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Nitric Oxide is a Physiological Substrate for Mammalian Peroxidases

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Running Title: Peroxidases are a catalytic sink for nitric oxide

Abbreviations: EPO, eosinophil peroxidase; Fe(III), ferric; Fe(II), ferrous; HRP, horseradish peroxidase; H₂O₂, hydrogen peroxide; LPO, lactoperoxidase; MPO, myeloperoxidase; NOS, nitric oxide synthase; NO, nitric oxide (nitrogen monoxide)

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ABSTRACT

We now show that NO serves as a substrate for multiple members of the mammalian peroxidase superfamily under physiological conditions. Myeloperoxidase (MPO), eosinophil peroxidase and lactoperoxidase all catalytically consumed NO in the presence of the co-substrate hydrogen peroxide (H$_2$O$_2$) at neutral pH. Near identical rates of NO consumption by the peroxidases were observed in the presence versus absence of plasma levels of Cl$^-$ (100 mM). Although rates of NO consumption in buffer were accelerated in the presence of a superoxide-generating system, subsequent addition of catalytic levels of a model peroxidase, MPO, to NO-containing solutions resulted in the rapid acceleration of NO consumption. The interaction between NO and Compounds I and II of MPO were further investigated during steady-state catalysis by stopped-flow kinetics. NO dramatically influenced the build-up, duration and decay of steady state levels of Compound II, the rate limiting intermediate in the classic peroxidase cycle, in both the presence and absence of Cl$^-$. Collectively, these results suggest that peroxidases may function as a catalytic sink for NO at sites of inflammation, influencing its bioavailability. They also support the potential existence of a complex and interdependent relationship between NO levels and the modulation of steady-state catalysis by peroxidases in vivo.
INTRODUCTION

Nitric oxide (NO, nitrogen monoxide) plays essential bioregulatory roles in a wide range of processes critical to normal functions in the cardiovascular, nervous, and immune systems (1,2). Under pathological conditions, such as during inflammation and vascular disease, rates of NO consumption become excessive and impaired response to endothelium-derived relaxing factor or NO are observed (1). Accordingly, factors which influence rates of NO removal following its synthesis by nitric oxide synthases (NOS) are of significant interest. The autoxidation of NO in aqueous solutions is slow at physiological concentrations of O₂ and NO (3). In the vascular compartment, a major pathway for NO removal is through near diffusion-controlled interaction with erythrocyte oxyhemoglobin yielding ferric (met)hemoglobin and nitrate (NO₃⁻) (4). However, during inflammation and vascular disease, oxyhemoglobin levels do not typically alter and enhanced production of NO by NOS is observed. These observations suggest that the rate NO consumption from the subendothelial space of atherosclerotic vessels and other inflammatory tissues is enhanced (1,2). However, the mechanisms accounting for accelerated removal of NO under these conditions are still unclear.

One potential pathway for accelerated NO consumption in tissues is through its rapid reaction with superoxide (O₂⁻) yielding peroxynitrite (ONOO⁻) (1,2,5-7). This reaction may be of particular importance wherever enhanced rates of NO and O₂⁻ production occur, such as in the subendothelial space and other sites of inflammation and phagocyte activation. However, NO consumption by reaction with O₂⁻ does not account for the complete loss of NO-dependent signaling to vascular smooth muscle cells (8), suggesting that alternative pathways exist for depletion of NO. In vitro studies demonstrate that NO is a potent scavenger of a variety of radical intermediates such as lipid peroxyl and alkoxyl radicals (9-12). Similar processes likely occur in atherosclerotic lesions where lipid oxidation products are enriched (13). Reactions of NO with radical intermediates of enzymes that catalyze electron transfer reactions are another potential pathway for NO consumption. For example, recent studies by O’Donnell and colleagues demonstrated turnover-dependent consumption of NO by
lipoxygenase (14), an enzyme implicated in atherogenesis (15,16). The role of these pathways in modulating NO-dependent signaling in vivo remains to be determined.

The ability of NO to react with hemoproteins at nearly diffusion-controlled rates, promoting activation of guanylate cyclase and possibly inhibition of many heme and non-heme proteins by interacting with their metal centers is well known (17-23). Likewise, a variety of studies have documented the ability of NO to bind to the heme moiety of peroxidases (24-27). Both spectroscopic and rapid kinetics measurements were recently used to demonstrate that NO rapidly binds to both ferric [Fe(III)] and ferrous [Fe(II)] forms of myeloperoxidase (MPO) (28), a hemoprotein which is present in abundance in neutrophils, monocytes and certain sub-populations of tissue macrophages, such as in atherosclerotic lesions (29,30). Although human neutrophils isolated from peripheral blood do not normally contain inducible NOS, neutrophils within human buffy coat preparations pre-treated with cytokines are reported to express inducible NOS (31). Moreover, immunohistochemical studies demonstrate that MPO and inducible NOS in cytokine-treated human neutrophils are both co-localized and secreted from the primary granules of activated leukocytes (31). Finally, numerous cell types generate NO at sites of inflammation. Hence, MPO typically performs its functions in environments where NO is formed. MPO uses H₂O₂ and a variety of co-substrates to generate reactive oxidants and diffusible radical species (32-37). Under physiological conditions, a major co-substrate is Cl⁻ yielding hypochlorous acid (HOCl), a potent chlorinating oxidant with microbicidal and viricidal properties (38). The reactive species formed are thought to play a key role in the ability of MPO to promote destruction of invading parasites and pathogens during the host response (39-41). However, MPO-generated oxidants are also linked to tissue oxidation in cardiovascular disease and other inflammatory disorders (42-44).

At ground state MPO exists in the ferric [Fe(III)] form. Like other peroxidases, MPO-Fe(III) uses H₂O₂ as a substrate and is in turn oxidized two electron (e⁻) equivalents forming a redox intermediate termed Compound I, a ferryl π cation radical (MPO-Fe(IV)=O⁺⁺π) with a formal heme charge of +5. The oxidation of halides (e.g. Cl⁻, Br⁻, I⁻) and pseudo halides (e.g. SCN⁻) by MPO Compound I occurs through a single 2 e⁻ transfer reaction where the heme of MPO is reduced to ferric
state and the corresponding hypohalous acid is formed (45,46). MPO Compound I can also oxidize numerous organic and inorganic substrates by two successive 1 e⁻ transfers generating Compound II (MPO-Fe(IV)=O) and MPO-Fe(III), respectively. Reduction of Compound II to ground state is the rate-limiting step in the typical peroxidase cycle and it can be accelerated by physiological reductants like O₂⁻ and ascorbic acid (45-48).

Recent studies suggest that NO can modulate the catalytic activity of MPO (28). High levels of NO inhibited MPO catalysis by the formation of a stable six coordinate low spin nitrosyl complex, MPO-Fe(III)-NO. In contrast, low levels of NO enhanced the initial rate of MPO-catalyzed peroxidation of substrate, presumably by accelerating the rate-limiting step in the peroxidase cycle, reduction of Compound II (28). The latter observation suggested that interactions between NO and Compounds I and II of mammalian peroxidases might serve as a mechanism for catalytic consumption of NO. Consistent with this hypothesis, Ischiropoulos and colleagues reported several years ago that the presence of NO in samples interferes with H₂O₂ determinations by ferrihorseradish peroxidase (HRP) (24), and during the preparation of this manuscript, the rate of reaction of NO with Compounds I and II of this plant peroxidase were reported (49). Although numerous studies have focused on the interactions of NO as a ligand for the heme group of mammalian peroxidases (24-28), neither its role as a substrate for mammalian peroxidases nor the potential for peroxidase-dependent modulation of NO bioavailability have been explored. The present studies evaluate whether mammalian peroxidases use NO as a substrate under physiological conditions and thus may limit its bioavailability at sites of inflammation.
**EXPERIMENTAL PROCEDURES**

*Materials:* NO gas was purchased from Matheson Gas products, Inc., and used without further purification. For each experiment, a fresh saturated stock of NO was prepared under anaerobic conditions. The extent of nitrite/nitrate (NO$_2$-/NO$_3$-) build-up in NO preparations over the time course used for the present studies was < 1-1.5% (per mol NO), as determined by anion exchange HPLC under anaerobic conditions (50). All other reagents and materials were of the highest purity grades available and obtained from Sigma Chemical Co (St. Louis, MO) or the indicated source.

Human EPO was isolated from porcine whole blood obtained fresh at the slaughterhouse according to the method of Jorg (51) employing guaiacol oxidation as the assay (38). Purity of EPO preparations was assured before use by demonstrating a $R_Z$ of $> 0.9$ ($A_{415}/A_{280}$), SDS PAGE analysis with Coomassie Blue staining, and in-gel tetramethylbenzidine peroxidase staining to confirm no contaminating MPO activity (52). MPO was initially purified from detergent extracts of human leukocytes by sequential lectin affinity and gel filtration chromatography as described (53). Trace levels of contaminating EPO were then removed by passage over a sulphopropyl Sephadex column (54). Purity of isolated MPO was established by demonstrating a $R_Z$ of 0.87 ($A_{430}/A_{280}$), SDS PAGE analysis with Coomassie Blue staining, and in-gel tetramethylbenzidine peroxidase staining to confirm no contaminating EPO activity (52). Enzyme concentrations were determined spectrophotometrically utilizing extinction coefficients of 89,000 and 112,000 M$^{-1}$cm$^{-1}$/heme of MPO (55) and EPO (56,57), respectively. The concentration of the MPO dimer was calculated as half the indicated concentration of heme-like chromophore. Bovine LPO was obtained from Worthington Biochemistry Corporation (Lakewood, NJ) and used without further purification. Purity was confirmed by demonstrating a $R_Z$ of 0.75 ($A_{412}/A_{280}$) and SDS PAGE analysis with Coomassie Blue staining.

*NO selective electrode measurements:* NO measurements were carried out using an NO-selective electrode (ISO-NO Mark II, World Precision Instruments, Sarasota, FL) connected to a chart recorder. Experiments were performed at 25°C by immersing the electrode in 10 ml of 0.2 M sodium phosphate
buffer, pH 7.0, under air. NO was added to continuously stirred buffer solution from an NO-saturated stock and the rise and fall in NO concentration was continuously monitored. To determine the effect of H$_2$O$_2$ and peroxidases on NO levels during steady state catalysis, 10 µl H$_2$O$_2$ (100 µM final) and 50 µl enzyme (150 nM final) were added to the reaction mixture. Where indicated, solutions were supplemented with NaCl (100 mM) and/or a cell-free O$_2$•-generating system comprised of lumazine (0.4 mm) and bovine milk xanthine oxidase (XO, Boehringer Mannheim). Superoxide generation under the conditions utilized was ~40µM/min, as measured by the superoxide dismutase-inhibitable reduction of ferricytochrome c (58).

**Spectroscopy:** Anaerobic spectra of MPO-Fe(III) were recorded at 25°C in septum-sealed quartz cuvettes that were equipped with a quick-fit joint for attachment to a vacuum system. MPO samples were made anaerobic by repeated cycles of evacuation and equilibrated with catalyst-deoxygenated N$_2$. Cuvettes were maintained under N$_2$ or NO atmosphere during spectral measurements.

**Stopped-flow Measurements:** The kinetics of Compound II formation and decay in the absence and presence of different NO concentrations were performed using a dual syringe stopped-flow instrument obtained from Hi-Tech Ltd (model SF-51). Measurements were carried out under anaerobic atmosphere, at 25°C and monitored at 455 nm (an isosbestic point of Compound I and MPO ground state) following rapid mixing of equal volumes of an H$_2$O$_2$-containing buffer solution and an MPO solution that contained different NO concentrations. The time course of absorbance change was fit to either single or double exponential functions as indicated. The rate constants for the formation (k$_{on}$) and decay (k$_{off}$) of the MPO-Fe(III)-NO complex in the presence of plasma levels of the alternative substrate Cl$^-$ (100 mM) were determined by monitoring absorbance change at 430 nm at 10°C. The time course was accurately fit to the first-order exponential equation (Y = 1 – e$^{-kt}$) using a nonlinear least-squares method provided by the instrument manufacturer. Signal to noise ratios for all kinetic analyses were improved by averaging at least six to eight individual traces.
RESULTS

NO is catalytically consumed by mammalian peroxidases under physiological conditions. Our initial experiments utilized an NO-selective electrode to determine whether NO serves as a general substrate for peroxidases. Reactions were performed under conditions where peroxidases were present in catalytic amounts. Following addition of an aliquot of NO-saturated buffer to the continuously stirred reaction mixture (6 µM NO final), the NO signal rose rapidly, achieved a maximum after ~30 s, and fell gradually to the origin as NO was depleted by autoxidation (Fig. 1A). Addition of H₂O₂ to the reaction mixture had no significant effect on the rate of NO decay (Fig. 1A), similar to prior reports (24). Subsequent addition of either MPO, EPO or LPO to the reaction mixture caused a rapid decay in the level of free NO (Fig. 1), indicating that NO is consumed as a substrate by mammalian peroxidases during steady-state catalysis. Reversal of the order of peroxidase and H₂O₂ addition demonstrated a modest brief decrement in NO concentration following addition of only the peroxidase (presumably due to heme Fe(III)-NO complex formation) and then a similar significant acceleration in NO consumption upon subsequent addition of H₂O₂ (Fig. 1B, only data for MPO shown).

To examine the potential physiological significance of these observations, we next determined the effect of additional substrates on peroxidase-catalyzed consumption of NO. MPO was initially used as a model peroxidase because of its abundance at sites of leukocyte recruitment and activation during inflammation (30), and its well-known use of the abundant halide Cl⁻ as substrate (32,58). Remarkably, the rates of NO consumption mediated by MPO in the presence versus absence of plasma levels of Cl⁻ were virtually indistinguishable (Fig. 2). These results are consistent with the fact that MPO is far from saturated at plasma levels of Cl⁻ (52). NO consumption by MPO was to a large extent prevented by pre-incubation of the enzyme solution with sodium azide, a peroxidase inhibitor (59) (Fig. 2). Similar results were observed with other mammalian peroxidases (data not shown).

Leukocyte activation in vivo is accompanied by MPO secretion and O₂•⁻ formation during the respiratory burst. Since O₂•⁻ interacts so rapidly with NO (60), it was unclear whether peroxidases could accelerate NO consumption under these conditions. Addition of NO to buffer containing a cell-
free $O_2^{\cdot-}$-generating system resulted in accelerated removal of NO, as detected by continuous monitoring with a NO-selective electrode (Fig. 3). Subsequent addition of catalytic amounts of a mammalian peroxidase (data for MPO shown) resulted in the enhanced removal of NO from the buffer (Fig. 3). Similar results were observed with other mammalian peroxidases (data not shown). Finally, addition of MPO to media containing plasma levels of Cl$^-$ (100 mM), NO and a $O_2^{\cdot-}$-generating system resulted in a marked acceleration in the rate of NO consumption above and beyond that observed with only the $O_2^{\cdot-}$-generating system (Fig. 3). Thus, peroxidases like MPO can effectively act as catalysts for NO consumption under conditions likely to be physiological in the phagolysosome or at sites of inflammation.

Spectroscopic and rapid-kinetics characterization of the interaction between NO and MPO Compounds I and II. As previously reported, addition of H$_2$O$_2$ to MPO-Fe(III) in the absence of co-substrates leads to the accumulation of Compound II via rapid initial formation of Compound I, and subsequent spontaneous one e$^-$ heme reduction, (45,48). Compound II, the rate-limiting intermediate in the peroxidase cycle, possesses a characteristic Soret absorbance peak at 455 nm that is easily distinguished from the Soret absorbance peaks of MPO-Fe(III) and the MPO-Fe(III)-NO, as shown in Fig. 4. MPO Compound II is unstable and converted gradually to the ground state, MPO-Fe(III), within minutes of initiating the reaction.

To examine the kinetics of interaction between NO and MPO Compounds I and II, we utilized stopped-flow spectroscopy. Rapid kinetic studies were initially performed under Cl$^-$-free conditions using 2 co-substrates, a fixed (low) level of H$_2$O$_2$ and variable levels of NO. These conditions were chosen to facilitate the direct examination of NO as a substrate for various forms of MPO in the absence of multiple competing co-substrates. The influence of NO on the kinetics of Compound II buildup, duration and decay during steady-state catalysis were examined under anaerobic conditions following rapid mixing of enzyme and various concentrations of NO (2.5, 12.5, 50 and 400 µM final) in the presence of physiological concentrations of H$_2$O$_2$ (10 µM final). Fig. 5A (Inset) shows the time course for the formation and decay of compound II in the absence of NO detected by monitoring the
absorbance change at 455 nm. The change in absorbance that takes place in the first 2 s of the reaction is shown in Fig. 5A and is attributed to the buildup of Compound II. The build-up of Compound II was best fit to a single exponential function, giving an apparent pseudo first-order rate constant of 3.2 s\(^{-1}\). The subsequent decrease in absorbance at 455 nm observed was also fit to a single exponential function with a rate constant of 0.008 s\(^{-1}\) and was attributed to the decay of compound II. Together, these results indicate that the buildup of MPO Compound II in the absence of NO is rapid, monophasic, and occurs with a much faster rate than its decay.

The addition of NO to reaction mixtures results in dramatic effects on the rates of MPO Compound II build-up, duration and decay, as assessed by stopped-flow spectroscopy (Fig. 6). NO was readily used as a one e\(^{-}\) substrate by Compound I, as indicated by the rapid buildup of MPO Compound II (Fig. 5B). The rate of Compound II accumulation was enhanced nearly 20 fold in the presence of NO and increased in a concentration-dependent and saturable manner (Fig. 7A). The presence of NO had a variable effect on the duration of steady state concentrations of Compound II which develop following H\(_2\)O\(_2\) addition (Fig. 6 and Fig. 7B). Finally, NO significantly accelerated the rate of MPO Compound II decay in a concentration-dependent fashion. A plot of NO concentration vs. rate of Compound II decay demonstrated linear kinetics and yielded a second order rate constant of 8 \(\times\) 10\(^3\) M\(^{-1}\)s\(^{-1}\) (Fig. 7C). The accelerated rate of Compound II decay in the presence of NO indicates that it also serves as a one e\(^{-}\) substrate for MPO Compound II.

In a parallel series of experiments, we examined the influence of NO on the kinetics of MPO Compound II buildup, duration and decay during steady-state catalysis in the presence of plasma levels of the competing substrate Cl\(^{-}\). Reactions were again performed under anaerobic conditions and the absorbance change at 455 nm monitored following rapid mixing of enzyme and various concentrations of NO (2.5, 12.5, 25 and 50 µM final) in the presence of H\(_2\)O\(_2\) (10 µM final) and Cl\(^{-}\) (100 mM final). Addition of NO to MPO, H\(_2\)O\(_2\) and Cl\(^{-}\) resulted in a significant increase in the amount of Compound II formed during steady state catalysis (Fig. 8). In the presence of physiologically relevant levels of the competing substrate Cl\(^{-}\), stopped-flow analysis of the NO concentration dependence on both the
amount of Compound II formed, and the rate of Compound II formation and decay, all reveal that NO serves as a substrate for MPO Compounds I and II during steady state catalysis (Fig. 9). Finally, to assist in the interpretation of these results (Discussion), stopped-flow methods were used to determine the association (kon) and dissociation (koff) rates of NO binding to MPO-Fe(III) in the presence of 100 mM Cl\. Analysis of stopped-flow traces collected when the enzyme solutions mixed with NO were accurately fit by a single exponential function (data not shown). The plots of the apparent rate constants as a function of NO concentration for MPO-Fe(III) were linear (r > 0.99, data not shown), consistent with NO binding to MPO-Fe(III) in a simple one step mechanism. The kon and koff calculated from the slope and intercept, respectively, for NO binding to MPO-Fe(III) in the presence of 100 mM Cl\ at 10°C were 0.15 µM\(^{-1}\)s\(^{-1}\) and 22.3 s\(^{-1}\), respectively.
DISCUSSION

Ischiropoulos first suggested that NO might serve as a substrate for plant peroxidases several years ago during investigations with HRP (24). The results of the present study extend these findings across phylogenetic boundaries and demonstrate that homologous members of the mammalian superfamily of peroxidases share this activity. During continuous monitoring of NO levels with an NO-selective electrode, rates of NO removal from media were dramatically accelerated by addition of mammalian (e.g. MPO, EPO, LPO) peroxidases and the co-substrate H$_2$O$_2$. Peroxidase-dependent consumption of NO was catalytic since only trace levels of peroxidases were required, relative to the concentrations of NO and the co-substrate H$_2$O$_2$ used. Furthermore, peroxidase-dependent consumption of NO occurred using concentrations of NO and H$_2$O$_2$ that span both the physiological and pathophysiological range. Studies using a model peroxidase, MPO, revealed that peroxidases efficiently consume NO in the presence of physiological levels of alternative substrates like Cl$^-$ (Fig. 2) and in the presence of a cell-free O$_2^*$-generating system both in the absence and presence of Cl$^-$ (Fig. 3). However, this process can be partially or completely blocked by pre-incubation of the enzyme sample with classic peroxidase inhibitors (Fig. 2). Finally, stopped-flow studies demonstrated that NO served as a one e$^-$ substrate for both MPO Compounds I and II during steady-state catalysis (Figs. 5-7), even in the presence of physiological levels of the alternative substrate Cl$^-$ (Figs. 8,9). Taken together, these studies suggest that mammalian peroxidases may serve as a catalytic sink for NO, regulating its bioavailability and function.

A model of how NO interacts with intermediates in the classic peroxidase cycle is proposed for MPO in Fig. 10. In general, NO may influence peroxidase steady-state catalysis by two distinct mechanisms - via acting either as a one e$^-$ peroxidase substrate or a heme ligand. The ability of NO to serve as a substrate for MPO was directly demonstrated in studies employing an NO-selective electrode (Figs. 1-3). Moreover, the ability of both MPO Compounds I and II to use NO as a one e$^-$ substrate is also illustrated by the NO-dependent increases in the rates of MPO Compound II formation and decay observed (Figs. 5-9). In the absence of Cl$^-$ and biologically relevant levels of NO (\( \leq 2.5 \))
µM) and H₂O₂ (10 µM), NO accelerated the overall transit time of the peroxidase cycle nearly three orders of magnitude by accelerating rates of Compound I and Compound II reduction approximately 20-fold and 44-fold, respectively. Studies performed in the presence of plasma levels of Cl⁻ demonstrate that NO will serve as physiological substrate for both MPO Compounds I and II, as reflected in accelerated rates of Compound II formation and decay, respectively (Figs 8 and 9).

The ability of NO to influence Compound II rates of formation and decay strongly supports the notion that NO undergoes a one rather than a two e⁻ oxidation transition following interaction with both Compounds I and II. The presumed intermediate formed, nitrosonium cation (NO⁺), is extremely labile (half life < 0.3 nsec) and is rapidly hydrolyzed in aqueous solutions forming nitrite (NO₂⁻) (61). The crystal structure of MPO demonstrates the presence of several ordered water molecules within the distal heme pocket (62). Although we provide no direct evidence for NO⁺ formation, we do note corresponding one e⁻ heme reduction steps for MPO Compounds I and II in the presence of NO. Mason and colleagues similarly concluded that NO⁺ is formed in their recent studies examining interactions of NO with the plant peroxidase HRP (49). Like them, we also note that the hydration product of NO⁺, NO₂⁻, is generated catalytically during peroxidase-mediated oxidation of NO (data not shown). Formation of NO₂⁻, a substrate for MPO and other heme peroxidases (36,37,63), at or near the heme moiety might thus also contribute to the increased overall transit time through the peroxidase cycle observed in the presence of NO. Moreover, it would generate a reactive nitrogen species capable of promoting protein nitration and lipid peroxidation, presumably nitrogen dioxide (•NO₂) (36,64,65) (Fig. 10). Thus, an overall net effect of NO consumption as a peroxidase substrate is likely to both limit NO bioavailability and to form more potent and bactericidal oxidants than NO.

One important methodological consideration was to ensure that the extent of NO₂⁻/NO₃⁻ accumulation in NO stock solutions was minimal so as not to contribute to observed rates of MPO Compound I and II reduction during rapid kinetics studies. To evaluate this possibility we used HPLC analysis (anion exchange) under strictly anaerobic conditions and observed a 1-1.5% accumulation of NO₂⁻/NO₃⁻ in NO stock solutions during the time course of use for each study. At the levels of NO that
produce dramatic effects on the rates of Compound II content, formation and decay (Figs. 6-9), the levels of NO\textsubscript{2} present are nominal. For example, significant effects are seen with as little as 2.5 µM NO on both the extent and rate of Compound II formation in both the absence (Fig. 6,7) and presence (Fig. 8,9) of Cl\textsuperscript{−}. At the levels of NO\textsubscript{2} contaminating these preparations (25-37.5 nM), no significant effect is observed on rates of MPO Compound II formation or decay (data not shown). Recent studies by Obinger and colleagues (66) consistent with these observations and demonstrate that significantly higher levels of NO\textsubscript{2} are required to promote comparable rates of reduction of MPO Compounds I and II.

Our rapid kinetics measurements indicate that accelerated MPO Compound II decay in the presence of NO (and/or NO\textsubscript{2}, not shown) is an irreversible process since the Y-intercept of the plots examining rates of NO-dependent decay of Compound II intersect at or near the rate observed in the absence of NO (Fig. 7C,9C). Consistent with this interpretation, addition of NO to MPO-Fe(III) fails to generate any detectable Compound II (Fig. 4). However, based upon the presented data, we can not exclude the possibility that the interaction of NO with Compound II is partially reversible. Compound II formation and decay were monitored at the isosbestic point for MPO-Fe(III) and Compound I; thus, if any Compound I (and NO\textsuperscript{−}) were formed following interaction of NO with Compound II, it would not have been observed. To address this question, a separate set of experiments were performed to look for evidence of Compound I accumulation during interaction of NO with Compound II. None was seen; rather, MPO-Fe(III) was formed (data not shown). These results strongly suggest that interaction of NO with Compound II is irreversible and does not form any Compound I. However, the present data still cannot exclude the possibility that there exists a slow finite rate of NO interaction with Compound II that yields Compound I and NO\textsuperscript{−}. If this hypothetical reverse reaction occurred at a slow rate relative to NO dependent reduction of Compound I generating Compound II, then accumulation of Compound I would not be observed. Recent studies with NO and various intermediate forms of HRP suggest that NO irreversibly promotes reduction of Compounds I and II of the plant peroxidase (49). We also note that recent studies by Obinger and colleagues conclude that
NO$_2^-$ irreversibly reduces MPO Compounds I and II (66). Collectively, our results strongly suggest that NO interactions with MPO Compounds I and II are irreversible and lead to one e$^-$ reduction steps of the respective heme groups.

NO also may serve as a ligand for MPO-Fe(III) leading to inhibition of peroxidase activity and formation of a MPO-Fe(III)-NO complex (Fig. 10) (28). Examination of the NO concentration-dependence for the rate of Compound II formation revealed saturable kinetics at levels of NO > 2.5 µM in both the absence (Fig. 7A) and presence (Fig. 9A) of the competitor Cl$^-$. The first order rate constant observed in the absence of Cl$^-$ and high levels of NO (53 s$^{-1}$, 25°C) is similar to that of the temperature adjusted dissociation rate reported for NO from the MPO-Fe(III)-NO complex (28). Thus, in the absence of Cl$^-$ and the presence of high levels of NO, the rate-limiting step in catalysis becomes the relatively slow dissociation of NO from the MPO-Fe(III)-NO complex, resulting in inhibition of peroxidase activity. In contrast, in the presence of physiological levels of the alternative substrate Cl$^-$, the NO concentration-dependence for the rate of Compound II formation yields a first order rate constant (6.10 s$^{-1}$, 25°C) that is significantly less than that of the dissociation rate (22.3 s$^{-1}$, 10°C) obtained for NO from the MPO-Fe(III)-NO complex (in the presence of 100 mM Cl$^-$) (Results). Thus, in the presence of Cl$^-$, the plateau in the curve for the NO concentration-dependence on the rate of Compound II formation (Fig. 9A) can not be explained by a rate-limiting dissociation of the MPO-Fe(III)-NO complex. The explanation for the plateau in the curve is still under investigation and may reflect a conformational alteration required for NO-dependent reduction of MPO Compound I in the presence of Cl$^-$ that is rate-limiting.

Although addition of plasma levels of the competing substrate Cl$^-$ have little effect on the capacity of MPO to consume NO as a substrate (Figs. 2,3), the present studies suggest that Cl$^-$ promotes significant alterations in the heme pocket of ground state MPO, as reflected in the $k_{on}$, $k_{off}$ and $K_{diss}$ describing interactions of NO with MPO-Fe(III). For example, in the absence of Cl$^-$, the $k_{on}$, $k_{off}$ and $K_{diss}$ at 10°C were recently determined to be 1.07 µM$^{-1}$s$^{-1}$, 10.8 s$^{-1}$, and 10 µM, respectively (28). This contrasts with the $k_{on}$, $k_{off}$ and $K_{diss}$ at 10°C obtained in the presence of Cl$^-$ (0.15 µM$^{-1}$s$^{-1}$,
22.3 s\(^{-1}\), and 148 µM, respectively) in the present studies. The binding of Cl\(^-\) to the enzyme thus significantly influences the interactions of NO with the distal heme moiety. Taken together, the present studies suggest that formation of a stable MPO-Fe(III)-NO complex is less likely to occur in the presence versus absence of Cl\(^-\).

Under all concentrations of NO examined, Compound II accumulated upon addition of H\(_2\)O\(_2\) during steady state catalysis. This is because the rate of Compound II formation always exceeds the rate of Compound II decay in both the absence (Fig. 7) and presence (Fig. 9) of Cl\(^-\). A predicted consequence of NO-dependent modulation of steady state levels of MPO Compound II will be to influence the ability of the enzyme to promote one vs. two e\(^-\) peroxidation reactions. In the presence of H\(_2\)O\(_2\), reduction of Compound II permits more of the peroxidase to be available for generation of Compound I, and enhanced chlorinating activity for MPO in the presence of other one e\(^-\) reductants (e.g. ascorbic acid, superoxide) has been reported (45,48).

Although the ability of ferrihorseradish peroxidase to use NO as a substrate has been known for several years (24), rapid kinetics analyses of the reaction between NO and Compounds I and II of the plant peroxidase were only recently reported (49). Like MPO, both Compounds I and II of HRP utilize NO as a one e\(^-\) substrate (49). Direct comparisons of the kinetics of MPO vs. HRP utilization of NO are difficult, however, since the two studies were carried out under different experimental conditions. Whereas our kinetic studies examined the interactions of NO with MPO intermediates during steady state catalysis under pseudo first order conditions, the HPR studies were carried out under non-steady state conditions in the presence of equimolar concentrations of enzyme and H\(_2\)O\(_2\) (49). Despite these differences in experimental design, several major distinctions between NO interactions with MPO and HRP are apparent: 1) NO dramatically accelerates the rate of MPO Compound II formation in the nanomolar to low micromolar (≤ 2.5 µM) range (Fig. 7), whereas significantly higher levels of NO are required with HRP (49); 2) NO reduces HRP Compound II faster than HRP Compound I (49) - the opposite was observed with MPO (Figs. 7,9); and 3) the rate constant for the reduction of HRP Compound II by NO appears to be much faster than the rate of NO-dependent reduction of MPO.
Compound II. Regardless of these differences, NO appears to serve as a general substrate for both plant and animal peroxidases.

An interesting feature of MPO - NO interactions is its parallel behavior to that observed during peroxidase interactions with another physiological diatomic ligand, $\text{O}_2^-\text{•}$ (45,48). Both serve as reductants for Compound II and lead to enhanced overall peroxidase activity due to acceleration of this rate limiting step in the peroxidase cycle. Both $\text{O}_2^-\text{•}$ and NO also serve as ligands for MPO-Fe(III) and generate inactive complexes, Compound III and MPO-Fe(III)-NO, respectively (Fig. 10). Formation of each is a reversible process and addition of $\text{H}_2\text{O}_2$ to each result in spectral changes consistent with formation of Compound II. Thus, both Compound III and MPO-Fe(III)-NO may still promote peroxidation reactions. This contrasts with the mechanism for inactivating MPO following reduction of the ground state ferric to ferrous form (Fig. 10). Here, heme reduction appears to be accompanied by collapse of the heme pocket, as defined by any conformational alteration, however subtle, that limits access of substrate to the distal heme center (28). An example would be the binding of a 6th axial ligand from an amino acid residue on the opposing side/wall of the heme pocket. The slow rate of NO binding to MPO-Fe(II) observed (28) is consistent with ligand replacement rather than direct binding of NO to the MPO heme iron. Changes in heme pocket geometry upon ligand binding (e. g. movement of a number of amino acid residues and a rearrangement of active site water molecules) have been described for cytochrome c peroxidase (26). Moreover, slower rates of NO binding to the Fe(III) forms of a number of heme proteins have been attributed to ligand replacement (21,67). Collapse in the heme pocket geometry upon heme reduction has also been reported for other heme proteins (68).

Another remarkable feature of the present studies is the demonstration that peroxidases catalytically consume NO under a variety of conditions that mimic those found in biological systems. For example, NO consumption rates were not inhibited by addition of physiologically relevant amounts of alternative substrates, such as plasma levels of $\text{Cl}^-\text{•}$ and the model peroxidase MPO (Fig. 2). Studies with MPO examining $\text{H}_2\text{O}_2$ consumption rates in the presence of plasma levels of $\text{Cl}^-\text{•}$ and
additional alternative substrates (e.g. thiocyanate) have revealed that MPO is far from saturated at plasma levels of Cl\(^-\) (52). It is also remarkable that peroxidases like MPO effectively act as catalysts for NO consumption, even in the presence of a \(\text{O}_2^{\bullet^-}\) - generating system (Fig. 3). The rate of NO interaction with \(\text{O}_2^{\bullet^-}\) is significantly greater than the rates observed for NO interaction with all of the various intermediates of MPO. The effectiveness of peroxidases in accelerating NO consumption in the presence of a \(\text{O}_2^{\bullet^-}\)-generating system was therefore surprising. However, it is easily rationalized once one recalls that \(\text{O}_2^{\bullet^-}\) fails to accumulate in aqueous solutions to any significant degree, but rather dismutates to form \(\text{H}_2\text{O}_2\), which can accumulate. Accelerated consumption of NO upon peroxidase addition is thus observed. The present results thus demonstrate that peroxidases may accelerate NO consumption even in the presence of alternative substrates and a \(\text{O}_2^{\bullet^-}\)-generating system.

Finally, the results of the present study raise the intriguing possibility that peroxidases may function as a catalytic sink for NO, limiting it bioavailability \textit{in vivo}. MPO is the single most abundant protein in neutrophils (30) and is present in large amounts at sites of inflammation where it is catalytically active (43). It is therefore tempting to speculate that this activity may be relevant, particularly at sites of inflammation where leukocyte peroxidases, NO and \(\text{H}_2\text{O}_2\) are present. In an analogous fashion, a wealth of clinical, pathological, biochemical and genetic data support the notion that atherosclerosis is a chronic inflammatory disorder (reviewed in ref. 69). The development of atherosclerosis is accompanied by impaired guanylate cyclase activation and vascular response to endothelium-derived relaxing factor or NO (1,2,5). Although NO consumption by oxyhemoglobin is likely a major pathway for NO consumption \textit{in vivo}, additional alternative pathways must also play a role in atherosclerosis since oxyhemoglobin levels are not changing. MPO is enriched (41) and catalytically active (42) in human atheroma. Mass spectrometry and immunohistochemistry studies have demonstrated that multiple distinct oxidation products formed by MPO are also enriched in human atheroma (reviewed in ref. 70). It is therefore tempting to speculate that peroxidases like MPO might play a role in altering guanylate cyclase activation as well as other NO-dependent signaling events during development of vascular disease. Our present and prior (28) studies also suggest that
NO may play a significant role in modulating peroxidase activity \textit{in vivo}. By influencing steady-state levels of MPO Compound II formation, duration and decay, NO affects the overall rate of peroxidation of substrates and the overall ability of the peroxidase to execute one vs. two e\textsuperscript{−} oxidation reactions. Thus, NO levels and peroxidase activity are apparently coupled through complex and interdependent pathways. The biological consequences of NO-peroxidase interactions may have broad implications for the regulation of local inflammatory, infectious and cardiovascular events \textit{in vivo}. 
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**FIGURE LEGENDS**

**Figure 1. Consumption of NO by mammalian peroxidases.** (Panel A, dotted line) A typical recording by an NO-selective electrode demonstrating the autoxidation of NO (6 µM) following addition to a stirred 0.2 M sodium phosphate buffer (pH 7.0) at 25°C under air. (Panel A, sold line) Addition of H₂O₂ followed by myeloperoxidase (MPO) results in a dramatic acceleration in the rate of NO consumption. (Panel B) Addition of MPO followed by addition of H₂O₂ results in a similar dramatic acceleration in NO consumption. (Panels C and D) Influence H₂O₂ followed by addition of eosinophil peroxidase (EPO, Panel C) and lactoperoxidase (LPO, Panel D). Tracings shown are from a typical experiment performed at least three times. The concentrations of additions were as follows: H₂O₂, 100 µM; MPO, EPO or LPO or 150 nM.

**Figure 2. Effect of chloride and azide on NO consumption by MPO during steady state catalysis.** Time course for NO (6 µM) autoxidation following the addition of 100 µM H₂O₂ in 0.2 M sodium phosphate buffer in the presence of either no other additions (N. A.) (...) or 150 nM MPO (—). Where indicated, reaction mixtures containing NO (6 µM) and H₂O₂ (100 µM) were also supplemented with 150 nM MPO incubated with 10 mM azide (---), or 150 nM MPO in the presence of 100 mM NaCl (••••).  

**Figure 3. Effect of MPO on NO consumption in the presence of a superoxide generating system.** A typical recording by an NO-selective electrode showing the autoxidation of NO (6 µM) following addition to a stirred 0.2 M sodium phosphate buffer (pH 7.0) at 25°C under air (N.A., no addition) in the absence (—) or presence (---) of 100 mM Cl⁻ is shown. Where indicated, a O₂⁻⁻-generating system (lumazine/xanthine oxidase) was included in the buffer solution at a level that produced a measured rate of O₂⁻⁻ production of 40 µM/min, as described under “Experimental Conditions.” MPO (150 nM) was added to reaction mixtures containing the O₂⁻⁻-generating system at the time indicated by the arrow. Note addition of MPO further accelerated the rate of NO consumption above that observed with the O₂⁻⁻-generating system both in the absence and presence of Cl⁻.
Figure 4. Absorbance spectra of MPO-Fe(III), MPO Compound II and MPO-Fe(III)-NO. Absorbance spectra of MPO-Fe(III) before (—) and after addition of either H$_2$O$_2$ (…) or NO (---). Experiments were performed under anaerobic conditions in sodium phosphate buffer (200 mM, pH 7.0) containing 0.86 µM MPO in the absence and presence of either 100 µM H$_2$O$_2$ or 100 µM NO at 25°C as described under “Experimental Conditions.”

Figure 5. Time course of MPO Compound II formation following addition of H$_2$O$_2$ in the absence and presence of NO. An anaerobic solution containing sodium phosphate buffer (200 mM, pH 7.0) supplemented with H$_2$O$_2$ (20 µM) was rapidly mixed with an equal volume of buffer supplemented with 0.86 µM of MPO-Fe(III) either in the absence (Panel A) or presence (Panel B) of NO (100 µM) at 25°C. Spectral changes were monitored at 455 nm, a characteristic wavelength for Compound II, as a function of time. Data was best fit to a single exponential function (solid line). (Inset) Expanded time course of Compound II formation and subsequent decay upon addition of MPO and H$_2$O$_2$ in the absence of NO as in Fig. 5A.

Figure 6. Effect of NO concentration on MPO Compound II formation, duration and decay. An anaerobic solution containing sodium phosphate buffer (200 mM, pH 7.0) supplemented with H$_2$O$_2$ (20 µM) was rapidly mixed with an equal volume of buffer containing 0.86 µM of MPO-Fe(III) and differing concentrations of NO at 25°C. MPO Compound II formation, duration and decay were monitored as a function of time by observing spectral changes at 455 nm. The final concentration of NO in mixtures is indicated.

Figure 7. Rate of MPO Compound II formation, duration and decay as a function of NO concentration. The observed rate of MPO Compound II formation, duration and decay (monitored at 455 nm) observed in Fig. 6 were plotted as a function of NO concentration. Data represent the mean of triplicate determinations from an experiment performed three times.

Figure 8. Effect of NO concentration on MPO Compound II formation during steady state catalysis in the presence of plasma levels of Cl$. An anaerobic solution containing sodium phosphate
buffer (200 mM, pH 7.0) supplemented with 100 mM NaCl and H₂O₂ (20 µM) was rapidly mixed with an equal volume of buffer containing 0.86 µM of MPO-Fe(III), 100 mM NaCl and differing concentrations of NO at 25°C. The time course of MPO Compound II formation during steady state catalysis was monitored by observing spectral changes at 455 nm. The final concentration of NO in mixtures is indicated. Note that even in the presence of physiological levels of the competing substrate Cl⁻, NO significantly enhances the overall level of MPO Compound II formed during steady state catalysis.

Figure 9. Rate of MPO Compound II formation and decay, and overall content of MPO

Compound II formed, as a function of NO concentration. The effect of varying NO concentration on the observed rate of MPO Compound II (Panel A) formation and (Panel C) decay, and (Panel B) steady state levels of MPO Compound II formed were monitored at 455 nm under experimental conditions identical to those described in Fig. 8. Data represent the mean of three independent experiments.

Figure 10. Working kinetic model for NO interactions with MPO
NO (µM)

Time (min)

H₂O₂

N. A.
+ MPO + NaN₃

+ MPO + Cl⁻
+ MPO
[NO] = 0 µM

2.5 µM

12.5 µM

50 µM

400 µM

Absorbance Change (%)

Time (s)
