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Reactions of Superoxide with Myeloperoxidase[†]

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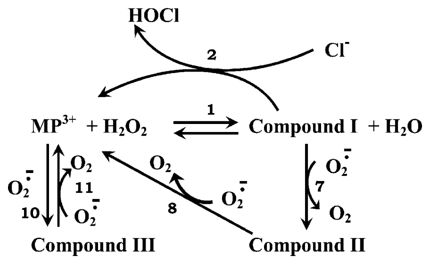
ABSTRACT: When neutrophils ingest bacteria, they discharge superoxide and myeloperoxidase into phagosomes. Both are essential for killing of the phagocytosed micro-organisms. It is generally accepted that superoxide is a precursor of hydrogen peroxide which myeloperoxidase uses to oxidize chloride to hypochlorous acid. Previously, we demonstrated that superoxide modulates the chlorination activity of myeloperoxidase by reacting with its ferric and compound II redox states. In this investigation we used pulse radiolysis to determine kinetic parameters of superoxide reacting with redox forms of myeloperoxidase and used these data in a steady-state kinetic analysis. We provide evidence that superoxide reacts with compound I and compound III. Our estimates of the rate constants for the reaction of superoxide with compound I, compound II, and compound III are 5×10^6 M^{-1} s^{-1}, $5.5 \pm 0.4 \times 10^6$ M^{-1} s^{-1}, and $1.3 \pm 0.2 \times 10^5$ M^{-1} s^{-1}, respectively. These reactions define new activities for myeloperoxidase. It will act as a superoxide dismutase when superoxide reacts consecutively with ferric myeloperoxidase and compound III. It will also act as a superoxidase by using hydrogen peroxide to oxidize superoxide via compound I and compound II. The favorable kinetics of these reactions indicate that, within the confines of a phagosome, superoxide will react with myeloperoxidase and affect the reactions it will catalyze. These interactions of superoxide and myeloperoxidase will have a major influence on the way neutrophils use oxygen to kill bacteria. Consequently, superoxide should be viewed as a cosubstrate that myeloperoxidase uses to elicit bacterial killing.

Myeloperoxidase is a fascinating enzyme because it possesses several catalytic activities and a plethora of physiological and exogenous substrates (see Scheme 1) (*1*, *2*). Its major activity is presumed to be oxidation of chloride to hypochlorous acid. However, it also uses hydrogen peroxide to oxidize thiocyanate and bromide to hypohalous acids (*3*, *4*), tyrosine to tyrosyl radical (*5*), nitrite to nitrogen dioxide (*6*), and phenols and anilines to free radicals (*1*). Nitric oxide is also a favorable substrate for the enzyme (*7*).

Of all the possible substrates of myeloperoxidase, superoxide is the one that continues to intrigue us. Superoxide and myeloperoxidase are used by neutrophils to kill a wide range of micro-organisms (*8*). They are both discharged into phagosomes where killing occurs. Apart from ubiquitous chloride, superoxide is the only other known reducing substrate for myeloperoxidase present within phagosomes at sufficient amounts and concentrations to influence the enzyme's activity. Superoxide enhances myeloperoxidase-dependent killing of *Staphylococcus aureus* by neutrophils (*9*), so it is conceivable that the two interact within phagosomes.

Scheme 1: Potential Reactions of Myeloperoxidase with Superoxide and Other Substrates[a]



[a] Only the major reactions of myeloperoxidase with superoxide and hydrogen peroxide that are relevant to this study are given. Numbers refer to the list of reactions given in Table 1. MP^{3+} is ferric myeloperoxidase.[1]

The possible reactions of superoxide with myeloperoxidase are shown in Scheme 1, and their associated rate constants are given in Table 1. Yamazaki and co-workers were the first to demonstrate that superoxide reacts with ferric myeloperoxidase to convert it to oxymyeloperoxidase, which is also called compound III (reaction 10) (*10*). Compound III is the predominant form of the enzyme within phagosomes (*11*). The formation of compound III by this route is fast ($k = 2 \times 10^6$ M^{-1} s^{-1}) and competes with conversion of the ferric enzyme to compound I by hydrogen peroxide (*12*). Superoxide also reacts with compound II of myeloperoxidase and boosts both the chlorination and catalytic activities of the enzyme (reaction 8) (*13*, *14*). Given that it reacts with compound II, it should react with highly oxidized compound I, as is the case with horseradish peroxide (*15*), lactoper-

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Table 1: Reactions of Myeloperoxidase (MP^{3+}) and Its Redox Intermediates with Hydrogen Peroxide, Superoxide, Organic Substrates (RH), and Chloride[a]

no.	reaction	rate constant	ref
1	$MP^{3+} + H_2O_2 \leftrightarrows$ compound I $+ H_2O$	$k_1 = 2 \times 10^7\ M^{-1}\ s^{-1}$ $k_{-1} = 58\ s^{-1}$	(*31*, *43*)
2	compound I $+ H^+ + Cl^- \rightarrow MP^{3+} + HOCl$	$k_2 = 2.5 \times 10^4\ M^{-1}\ s^{-1}$	(*39*)
3	compound I $+ H_2O_2 \rightarrow MP^{3+} + H_2O + O_2$	$k_3 = 2 \times 10^6\ M^{-1}\ s^{-1}$	(*14*)
4	compound I $+ H_2O_2 \rightarrow$ compound II $+ O_2^{\bullet-} + H^+$	$k_4 = 8 \times 10^4\ M^{-1}\ s^{-1}$	(*31*)
5	compound I $+$ RH $\rightarrow$ compound II $+ R^\bullet + H^+$	see text	
6	compound II $+$ RH $+ H^+ \rightarrow MP^{3+} + R^\bullet + H_2O$	see text	
7	compound I $+ O_2^{\bullet-} \rightarrow$ compound II $+ O_2$	$k_7 \sim 5 \times 10^6\ M^{-1}\ s^{-1}$	this study
8	compound II $+ O_2^{\bullet-} + H^+ \rightarrow MP^{3+} + O_2 + H_2O$	$k_8 = 5 \times 10^6\ M^{-1}\ s^{-1}$	this study
9	compound II $+ H_2O_2 \rightarrow$ compound III $+ H_2O$	$k_9 = 78\ M^{-1}\ s^{-1}$	(*31*)
10	$MP^{3+} + O_2^{\bullet-} \leftrightarrows MP^{2+}O_2$ (compound III)	$k_{10} = 2 \times 10^6\ M^{-1}\ s^{-1}$ $k_{-10} = 1.4 \times 10^{-3}\ s^{-1}$	(*12*) (*11*)
11	compound III $+ O_2^{\bullet-} + 2H^+ \rightarrow MP^{3+} + H_2O_2 + O_2$ or compound III $+ O_2^{\bullet-} + 2H^+ \rightarrow$ compound I $+ H_2O + O_2$	$k_{11} = 1.3 \times 10^5\ M^{-1}\ s^{-1}$	this study
12	$MP^{3+} + Cl^- \leftrightarrows MP^{3+}\cdot Cl^-$	$K_{12} = 0.44$ M	(*44*)
13	$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$	$k_{13} = 2.4 \times 10^5\ M^{-1}\ s^{-1}$	(*45*, *46*)
14	$2O_2^{\bullet-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$	$k_{14} = 2 \times 10^9\ M^{-1}\ s^{-1}$	(*47*)

[a] All rate constants are for reactions at pH 7.4 and approximately 20 °C.

oxidase (*16*), and catalase (*17*). Compound III has also been proposed to be reduced by superoxide, which may provide a route to compound I that is independent of hydrogen peroxide (reaction 11) (*18*).

The different interactions of superoxide with myeloperoxidase may influence the killing mechanisms operative within phagosomes. Superoxide may boost hypochlorous acid production by preventing the accumulation of compound II (reaction 8). In this way superoxide would optimize the oxidative killing mechanism of neutrophils. Alternatively, myeloperoxidase could act as a superoxide dismutase (reactions 10 and 11) or a superoxidase by using hydrogen peroxide to oxidize superoxide to oxygen (reactions 1, 7, and 8). If these activities were to dominate, then myeloperoxidase could be viewed as a terminal oxidase that degrades hydrogen peroxide and superoxide to oxygen and water. These latter reactions would support the recent proposal that superoxide has more of an electrogenic role than a source of oxidants (*19*, *20*).

In this investigation we have used pulse radiolysis and steady-state kinetic analysis to determine the rate constants for the reactions of superoxide with the redox intermediates of myeloperoxidase. We show that superoxide reacts rapidly with compound I, compound II, and compound III. Consequently, all the reactions of superoxide with myeloperoxidase must be considered when assessing the role superoxide plays in bacterial killing by neutrophils.

EXPERIMENTAL PROCEDURES

Materials. Myeloperoxidase was purified from human leukocytes as described previously and its concentration determined by measuring its absorbance at 430 nm (ϵ_{430} 89 000 $M^{-1}\ cm^{-1}\ heme^{-1}$) (*21*). Its purity index (A_{430}/A_{280}) was 0.82. The enzyme was also purchased from Planta (Austria). The two sources of myeloperoxidase gave similar results. Xanthine oxidase, cytochrome *c*, superoxide dismutase, catalase, taurine, diethylenetriaminepentaacetic acid (DTPA), *p*-hydroxyphenylacetic acid, and 3,3′,5,5′-tetramethylbenzidine were purchased from the Sigma Chemical Company (St. Louis, MO). Hydrogen peroxide solutions were prepared daily by diluting a 30% stock solution, and the concentration was determined by measuring its absorbance at 240 nm (ϵ_{240} 43.6 $M^{-1}\ cm^{-1}$) (*22*). Acetaldehyde was purchased from BDH.

Methods. Pulse Radiolysis Experiments. Pulse radiolysis was performed using the Dynaray 4 MV linear accelerator facility at the University of Auckland, New Zealand, which has an adjunct computer-controlled optical detection system fitted with Supracil optics (*23*). Electron pulses of 200 ns to 3 μs duration were used to deliver radiolytic doses of 2−100 Gy (J/kg) to the temperature-controlled reaction cell (set at 22 °C), as determined by using the standard potassium thiocyanate dosimeter to calibrate a surrounding charge collection plate to correct for small variations in the delivered doses (*24*). Typically reactions contained 1 μM myeloperoxidase (heme) in 25 mM phosphate buffer pH 7.4 containing 100 μM DTPA, and 10 mM formate or 100 mM tertiary butanol. Adding formate is preferred as a “clean” system to produce superoxide radiolytically. Using tBuOH to scavenge hydroxyl radicals in oxygenated solutions results in the tBu-hydroperoxyl radical being formed, which can undergo additional redox reactions. The concentration of superoxide produced in oxygenated solutions containing formate ions is taken as 0.68 $\mu M\cdot Gy^{-1}$. For pulse radiolysis experiments, all solutions were aerated.

Measurement of Hypochlorous Acid Formation by Myeloperoxidase. Hypochlorous acid production by myeloperoxidase was determined in phosphate buffer containing chloride and 5 mM taurine. Accumulated taurine chloramine was assayed by measuring the iodide catalyzed oxidation of 3,3′,5,5′-tetramethylbenzidine (*25*).

Measurement of Superoxide Generation by Xanthine Oxidase. Superoxide production by xanthine oxidase was measured as superoxide dismutase-inhibitable cytochrome *c* reduction. Reactions were carried out in the presence of 10 μg/mL of catalase, and the rate of reduction of cytochrome *c* was recorded at 550 nm (ϵ_{550} 21 100 $M^{-1}\ cm^{-1}$) (*26*).

Spectral Analysis of the Reaction of Compound II with Superoxide Generated by Xanthine Oxidase. Myeloperoxidase was partially converted to compound II by adding hydrogen peroxide to the native enzyme in the absence of chloride. This ensured that compound II would compete with

Table 2: Extinction Coefficients Used for Modeling Reactions of Myeloperoxidase[a]

	430 nm ($\Delta\epsilon$)	456 nm ($\Delta\epsilon$)
MP^{3+}	89,000 M^{-1} cm^{-1}	27,000 M^{-1} cm^{-1}
compound I	44,000 M^{-1} cm^{-1} (45,000)	27,000 M^{-1} cm^{-1} (0,000)
compound II	45,000 M^{-1} cm^{-1} (44,000)	82,300 M^{-1} cm^{-1} (55,300)
compound III	56,000 M^{-1} cm^{-1} (33,000)	76,000 M^{-1} cm^{-1} (49,000)

[a] Data were obtained from papers by Hoogland et al. (*27*) and Marquez et al. (*31*) and represent absorbance per heme group. $\Delta\epsilon$ is the difference in extinction coefficients from that of the native enzyme (MP^{3+}).

ferric enzyme for superoxide. Catalase was then added to scavenge excess hydrogen peroxide. Reaction of superoxide with myeloperoxidase was promoted by the subsequent addition of xanthine oxidase and acetaldehyde. Chloride was added with xanthine oxidase to prevent conversion of any compound I to compound II. Reactions were monitored using a Beckman 7500 diode array spectrophotometer to record spectral changes of myeloperoxidase at discrete time points. The spectral data were then used to calculate the temporal changes in the concentrations of the redox intermediates of myeloperoxidase. Concentrations of mixtures of ferric enzyme, compound II, and compound III were calculated using the absorption coefficients given in Table 2 and the following simultaneous equations for the absorbance (A) of myeloperoxidase at the indicated wavelengths. Absorption coefficients (units = mM^{-1} cm^{-1}) were obtained from the published spectra of Hoogland et al. (*27*).

$$A_{430} = 89[\mathrm{MP}^{3+}] + 45[\text{compound II}] + 56[\text{compound III}]$$

$$A_{456} = 27[\mathrm{MP}^{3+}] + 82.3[\text{compound II}] + 76[\text{compound III}]$$

$$A_{625} = 10.2[\mathrm{MP}^{3+}] + 18[\text{compound II}] + 32.5[\text{compound III}]$$

Simultaneous equations were solved using the transform equation editor in SigmaPlot (Jandel Scientific).

Measurement of Hydrogen Peroxide Utilization by Myeloperoxidase. The activity of myeloperoxidase was measured by continuously monitoring the hydrogen peroxide concentration with a YSI 2510 oxidase probe fitted to a YSI model 25 oxidase meter (Yellow Springs Instrument Co., Yellow Springs, OH) (*28*). The electrode was covered with a single layer of dialysis tubing and calibrated against known concentrations of hydrogen peroxide. For experiments using xanthine oxidase, the electrode was calibrated in the presence of 10 mM acetaldehyde.

Kinetic Analysis. Kinetic analysis of experimental data to estimate unknown rate constants was performed with the software package Berkeley Madonna (Version 8; http://www.berkeleymadonna.com) using the Rosenbrock (stiff) integration method.

RESULTS

Reaction of Compound I with Superoxide. When aqueous solutions are irradiated, the primary radicals $HO^\bullet$, $H^\bullet$, and e_{aq}^- are generated along with H_2O_2 and H_2. In the presence of excess formate and oxygen all the primary radicals are converted to superoxide, which is generated in a 9-fold excess over H_2O_2 (*29*). This system is ideal for investigating fast reactions of superoxide with myeloperoxidase. However, Klebanoff and Clarke found that formate is oxidized to carbon dioxide by myeloperoxidase (*30*). Therefore, we determined how readily formate is oxidized by myeloperoxidase to assess whether it would influence the reactions of superoxide with the enzyme (see Supporting Information). We found that formate competes with chloride for oxidation by myeloperoxidase and that its rate constant for reaction with compound I is 2.8×10^4 M^{-1} s^{-1}. Pulse radiolysis of myeloperoxidase in the presence of superoxide dismutase and formate did not result in the formation of compound II. This indicates that formate must reduce compound I in a two electron reaction to regenerate the ferric enzyme. Reaction of formate with compound I was taken into account when interpreting subsequent kinetic data. Formate did not undergo other appreciable reactions with myeloperoxidase (see Supporting Information).

Our first aim was to establish whether superoxide reacts with compound I. This redox intermediate of myeloperoxidase is extremely unstable and short-lived (*31*), making observations of its reactions under turnover conditions difficult but suitable for study using pulse radiolysis. We used concentrations of superoxide which were at least 6-fold in excess of myeloperoxidase to observe sequential reactions of the redox intermediates of myeloperoxidase. When solutions of myeloperoxidase and formate are irradiated, the ferric enzyme will undergo parallel reactions with superoxide and hydrogen peroxide (reactions 1 and 10). These reactions should occur at approximately the same rate because the rate constant for reaction 1 ($k \sim 2 \times 10^7$ M^{-1} s^{-1}) (*31*, *32*) is about 10-fold greater than that for reaction 10 ($k \sim 2 \times 10^6$ M^{-1} s^{-1}) (*12*). Reactions were monitored at 430 nm, which is the absorbance maximum for ferric myeloperoxidase and compound I, as well as at 456 nm, which is the absorbance maximum for compound II and close to that for compound III (see Table 2) (*27*, *33*). Ferric myeloperoxidase and compound I have an isosbestic point at 456 nm, so conversion of the enzyme to compound I is not observable at this wavelength (*33*). Upon pulsing myeloperoxidase with radiation there was an exponential loss in absorbance at 430 nm (Figure 1A) and a corresponding increase in absorbance at 456 nm (Figure 1B). The rate of change in absorbance increased with increasing doses of radiation. Both absorbance changes were best fitted to double exponential equations (Figure 2A). This indicates that consecutive reactions of myeloperoxidase were occurring. When the two phases of the absorbance changes were extracted, it was apparent that there was an initial rapid formation of a redox intermediate that absorbs at 456 nm. In the second reaction there was a slower accumulation of the same or another intermediate that absorbs at this wavelength. Based on the known reactions outlined in Scheme 1, the first phase recorded at 456 nm should represent conversion of ferric myeloperoxidase to compound III (reaction 10). The second slower phase most likely represents reduction of compound I to compound II via reactions 1 and 7.

Spectral changes relative to unirradiated ferric myeloperoxidase were also recorded at discrete wavelengths

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[1] Abbreviation: MP^{3+}, ferric myeloperoxidase.

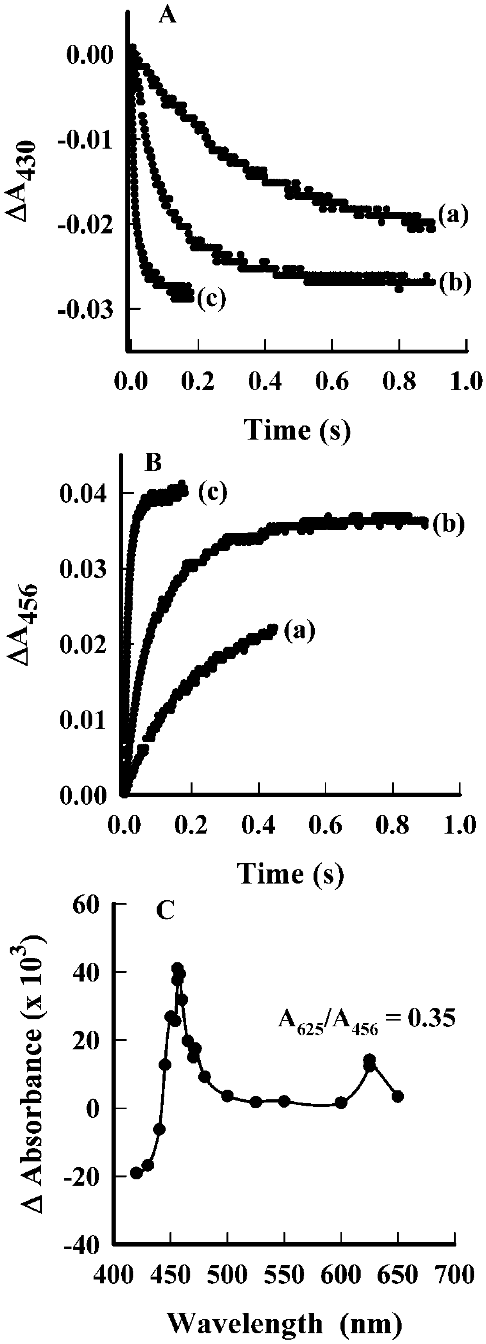

FIGURE 1: Reaction of ferric myeloperoxidase with superoxide. Solutions of 1 μM myeloperoxidase in 25 mM phosphate buffer pH 7.4 containing 10 mM formate and 100 μM DTPA at 20 °C were pulsed with varying doses of radiation to give superoxide and hydrogen peroxide at the following initial concentrations: (a) 6 μM and 0.6 μM; (b) 9.5 μM and 1 μM; (c) 52 μM and 5.5 μM, respectively. Absorbances were recorded at either (A) 430 nm or (B) 456 nm, respectively. Results are typical of 16 experiments. (C) The difference spectrum against 1 μM ferric myeloperoxidase was obtained 100 ms after the pulse radiolysis of solutions to give initial concentrations of 37 μM $O_2^{\cdot-}$ and 4 μM H_2O_2. Other conditions were as described above.

(Figure 1C). The ratio of absorbances at 625 and 456 nm was 0.35 ± 0.1 ($n = 3$). When the enzyme is completely converted to either compound II or compound III, this ratio is 0.17 or 0.54, respectively (*11*, *27*). Thus, the spectral data confirm that ferric myeloperoxidase was converted to a mixture of compound II and compound III.

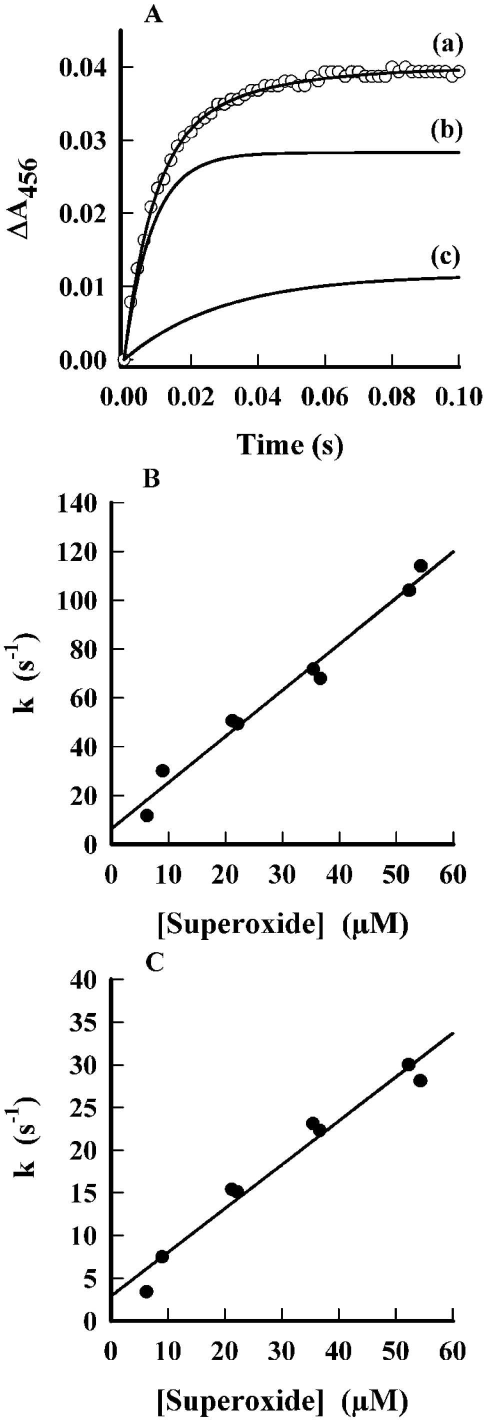

FIGURE 2: Kinetic analysis of the reactions of superoxide with myeloperoxidase. (A) A double exponential equation (a) was fitted to the absorbance changes at 456 nm when 1 μM myeloperoxidase was subjected to pulse radiolysis to give 52 μM superoxide and 5.5 μM hydrogen peroxide. The two components of this equation are shown in (b) and (c). Every 10th data point is shown by (○), while fitted data are given as solid lines. (B) The observed rate constants for the first exponential and (C) the second exponential obtained from data collected at 456 nm were plotted against the initial concentration of superoxide. Reaction conditions were described in Figure 1.

The observed rate constants for both reactions, obtained from the fitted exponentials to absorbance changes at 456 nm against time, were dependent on the initial concentration of superoxide (Figure 2B and C). For the first of the two consecutive reactions the rate constants obtained from the plots had values of 2.8 ± 0.2 × 10^6 M^{-1} s^{-1} at 430 nm (not shown) and 1.9 ± 0.1 × 10^6 M^{-1} s^{-1} at 456 nm (Figure 2B). The reactions monitored at 430 nm are those of the ferric enzyme with superoxide and hydrogen peroxide. Hence the rate constant obtained at 430 nm will be composed of k_1 and k_{10}. The fast reaction monitored at 456 nm will be formation of compound III (reaction 10). The rate constant obtained for this reaction is close to that previously measured for reaction of superoxide with ferric myeloperoxidase (2.1 ± 0.2 × 10^6 M^{-1} s^{-1}) (*12*). The magnitude of the difference between the rate constants obtained at 430 and 456 nm, 0.9 ± 0.3 × 10^6 M^{-1} s^{-1}, should account for the reaction of hydrogen peroxide with ferric myeloperoxidase (reaction 1). Since the initial concentration of hydrogen peroxide was one-ninth the concentration of superoxide, this value gives an apparent rate constant for reaction 1 of 8.2 ± 0.3 × 10^6 M^{-1} s^{-1}. This value is lower than those obtained by stopped flow techniques (~2 × 10^7 M^{-1} s^{-1}) (*31*, *32*) and can be explained by recycling of compound I by formate.

The rate constants obtained at the two wavelengths for the second of the consecutive reactions were of the same magnitude: 5.5 ± 0.7 × 10^5 M^{-1} s^{-1} at 456 nm (Figure 2C) and 5.9 ± 0.4 × 10^5 M^{-1} s^{-1} at 430 nm (not shown). The rate constant most likely reflects the reduction of compound I to compound II by superoxide. It is an apparent rate constant that will be a function of rate constants for formation of compound I (k_1) and compound II (k_7), turnover of compound II (k_8), and reduction of compound I by formate. Hence, it represents a lower limit for the reaction of superoxide with compound I.

When superoxide dismutase was added to the reaction system, it accelerated the loss in absorbance at 430 nm (Figure 3A), whereas at 456 nm it dramatically inhibited the increase in absorbance (Figure 3B). This is compelling evidence that, in the presence of superoxide dismutase, compound I was formed and was stable for at least 20 ms. Superoxide dismutase would scavenge superoxide and increase the initial concentration of hydrogen peroxide. This would increase the rate of reaction 1, which explains the increased rate of loss in absorbance at 430 nm. Its ability to prevent the fast increase in absorbance at 456 nm, observed in Figure 2A, is confirmation that superoxide reacts with the ferric enzyme to form compound III. Furthermore, inhibition of the slower increase in 456 nm observed in Figure 2A demonstrates that superoxide also reduces compound I to compound II. The gradual and much slower increase in absorbance at 456 nm in Figure 3B was independent of superoxide which would have been eliminated by superoxide dismutase within a few milliseconds.

It was possible to use a kinetic model of reactions in Scheme 1 and Table 2 to estimate the rate constant for the reaction of superoxide with compound I. This model took account of the two electron reduction of compound I by formate. In the reaction systems with superoxide dismutase, this enzyme will compete with myeloperoxidase for superoxide. From simulations it was apparent that the reaction of superoxide with compound I must have a rate constant of less than 1 × 10^7 M^{-1} s^{-1}. With rate constants larger than this value, compound I would compete with superoxide dismutase for superoxide. This would have resulted in a much greater initial increase in absorbance at 456 nm than that shown in Figure 3B. We also used the kinetic model to estimate the rate constants for reduction of compound I and compound II by superoxide to give best fits to the absorbance changes at 456 nm as shown in Figure 1B. Rate constants were set to the values given in Table 1 except that k_1, k_8, k_{10}, and k_{11} were constrained between 1−3 × 10^7 M^{-1} s^{-1}, 2−6 × 10^6 M^{-1} s^{-1}, 1−3 × 10^6 M^{-1} s^{-1}, and 1−3 × 10^5 M^{-1} s^{-1}, respectively. The value for k_7 was allowed to vary between 0.1 × 10^7 M^{-1} s^{-1} and 1 × 10^7 M^{-1} s^{-1}. Estimates for k_7 and k_8 were 3.1 ± 1.9 × 10^6 M^{-1} s^{-1} and 3.4 ± 1.6 × 10^6 M^{-1} s^{-1} (n = 5). Based on these values, we conclude that the rate constants for reduction of compound I and compound II by superoxide are of a similar magnitude.

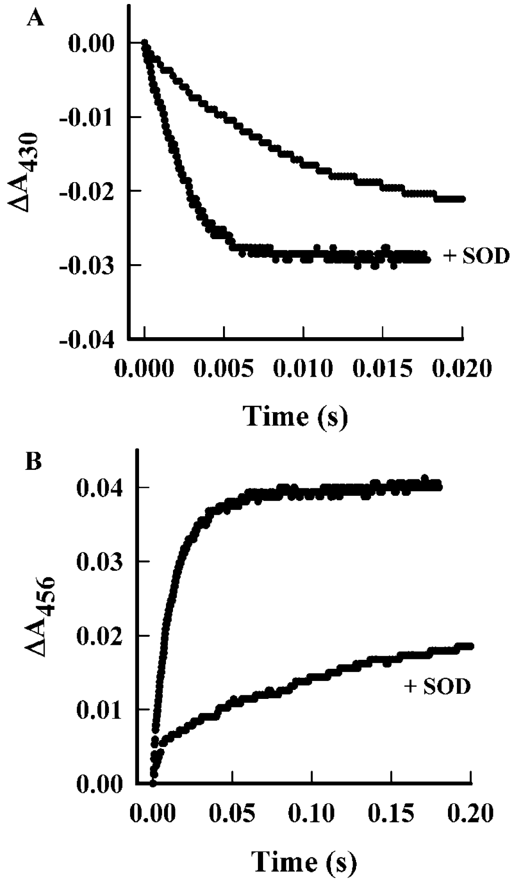

FIGURE 3: Effects of superoxide dismutase on the reaction of superoxide with myeloperoxidase. Solutions contained 1 μM myeloperoxidase in 25 mM potassium phosphate buffer (pH 7.4) with 10 mM formate, 100 μM DTPA, and where indicated 0.50 μM superoxide dismutase. Solutions were subjected to pulse radiolysis to give initial concentrations of superoxide and hydrogen peroxide of 52 μM and 5.5 μM, respectively. The absorbance changes were recorded at (A) 430 nm and (B) 456 nm.

Reaction of Superoxide with Compound II. We investigated the reaction of superoxide with compound II (reaction 8) by using either pulse radiolysis to generate an instantaneous concentration of superoxide or xanthine oxidase to produce a steady flux of superoxide. For pulse radiolysis, tertiary butanol was used to scavenge hydroxyl radicals. Compound II was first prepared by adding hydrogen peroxide to the ferric myeloperoxidase. After approximately 1 min, solutions were irradiated and absorbance changes were monitored at 430 and 456 nm. At this time most of the added hydrogen peroxide would have been consumed by the catalase activity of myeloperoxidase (*14*). Upon irradiation there was a rapid loss in absorbance at 456 nm and an equally rapid increase in absorbance at 430 nm (Figure 4). In both cases the

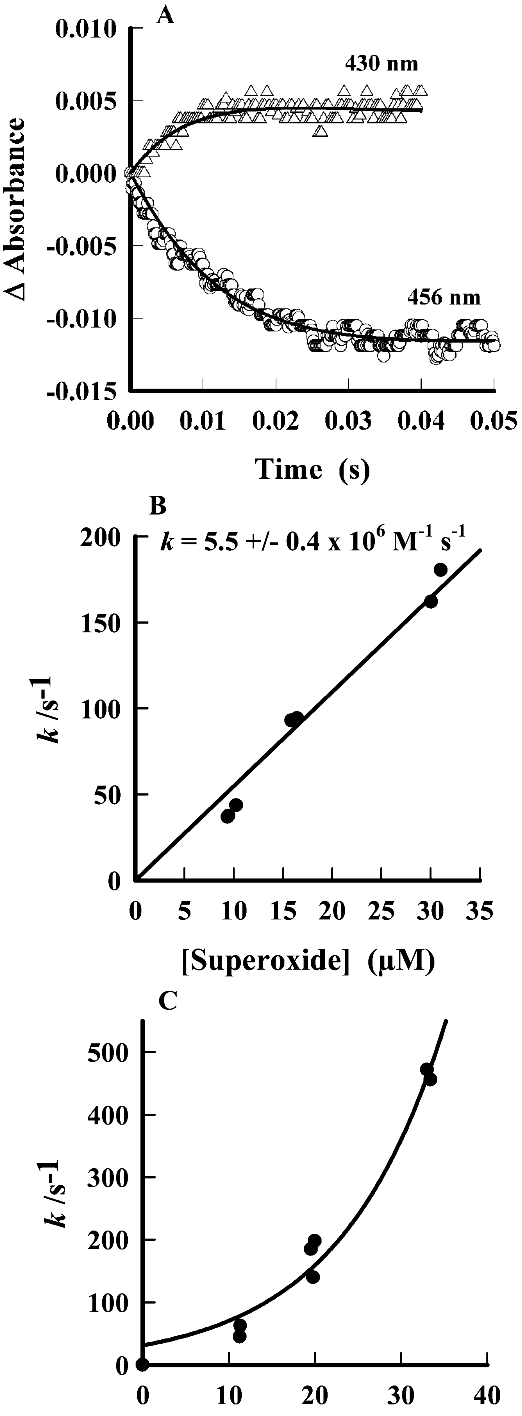

FIGURE 4: Reaction of compound II with superoxide generated by pulse radiolysis. (A) Compound II was formed by adding 30 μM hydrogen peroxide to 1 μM myeloperoxidase in 25 mM phosphate buffer (pH 7.4) containing 100 mM t-butanol and 100 μM DTPA. After 1 min, solutions were irradiated and absorbance changes were recorded at 430 nm and 456 nm. Data were fitted using the kinetic model of reactions in Scheme 1. Single-exponential equations were fitted to the data in (A) and the observed rate constants obtained at (B) 456 nm or (C) 430 nm were plotted against the initial concentration of superoxide.

absorbance changes were small. The magnitude of the change in absorbance was much smaller at 430 nm than at 456 nm. No changes in absorbance occurred when 0.5 μM superoxide dismutase was added to the reaction system (not shown). These spectral changes are indicative of reduction of compound II to ferric enzyme by superoxide. The absorbance changes were fitted by single exponentials. The observed rate constants obtained from these fits were plotted against the initial concentration of superoxide (Figures 4B and C). At 456 nm the observed rate constant was proportional to the initial concentration of superoxide (Figure 4B). The slope of the graph indicates that the rate constant for reduction of compound II by superoxide was $5.5 \pm 0.4 \times 10^6$ M^{-1} s^{-1}. However, at 430 nm the observed rate constant increased exponentially with the concentration of superoxide (Figure 4C). The most likely explanation for these results is that ferric myeloperoxidase was reacting rapidly with superoxide and hydrogen peroxide as it was formed from compound II.

To verify the rate constant for reduction of compound II by superoxide, we used xanthine oxidase as a source of superoxide and reacted it with a mixture of compound II and ferric enzyme (Figure 5). Upon generation of superoxide, there was a loss in absorbance at 456 nm and a corresponding increase at 430 nm (Figure 5A). Analysis of these spectral changes indicated that preformed compound II decayed and there was concomitant formation of ferric myeloperoxidase and compound III (Figure 5B). After about 1 min, formation of ferric myeloperoxidase peaked, then declined as it was converted to compound III. In agreement with our previous result (*13*), superoxide dismutase inhibited the rate of decay of compound II to ferric myeloperoxidase by 80%, which confirms that superoxide was responsible for promoting the loss of compound II (data not shown).

From the data presented in Figure 5B we constructed a secondary plot from which k_8 was calculated (Figure 5C). We assumed that during the initial 20 s of the reaction superoxide reacted chiefly with ferric myeloperoxidase and compound II (reactions 10 and 8). The high concentration of chloride should have prevented superoxide reacting with compound I and, in the early stages, reaction with compound III would be too slow to influence the analysis.

Based on these assumptions the rates of change of compound II and ferric myeloperoxidase are given by eqs i and ii.

$$-d[\text{compound II}]/dt = k_8[\text{compound II}][O_2^{\bar{\bullet}}] \quad \text{(i)}$$

$$-d[\text{MP}^{3+}]/dt = k_{10}[\text{MP}^{3+}][O_2^{\bar{\bullet}}] - k_8[\text{compound II}][O_2^{\bar{\bullet}}] \quad \text{(ii)}$$

Dividing eq ii by eq i gives the following expression:

$$(-d[\text{MP}^{3+}]/dt)/(-d[\text{compound II}]/dt) = (k_{10}[\text{MP}^{3+}]/k_8[\text{compound II}]) - 1 \quad \text{(iii)}$$

From eq iii, a plot of the ratio of rates of change of ferric myeloperoxidase and compound II versus the ratio of their concentrations should produce a straight line. Its slope will equal k_{10}/k_8, and it will have an intercept of -1. The rates of formation of ferric myeloperoxidase and decay of compound II were calculated by differentiating the third-order polynomials that fitted the plots of their concentrations against time. These rates were corrected for the spontaneous decay of compound II to ferric myeloperoxidase. This was determined by following the reaction in the presence of

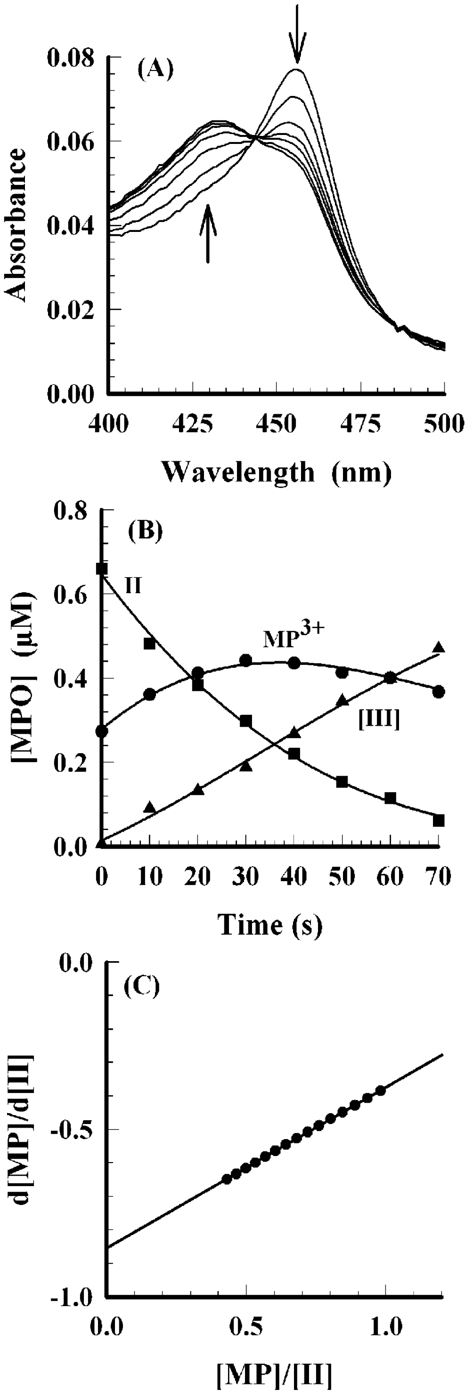

FIGURE 5: Reaction of superoxide with a mixture of compound II and ferric myeloperoxide. (A) Compound II was formed by adding 50 μM hydrogen peroxide to 1 μM ferric myeloperoxidase in 50 mM phosphate buffer pH 7.4 containing xanthine oxidase and 100 μM DTPA at 21 ± 2 °C. After 10 s, 10 μg/mL of catalase was added to scavenge excess hydrogen peroxide. Sodium chloride (100 mM) and 1 mM methionine were also added, and 30 s later 10 mM acetaldehyde was added. The rate of superoxide production was 1 μM/min. Spectral changes were then recorded every 10 s. Each spectrum is the average of 10 recordings taken over 1 s. Arrows indicate the direction of spectral changes. Only data between 400 and 500 nm are shown. (B) Data in (A) were used to calculate the absolute concentrations of ferric myeloperoxidase (●), compound II (■), and compound III (▲) (see Experimental Procedures). Lines through data are polynomial fits to temporal concentration changes. (C) Rates of change for compound II and ferric myeloperoxidase were calculated from the polynomial fits over the first 20 s of the reaction and plotted according to eq iii (●). The solid line is the regression line through the calculated data.

superoxide dismutase (not shown). We conducted four experiments as illustrated in Figure 5, and the means and standard deviations for the slope and intercept were 0.5 ± 0.1 and −0.9 ± 0.1 respectively. The correlation coefficients for the secondary plots were at least 0.96. From these data and based on a value of 2×10^6 M^{-1} s^{-1} for k_{10}, k_8 was calculated to be $4.0 \pm 0.4 \times 10^6$ M^{-1} s^{-1}. This value is close to that measured directly by pulse radiolysis.

Reactions of Compound III with Superoxide. Reduction of compound III by superoxide has been proposed (*18*). To confirm this finding we undertook both spectral and kinetic investigations. It is known that superoxide converts myeloperoxidase to compound III when catalase is present to scavenge hydrogen peroxide and substrates such as chloride or thiocyanate are available to prevent formation of compound II. Decay back to the native enzyme is slow (*11*), so full conversion of the enzyme to compound III would be expected unless compound III reacts with superoxide. Therefore, we undertook spectral studies to determine the percent conversion of myeloperoxidase to compound III in the presence of a superoxide generating system. Catalase and thiocyanate were included to maximize conversion of myeloperoxidase to compound III. Spectral changes of myeloperoxidase occurred rapidly, and a steady state was reached with a maximum of 90% conversion to compound III (Figure 6A). This result demonstrates that superoxide is likely to react with compound III. No other potential substrates of compound III were present in the reaction system to account for its reduction.

The rate of loss of compound III is described by eq iv:

$$-\mathrm{d}[\text{compound III}]/\mathrm{d}t = \mathrm{k}_{11}[\text{compound III}][\mathrm{O}_2^{\bullet -}] + k_{-10}[\text{compound III}] - k_{10}[\mathrm{MP}^{3+}][\mathrm{O}_2^{\bullet -}] \quad \text{(iv)}$$

Assuming the decay makes a minor contribution to the turnover of compound III, the steady-state situation simplifies to

$$k_{11}[\text{compound III}][\mathrm{O}_2^{\bullet -}] = k_{10}[\mathrm{MP}^{3+}][\mathrm{O}_2^{\bullet -}] \quad \text{(v)}$$

or

$$k_{11}/k_{10} = [\mathrm{MP}^{3+}]/[\text{compound III}] \quad \text{(vi)}$$

Using a value of 2×10^6 M^{-1} s^{-1} for k_{10}, and 90% conversion of myeloperoxidase to compound III in the presence of a superoxide generating system, k_{11} is determined to be approximately 2×10^5 M^{-1} s^{-1}.

We also estimated k_{11} using a kinetic model of all the reactions in Scheme 1 to fit progress curves for hydrogen peroxide accumulation in reaction systems containing myeloperoxidase, xanthine oxidase, and chloride (Figure 6B). Reaction conditions were chosen to ensure that compound III was the predominant form of the enzyme present at steady state; i.e., low fluxes of superoxide and hydrogen peroxide, and high concentrations of chloride to prevent formation of compound II. Progress curves for hydrogen peroxide accumulation were followed at varying fluxes of hydrogen peroxide and concentrations of myeloperoxidase. Kinetic fits to these curves were independent of k_7 and k_8 within a relatively broad range around the values given in Table 1 (not shown). Best fits to the experimental data were obtained

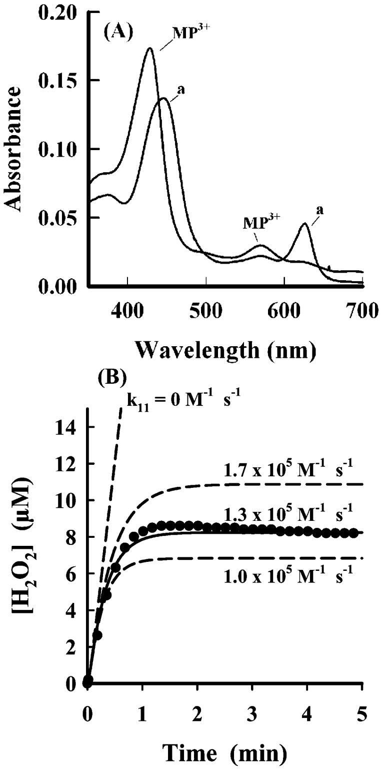

FIGURE 6: Estimation of the rate constant for reaction of superoxide with compound III. (A) Myeloperoxidase (MP^{3+}) (2 μM) was incubated in 50 mM phosphate buffer pH 7.4 containing, xanthine oxidase, 1 mM thiocyanate, 50 μg/mL of catalase, and 100 μM DTPA at 21 ± 2 °C. Reactions were started by adding 10 mM acetaldehyde. The rate of superoxide generation was 2.9 μM/min. The final spectrum (a) was attained within 1 min and was stable for at least 7 min. (B) Hydrogen peroxide accumulation was monitored (●) after adding xanthine oxidase to a solution of 10 mM acetaldehyde and 44 nM myeloperoxidase. Reactions were carried out at 21 ± 2 °C in 10 mM phosphate buffer pH 7.4 containing 140 mM sodium chloride and 10 mM taurine. Fluxes of superoxide and hydrogen peroxide were 26 μM/min and 12.5 μM/min, respectively Results are typical of at least three experiments. The value of k_1 was set at 2×10^7 M^{-1} s^{-1}, and k_{11} was allowed to vary between 0.5×10^5 M^{-1} s^{-1} and 5×10^5 M^{-1} s^{-1}. The best fit (solid line) was determined by iterative regression analysis using Berkeley Madonna. Dashed lines are simulated fits at the indicated values of k_{11}. Values for other rate constants in the kinetic model are given in Table 1.

with an average value for k_{11} of $1.3 \pm 0.2 \times 10^5$ M^{-1} s^{-1} ($n = 3$) (Figure 6B). Small changes in the value of k_{11} resulted in significant deviations from the best fit (Figure 6B dashed lines), which demonstrates that turnover of compound III was rate determining under the reaction conditions analyzed. When k_{11} was set to zero, simulations revealed that myeloperoxidase would not consume hydrogen peroxide. This demonstrates that reaction of superoxide with compound III is essential for turnover of myeloperoxidase. Its decay to ferric enzyme is too slow to account for the hydrogen peroxide consumption in the absence of chloride.

DISCUSSION

In this investigation we have shown that superoxide reacts rapidly with the redox intermediates of myeloperoxidase. These reactions confer additional activities on myeloperoxidase that should be considered when assessing the physiological function of the enzyme. Its sequential reactions with ferric myeloperoxidase and compound III result in dismutation to hydrogen peroxide and oxygen (reactions 10 and 11). Hence, myeloperoxidase has the potential to act as a superoxide dismutase. Myeloperoxidase can also use hydrogen peroxide to oxidize superoxide to oxygen via a superoxidase activity (reactions 1, 7, and 8). Both these newly described activities should be relevant to how neutrophils use superoxide to kill bacteria because the high concentrations of myeloperoxidase and superoxide within phagosomes will favor reactions between them. Accordingly, superoxide should be viewed as a cosubstrate used by myeloperoxidase during bacterial killing.

It is apparent from this work and earlier studies (*11*, *18*, *34*) that compound III is not a dead-end intermediate. Otherwise the enzyme would not produce hypochlorous acid in the presence of a superoxide generating system. Our results support the earlier proposal that superoxide reduces compound III (*18*), and we conclude that this is the major mechanism for compound III turnover. We have excluded alternatives such as reaction of hydrogen peroxide with compound III (*13*) or ferrous myeloperoxidase (*35*) and the decay of compound III to ferric or ferrous myeloperoxidase (*36*, *37*) because they are too slow to account for turnover. They did not influence kinetic fits to the experimental data.

The sequential reactions of superoxide with ferric myeloperoxidase and compound III constitute superoxide dismutase activity. The magnitude of the rate constant indicates that myeloperoxidase is a weak superoxide dismutase. It would be expected to be an effective superoxide dismutase only at the high concentrations of enzyme that exist within neutrophil phagosomes. Wever and co-workers (*18*) originally proposed that superoxide reduces compound III to compound I and suggested that myeloperoxidase could produce hypochlorous acid via reactions 10 and 11b. However, catalase inhibits hypochlorous acid production by myeloperoxidase and xanthine oxidase by 95% (*13*). Thus, it is more likely that when superoxide reacts with compound III the products are ferric myeloperoxidase and hydrogen peroxide.

By using high concentrations of superoxide in the pulse radiolysis experiments we were able to demonstrate that superoxide also reacts with compound I and reduces it to compound II. This result was supported by the ability of superoxide dismutase to slow the conversion of compound I to compound II. The lower limit of the apparent rate constant for reaction of superoxide with compound I was approximately 6×10^5 M^{-1} s^{-1}. Competition between myeloperoxidase and superoxide dismutase indicated that the upper limit for the absolute rate constant is less than 1×10^7 M^{-1} s^{-1}. Estimation of the rate constant by kinetic modeling had relatively large errors but indicated that its value was similar to that for the reduction of compound II by superoxide. Due to the inherent instability of compound I, accurate measurement of this rate constant will require a combination of stopped flow and pulse radiolysis to ensure that reduction of compound I by superoxide is the dominant reaction that is monitored. Our current results imply that only serotonin and thiocyanate have similar reactivity to superoxide (*38*, *39*). Other substrates such as bromide, ascorbate, tyrosine, and chloride react at least an order of magnitude more slowly (*40*, *41*). Physiologically, substrate preference for compound I will be determined by these rate constants and the relative concentrations of the substrates.

Reduction of compound II by superoxide prevents accumulation of this inactive intermediate and optimizes production of hypochlorous acid (*13*). We measured the rate constant for this reaction using two different techniques and show that superoxide is by far the best substrate for compound II to be identified to date ($k = 5.5 \pm 0.4 \times 10^6$ M^{-1} s^{-1}). This is 4-fold greater than for serotonin ($1.4 \pm 0.1 \times 10^6$ M^{-1} s^{-1}) (*38*) and well over two orders of magnitude greater than for tyrosine ($1.6 \pm 0.1 \times 10^4$ M^{-1} s^{-1}) (*41*) and ascorbate ($1.1 \pm 0.1 \times 10^4$ M^{-1} s^{-1}) (*40*). Our results demonstrate that superoxide is an excellent substrate for the classical peroxidase activity of myeloperoxidase, which involves consecutive reactions of compound I and compound II (reactions 1, 7, and 8). However, this activity is constrained by compound III formation. It may be significant only when there is a high concentration of superoxide to make it competitive with oxidation of chloride. This discovery has important ramifications for understanding how neutrophils use superoxide to kill bacteria (*42*). At high concentrations of chloride, superoxidase activity would be blocked because chloride would prevent reaction of superoxide with compound I. However, superoxide would be able to prevent accumulation of compound II and maintain the chlorination activity of myeloperoxidase. Alternatively, at low concentrations of chloride, myeloperoxidase would have more of an antioxidant activity in breaking down hydrogen peroxide and superoxide to oxygen and water.

The reactions of superoxide with myeloperoxidase that we have investigated have the potential to influence the chlorination activity of the enzyme. The extent to which they occur will depend on the precise conditions within phagosomes. In a related paper, we have used the rate constants measured in this study to develop a kinetic model to assess the impact superoxide has on the chlorination activity of myeloperoxidase within neutrophil phagosomes (*42*). Predictions from the model indicate that myeloperoxidase dismutates the majority of superoxide generated. Most of the resulting hydrogen peroxide can be converted to hypochlorous acid, unless the chloride concentration falls below about 20 mM when the superoxidase activity comes more into play. Superoxide will prevent poor peroxidase substrates, such as nitrite, from inhibiting myeloperoxidase by reducing compound II back to the active enzyme. Therefore, superoxide should be viewed as a major cosubstrate used by myeloperoxidase during bacterial killing.

SUPPORTING INFORMATION AVAILABLE

Information on the oxidation of formate. This material is available free of charge via the Internet at http://pubs.acs.org.

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