THIOCYANATE MODULATES THE CATALYTIC ACTIVITY OF MAMMALIAN PEROXIDASES

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Running Title: Differences in the Mechanisms of Mammalian Peroxidases

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We investigated the potential role of the co-substrate, thiocyanate (SCN-), in modulating the catalytic activity of myeloperoxidase (MPO) and other members of mammalian peroxidase superfamily (lactoperoxidase (LPO) and eosinophil peroxidase (EPO)). Pre-incubation of SCN- with MPO generates a more complex biological setting, because SCN- serves as either a substrate or inhibitor, causing diverse impacts on MPO heme iron microenvironment. Consistent with this hypothesis, the relationship between the association rate constant of nitric oxide (NO) binding to MPO-Fe(III) as a function of SCN- concentration is bell-shaped, with a trough comparable to normal SCN- plasma levels. Rapid kinetic measurements indicate that MPO, EPO, and LPO Compound I formation occur at rates slower than complex decay, and its formation serves to simultaneously catalyze SCN- via 1e- and 2e- oxidation pathways. For the three enzymes, Compound II formation is a fundamental feature of catalysis, and allows the enzymes to operate at a fraction of their possible maximum activities. MPO and EPO Compound II is relatively stable and decays gradually within minutes to ground state upon H2O2 exhaustion. In contrast, LPO Compound II is unstable and decays within seconds to ground state, suggesting that SCN- may serve as a substrate for Compound II. Compound II formation can be partially or completely prevented by increasing SCN- concentration, depending on the experimental conditions. Collectively, these results illustrate for the first time the potential mechanistic differences of these three enzymes.

A modified kinetic model, which incorporates our current findings with the mammalian peroxidases classic cycle, is presented.

Myeloperoxidase (MPO) and other members of mammalian peroxidases superfamily (eosinophil peroxidase (EPO) and lactoperoxidase (LPO)) display a crucial difference (within a wide range of biological processes) in their unique ability in catalyzing the H2O2 - dependent peroxidation of halides and pseudo halides to produce anti-microbial agents, hypohalous acids (1-7). These heme-containing enzymes share 50-70% overall amino acid sequences homology, but differ from each other with respect to their sites of expression, their primary sequences, and their substrate specificities (1,2,8-10). MPO is a 150-165 kDa molecule synthesized during myeloid differentiation that constitutes the major component of the neutrophil azurophilic granules (11,12). The enzyme is a homodimer comprising of a pair of light- and heavy-chain derived from a single gene product (12,13) with its subunits joined by a single disulfide bridge (12). The heavy chain contains an iron bound by a novel protoporphyrin IX derivative, which is covalently linked to the heavy chain polypeptide (14-16).

EPO is a monomeric molecule comprised of a pair of light- and heavy-chain derived from a single gene product (12,13) with its subunits joined by a single disulfide bridge (12). The heavy chain contains an iron bound by a novel protoporphyrin IX derivative, which is covalently linked to the heavy chain polypeptide (14-16). EPO is a monomeric molecule comprised of a light and heavy chain with a molecular weight of 15.5 and 50 kDa, respectively (17). This enzyme is stored in eosinophil granules and catalyzes the formation of antimicrobial species from the oxidation of Br- and SCN- (17-19). LPO is a monomeric single polypeptide chain with a molecular weight of 78.5 kDa (20-23). LPO has been identified as an antimicrobial agent within exocrine gland secretions such as milk, saliva, and
tears through the oxidation of thiocyanate by H₂O₂ to yield the intermediary oxidation product hypothiocyanate (OSCN⁻) (24,25).

The properties of the heme in MPO, EPO and LPO have been characterized by a wide variety of spectroscopic techniques, including optical absorption, stopped-flow, electron paramagnetic resonance, and resonance Raman spectroscopy (26-37). These techniques, with a combination of structure analysis (16,38-39) and advanced computer modeling (40), have shown that the heme pockets of mammalian peroxidases are envisioned to form the catalytic site where the stepwise reduction of H₂O₂ takes place. The simplified mechanism that governs the catalytic activity of mammalian peroxidase superfamily can be represented by the classic peroxidases catalytic cycle (Equations 1-4). In this cycle, H₂O₂ reacts rapidly and reversibly with ground state

\[
\text{E-Fe(III)} + \text{H}_2\text{O}_2 \rightarrow \text{E-Fe(IV)} + \text{H}_2\text{O} \quad \text{(Eq. 1)}
\]

\[
\text{E-Fe(IV)} + \cdot \text{Fe} + \cdot \text{O} + \text{SCN}^- + \text{H}^+ \rightarrow \text{E-Fe(III)} + \text{HOSCN} \quad \text{(Eq. 2)}
\]

\[
\text{E-Fe(IV)} + \cdot \text{Fe} + \cdot \text{O} + \text{AH}_2 \rightarrow \text{E-Fe(III)} + \cdot \text{AH} \quad \text{(Eq. 3)}
\]

\[
\text{E-Fe(IV)} + \cdot \text{Fe} + \cdot \text{O} + \text{AH}_2 \rightarrow \text{E-Fe(III)} + \cdot \text{AH} \quad \text{(Eq. 4)}
\]

The (E-Fe(III) state) of mammalian peroxidases and generating a ferryl \( \pi \) cation radical (E-Fe(IV)=O*\( \pi \)) intermediate named Compound I (Equation 1) (33,41). Compound I is capable of oxidizing either halides and pseudo halides through a 2 e⁻ transition generating ground state and the corresponding hypohalous acid (Equation 2), or oxidizing multiple organic and inorganic molecules (AH₂) by two successive sequential 1 e⁻ transition generating their corresponding cation (\( \cdot \text{AH} \)) and the peroxidase intermediates Compound II (E-Fe(IV)=O) and E-Fe(III), respectively (Equations 3 and 4) (33,41-43). Compound II is a longer lived intermediate whose decay to ground state is considered to be the rate limiting step during steady state catalysis (33,44). Acceleration in Compound II formation and decay has been noted with a series of organic and inorganic substrates, (28,30,33,41-43) and physiological reductants like nitric oxide (NO) and superoxide (O₂\( \cdot \)) (28,30,33,41-43).

Despite the potential significance of mammalian peroxidases to both human health and disease, little is known about the factors that govern their substrate selectivity and specificity. Previous kinetic studies with mammalian peroxidases have been limited in scope by focusing primarily on the direct reaction of SCN⁻ with Compound I (36,37,44,46,47). In the present studies, we utilize a combination of optical absorbance, rapid kinetics measurements, and NO binding studies to assess the distinct conformational changes that occur upon SCN⁻ binding, and its influence on the catalytic activity of MPO and other members of mammalian peroxidase superfamily. Our results indicate that SCN⁻ binding regulates the catalytic activity of MPO, EPO and LPO, which appears to result from significant electronic and/or conformational alterations in their catalytic sites that are caused by SCN⁻.

MATERIAL AND METHODS

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 (NO₂⁻/NO₃⁻) 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 (48). 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.  

Enzyme Purification — MPO was purified from detergent extracts of human leukocytes as described (49). Trace levels of contaminating eosinophil peroxidase were then removed by passage over a sulphotropyl Sephadex column (50). Purity of isolated MPO was established by demonstrating a Reinheitzahl (RZ) value of > 0.85 (A_{430}/A_{280}), SDS PAGE analysis with Coomassie blue staining, and in-gel tetramethylbenzidine peroxidase staining to confirm no observable contaminating eosinophil peroxidase activity (51). EPO was purified from porcine whole blood as previously described (52). The purity of EPO was confirmed by demonstrating an RZ of > 0.9 (A_{415}/A_{280}), SDS-PAGE analysis with Coomassie blue staining, and in-gel tetramethylbenzidine peroxidase staining (53). Enzyme concentrations were determined spectrophotometrically utilizing extinction coefficients of 89,000 and 112,000 M^{-1}cm^{-1}/heme of MPO (51) and EPO (54,55), respectively. LPO was obtained from Worthington Biochemical Corporation (Lakewood, NJ) and used without further purification. Purity was confirmed by demonstrating a RZ of 0.75 (A_{412}/A_{280}) and SDA PAGE analysis with Coomassie blue staining.  

Stopped-Flow and Absorbance Measurements — Rapid kinetic measurements were performed using a dual syringe stopped-flow instrument obtained from Hi-Tech Ltd (model SF-61). Measurements were carried out at 10°C following rapid mixing of equal volumes of an H_2O_2-containing buffer solution and a peroxidase solution. MPO Compound I and II formation and their decay to ground state were monitored at 435 and 455 nm, respectively. EPO and LPO Compound I and II formation and their decay to ground state were monitored at 412 and 432 nm, respectively. For NO binding to MPO, measurements were carried out by rapidly mixing equal volumes of the enzyme solutions (0.86 µM) with buffer solution supplemented with increasing concentrations of NO. The reactions for NO binding to MPO-Fe(III) were monitored under anaerobic conditions at wavelengths determined based on the spectral changes that occur upon NO binding to enzyme, as indicated. The time course of absorbance change was fit to either single (Y = 1 - e^{-kt}) or double exponential (Y= Ae^{-k_1t} + Be^{-k_2t}) functions as indicated. Signal to noise ratios for all kinetic analyses were improved by averaging at least six to eight individual traces. In some experiments the stopped-flow instrument was attached to a rapid scanning diode array device (Hi-Tech) designed to collect a multiple number of complete spectrum (200-800 nm) at specific time ranges. The detector was automatically calibrated relative to a holmium oxide filter, as it has spectral peaks at 360.8, 418.5, 446.0, 453.4, 460.4, 536.4 and 637.5 nm, which were used by the software to correctly align pixel positions with wavelength. Rapid scanning stopped-flow experiments involve mixing solutions of MPO (3 µM) with buffer solutions containing 40 µM H_2O_2 in the absence and presence of increasing SCN^- concentration, at 10°C.  

Spectroscopy — Optical spectra were recorded on a Cary 100 Bio UV-visible spectrophotometer at 25°C. Anaerobic spectra were recorded using septum-sealed quartz cuvettes that could be attached through a quick-fit joint to an all-glass vacuum system. MPO samples were made anaerobic by several cycles of evacuation and equilibrated with catalyst-deoxygenated N_2. Separate buffer solutions were evacuated, gassed with N_2 and anaerobically transferred either to the stopped-flow instrument or to anaerobic cuvettes using gas-tight syringes. Cuvettes were maintained under N_2, or NO positive pressure during spectral measurements.  

Solution Preparation — A fresh saturated stock of NO was prepared under anaerobic conditions. Anaerobic 0.2 M sodium phosphate buffer solutions, pH 7.0, containing various concentrations of NO were prepared by mixing different volumes of buffer saturated with NO gas at 21°C with anaerobic buffer solution. A saturating concentration of NO at 21°C is approximately 2 mM.  

H_2O_2 Selective Electrode Measurements — H_2O_2 measurements were carried out using an H_2O_2-selective electrode (Apollo 4000 Free Radical Analyzer; World Precision Instruments, Sarasota, FL). 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. 20 µM H_2O_2 was added to a continuously stirred buffer solution.
during which the rise and fall in H$_2$O$_2$ concentration was continuously monitored. Where indicated, 50 µl MPO (EPO or LPO) (200 nM final) was added to the reaction mixture. To determine the effect of SCN$^-$ on H$_2$O$_2$ consumption by mammalian peroxidases, similar experiments were repeated by adding 50 µl of the enzyme solution (200 nM final) pre-incubated with 40 µM SCN$^-$ to the reaction mixture.

RESULTS

Effects of SCN$^-$ on NO Binding to MPO Heme Iron — In order to assess the influence of SCN$^-$ on the catalytic activity of MPO, the rates of NO binding to the Fe(III) form of MPO have been determined in the presence of increasing SCN$^-$ concentrations. Investigations were carried out under two different circumstances: a fixed amount of NO and varying concentrations of SCN$^-$, or a fixed amount of SCN$^-$ and varying levels of NO. As shown in Fig. 1, the plots of the apparent rate constants for NO binding as a function of NO concentration were linear, consistent with a simple one-step mechanism. The positive intercepts confirm that NO binds to MPO-Fe(III) by a reversible process, as shown in Equation 5. These kinetic parameters suggest that SCN$^-$ modulates the affinity of MPO-Fe(III) towards NO. The second-order combination rate constants ($k_{on}$) calculated from the slopes were plotted as a function of SCN$^-$ concentration and showed a bell-shaped relationship (Fig. 2), with a minimum centered at biologically relevant levels of SCN$^-$ (60 µM). Collectively, these results indicate that SCN$^-$ binds within the enzyme system and modulates the affinity of MPO-Fe(III) towards NO.

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SCN-MPO-Fe(III) + NO \rightleftharpoons SCN-MPO-Fe(III)-NO \quad (\text{Eq. 5})
\]

Formation of MPO, EPO, and LPO Compound II During the Metabolism of SCN$^-$ — We next utilized diode array spectrophotometry to study the effect of SCN$^-$ on MPO, EPO, and LPO intermediates formation, duration, and decay as they occur during steady state catalysis. Investigation was carried out by rapid mixing of a buffer solution supplemented with 1.2 µM MPO (EPO or LPO) and 40 µM SCN$^-$ against an equal volume of a buffer solution supplemented with 40 µM H$_2$O$_2$, at 10$^\circ$ C. Spectral analysis indicated that the majority of MPO-Fe(III) (80-90%) converted immediately to an MPO species whose Soret absorbance peak along with the visible bands are identical to those reported for MPO Compound II. This species was stable and occurs immediately after initiating the reaction without any indication of prior accumulation of MPO Compound I. Increasing SCN$^-$ concentration decreased the amount of the MPO Compound II accumulation up to 50%, as judged by the decrease in the amplitude of the Soret absorbance peak at 455 nm. Similar results were obtained for EPO. Indeed, the majority of EPO-Fe(III) converted immediately to a stable EPO Compound II without any sign of the accumulation of Compound I. Fig. 3 shows spectral traces collected at 0.027, 0.054, 0.081, 0.110 and 0.135 seconds after initiating the reaction. Similarly, Compound II LPO accumulation during catalysis occurs without prior accumulation of LPO Compound I, but to a lesser extent, and decay immediately to ground state upon H$_2$O$_2$ exhaustion. Fig. 4, panel A, shows spectra collected at 0.035, 0.07, 0.105, 0.14 and 0.244 seconds after initiating the reaction, while panel B shows spectra collected at 0.45, 0.75, 0.90, 1.05, 1.20 and 2.20 seconds after initiating the reaction. However, when the similar reaction was repeated in the presence of 200 µM SCN$^-$, ground state LPO-Fe(III) predominated with a small a portion of Compound II (3-5% of the total LPO); its decay to ground state occurs immediately after H$_2$O$_2$ exhaustion (data not shown). Collectively, our results indicate for the first time that MPO, EPO, and LPO Compound II is a major component in the catalytic activity of these three enzymes, and its formation allow the enzymes to operate at only a fraction of their maximum activity.

Effect of SCN$^-$ on the Formation, Duration, and Decay of MPO and EPO Compound II Formation During Steady State Catalysis — To take a closer look at the reaction mechanism of the metabolism of SCN$^-$ by MPO, we next investigated the influence of SCN$^-$ on the kinetics of MPO compound II buildup, duration, and decay using single wavelength stopped-flow methods. Experiments were carried out under aerobic conditions following rapid mixing of (40 µM) H$_2$O$_2$.
and (0.85 µM) enzyme solution pre-incubated with various concentrations of SCN⁻, ranging from 10-160 µM. The time courses for the formation, duration, and decay of MPO steady state catalysis were examined by following the absorbance changes at 434 and 455 nm. As shown in Fig. 5, we observed a rapid increase in absorbance at 455 nm, followed by a steady state in which the intermediate formation remained relatively constant, with a final decay to the origin after MPO had oxidized all of the available substrate, H₂O₂. The decay of the steady state reaction is biphasic. There is a rapid initial decrease in absorbance (Fig. 5) followed by a much slower decrease in absorbance over the next 500 seconds (data is not included in Fig. 5). As summarized in Fig. 6, the addition of SCN⁻ to MPO reaction results in dramatic effects on the steady state accumulation rates duration and decay. The rate of steady state accumulation was decreased in the presence of SCN⁻ in a concentration-dependent and saturable manner (Fig. 6A). The rate of its initial decay increased in a linear manner as a function of SCN⁻ concentration (Fig. 6B), yielding a second-order combination rate constant (k_\text{on}) of 1.0 \times 10^4 M^{-1} s^{-1} calculated from the slope. In addition, SCN⁻ influences the level of the steady state accumulation and stability as reflected by the amplitude and duration of the stopped-flow traces, respectively. As the concentration of SCN⁻ present in the reaction mixture increased, both the steady-state level and the amount of the intermediates generated progressively decreased. When the reactions were monitored at 434 nm, the direction of absorbance change was reversed and the signal amplitudes declined, but otherwise proceeded with identical kinetics (data not shown). Collectively, the spectra of the intermediates initially formed following initiation of the reaction indicate that the majority of MPO exists as a Compound II, and its formation occurs without the accumulation of Compound I. Similar results were obtained for EPO (data not shown) indicating that both MPO and EPO display similar kinetic mechanism in metabolizing SCN⁻.

**Effect of SCN⁻ on the Formation, Duration, and Decay of LPO Steady State Catalysis** — The time courses for the formation, duration, and decay of LPO steady state catalysis were examined by following the decrease in absorbance changes at 412 nm. At each SCN⁻ concentration tested, there is a rapid decrease in absorbance, followed by a steady state in which the intermediate formation remained relatively constant, and finally decay exponentially to the origin after LPO had oxidized all of the available substrate, H₂O₂. However, it is apparent that as SCN⁻ concentration increased there was a decrease in rate of formation, decrease in the duration of steady state, and an increase in the decay rate (Fig. 7). When the reactions were monitored at 434 nm, the direction of absorbance change were reversed, the signal amplitudes were reduced, but otherwise proceeded with identical kinetics (data not shown). This behavior suggests that the spectral changes observed should reflect the change in LPO Compound II during catalysis. As summarized in Fig. 8, the addition of SCN⁻ to LPO reaction results in dramatic effects on the steady state accumulation rates duration and decay. The rate of steady state accumulation was decreased in the presence of SCN⁻ in a concentration-dependent and saturable manner (Fig. 8). The rate of its decay increased in a linear manner as a function of SCN⁻ concentration (Fig. 8) yielding a second-order combination rate constant (k_\text{on}) of 7.0 \times 10^3 M^{-1} s^{-1} and a first order dissociation rate constant of 1 s^{-1} calculated from the slope and the intercept, respectively. In addition, SCN⁻ influences the level of the steady state accumulation and stability as reflected by amplitude and duration of the stopped-flow traces, respectively. As the concentration of SCN⁻ present in the reaction mixture was increased, both the steady-state level and the amount of the intermediates generated progressively decreases.

**Direct H₂O₂ Consumption by MPO Using H₂O₂-Selective Electrode** — Following addition of 20 µM of H₂O₂ to a continuously stirred phosphate buffer, the H₂O₂ signal rose rapidly, achieved a maximum after ~30s, and fell gradually to the origin as H₂O₂ was depleted by autoxidation. Addition of MPO to the reaction mixture caused an immediate rapid decay in the level of free H₂O₂, followed by a slow decay indicating that H₂O₂ is consumed as a substrate by MPO during steady state catalysis (Fig. 9, Panel A). The first step occurs immediately after the enzyme addition and is attributable to the formation of MPO Compound I. The second step is much slower and is attributable to the reaction of MPO with H₂O₂ after the conversion of MPO Compound II to ground state. When the same reaction was repeated by
adding MPO sample saturated with SCN\(^-\) solution to a stirred H\(_2\)O\(_2\) solution, only the first step was observed (Fig. 9, Panel B). Similar results were obtained for both EPO and LPO.

**DISCUSSION**

Pre-incubation of SCN\(^-\) with MPO and other members of mammalian peroxidase superfamily (e.g. EPO and LPO) causes multiple and sequential reactions, and suggests a multifunctional role for SCN\(^-\) pre and during steady state catalysis. Indeed, pre-incubation with SCN\(^-\) prior to initiating peroxidation generates a more complex biological setting, through its potential capacity to bind within the enzyme systems, above the heme prosthetic group, or directly to the heme iron generating a six coordinate low spin complex. SCN\(^-\) binds within the MPO system and serves as a substrate or inhibitor and modulates the heme iron microenvironment causing significant alteration in its catalytic site, thereby altering the enzyme’s affinity towards H\(_2\)O\(_2\). Rapid kinetic measurement utilizing sequential stopped-flow methods have indicated that the direct reaction between MPO, EPO, and LPO Compound I and SCN\(^-\) is extremely fast and occurs with second order rate constants ranging from 9.6 \(\times\) 10\(^7\) to 2 \(\times\) 10\(^8\) M\(^{-1}\)s\(^{-1}\). Thus, the order of addition (the enzyme and H\(_2\)O\(_2\) mixed first and SCN\(^-\) second, or the enzyme and SCN\(^-\) mixed first and H\(_2\)O\(_2\) second) may have created a considerable degree of controversy, ambiguity, and diverse results in predicting the preferred biological substrate of the mammalian peroxidase superfamily.

Because MPO, EPO, and LPO Compound I formation rates are slower than the 2e\(^-\) oxidation of SCN\(^-\), Compound I accumulation cannot be detected during steady state catalysis (27,33,36,37,44,46,47). Thus, the observed absorbance changes during SCN\(^-\) metabolism should reflect the alteration in Compound II accumulation, duration, and decay. Rapid kinetic measurements indicated that MPO, EPO, and LPO Compound II is the predominant species formed, providing the first direct evidence for the involvement of Compound II in the catalytic inhibition of mammalian peroxidases. The kinetic parameters of MPO and EPO Compound II formation, duration and decay were similar, but were different from that obtained for LPO. The degree of MPO and EPO Compound II complex accumulation was surprisingly high, in that 80-90% of the total enzymes were estimated to be in Compound II form and attenuated in a saturable manner to a level of saturation approaching 50% upon increasing SCN\(^-\) concentration. Because Compound II is a catalytically inactive complex, a steady but suboptimal rate of SCN\(^-\) oxidation and HOSCN synthesis is maintained which is proportional to the percentage of MPO and EPO cycling through 2e\(^-\) oxidation pathway. Indeed, the presence SCN\(^-\) in the milieu resulted in a dramatic acceleration in H\(_2\)O\(_2\) consumption by both MPO and EPO, as detected by continuous monitoring with an H\(_2\)O\(_2\)-selective electrode. In contrast, LPO Compound II is unstable and converted to ground state immediately after H\(_2\)O\(_2\) exhaustion, indicating that SCN\(^-\) may serve as a 1e\(^-\) substrate for LPO Compound II. At a low SCN\(^-\) concentration, LPO Compound II was also maintained at a constant level while the enzyme continued to catalyze SCN\(^-\) oxidation and HOSCN synthesis. We observed that increasing the SCN\(^-\) concentration was accompanied by a modest decrease in the rate of Compound II formation, but with a significant decrease in the accumulation, decrease in the duration, and a significant increase in the decay rate. LPO Compound II formation and accompanying inhibition did not occur at a higher SCN\(^-\) concentration. This behavior can be interpreted by either or both of the following: 1) the Compound II formation rate becomes slower than the complex decay, or 2) the majority of the reaction proceeds through a 2e\(^-\) oxidation pathway.

A general kinetic scheme of how SCN\(^-\) interacts with mammalian peroxidases intermediates incorporated into the classic catalytic cycle is illustrated in Fig. 10. This working kinetic model consists of two major pathways: (Pathway A; Fig. 10) the classical peroxidase cycle; and (Pathway B; Fig. 10) the binding of co-substrate to E-Fe(III) and the formation of E\(_{\text{inh}}\)-Fe(II) complex and the effect of these binding on the formation of Compound I. Compound I formation is rapid and occurs with a second-order rate constant ranging from 1 \(\times\) 10\(^7\) to 4.3 \(\times\) 10\(^7\) M\(^{-1}\) s\(^{-1}\) (27,33,36,37,44,46,47). Mammalian peroxidases Compound I oxidizes
halides and pseudo halides by 2 e\(^{-}\) oxidation process yielding the corresponding hypohalous acid and the ground state (E-Fe(III)) (33,41-43). Alternatively, Compound I oxidizes organic and inorganic substrates by two sequential 1e\(^{-}\) oxidation to ground state, through the formation of Compound II, the rate limiting step in the classic cycle of peroxidases (33,44).

NO, like CN\(^{-}\), binds to the MPO heme iron and displaces the water molecule (W1), which is hydrogen bonded to the distal His 95 in the native enzyme. The bent mode of the Fe-N-O may allow a perturbation in the hydrogen bonding within the distal cavity. Formation of such a complex is accomplished with the movement of the iron atom into the porphyrin ring generating its respective low-spin six-coordinate complex. SCN\(^{-}\) is a relatively bulky molecule; its binding above the heme moiety of MPO may constrain NO binding by either filling the space directly above the heme, or promoting a protein conformational change that constrains the distal heme pocket (Fig. 11). The ability of SCN\(^{-}\) to modify the heme pocket of MPO and/or shield the catalytic site of the enzyme is directly reflected by the modulation of NO binding to the enzyme heme moiety. Varying the concentration of SCN\(^{-}\) displays different impact on the MPO heme iron microenvironment. Indeed, the plot of the second-order combination rate constant (k\(_{on}\)) of NO binding to MPO-Fe(III) as a function of SCN\(^{-}\) concentration displayed a bell-shaped, with a negative slope over the range where SCN\(^{-}\) concentrations were < 60 µM and a positive slope over the range where SCN\(^{-}\) concentrations were > 60 µM, and a saturation above 300 µM. This behavior may have a broader link effect on MPO activity, since the initial decrease in the second order rate constant of NO combination (k\(_{on}\)) occurs within the range where SCN\(^{-}\) binds to distal cavity located in MPO Compound I and allows the direct contact with the oxyferryl oxygen. Under these circumstances, Compound I appears to act favorably in triggering electron transfer to the heme with subsequent conversion to hypoiodite/hypophosphite as a final reaction product (16,38,39). This decrease was reversed and reaches saturation at higher SCN\(^{-}\) concentrations where SCN\(^{-}\) binds to MPO at both the distal cavity and the proximal helix sites and forms an inactive complex (SCN-E-Fe(III)\(_{(inh)}\)). Indeed, Blair-Johson et al. have shown that the inhibitory complex with thiocyanate indicates replacement of chloride at the proximal helix halide binding site in addition to binding in the distal cavity in an orientation parallel with the heme (Fig. 11). It was thought that halides binding at the distal cavity site inhibit the enzyme by interfering with the deprotonation of H\(_{2}\)O\(_{2}\) by the adjacent distal His 95 (16,38,39), a mechanism consistent with previous reports that inhibition by halides is competitive with respect to H\(_{2}\)O\(_{2}\) (56-59). Thus, the saturation in bell-shaped curve occurs, at high concentration of SCN\(^{-}\) (> 300 µM), when the two sites are occupied. Therefore, the plasma levels of SCN\(^{-}\) (60 µM) is crucial in saturating the substrate site of the enzyme, as judged by the inflection point in the bell-shaped curve that is illustrated in Fig. 2. Consequently, the plasma level of SCN\(^{-}\) may govern the catalytic reaction of MPO, both in vivo and in vitro.

Identifying similarities and differences in the interactions between MPO and various members of mammalian peroxidases superfamily yields valuable mechanistic insights into the role of Compound II formation in modulating the catalytic activity and function. MPO, EPO, and LPO Compound I simultaneously catalyzes SCN\(^{-}\) via 2 and 1e\(^{-}\) oxidation pathways which leads to the formation of HOSCN and sulfer-centered thiocyanate radicals (SCN\(^{-}\)), respectively (60,61). The partitioning between the two pathways depends in part on the rate of 2e\(^{-}\) versus 1e\(^{-}\) oxidation of SCN\(^{-}\), and the concentration of SCN\(^{-}\). It appears that as the SCN\(^{-}\) concentration increases, Compound I is converted rapidly and more efficiently to both ground state and Compound II. This behavior is illustrated by the significant alteration in the intermediate distributions during steady state catalysis, as reflected by the decrease in the amplitude of Compound II formation observed in a SCN\(^{-}\)-dependent and saturable manner (Fig. 6C). Thus, the plateau in the curve is governed by the rate of the 1e\(^{-}\) oxidation reaction of SCN\(^{-}\) by Compound I, which would then result from the cleavage of SCN\(^{-}\) from the SCN-MPO\(_{(inh)}\)-Fe(III) complex. Thus, the plot of the formation rate of Compound II plateaus at a rate that should be comparable to the rate of SCN-E-Fe(III)\(_{(inh)}\) conversion to the active form, which under these circumstances is
the formation of E-Fe(III) and/or SCN-E-Fe(III) (Fig. 10). The low conversion rate constant limits E-Fe(III) bioavailability, and subsequently attenuates the turnover number of peroxidation.

The time course for MPO Compound II decay monitored in the absence of SCN⁻, detected by monitoring the absorbance changes at 445 nm, is extremely slow and occurs with a rate constant of 0.008 s⁻¹, at 10°C (28-30). In the presence of SCN⁻, and after the reaction had ceased, there is an initial rapid decay which is followed by a slower decay regenerating ferric MPO. The initial rapid phase may be attributed to a rapid decay of Compound I to ground state, which increases in a linear manner as a function of SCN⁻ concentration with a second order rate constant of 1 X 10⁶ M⁻¹ s⁻¹, while the second phase was attributed to the decay of MPO Compound II to ground state. Compound II does not possess suitable redox potential to oxidize halides and pseudo-halides (33,41). Rather, Compound II uses a variety of organic and inorganic compounds as substrates (33). Similar results were observed for EPO, indicating that both enzymes display similar mechanism in metabolizing SCN⁻. Van Dalen and Kettle have recently reported similar spectral changes during turnover metabolism of SCN⁻ by EPO and MPO (62). Like them, we also noted that the addition of GSH, a hypothiocyaite scavenger, prevented the buildup of Compound II intermediate, but at the same time, GSH would not have contributed to the turnover of Compound II because it did not reduce preformed MPO Compound II (data not shown). Furthermore, Compound II formation can be destabilized in the presence of organic and inorganic substrates (e.g. low levels of NO) (data not shown).

In contrast, LPO Compound II formation and the accompanying LPO inhibition did not occur under conditions where SCN⁻ is in large excess. Thus, a majority of Compound I quickly generates its ferric complex upon initiating the reaction. The apparent ability of LPO to oxidize SCN⁻ through two successive 1 e⁻ transfers generating Compound II and LPO-Fe(III) is unprecedented. Increasing SCN⁻ concentration accelerates the decay of the Compound II, rate-limiting step in the peroxidase cycle, and enhances the overall rates of catalysis. Enhancement of peroxidase catalysis due to reduction of LPO Compound II to LPO-Fe(III) has been noted with other physiological reductants such as NO, superoxide and ascorbic acid (28-30,33). That the rate of LPO Compound II formation remained relatively unchanged at high SCN⁻ concentrations studied (> 50 µM SCN⁻) is consistent with the reaction being limited by the release of SCN⁻ from the SCN-LPOᵒh complex. Consistent with this interpretation, the failure of the higher SCN⁻ concentration to alter the LPO spectrum appeared to be linked to the fact that the formation of Compound II becomes slower than complex decay. Collectively, the formation of LPO Compound II during steady state catalysis is a fundamental feature of catalysis, and functions to down-regulate the peroxidation process by the enzyme.

The ability of MPO, EPO, and LPO to employ SCN⁻ as a 1 e⁻ substrate influences the nature of the end products of the SCN⁻ oxidation reaction. For example, 1 e⁻ oxidation of SCN⁻ generates a thiocyanate radical, which dimerized to yield a labile short-lived intermediate, thiocyanogen (SCN)₂ (61,63). This intermediate is rapidly hydrolyzed to generate a combination of HOSCN and OSCN⁻ as final end products (61,63). These observations are consistent with earlier studies by Modi et al., which suggested that LPO catalyzes SCN⁻ oxidation at pH 6.1 to produce HOSCN and OSCN⁻ (60). Alternatively, (SCN)₂ is hydrolyzed to produce CN⁻ as an end product without the formation of HOSCN (64,65). In the presence of plasma level of SCN⁻, SCN⁻ may be directly oxidized to OSCN⁻ (60,63,66). It has been suggested that other different products besides hypothiocyanite and CN⁻ might be formed during the metabolism of SCN⁻. For example, Pruitt et al. have proposed that hypothiocyanous acid might be oxidized by excess H₂O₂ or MPO to yield a short-lived intermediate oxidants, cyanosulphurous acid (HO₂SCN) and cyanosulphuric acid (HO₃SCN), which then breakdown in the presence of H₂O₂ to generate cyanate (OCN⁻) (67-69).

In related studies, Furtmüller et al. have demonstrated that the apparent second-order rate constants of the reactions between Compound I of LPO, EPO and MPO with SCN⁻ are extremely high, with apparent second-order constants of 2 x 10⁸, 1 x 10⁷ and 9.6 x 10⁷ M⁻¹ s⁻¹, respectively (36,44,47). These results are consistent with the
direct reaction between Compound I and SCN\(^-\), and to a large extent prevent the potential alteration in the heme pocket and thereby the formation of the inactive form of the enzyme that occur with prior incubation of SCN\(^-\) with the three enzymes.

Binding of the co-substrate to the heme moiety generating the E-Fe(III)-SCN complex (Fig. 10) may considerably restrict \(\text{H}_2\text{O}_2\) access to the heme iron, and the slow rate of \(\text{H}_2\text{O}_2\) binding observed would then result from ligand replacement processes. There is no data available to support the notion that SCN\(^-\) serves as a ligand in mammalian peroxidase when present at plasma levels. However, the presence of higher levels of SCN\(^-\) (e.g. > 1mM) may promote the formation of such a complex (45). Formation of E-Fe(III)-SCN can be characterized based on its characteristic absorbance feature, which displays a large red shift in the Soret absorbance region compared to native enzyme (45). Whether the slow binding of \(\text{H}_2\text{O}_2\) to the enzyme occurs through conformational changes in the heme pocket geometry, or due to the replacement reaction, the rate limited step becomes the binding of \(\text{H}_2\text{O}_2\) to the enzymes. Under all circumstances, the catalytic activity of the peroxidases will depend on multiple factors including the concentration of SCN\(^-\) versus \(\text{H}_2\text{O}_2\), the affinity of the enzymes towards \(\text{H}_2\text{O}_2\) versus SCN\(^-\) and the rate of SCN-E-Fe(III) and E-Fe(III)-SCN breakdown (Fig. 10). Changes in heme pocket geometry upon ligand binding have been described for cytochrome C peroxidase (70), and a slower rate of NO binding to the Fe(III) forms of a number of hemoproteins have been attributed to ligand replacement (71-73). Ligation of SCN\(^-\) to the distal heme would prevent access of \(\text{H}_2\text{O}_2\) to the catalytic site of the enzymes.

In summary, our current studies reveal for the first time that the formation of Compound II during steady state catalysis is a fundamental feature of the kinetic reaction of mammalian peroxidases. Its formation during steady state catalysis operates to down-regulate the catalytic activity of MPO, EPO, and LPO. Our results also demonstrate that pre incubation of SCN\(^-\) with mammalian peroxidases significantly affect their catalytic sites, subsequently altering heme iron reactivity, decreasing the affinity towards \(\text{H}_2\text{O}_2\), and disturbing the intermediates distribution in a distinct and different manner. These findings suggest a regulatory role for SCN\(^-\) in mediating particular structure/function performance, regardless of its bioavailability and function, which have the ultimate effect of promoting substrate selectivity and specificity. Thus, the metabolism of SCN\(^-\) by mammalian peroxidases is determined by four major factors: 1) the concentration of \(\text{H}_2\text{O}_2\), 2) the concentration of SCN\(^-\), 3) the stability of Compound II that formed during steady state catalysis, and 4) the order of addition between the enzymes and their substrates (E and \(\text{H}_2\text{O}_2\) mixed first and SCN\(^-\) second, or E and SCN\(^-\) mixed first and \(\text{H}_2\text{O}_2\) second).

REFERENCES


FOOTNOTES

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1The abbreviations used are: MPO, myeloperoxidase; EPO, eosinophil peroxidase; LPO, lactoperoxidase; H2O2, hydrogen peroxide; NO, nitric oxide (nitrogen monoxide).

2Key Words: Inflammation, myeloperoxidase, eosinophil peroxidase, lactoperoxidase, thiocyanate, stopped-flow.

FIGURE LEGENDS

Fig. 1. **SCN\(^{-}\) modulates NO binding to MPO heme iron.** Plots of the observed rates of NO binding to MPO-Fe(III) as a function of NO and SCN\(^{-}\) concentrations. An anaerobic solution containing 0.86 \(\mu\)M MPO-Fe(III) supplemented with varying concentrations of SCN\(^{-}\) was rapidly mixed with an equal
volume of sodium phosphate buffer (200 mM, pH 7.0) supplemented with varying concentration of NO at 10° C. The high concentration of the phosphate buffer is to keep the pH of the solution unaltered, after the addition of NO. The observed rates of MPO-Fe(III)-NO were plotted as a function of NO concentration.

**Fig. 2.** Relationship between the second-order combination rate ($k_{on}$) of NO binding to MPO-Fe(III) obtained from Fig. 1 as a function of SCN$^-$ concentration used.

**Fig. 3.** Formation of EPO Compound II during steady state catalysis of SCN$^-$. Absorbance change over time for the reaction that was initiated by rapidly mixing a buffer solution containing 40 µM H$_2$O$_2$ with an equal volume of buffer solution containing 1.2 µM EPO and 40 µM SCN$^-$, at 10° C. Spectra traces were recorded at 0.027, 0.054, 0.081, 0.110 and 0.135 seconds after initiating the reaction. Arrows in the panels indicate the direction of spectral change over time. Experiments were carried out under anaerobic conditions in sodium phosphate buffer (200 mM pH 7.0).

**Fig. 4.** Absorbance changes for the metabolism of SCN$^-$ by LPO as it occurs during steady state catalysis. Absorbance change over time for the reaction that was initiated by rapidly mixing a buffer solution containing 40 µM H$_2$O$_2$ with an equal volume of buffer solution containing 1.0 µM LPO and 40 µM SCN$^-$ using stopped-flow diode array methods, at 10° C. *Panel A,* spectra collected at 0.035, 0.07, 0.105, 0.14 and 0.244 seconds after initiating the reaction. *Panel B,* spectra collected at 0.45, 0.75, 0.90, 1.05, 1.20 and 2.20 seconds after initiating the reaction. Arrows indicate the direction of spectral change over time.

**Fig 5.** Effect of SCN$^-$ concentration on MPO Compound II formation, duration, and decay as they occur during steady state catalysis. Formation, duration, and decay of steady state catalysis of MPO were monitored as a function of time at 455 nm. An aerobic solution containing 0.86 µM of MPO-Fe(III) pre-incubated with different concentrations of SCN was rapidly mixed with an equal volume of sodium phosphate buffer (200 mM, pH 7.0) supplemented with 40 µM H$_2$O$_2$, at 10° C. The initial concentration of SCN$^-$ in the mixtures is 10, 20, 40, 80 and 160 µM from bottom to top.

**Fig. 6.** Rate of MPO Compound II formation, duration and decay as a function of SCN$^-$ concentration. The observed rates of MPO Compound II formation (top panel), duration (middle panel), and decay (bottom panel) (monitored at 455 nm) observed in Fig. 4 were plotted as a function of SCN$^-$ concentration. The percent absorbance change at 455 nm is also shown for MPO steady state as a function of SCN$^-$ concentration (middle panel).

**Fig. 7.** Spectral changes of SCN$^-$ metabolism by LPO as they occur during steady state catalysis at four different SCN$^-$ concentrations. Formation, duration, and decay of steady state catalysis of LPO were monitored as a function of time at 412 nm. An aerobic solution containing 0.86 µM of LPO-Fe(III) pre-incubated with different concentrations of SCN was rapidly mixed with an equal volume of sodium phosphate buffer (200 mM, pH 7.0) supplemented with 40 µM H$_2$O$_2$, at 10° C. The initial concentration of SCN$^-$ in the mixtures is 10, 20, 40, 80 and 160 µM from bottom to top.

**Fig. 8.** Rate of LPO steady state formation, duration and decay as a function of SCN$^-$ concentration. The observed rates of LPO steady state formation (top panel), duration (middle panel), and decay (bottom panel) (monitored at 412 nm) observed in Fig. 7 were plotted as a function of SCN$^-$ concentration. The percent absorbance change at 412 nm is also shown for LPO steady state as a function of SCN$^-$ concentration (middle panel).
Fig. 9. A typical recording by H$_2$O$_2$-selective electrode for H$_2$O$_2$ consumption by MPO. *Panel A*, addition of H$_2$O$_2$ followed by MPO to a stirred 0.2 M sodium phosphate buffer (pH 7) results in biphasic dramatic acceleration in the rate of H$_2$O$_2$ consumption, at 25º C. *Panel B*, addition of 20 µM of H$_2$O$_2$ followed by MP, only the fast phase has been seen. Tracings shown are from typical experiment performed at least three times. The H$_2$O$_2$ traces were corrected by subtracting the autooxidation of H$_2$O$_2$.

Fig. 10. Modified working kinetic model for SCN$^-$ interaction with mammalian peroxidases (E = Enzyme MPO, EPO, or LPO).

Fig. 11. Stereo view of the MPO distal heme cavity of human MPO with bound thiocyanate. The figure was created using the coordinates deposited in the Protein Data Bank (accession code 1D7W).
Figure 1
Figure 3
Figure 5
Figure 8

A

B

C

Formation Rate (s⁻¹)

Decay Rate (s⁻¹)

Duration (s)

% Absorbance Change

SCN⁻ (µM)

10

2

6

0

5

3

0

1

3

6

50

100

0

50

100

0
Figure 9
Figure 10