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A predictive kinetic model for inhibitory effect of nitrite on myeloperoxidase catalytic activity towards oxidation of chloride

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ABSTRACT

Myeloperoxidase (MPO) is a neutrophil enzyme that employs hydrogen peroxide (H_2O_2) to catalyze the oxidation of chloride (Cl^-) to hypochlorous acid (HOCl*)*. Accepted mechanism is based on rapid reaction of native MPO with H_2O_2 to produce Compound I (MPO-I) which oxidizes Cl^- through a $2e^-$ transition generating MPO and HOCl. MPO-I also reacts with H_2O_2 to generate Compound II (MPO-II) which is inactive in 2e oxidation of Cl^-. Nitrite (NO_2^-) inhibits the 2e oxidation of Cl^- by reaction with MPO-I through 1e transition generating MPO-II and nitrite radical. H_2O_2 consumption during steady-state catalysis was monitored amperometrically by a carbon fiber based H_2O_2-biosensor at 25˚C. Results demonstrated that in absence of NO_2^- reactions were monophasic and rapid (complete H_2O_2 consumption occurs in <10 s). As concentration of NO_2^- increases, reactions change to biphasic (rapid step followed by a slow step) and both steps have been inhibited by NO_2^-. A predictive kinetic model describing the inhibittory effect of NO_2^- was developed and applied to experimental results. The model is based on the assumption that MPO-I cannot be detected during steady-state catalysis. Calculated rate constants are in agreement with those obtained from pre-steady state kinetic methods.

Keywords: MPO-Hydrogen Peroxide-Chloride System; Nitrite Inhibitor; $\frac{k_4}{k_3}$, $\frac{k_4}{k_2}$, and $\frac{k_2}{k_3}$ Ratios; Theoretical Kinetic Model

1. INTRODUCTION

Myeloperoxidase (MPO) is a human peroxidase enzyme and a lysosomal protein stored in azurophilic granules of the neutrophil. Its deficiency can severely cause quantitative or functional genetic disorder [1]. The major role of MPO is to aid in microbial killing. It oxidizes tyrosine to tyrosyl radical using hydrogen peroxide as oxidizing agent [2]. Furthermore, the MPO catalyzed production of HOCl from hydrogen peroxide (H_2O_2) and chloride ion (Cl^-), together with tyrosyl radical, are aimed at killing bacteria and other pathogens. The MPO-hydrogen peroxide-chloride system has been considered an important pathophysiologic factor in kidney disease [3], to enhance lipid oxidation in LDL in presence of SCN^- catalyst [4], to lead to oxidative damage of apolipoprotein A-I [5], to oxidizes free α-amino acids to aldehydes [6], leading to advanced glycation products present in human lesion material [7].

Vast range of inflammatory diseases was found to be correlated with products of MPO nitration of tyrosine residues. Myeloperoxidase can oxidize nitrite ions to an intermediate capable of nitrating tyrosine and tyrosyl residues in proteins [8-10].

The simplified mechanism that governs the catalytic activity of MPO can be represented by the classic peroxidases catalytic cycle as follows:

Chloride oxidation starts by rapid reaction of ground state MPO with H_2O_2 to form Compound I (MPO-I). Compound I is capable of oxidizing chloride (Cl^-) through a $2e^-$ transition generating the ground state MPO and hypochlorous acid (HOCl). During turnover, some MPO-I is converted to Compound (II) (MPO-II) by reaction with $1e^-$ donors such as nitrite (NO_2^-) or (H_2O_2). As could be seen in equations 1 to 4 [11,12,17].

$$\mathrm{MPO} + \mathrm{H_2O_2} \xrightarrow{k_1} \mathrm{MPO\text{-}I} + \mathrm{H_2O} \qquad (1)$$

$$\text{MPO-I} + \text{Cl}^- + \text{H}^+ \xrightarrow{k_2} \text{MPO} + \text{HOCl} \quad (2)$$

$$\text{MPO-I} + \text{NO}_2^- \xrightarrow{k_3} \text{MPO-II} + \text{NO}_2 \quad (3)$$

$$\text{MPO-I} + \text{H}_2\text{O}_2 \xrightarrow{k_4} \text{MPO-II} + \text{H}_2\text{O}_2 \quad (4)$$

MPO-II is believed to be inactive in chloride oxidation. The decay of MPO-II to ground state is considered as the rate-limiting step during steady-state catalysis [11,12].

Pre-steady-state and steady-state studies based on stopped-flow mixing and optical detection were employed for studies of MPO-hydrogen peroxide-chloride system in presence and absence of nitrite [13,14]. In such studies, larger than physiological plasma concentrations of MPO and/or NO_2^- were employed to monitor measurable changes in absorbance at selected wavelengths. Steady-state methods with amperometric monitoring have advantages over optical methods. In such methods, the oxidation or reduction of a targeted reactant or product is directly monitored at the surface of a selective electrochemical biosensor. However, lack of biosensors with enough sensitivity, selectivity and short response time limited their role to initial rate measurements [13-17].

Recently, combination H_2O_2-biosensors with adequate sensitivity (2 pA/nM) and a relatively short response time (<2 s) were developed [18-20].

In a previous study [20], we employed a carbon fiber based H_2O_2-biosensor to study the effect of NO_2^- on catalytic activity of MPO towards oxidation of chloride under respective physiological concentrations. Our results confirmed the inhibitory nature of NO_2^-. In this study, we utilized experimental data to develop a kinetic model capable of explaining the monophasic and biphasic phenomena. Additionally, $\frac{k_4}{k_3}$, $\frac{k_4}{k_2}$, and $\frac{k_2}{k_3}$ ratios were estimated and the dependence of k_1 on [Cl^-] and [NO_2^-] was determined.

2. EXPERIMENTAL

2.1. Reagents

Chemicals used for preparation of buffer, stock and standard solutions were of analytical grade reagents and purchased from Sigma Chemical Co. (St. Louis, MO, USA). Phosphate buffer, 100 mM and pH 7.00, was prepared by mixing appropriate volumes of 0.10 M NaH_2PO_4 and 0.10 M Na_2HPO_4 to achieve pH 7.00. A 3.00 mM H_2O_2 solution was freshly prepared from stock solutions prepared by sequential dilutions from 30% H_2O_2 solution. Standard solutions of chlorides and NO_2^- were prepared by sequential dilutions from their respective sodium salts. All solutions were bubbled with high purity N_2 gas before use. MPO was purified from human leukocytes [21-23]. A 30 μM MPO solution was freshly prepared by diluting measured amounts with buffer.

2.2. Electrochemical Measurements

The Amperometric system consisted from an Apollo 4000 free radical analyzer, ISO-HPO-100 H_2O_2-biosensor and a thermostated measurement chamber (WPI, Sarasota, FL, USA). All experiments were performed at room temperature.

For each experiment, 3.00 mL of 100 mM phosphate buffer solution containing 30 μM EDTA were placed in the measurement chamber. For effect of nitrite measurements, a 100 mM Cl^- and varied concentrations of NO_2^- (0 - 100) μM were pre-incubated with buffer solution in the chamber. For effect of chloride measurements, a 100 μM NO_2^- and varied concentrations of Cl^- (0 - 100) mM were pre-incubated with buffer solution in the chamber. The electrode was immersed and magnetic stirrer was turned on at fixed moderate speed. Continuous amperometric monitoring started after addition of 30 μL H_2O_2 (10 μM). Reactions started with addition of 5.0 μL MPO solution (50 nM) and allowed to proceed until complete decay of initial current signal. H_2O_2 concentrations (μM) versus time (s) plots were obtained by setting the initial current signal to 10 μM H_2O_2 [19-20].

3. RESULTS AND DISCUSSION

Due to its much higher concentration (100 - 140 mM) relative to other halides, chloride is assumed to be the physiological substrate for MPO. Time course H_2O_2-decay plots for MPO-catalyzed oxidation of Cl^-, at a selected normal plasma level (100 mM), in presence of increasing NO_2^- concentrations were studied by continuous amperometric monitoring of H_2O_2 consumption (**Figure 1**) [20]. A monophasic plot prevails in absence of NO_2^- (**Figure 1(a)**) which is demonstrated by a rapid consumption of H_2O_2. As NO_2^- concentration increases, plots became biphasic and the rapid step is followed by a slower second step which is observed as an exponential decay of H_2O_2 signal (**Figures 1(b)-(d)**). The second step dominates at larger NO_2^- concentration (**Figure 1(d)**).

Further prevailing of second phase was observed, when Cl^- was decreased in presence of 100 μM NO_2^- (**Figure 2**). Both steps are observed in **Figure 2(e)** which is actually **Figure 1(d)**. As Cl^- concentration continues to decrease, the second step is further extended and first step is disappeared. (**Figures 2 (a)-(c)**). Extension of second step is accompanied by increase in reaction time.

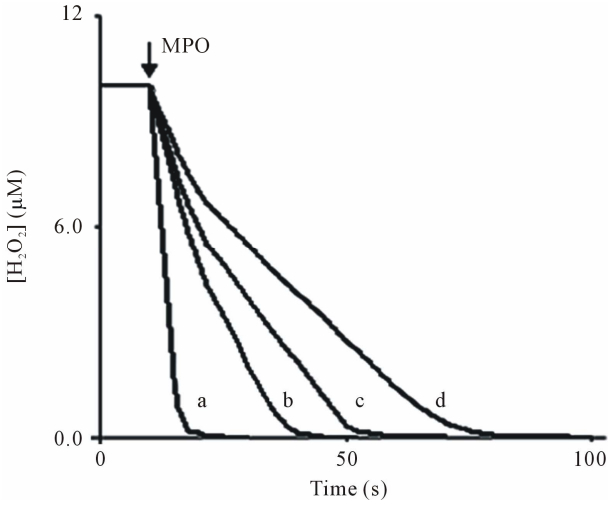

Figure 1. Effect of NO_2^- on MPO-catalytic activity towards oxidation of Cl^- (Courtesy of portugaliae electrochimica acta with permission). H_2O_2 consumption plots as a function of [NO_2^-]. Reactions were started by the addition of 50 nM MPO to 10 μM H_2O_2 in 100 mM phosphate buffer, pH 7.0, containing 30 μM EDTA pre-incubated with 100 mM Cl^- (a) and 25 (b), 50 (c), 100 μM NO_2^- (d). Reactions were carried at 25˚C. Plots are average of four replicates.

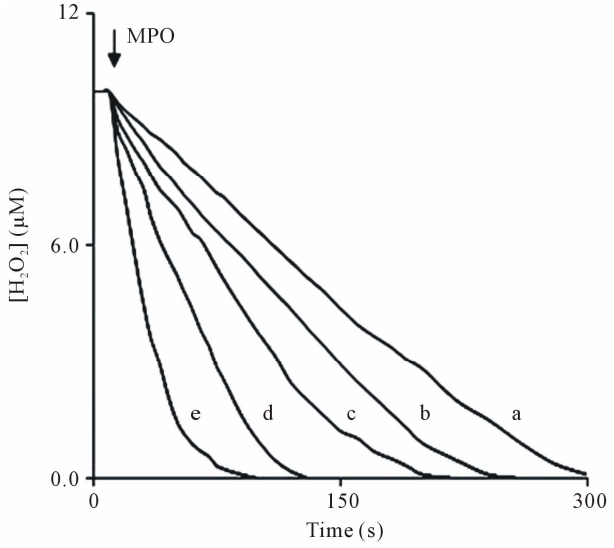

Figure 2. Effect of Cl^- on MPO-catalytic activity towards oxidation of Cl^- in presence of NO_2^-. H_2O_2 consumption plots as a function of [Cl^-]. Reactions were started by the addition of 50 nM MPO to 10 μM H_2O_2 in 100 mM phosphate buffer, pH 7.0, containing 30 μM EDTA, pre-incubated with 100 μM NO_2^- and 5 (a), 10 (b), 25 (c), 50 (d) and100 mM Cl^- (e) Reactions were carried at 25˚C. Plots are average of four replicates.

3.1. Proposed Kinetic Model

Referring to mechanism in introduction, reaction 1 is known to be very fast, k_1 is in the order of 10^7 [24], whereas reaction 2 is slower with $k_2 \sim 10^4$ [25]. Interestingly, reaction 3 is found to be extremely fast, k_3 in the order of 10^7 [13] and k_4 (10^2 - 10^4) consequently much slower than k_1 [17,26-28].

Let rate of consumption of H_2O_2 be written as:

$$-\frac{d[H_2O_2]}{dt} = k_1[\text{MPO}][H_2O_2] + k_4[\text{MPO-I}][H_2O_2] \quad (5)$$

On Parallel, the rate of consumptions of MPO, Cl^-, and NO_2^- ions together with rate of formation of HOCl can be written as:

$$-\frac{d[Cl^-]}{dt} = \frac{d[\text{HOCl}]}{dt} = k_2[\text{MPO-I}][Cl^-] \quad (6)$$

$$-\frac{d[\text{MPO}]}{dt} = k_1[\text{MPO}][H_2O_2] - k_2[\text{MPO-I}][Cl^-] \quad (7)$$

$$-\frac{d[NO_2^-]}{dt} = k_3[\text{MPO-I}][NO_2^-] \quad (8)$$

Furthermore the rate of formation of MPO-II is:

$$\frac{d[\text{MPO-II}]}{dt} = k_3[\text{MPO-I}][NO_2^-] + k_4[\text{MPO-I}][H_2O_2] \quad (9)$$

All **Eqs.5-9** contain an unstable MPO-I intermediate. Thus the rate of consumption of MPO-I intermediate can be obtained from the use of steady-state method as follows:

$$-\frac{d[\text{MPO-I}]}{dt} = -k_1[\text{MPO}][H_2O_2] + k_2[\text{MPO-I}][Cl^-] + k_3[\text{MPO-I}][NO_2^-] + k_4[\text{MPO-I}][H_2O_2] = 0 \quad (10)$$

Then

$$[\text{MPO-I}] = \frac{k_1[\text{MPO}][H_2O_2]}{k_2[Cl^-] + k_3[NO_2^-] + k_4[H_2O_2]} \quad (11)$$

Pre-steady states studies reported that k_4 is much smaller than k_2 and k_3 [17] and thus reaction 4 is very slow with respect to reactions 1, 2 and 3 respectively, then $k_4[H_2O_2]$ could be neglected and omitted from denominator and thus, **Eq.11** becomes:

$$[\text{MPO-I}] = \frac{k_1[\text{MPO}][H_2O_2]}{k_2[Cl^-] + k_3[NO_2^-]} \quad (12)$$

The reaction could be studied by monitoring the rate of consumption of H_2O_2 and/or the rate of formation of MPO-II. In our case we were able to experimentally

monitor the rate of consumption of H_2O_2. Thus, substitution of **Eq.12** in **Eq.5** yields the net rate of consumption of H_2O_2 through the entire reaction:

$$-\frac{d[H_2O_2]}{dt} = \underbrace{k_1[MPO][H_2O_2]}_{\text{First part}} + \underbrace{\frac{k_4k_1[MPO][H_2O_2]^2}{k_2[Cl^-]+k_3[NO_2^-]}}_{\text{Second part}} \quad (13)$$

Eq.13 illustrates the rate change of consumption of H_2O_2 through time scale. At the very early time of reaction (first 10 s) fast drop of H_2O_2 signal was observed (first phase), indicating domination of first part which is attributed to no significant formation of MPO-II (enzyme is swinging between MPO-1 and ground state MPO). Afterwards, and with presence of increasing concentrations of NO_2^-, part of MPO-I is reduced to MPO-II causing the enzyme to work under partial activity (second phase) indicating domination of second part of **Eq.13**.

Consequently, at the early time of the reaction, the rate of consumption of H_2O_2 depends only on the first part which is a second order reaction that depends entirely on initial concentrations of MPO and H_2O_2 and conclusively, the rate constant (k_1) could be estimated from slope of curve.

$$-\frac{d[H_2O_2]}{dt} = k_1[MPO][H_2O_2] \quad (14)$$

After the passage of few seconds (10 s), second phase is dominated and H_2O_2 consumption will follow the second part of **Eq.13** which is observed as exponential decay of H_2O_2 signal **(Figures 1(b)-(d))**. The second part of **Eq.13** introduces new variables such as $[Cl^-]$, $[NO_2^-]$ together with [MPO], $[H_2O_2]$ and rate constant values k_1, k_2, k_3 and k_4 , that affect the rate of consumption of H_2O_2. Collectively, **Eq.13** expresses a two-step sequential decay of H_2O_2 signal.

By applying extreme values for NO_2^- concentration in **Eq.13** assuming that $[NO_2^-] = \infty$, then [MPO] approaches [MPO-II] and the rate of consumption of H_2O_2 will be zero indicating complete inhibition of reaction 2. Additionally if $[NO_2^-] = 0$ then $k_3[NO_2^-] = 0$, and **Eq.13** could still be used even in the absence of NO_2^- ions. Biphasic plots were observed for $[Cl^-] < 20$ mM (data not shown). This confirms the solidarity and consistency of **Eq.13** to present the rate of consumption of H_2O_2 in the presence of MPO, Cl^- and presence or absence of NO_2^-.

Furthermore **Eq.13** is a separable differential equation, which can re-write as:

$$-\frac{d[H_2O_2]}{dt} = A[H_2O_2] + B[H_2O_2]^2 \quad (15)$$

where A and B are constants, $A = k_1[MPO]$ and $B = \frac{k_4k_1[MPO]}{k_2[Cl^-]+k_3[NO_2^-]}$. This differential equation can be solved by having different $-\frac{d[H_2O_2]}{dt}$ and $[H_2O_2]$ values at increasing time and then by using ordinary differential computer program namely *Mathematica*, A and B values could be determined. The negative B value explains the decrease in the overall rate of consumption of H_2O_2 at prolonged time. This finding strengthen the discussion of the solidarity of **Eq.13** to present the situation, because it simply states that the second part of **Eq.13** is the parameter responsible for the altering H_2O_2 consumption from a rapid step to a slower step. Finally, calculated B values were used to estimate the ratio of rate constants $\frac{k_2}{k_3}$, $\frac{k_4}{k_3}$ and $\frac{k_4}{k_2}$ values. Rate constant ratios are important, because they show the competitiveness of Cl^- and NO_2^- towards reaction with MPO-I, and further explain the slowness of reaction 4 relative to reactions 2 and 3.

Table 1 and **Figure 3** show the change of k_1 values, deduced from **Eq.14** with respect to changes in Cl^- concentrations.

Furthermore, **Table 2** and **Figure 4** show the change of k_1 value, deduced from **Eq.14** with respect to changes in NO_2^- concentrations.

After the passage of 10 s, H_2O_2 consumption pattern changes entirely, where the second part of **Eq.13** became the major factor. This factor introduces new parameters that affect the rate of consumption of H_2O_2 such as Cl^-, NO_2^- and reaction 4.

Mathematical salvation of **Eq.15** at variant consumption rates of H_2O_2 versus H_2O_2 concentrations under variant chloride and nitrite concentrations (*i.e.* **Tables 1** and **2**) yielded A and B constant values, and consequently the determined B constant values were employed to estimate $\frac{k_4}{k_3}$, $\frac{k_4}{k_2}$ and $\frac{k_2}{k_3}$ ratios (**Table 3**).

Rate constant ratios tell explicitly which reaction is faster or slower in the mechanism, and hence several conclusive remarks could be concluded from **Table 3**. Firstly, is that k_3 is much larger than k_2, which emphasizes that the nitrite inhibition reaction 3 producing MPO-II, is faster than the catalyzing reaction 2 producing HOCl, (*i.e.* $\frac{k_2}{k_3} = 1.15 \times 10^{-3}$), and that confirms the inhibition nature of NO_2^- in the mechanism.

Secondly, the previously assumed that $k_3 \gg k_4$ has been mathematically justified and it was also proven that k_4 is very much smaller than k_3 or k_2. Finally, reaction 4

Table 1. Change of rate constant (k_1) versus chloride ion concentration [Cl^-].

k_1 ($M^{-1}\cdot s^{-1}$)	[Cl^-], mM
2.33×10^5	5
2.67×10^5	10
3.02×10^5	25
3.96×10^5	50
5.23×10^5	100

[MPO] = 50 nM, [H_2O_2] = 10 μM, [NO_2^-] = 100 μM, in 100 mM phosphate buffer, pH = 7.0, containing 30 μM EDTA at 25˚C.

Table 2. Change of rate constant (k_1) versus nitrite ion concentration [NO_2^-].

k_1 ($M^{-1}\cdot s^{-1}$)	[NO_2^-], μM
1.5×10^6	0
1.4×10^6	25
7.13×10^5	50
5.75×10^5	100

[MPO] = 50 nM, [H_2O_2] = 10 μM, [Cl^-] = 100 mM, in 100 mM phosphate buffer, pH = 7.0, containing 30 μM EDTA at 25˚C.

Table 3. Rate constant ratios in the presence and absence of (NO_2^-) inhibitor.

Rate constant ratios	$\frac{k_2}{k_3}$	$\frac{k_4}{k_3}$	$\frac{k_4}{k_2}$
Presence of (NO_2^-) inhibitor	1.15×10^{-3}	7.0×10^{-8}	6.09×10^{-5}
Absence of (NO_2^-) inhibitor	-	-	4.4×10^{-3}

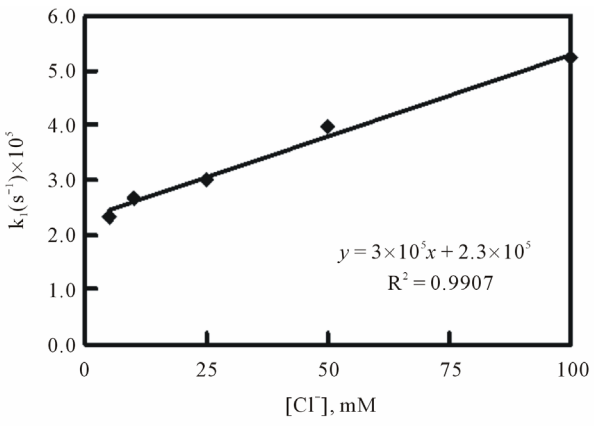

Figure 3. Change of rate constant (k_1) versus chloride ion concentration [Cl^-].

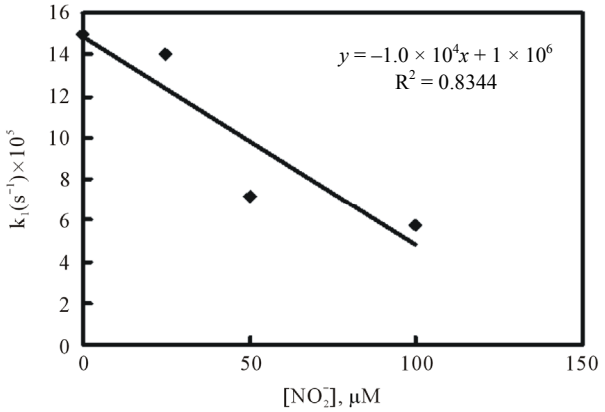

Figure 4. Change of rate constant (k_1) versus nitrite ion concentration [NO_2^-].

becomes significant in absence of NO_2^-.

4. CONCLUSIONS

Assessment of MPO-catalytic activity towards oxidation of chloride and other halides is a complex and multifunctional process [24,29]. MPO catalytic activity is dependent on initial concentrations of MPO, H_2O_2, Cl^-, pH, H_2O_2 to MPO concentration ratio and order of mixing. Thus, development of a comprehensive kinetic model is a complex task. We acknowledge that our proposed kinetic model is limited to describing our experimental data.

Proposed kinetic steady-state model was able to explain the monophasic and biphasic phenomena in absence and presence of nitrite. Additionally, the model was able to estimate values for k_1 and $\frac{k_4}{k_3}$, $\frac{k_4}{k_2}$ and $\frac{k_2}{k_3}$ rate constant ratios. Interestingly, this model supports previous findings that MPO has two binding sites that have distinct impact on the heme iron microenvironment [29]. Chloride occupied one site as substrate and nitrite occupied the other site as inhibitor.

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