of nitrite, nitration reactions become more and more independent of co-substrates. It was shown that above 250 μM nitrite, a co-substrate was no longer required to promote nitration reactions (27). 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. It is known that high levels of nitrite are present in the rheumatoid joint of patients with rheumatoid arthritis (9) and in synovial fluids of rheumatoid arthritis patients (30). Interestingly, it was recently reported that NO synthase is induced in cytokine-stimulated human neutrophils and found to be co-localized with MPO in primary granules (7). Thus nitrite may play a role in phagocyte-mediated oxidative reactions at sites of inflammation and infection, and formation of NO2 may represent an additional mechanism of host defense.

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