# A Kinetic Analysis of the Catalase Activity of Myeloperoxidase †

**2 AUTHORS:**

Anthony J Kettle
University of Otago
**134** PUBLICATIONS **6,141** CITATIONS

Christine Winterbourn
University of Otago
**363** PUBLICATIONS **19,772** CITATIONS

# A Kinetic Analysis of the Catalase Activity of Myeloperoxidase[†]

Anthony J. Kettle* and Christine C. Winterbourn

*Free Radical Research Group, Biomedical Research Unit, Department of Pathology, Christchurch School of Medicine, P.O. Box 4345, Christchurch, New Zealand*
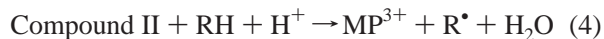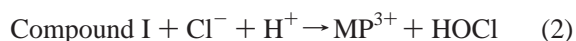
ABSTRACT: The predominant physiological activity of myeloperoxidase is to convert hydrogen peroxide and chloride to hypochlorous acid. However, this neutrophil enzyme also degrades hydrogen peroxide to oxygen and water. We have undertaken a kinetic analysis of this reaction to clarify its mechanism. When myeloperoxidase was added to hydrogen peroxide in the absence of reducing substrates, there was an initial burst phase of hydrogen peroxide consumption followed by a slow steady state loss. The kinetics of hydrogen peroxide loss were precisely mirrored by the kinetics of oxygen production. Two mols of hydrogen peroxide gave rise to 1 mol of oxygen. With 100 $\mu$M hydrogen peroxide and 6 mM chloride, half of the hydrogen peroxide was converted to hypochlorous acid and the remainder to oxygen. Superoxide and tyrosine enhanced the steady-state loss of hydrogen peroxide in the absence of chloride. We propose that hydrogen peroxide reacts with the ferric enzyme to form compound I, which in turn reacts with another molecule of hydrogen peroxide to regenerate the native enzyme and liberate oxygen. The rate constant for the two-electron reduction of compound I by hydrogen peroxide was determined to be 2 $\times$ 10$^6$ M$^{-1}$ s$^{-1}$. The burst phase occurs because hydrogen peroxide and endogenous donors are able to slowly reduce compound I to compound II, which accumulates and retards the loss of hydrogen peroxide. Superoxide and tyrosine drive the catalase activity because they reduce compound II back to the native enzyme. The two-electron oxidation of hydrogen peroxide by compound I should be considered when interpreting mechanistic studies of myeloperoxidase and may influence the physiological activity of the enzyme.

Myeloperoxidase is a green heme enzyme that is the most abundant protein in neutrophils. It plays a central role in infection and inflammation (*1*). It is required for neutrophils to kill a variety of microorganisms (*2*) and has been implicated in promoting tissue damage in numerous inflammatory diseases including rheumatoid arthritis (*3*), adult respiratory distress syndrome (*4*), multiple sclerosis (*5*), cystic fibrosis (*6*), and neonatal lung injury (*7*). Myeloperoxidase has also been proposed to trigger atherosclerosis by oxidizing low-density lipoproteins (*8*) and is implicated in acute myeloid leukemia (*9*) and lung cancer (*10*).

Myeloperoxidase is a promiscuous enzyme that has several different activities (*11*). The physiological milieu in which the enzyme functions will determine the substrates it uses and the activity it exhibits. Its main physiological activity is generally accepted to be production of hypochlorous acid. This strong oxidant is generated when hydrogen peroxide reacts with the native ferric enzyme (MP$^{3+}$) to form the redox intermediate compound I, which then oxidizes chloride (reactions 1 and 2). Hydrogen peroxide is supplied by the NADPH-oxidase of neutrophils, which produces superoxide as its primary product. Myeloperoxidase also oxidizes the pseudo-halide thiocyanate by a two-electron mechanism (*12*). Additional substrates include tyrosine, nitrite, urate, and a plethora of xenobiotics, which are oxidized via the classical peroxidation mechanism (reactions 1, 3, and 4) to free radical intermediates (R$^\bullet$). Myeloperoxidase can use superoxide to hydroxylate salicyate (*13*) in an analogous activity to that of the cytochrome P450s.

$$MP^{3+} + H_2O_2 \rightarrow \text{Compound I} + H_2O \qquad (1)$$

$$\text{Compound I} + Cl^- + H^+ \rightarrow MP^{3+} + HOCl \qquad (2)$$

$$\text{Compound I} + RH \rightarrow \text{Compound II} + R^\bullet + H^+ \quad (3)$$

$$\text{Compound II} + RH + H^+ \rightarrow MP^{3+} + R^\bullet + H_2O \quad (4)$$
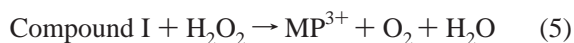
Agner (*14*) found that myeloperoxidase displays a catalase-like activity in which it degrades hydrogen peroxide to oxygen and water. Surprisingly, the mechanism of this activity and its physiological significance has received scant attention. Winterbourn et al. (*15*) proposed a mechanism in which the enzyme is turned over and oxygen liberated by a reaction between compound II and compound III. Later Iwamato (*16*) claimed the enzyme has true catalase activity in which hydrogen peroxide reacts with both the ferric enzyme and compound I (reactions 1 and 5). This proposal was dismissed by Marquez et al. (*17*) because they could find no evidence of the two electron reduction of compound I by hydrogen peroxide when they observed the reaction using stopped flow spectrophotometry. Rather, they found that hydrogen peroxide reduced compound I to compound

II (reaction 6), which inhibits the chlorination activity of myeloperoxidase as demonstrated earlier by Wever and co-workers (*18*).

$$Compound\ I + H_2O_2 \rightarrow MP^{3+} + O_2 + H_2O \quad (5)$$

$$Compound\ I + H_2O_2 \rightarrow Compound\ II + O_2^- + 2H^+ \quad (6)$$

Given the potential importance of myeloperoxidase acting as a catalase and attenuating oxidative reactions during inflammation, we have investigated the mechanism responsible for degradation of hydrogen peroxide. We conclude that hydrogen peroxide reacts with compound I to liberate oxygen but also slowly reduces it to compound II, which inhibits the catalase activity. The catalase activity is efficiently maintained by superoxide and other one-electron donors that act by reducing compound II to the ferric enzyme.

## EXPERIMENTAL PROCEDURES

*Materials.* Myeloperoxidase was purified from human leukocytes as described previously and its concentration determined by measuring its absorbance at 430 nm ($\epsilon_{430} = 89\ 000\ M^{-1}\ cm^{-1}\ heme^{-1}$) (*19*). Two enzyme preparations with purity indexes ($A_{430}/A_{280}$) of 0.82 and 0.66 were used in this study. Hemi-myeloperoxidase was prepared by reductive alkylation essentially as described by Zuurbier and co-workers (*20*). Xanthine oxidase, cytochrome *c*, superoxide dismutase, catalase, taurine, diethylenetriaminepentaacetic acid (DTPA), and 5,5′-dithiobis-2-nitrobenzoic acid 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 ($\epsilon_{240} = 43.6\ M^{-1}\ cm^{-1}$) (*21*). 5-Thio-2-nitrobenzoic acid was prepared from 5,5′-dithiobis-2-nitrobenzoic acid as outlined previously (*22*). Acetaldehyde was purchased from BDH.

*Methods. Measurement of Hydrogen Peroxide Utilization and Oxygen Production 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) (*22*). 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. Oxygen evolution was measured with a YSI oxygen electrode fitted to a YSI model 53 oxygen meter. In these experiments the initial oxygen concentration in the buffer was lowered to about 100 $\mu$M by bubbling with nitrogen.

*Measurement of Hypochlorous Acid Formation by Myeloperoxidase.* Hypochlorous acid production was determined in the presence of 10 mM taurine by measuring the accumulation of taurine chloramine using 5-thio-2-nitrobenzoic acid (*22*).

*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 with 10 $\mu$g/mL of catalase and the rate of reduction of cytochrome *c* at 550

nm ($\epsilon_{550} = 21\ 000\ M^{-1}\ cm^{-1}$) was measured in the presence and absence of 15 $\mu$g/mL of superoxide dismutase (*23*).

*Spectral Analysis of Myeloperoxidase.* The visible absorption spectra of myeloperoxidase were recorded during its reactions with hydrogen peroxide and superoxide using a Beckman 7500 diode array spectrophotometer. Each spectrum was recorded from 350 to 700 nm for 2s and was an average of 20 spectra.

*Kinetic Analysis.* Kinetic analysis of experimental data was performed using the software package MATLAB which was purchased from MathWorks, Inc (Natick, MA).

## RESULTS

*Hydrogen Peroxide Loss and Oxygen Formation Catalyzed by Myeloperoxidase.* There have been numerous studies looking at the kinetics of reactions of the redox intermediates of myeloperoxidase with hydrogen peroxide (*17, 18, 24–28*). However, there have been no detailed kinetic studies on the fate of hydrogen peroxide. To address this shortfall, we used a hydrogen peroxide electrode to monitor hydrogen peroxide loss catalyzed by myeloperoxidase in the absence of other substrates. Initially, we chose similar conditions to those used by Marquez et al. when they monitored conversion of the enzyme to compound II (*17*). These investigators found that at a 100-fold excess of hydrogen peroxide over enzyme, myeloperoxidase was converted to compound II within 2 s in a monophasic reaction. However, with a 20-fold excess of hydrogen peroxide, the reaction was distinctly biphasic, with 90% compound II formed after 5 s. When myeloperoxidase was added to a 100-fold excess of hydrogen peroxide there was a rapid and distinct burst of hydrogen peroxide loss over about five seconds, which was followed by a much slower steady state decline (Figure 1a). At a 20-fold excess of hydrogen peroxide, most of the hydrogen peroxide was lost during the burst phase (Figure 1b). Our results demonstrate that at least initially myeloperoxidase is able to degrade hydrogen peroxide efficiently in the absence of reducing substrates.

The burst phase for loss of hydrogen peroxide was dependent on the concentration of myeloperoxidase (Figure 2a) and approached a plateau at 100 $\mu$M hydrogen peroxide (Figure 2b). The latter result indicates that activity during the burst phase could be saturated by hydrogen peroxide. The steady-state loss of hydrogen peroxide was dependent on the enzyme and hydrogen peroxide concentrations (not shown).

To determine whether myeloperoxidase was converting hydrogen peroxide to oxygen by a catalase-like activity, we monitored hydrogen peroxide loss and oxygen production in parallel experiments conducted under identical conditions. The kinetics of oxygen evolution precisely mirrored the loss of hydrogen peroxide (Figure 3). In both cases, there was a distinct burst phase followed by a slower steady-state activity. Under the conditions described in Figure 1, myeloperoxidase converted 50 $\mu$M hydrogen peroxide to 22.9 $\pm$ 0.5 $\mu$M oxygen (mean $\pm$ range of duplicate experiments). The burst phases for hydrogen peroxide loss and oxygen evolution were 9.8 $\pm$ 0.2 and 5.5 $\pm$ 0.5 $\mu$M, respectively. The corresponding steady-state rates were 2.0 $\pm$ 0.2 and 1.1 $\pm$ 0.3 $\mu$M/min. Thus, at each stage of the reaction about two molecules of hydrogen peroxide were degraded to one molecule of oxygen.

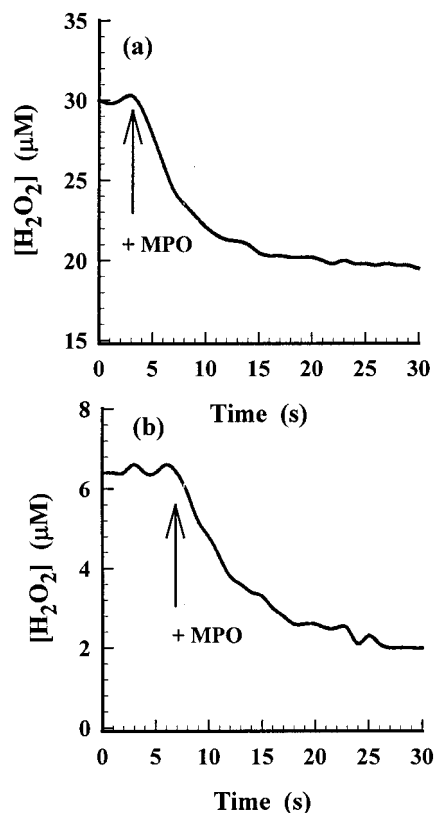FIGURE 1: Loss of hydrogen peroxide catalyzed by myeloperoxidase. Myeloperoxidase (300 nM) was added to either (a) 30 $\mu$M or (b) 6 $\mu$M hydrogen peroxide in 50mM phosphate buffer, pH 7.4, containing 100 $\mu$M DTPA at 21 °C. Loss of hydrogen peroxide was measured continuously with a hydrogen peroxide electrode and corrected for the dilution due to the addition of enzyme.

The burst phase of oxygen production became independent of the initial hydrogen peroxide concentration above 100 $\mu$M hydrogen peroxide, whereas the steady state rate of oxygen evolution was directly proportional to hydrogen peroxide concentration and showed no sign of saturation up to 1.5 mM hydrogen peroxide (not shown). This latter result is in agreement with those obtained earlier for steady-state hydrogen peroxide consumption (*15*).

There was minimal loss of hydrogen peroxide or formation of oxygen in the absence of myeloperoxidase (not shown). A possible explanation for the catalase activity is that contaminating halides were oxidized to hypohalous acids that then reacted with hydrogen peroxide to produce singlet oxygen (*29−31*). However, addition of methionine, which scavenges hypohalous acids, had no effect on the burst phase (not shown). The burst phase also occurred to the same extent with hemi-myeloperoxidase, which is a reductively cleaved and alkylated form of the enzyme that contains only one heme group per molecule of protein (*32, 33*) (not shown). This result rules out the possibility that the catalase activity was due to an intramolecular reaction between the two hemes of myeloperoxidase as proposed by Agner (*14*).

Under the conditions of the experiments in Figures 1−3, we monitored the absorption spectrum of 400 nM myeloperoxidase 20 s and 1 min after adding hydrogen peroxide. With 5 $\mu$M hydrogen peroxide, there was only a 50% conversion to compound II. Increasing the concentration of hydrogen peroxide to 250 $\mu$M resulted in 100% conversion of myeloperoxidase to compound II within the first 20 s,
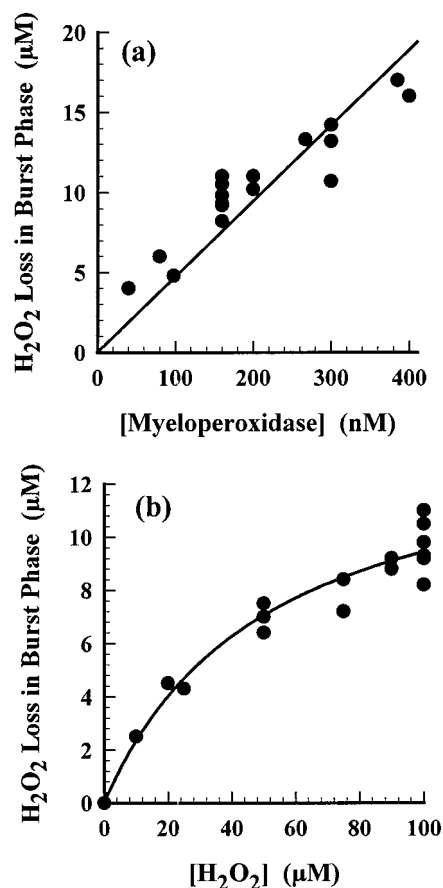


FIGURE 2: Effects of hydrogen peroxide and myeloperoxidase on the kinetics of hydrogen peroxide loss catalyzed by myeloperoxidase. The burst phase for loss of hydrogen peroxide was measured after adding 100 $\mu$M hydrogen peroxide to varying concentrations of myeloperoxidase (a) or after adding varying concentrations of hydrogen peroxide to 160 nM myeloperoxidase (b).

which was then slowly converted to compound III. These results are similar to those reported previously (*17, 18*) and demonstrate that during the burst phase myeloperoxidase was converted from its native form to compound II.

*Effects of One-Electron Reductants on Hydrogen Peroxide Loss Catalyzed by Myeloperoxidase.* As shown in Figures 1 and 3, catalatic breakdown of hydrogen peroxide by myeloperoxidase alone was short-lived. However, sustained hydrogen peroxide consumption and oxygen evolution could be achieved by adding a low concentration of tyrosine. When added after the burst phase tyrosine caused an immediate 3−4-fold increase in both the rate of hydrogen peroxide loss (Figure 3a) and formation of oxygen (Figure 3b). The concomitant increase in oxygen evolution with hydrogen peroxide loss indicates that tyrosine was acting by promoting the catalase-like activity of myeloperoxidase rather than being oxidized by the peroxidase cycle (reactions 1, 3, and 4). This conclusion is supported by the fact that within 4 min of adding 5 $\mu$M tyrosine, an additional 13 $\mu$M hydrogen peroxide was consumed compared to what would have been lost in the absence of tyrosine. If tyrosine had acted by reducing compound I and compound II, it would have given rise to only an additional loss of 2.5 $\mu$M hydrogen peroxide.

When tyrosine or tryptophan was added to the reaction system before the reaction was started, they inhibited the burst phase (Figure 4). In contrast, tyrosine accelerated the steady state loss of hydrogen peroxide but tryptophan was
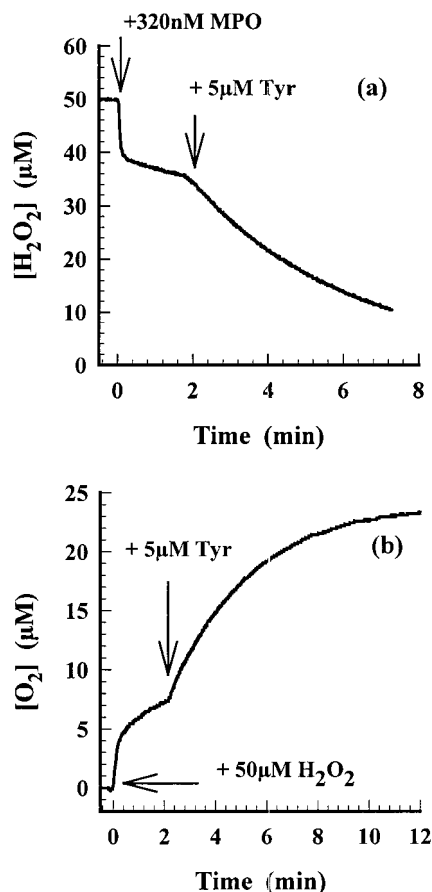
FIGURE 3: Conversion of hydrogen peroxide to oxygen by myeloperoxidase. (a) Myeloperoxidase (320 nM) was added to 50 $\mu$M hydrogen peroxide in 50 mM phosphate buffer (pH 7.4) containing 100 $\mu$M DTPA at 21 °C and hydrogen peroxide loss was monitored with a hydrogen peroxide electrode. Tyrosine (5 $\mu$M) was added at the time indicated by the arrow. (b) Oxygen evolution was monitored with an oxygen electrode when hydrogen peroxide (50 $\mu$M) was added to 320 nM myeloperoxidase. Results are typical of three experiments.
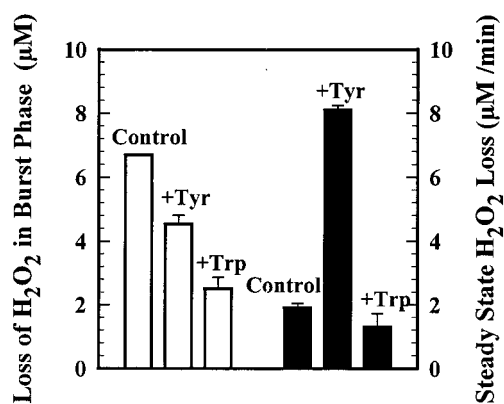
FIGURE 4: Effects of tyrosine and tryptophan on hydrogen peroxide consumption catalyzed by myeloperoxidase. Myeloperoxidase (195 nM) was added to 50 mM phosphate buffer, pH 7.4, containing 50 $\mu$M hydrogen peroxide and 5 $\mu$M of tyrosine or tryptophan. The burst phase (clear bars) and steady state rate of hydrogen peroxide loss (filled bars) were measured continuously with a hydrogen peroxide electrode. Results are means and ranges of duplicate experiments.

without effect (Figure 4). Both these substrates rapidly reduce compound I to compound II but only tyrosine is able to efficiently reduce compound II back to the ferric enzyme (*34, 35*). In total, these results demonstrate that formation
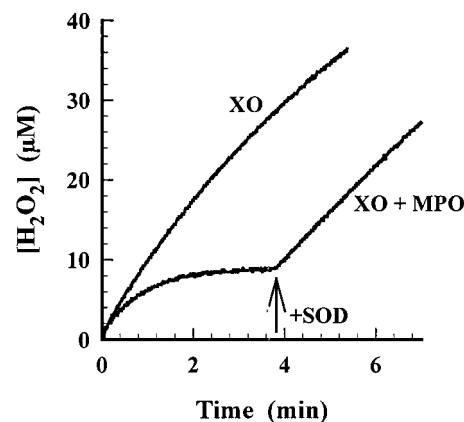
FIGURE 5: Effect of superoxide on hydrogen peroxide consumption catalyzed by myeloperoxidase. Xanthine oxidase (XO) was added to 50 mM phosphate buffer (pH 7.4, 21 °C) containing 10mM acetaldehyde and 100 $\mu$M DTPA plus or minus 150 nM myeloperoxidase. Hydrogen peroxide concentration was monitored with a hydrogen peroxide electrode and 15 $\mu$g/mL of superoxide dismutase (SOD) was added as indicated by the arrow. The initial rate of superoxide production was 5.4 $\mu$M/min. Results are typical of four experiments.

of compound II inhibits the burst phase, and its conversion back to the native enzyme enhances the steady-state activity.

*Effects of Superoxide on Hydrogen Peroxide Loss Catalyzed by Myeloperoxidase.* Superoxide is an obligatory product in reaction 6, and has been shown to react with both the ferric enzyme (reaction 7) and compound II (reaction 8) (*36, 37*).

$$MP^{3+} + O_2^- \rightarrow Compound\ III \tag{7}$$

$$Compound\ II + O_2^- + H^+ \rightarrow MP^{3+} + O_2 + OH^- \tag{8}$$
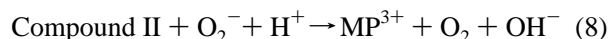
We initially determined whether endogenously generated superoxide influenced the burst phase and the steady-state loss of hydrogen peroxide by adding 10 $\mu$g/mL of superoxide dismutase to a reaction system containing 100 $\mu$M hydrogen peroxide and 160 nM myeloperoxidase. Superoxide dismutase had little effect on the magnitude of the burst phase but significantly decreased the steady-state rate of hydrogen peroxide loss from 1.5 to 0.7$\mu$M/min ($p < 0.003$; $n = 3$). This result suggested that superoxide was generated by myeloperoxidase and hydrogen peroxide and contributed to the turnover of compound II.

To explore this possibility further, we examined the catalase-like activity of myeloperoxidase using xanthine oxidase and acetaldehyde as a source of superoxide and hydrogen peroxide. Acetaldehyde was used as a substrate for xanthine oxidase because xanthine and hypoxanthine are both converted to uric acid, which is a substrate for myeloperoxidase (*38*). Xanthine oxidase and acetaldehyde gave steady accumulation of hydrogen peroxide (Figure 5). With myeloperoxidase present, there was an immediate increase in the concentration of hydrogen peroxide, but its accumulation slowed until a constant concentration was maintained. At this point, the rate of hydrogen peroxide production by xanthine oxidase was equal to the rate of breakdown by myeloperoxidase. On addition of superoxide dismutase to the reaction system, hydrogen peroxide accumulated at the same rate as when myeloperoxidase was
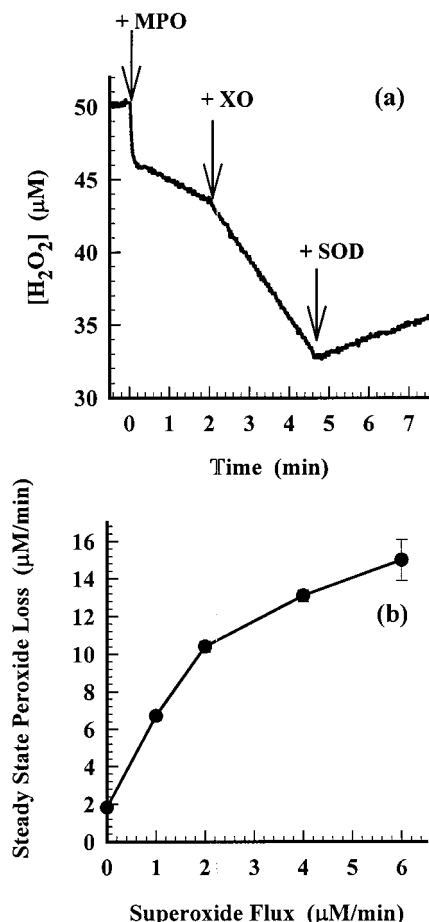
FIGURE 6: Effect of superoxide on the steady-state rate of loss of hydrogen peroxide catalyzed by myeloperoxidase. (a) Myeloperoxidase (100 nM) was added to 50 $\mu$M hydrogen peroxide in 50 mM phosphate buffer (pH 7.4, 21 °C) containing 10 mM acetaldehyde and 100 $\mu$M DTPA. After steady-state had been reached, xanthine oxidase was added to generate 2 $\mu$M/min of superoxide and 3.3 $\mu$M/min of hydrogen peroxide. Superoxide dismutase (15 $\mu$g/mL) was added at the indicated time. (b) The steady-state rate of hydrogen peroxide loss after varying amounts of xanthine oxidase had been added to myeloperoxidase and hydrogen peroxide were calculated by adding the rate of loss for the three steady-state phases shown in panel a. Results are means and ranges of duplicate experiments.

absent. This indicates that the reaction responsible for breakdown of hydrogen peroxide had been inhibited by superoxide dismutase. Spectral investigations of myeloperoxidase were performed during breakdown of hydrogen peroxide. In the presence of superoxide the Soret band of the ferric enzyme, which has a characteristic maximum of 430 nm, was shifted to 454 nm. There was also a small band at 630 nm. The ratio of absorbances at 452 and 630 nm indicated that the enzyme was converted to its compound II form (*39*). After 3 min of reaction, there was a decline in absorbance at 454 nm but the spectrum still indicated that the enzyme existed as predominantly compound II (spectra not shown).

To determine the extent to which superoxide increases the rate of hydrogen peroxide loss, xanthine oxidase and acetaldehyde were added to myeloperoxidase and hydrogen peroxide after steady state had been attained (Figure 6a). Under these conditions, additional hydrogen peroxide was generated by xanthine oxidase as seen by the increase in hydrogen peroxide concentration in the presence of super-
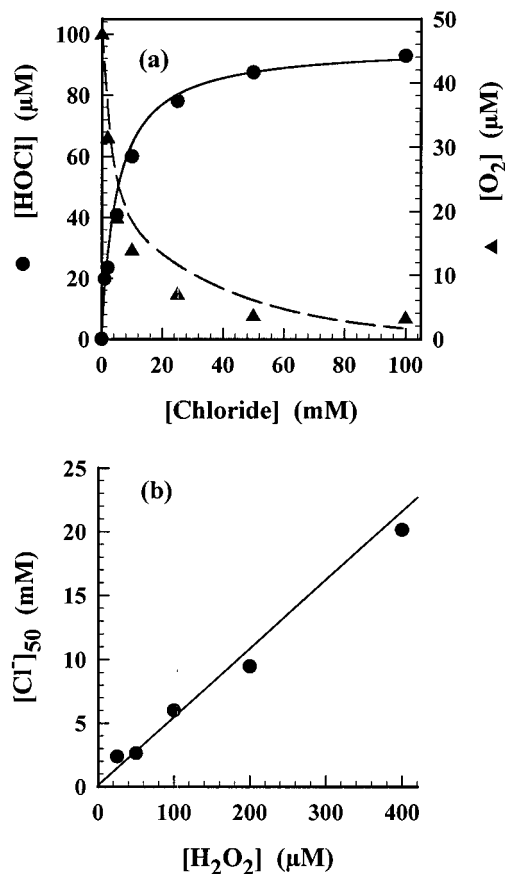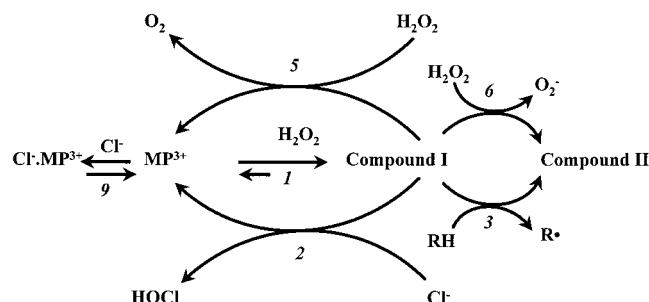


FIGURE 7: Effect of chloride on oxygen and hypochlorous acid production by myeloperoxidase. (a) Hydrogen peroxide (100 $\mu$M) was added to 400 nM myeloperoxidase in 50 mM phosphate buffer (pH 7.4, 21 °C) containing 10 mM taurine, 100 $\mu$M DTPA, and varying concentrations of chloride. Oxygen production (5) was monitored with an oxygen electrode and in parallel experiments the concentration of accumulated taurine chloramine was assayed as a measure of hypochlorous acid formation (●). Results are representative of two experiments and lines are curves of best fit through the data. (b) The concentration of chloride that gave 50% of maximum conversion of hydrogen peroxide to hypochlorous acid ([Cl$^-$]$_{50}$) was determined at varying concentrations of hydrogen peroxide. At all concentrations of hydrogen peroxide at least 90% of it was converted to HOCl at the highest concentration of chloride. The fitted line was determined by simulating the utilization of hydrogen peroxide using a kinetic model for Scheme 1.

oxide dismutase. In the absence of superoxide dismutase, superoxide substantially increased the rate of hydrogen peroxide loss. This corresponds to not only enhanced consumption of the initial hydrogen peroxide but also consumption of that generated by xanthine oxidase. As the flux of superoxide was increased, there was a corresponding increase in the steady-state rate of hydrogen peroxide loss (Figure 6b). A flux of 2 $\mu$M/min of superoxide increased the steady-state rate of hydrogen peroxide consumption by 8.5 $\mu$M/min. Thus, superoxide was acting in a super-stoichiometric fashion and not just promoting the peroxidatic breakdown of hydrogen peroxide via reactions 1, 6, and 8.

*Hydrogen Peroxide and Chloride as Competing Substrates for Myeloperoxidase.* To determine how effectively hydrogen peroxide competes with chloride for oxidation by myeloperoxidase, we measured formation of oxygen and hypochlorous acid when a constant concentration of hydrogen peroxide was consumed in the presence of varying concentrations of chloride (Figure 7). With 100 $\mu$M hydrogen

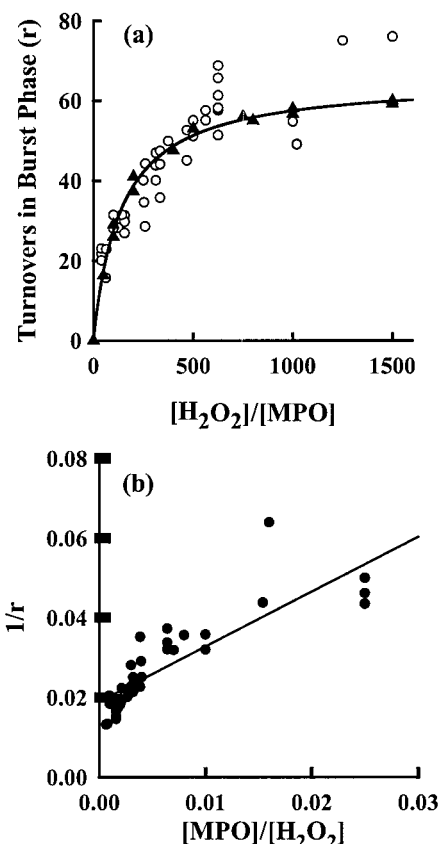Scheme 1: Reaction Mechanism for the Catalase Activity of Myeloperoxidase





FIGURE 8: Effect of hydrogen peroxide and enzyme concentrations on the turnover number of myeloperoxidase during the burst phase of hydrogen peroxide loss. (a) The turnover number or partition ratio, $r$, was calculated by dividing the burst phase by the enzyme concentration. In these experiments the concentration of myeloperoxidase varied between 50 and 400 nM and concentration of hydrogen peroxide ranged from 10 to 150 $\mu$M. Open circles represent experimental data and closed triangles are simulated data through which the solid curve of best fit is drawn. (b) The double reciprocal plot data in panel a. The intercept was 0.018 and the slope 1.38. Other conditions were as described in Figure 1.

peroxide, very little oxygen was produced when the chloride concentration was above 50 mM (Figure 7a). However, there was appreciable formation of oxygen as the chloride concentration was decreased. The concentration of chloride ($[Cl^-]_{50}$) that gave a 50:50 split of hydrogen peroxide to hypochlorous acid and oxygen was directly proportional to the initial concentration of hydrogen peroxide (Figure 7b).

*Mechanistic Interpretation of Kinetic Data.* The burst phase for conversion of hydrogen peroxide to oxygen indicates that the reaction mechanism for the catalase activity of myeloperoxidase involves a branched chain that leads to the accumulation of an inactive intermediate (*40*). This inactive intermediate must be compound II because we showed that it is formed during the burst phase. Also, substrates that promoted compound II formation inhibited the burst phase while substrates that turned it over enhanced the steady state breakdown of hydrogen peroxide. Any mechanism proposed for the catalase activity must account for our finding that the burst phase (Figure 2b) is dependent on the concentration of hydrogen peroxide up to a maximum value. It must also accommodate results from previous studies in which it was demonstrated that the rate of compound II formation is dependent on the concentration of hydrogen peroxide with no apparent saturation (*17, 18, 28*).

We propose the mechanism given in Scheme 1 is responsible for the catalase activity of myeloperoxidase. This scheme unifies the previous mechanisms proposed by Iwamoto et al. (*16*) and Marques et al. (*17*) and accounts for the steady-state and pre-steady-state results obtained in this and other studies. In this mechanism, the dominant catalatic route starts with two-electron oxidation of the ferric myeloperoxidase to give compound I, which then undergoes a two-electron reduction by a second molecule of hydrogen peroxide to regenerate the native enzyme (reactions 1 and 5). Hydrogen peroxide also reduces compound I in a slower one-electron reaction to trap the enzyme as compound II (reaction 6). This reaction accounts for the burst phase we observed. Reduction of compound I to compound II by endogenous donors (reaction 3) is necessary to fit the data, otherwise the burst phase would not depend on the concentration of hydrogen peroxide. At low concentrations of hydrogen peroxide, endogenous donors would decrease the burst phase by converting the enzyme to compound II. However, their effect would be overwhelmed with increasing concentration of hydrogen peroxide as reaction 6 became the main route by which compound II is formed. Endogenous donors have previously been invoked to account for anomalous kinetics of peroxidases (*41*). Given the high reduction

potential of myeloperoxidase compound I (*42*), it is not unrealistic to expect that some impurities were present with the enzyme and affected its activity. This would be particularly evident when no reducing substrate was added to react with compound I.

On the basis of the reaction mechanism given in Scheme 1, a kinetic expression can be derived for the partition ratio, $r$, for hydrogen peroxide breakdown by the catalatic cycle (reactions 1 and 5) compared to its reactions that promote accumulation of compound II (reactions 1 and 3 or 6) (see Appendix).

$$r = 2k_5([H_2O_2]/[MPO])/(1.5k_6([H_2O_2]/[MPO]) + nk_3) \text{ or}$$

$$1/r = 0.75k_6/k_5 + (0.5nk_3/k_5)[MPO]/[H_2O_2]$$

All data for the burst phase were plotted according to the above expressions for the partition ratio (Figure 8). From the double reciprocal plot (Figure 8b) and a value of $k_6$ based on published data (see Table 1), a value of $2.9 \times 10^6$ M$^{-1}$ s$^{-1}$ for $k_5$ was obtained from the intercept. A plot of the partition ratio against enzyme concentration at a fixed concentration of hydrogen peroxide had a negative slope (not shown). This result indicates that myeloperoxidase was less

Table 1: Reaction Constants for the Redox Intermediates of Myeloperoxidase

| reaction | reaction constant |
|---|---|
| 1 | $1.8 \times 10^7$ M$^{-1}$ s$^{-1}$ (*17*), $2.3 \times 10^7$ M$^{-1}$ s$^{-1}$ (*24*), $1.4 \times 10^7$ M$^{-1}$ s$^{-1}$ (*25*), and $3.1 \times 10^7$ M$^{-1}$ s$^{-1}$ (*26*) |
| −1 | 58 s$^{-1}$ (*17*) |
| 2 | $2.5 \times 10^4$ M$^{-1}$ s$^{-1}$ (*25*) |
| 3 | $2.0 \times 10^6$ M$^{-1}$ s$^{-1}$ (assumed in this study) |
| 5 | $2.2 \times 10^6$ M$^{-1}$ s$^{-1}$ and $1.7 \times 10^6$ M$^{-1}$ s$^{-1}$ (determined in this study) |
| 6 | $8.1 \times 10^4$ M$^{-1}$ s$^{-1}$ (*17*), $3.5 \times 10^4$ M$^{-1}$ s$^{-1}$ (*18*), $3.3 \times 10^4$ M$^{-1}$ s$^{-1}$ (*28*), and $7 \times 10^4$ M$^{-1}$ s$^{-1}$ (used in simulations) |
| 9 | 0.4 M (*24*) |

efficient at higher concentrations. Presumably, this was due to the presence of impurities in the enzyme preparations that acted as endogenous donors (RH) and promoted formation of compound II. Therefore, assuming the concentration of endogenous donor was a function of the concentration of myeloperoxidase, a value of $1.6 \times 10^6$ M$^{-1}$ s$^{-1}$ for $k_3$ was obtained from the slope of the double reciprocal plot (Figure 8b). By using these values as initial estimates along with literature values of rate constants for the other reactions in Scheme 1, and the dissociation constant for reversible binding of chloride to the native enzyme (reaction 9) (Table 1), a kinetic model of Scheme 1 was used to fit the data in Figure 8. The best fit, shown in Figure 8 by the solid curve, was obtained using values of $2.2 \times 10^6$ for $k_5$, $2 \times 10^6$ M$^{-1}$ s$^{-1}$ for $k_3$, and [RH] = 5 × [MPO]. Conversion of compound II back to the ferric enzyme (reaction 4) had little effect on simulation of the burst phase.

To check the validity of the reaction scheme, we also examined how well simulations fitted previous data obtained by stopped flow techniques. The observed rate constant for reaction 6 was found to be dependent on the concentration of hydrogen peroxide, with no saturation evident (not shown). The plot of $k_{6obs}$ had a slope of $6.4 \times 10^4$ M$^{-1}$ s$^{-1}$, which is in good agreement with apparent values obtained experimentally (see Table 1) (*18*, *25*). The intercept was dependent on the amount of endogenous donor assumed to be present with the myeloperoxidase and was similar to intercepts determined experimentally (*18*). Furthermore, we could accurately simulate the biphasic accumulation of compound II that Marquez et al. (*17*) observed with a 20-fold excess of hydrogen peroxide over enzyme, if we assumed an endogenous donor was present at 1.2 $\mu$M or 1.5 times the enzyme concentration (not shown).

We found that chloride and hydrogen peroxide are competing substrates for oxidation by myeloperoxidase (Figure 7). In the mechanism shown in Scheme 1, they would compete by reacting with compound I. Therefore, we used the model to simulate the competition between chloride and hydrogen peroxide as reducing substrates for compound I. The best fit to the experimental data, which is given by the solid line in Figure 7b, was obtained using a value of $1.7 \times 10^6$ M$^{-1}$ s$^{-1}$ for $k_5$. Thus, the two independent approaches for determining $k_5$ gave very similar values.

## DISCUSSION

By undertaking a kinetic investigation of the fate of hydrogen peroxide when it is degraded by myeloperoxidase, we have demonstrated that hydrogen peroxide reacts with both the ferric enzyme and compound I in two electron reactions that liberate water and oxygen. Our findings establish myeloperoxidase as a catalase-peroxidase and add another activity to the reactions catalyzed by this complex and promiscuous enzyme. The two-electron oxidation of hydrogen peroxide by compound I has important implications for interpreting mechanistic studies of myeloperoxidase and may affect the physiological activity of the enzyme. In exhibiting catalase activity, myeloperoxidase is no different from horseradish peroxidase (*25*) and chloroperoxidase (*43*). Only the rate at which hydrogen peroxide reacts with compound I differs. The rate constants for reaction 5 with horseradish peroxidase, chloroperoxidase, and catalase are $5 \times 10^2$ M$^{-1}$ s$^{-1}$ (*44*), $2 \times 10^5$ M$^{-1}$ s$^{-1}$ (*43*), and $3.5 \times 10^7$ M$^{-1}$ s$^{-1}$ (*45*), respectively, compared with the averaged value of $2 \times 10^6$ M$^{-1}$ s$^{-1}$ we measured for myeloperoxidase. Thus, myeloperoxidase is potentially a better catalase than other peroxidases but clearly not as efficient as catalase itself. The magnitude of $k_5$ also points to hydrogen peroxide as a physiological reducing substrate for myeloperoxidase. Only thiocyanate and iodide are known to have greater rate constants for their reaction with compound I (*25*).

Iwamoto and co-workers (*16*) proposed essentially the same mechanism we have outlined in Scheme 1, except that they did not include a reaction of compound I with endogenous donors. Also, they suggested that hydrogen peroxide reacts faster with compound I than it does with the ferric enzyme. Marquez et al. (*17*) rightly dismissed this suggestion because, if it was correct, at least a 300-fold excess of hydrogen peroxide over enzyme would be required for 100% conversion of myeloperoxidase to compound II. It is clear from this and other studies that a 20-fold excess of hydrogen peroxide is sufficient to convert all the enzyme to compound II (*17*, *18*, *25*). Marquez et al. (*17*) observed only reduction of compound I to compound II in their stopped flow experiments, which is consistent with our finding that reaction 5 is an order of magnitude slower than reaction 1. Thus, in the presence of an excess of hydrogen peroxide, any ferric myeloperoxidase formed via reaction 5 would be rapidly converted back to compound I and not observed by stopped flow.

The one electron reduction of compound I to compound II by hydrogen peroxide (reaction 6) and endogenous donors (reaction 3) has a profound effect on the catalase activity because it diverts the enzyme from its catalatic cycle (see Scheme 1). These reactions give rise to the burst phase and subsequent turnover of compound II determines the steady-state rate of conversion of hydrogen peroxide to oxygen. Interestingly, catalase displays similar burst phase kinetics to myeloperoxidase, which Chance (*46*) also attributed to gradual formation of compound II. Comparison of the one and two-electron rate constants for reduction of compound I by hydrogen peroxide reinforce our proposal that, in the absence of chloride, the catalatic cycle will be the dominant route by which myeloperoxidase degrades hydrogen peroxide (see Table 1).

Our findings that superoxide and tyrosine gave super-stoichiometric enhancement of the catalase activity can be explained by their ability to reduce compound II and push the enzyme back to the faster and more efficient cycle of hydrogen peroxide breakdown. An alternative explanation for the stimulatory effects of tyrosine and superoxide might

be that they enable hydrogen peroxide consumption and oxygen production to proceed via a cycle consisting of reactions 1, 6, and 4 or 8. However, this can be discounted for tyrosine because the stoichiometry of the overall reaction (reaction 10) would be 3 mol of hydrogen peroxide consumed for each mole of oxygen produced. This is not consistent with our finding that 2 mol of hydrogen peroxide were lost for each mole of oxygen produced (Figure 3). Also, more than twice as much hydrogen peroxide was consumed than the 7.5 $\mu$M permissible with this mechanism.

$$3H_2O_2 + 2Tyrosine \rightarrow Dityrosine + O_2 + 4H_2O \quad (10)$$

Likewise, with superoxide, the rate of hydrogen peroxide consumption could not exceed the rate of superoxide generation because the rate-determining step in this cycle would be reaction 8. This is not consistent with our finding that in a superoxide generating system the rate of hydrogen peroxide breakdown was as much as five times that of the flux of superoxide (Figure 6).

The effects of tyrosine and superoxide are analogous to that of NADPH in maintaining the activity of catalase (*47*). These reductants prevent accumulation of inactive compound II and ensure that the enzymes are kept in their catalitic cycles. Thus, myeloperoxidase can be expected to display significant catalase activity whenever superoxide, or a similar reducing cofactor such as tyrosine, is available. In the absence of such cofactors, myeloperoxidase is clearly a poor catalase.

Given that myeloperoxidase and superoxide are discharged in tandem by stimulated neutrophils, the catalase activity may influence oxidant production by these inflammatory cells. This may be especially important in the immediate environment of stimulated neutrophils as it becomes highly oxidizing so that reducing substrates are consumed. For example, our data indicates that the catalase activity of myeloperoxidase is most likely to be physiologically relevant where chloride concentrations are low. These conditions may be achieved in the neutrophil phagosome during bacterial killing. The chloride concentration in the narrow confines of the phagosome is unknown. If it is maintained at about 140 mM, myeloperoxidase would degrade little hydrogen peroxide to oxygen. The majority of it would be converted to hypochlorous acid. However, if chloride is rapidly consumed by incorporation into chloramines, then its concentration may decline low enough so that the catalase activity of myeloperoxidase dominates.

The relevance of the catalase activity in experimental studies of myeloperoxidase is more certain. The rate constant we obtained for reaction 5 indicates that hydrogen peroxide is an excellent substrate for compound I, reacting with it at least as fast as tyrosine (*34*) and nitrite (*48*). Thus, it is certain that hydrogen peroxide will compete with these types of substrates for oxidation by compound I, and their relative concentrations will influence product yields. Studies of myeloperoxidase should be interpreted with this knowledge in mind.

## ACKNOWLEDGMENT

## APPENDIX

*Determination of the Partition Ratio.* The partition ratio, $r$, is defined as the number of times myeloperoxidase turns over during the burst phase.

$$r = \Delta[H_2O_2]/[MPO]$$

In Scheme 1, hydrogen peroxide can be consumed via three different reaction sequences. Turnover of compound II is assumed to be too slow to make a significant contribution to the loss of hydrogen peroxide in the burst phase.
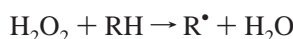(1) Reactions 1 and 5

$$2H_2O_2 \rightarrow O_2 + 2H_2O$$
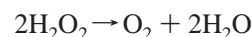
$$-d[H_2O_2]/dt = 2k_5[\text{Compound I}][H_2O_2]$$

(2) Reactions 1 and 6, with superoxide undergoing spontaneous dismuation

$$1.5H_2O_2 \rightarrow 1/2\, O_2 + H_2O$$
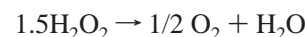
$$-d[H_2O_2]/dt = 1.5k_6[\text{Compound I}][H_2O_2]$$

(3) Reactions 1 and 3

$$H_2O_2 + RH \rightarrow R^\bullet + H_2O$$
$$(\text{where RH is an endogenous donor})$$

$$-d[H_2O_2]/dt = k_3[\text{Compound I}][RH]$$

Therefore, the amount of hydrogen peroxide consumed in the burst phase via the catalase activity as opposed to peroxidative breakdown is described by the equation

$$r = 2k_5[H_2O_2]/(1.5k_6[H_2O_2] + k_3[RH])$$

If it is assumed that $[RH] = n[MPO]$, then

$$r = 2k_5[H_2O_2]/(1.5k_6[H_2O_2] + nk_3[MPO]) \text{ or}$$

$$r = 2k_5([H_2O_2]/[MPO])/(1.5k_6([H_2O_2]/[MPO]) + nk_3)$$

$$\therefore\ 1/r = 0.75k_6/k_5 + (0.5nk_3/k_5)[MPO]/[H_2O_2]$$

Thus, a plot of $1/r$ vs $[MPO]/[H_2O_2]$ should give a straight line with an intercept of $0.75k_6/k_5$ and a slope of $0.5nk_3/k_5$.

## REFERENCES

1. Winterbourn, C. C., Vissers, M. C. M., and Kettle, A. J. (2000) *Curr. Opin. Hematol. 7*, 53−58.
2. Klebanoff, S. J. (1999) in *Inflammation: Basic Principles and Clinical Correlates* (Gallin, J. I., and Snyderman, R., Eds.) pp 721−768, Lippincott Williams & Wilkins, Philadelphia.
3. Edwards, S. W., and Hallett, M. B. (1997) *Immunol. Today 18*, 320−324.
4. Chabot, F., Mitchell, J. A., Gutteridge, J. M., and Evans, T. W. (1998) *Eur. Respir. J. 11*, 745−757.
5. Nagra, R. M., Becher, B., Tourtellotte, W. W., Antel, J. P., Gold, D., Paladino, T., Smith, R. A., Nelson, J. R., and Reynolds, W. F. (1997) *J. Neuroimmunol. 78*, 97−107.
6. Regelmann, W. E., Siefferman, C. M., Herron, J. M., Elliot, G. R., Clawson, C. C., and Gray, B. H. (1995) *Pediatr. Pulmonol. 19*, 1−9.
7. Buss, I. H., Darlow, B. A., and Winterbourn, C. C. (2000) *Pediatr. Res. 47*, 640−645.

8. Heinecke, J. W. (1994) *Coronary Artery Dis. 5*, 205−210.

9. Reynolds, W. F., Chang, E., Douer, D., Ball, E. D., and Kanda, V. (1997) *Blood 90*, 2730−2737.

10. London, S. J., Lehman, T. A., and Taylor, J. A. (1997) *Cancer Res. 57*, 5001−5003.

11. Kettle, A. J., and Winterbourn, C. C. (1997) *Redox Rep. 3*, 3−15.

12. Van Dalen, C. J., Whitehouse, M., Winterbourn, C. C., and Kettle, A. J. (1997) *Biochem. J. 327*, 487−492.

13. Kettle, A. J., and Winterbourn, C. C. (1994) *J. Biol. Chem. 269*, 17146−17151.

14. Agner, K. (1963) *Acta Chem. Scand. 17 (Suppl. 1)*, S332−S338.

15. Winterbourn, C. C., Garcia, R., and Segal, A. W. (1985) *Biochem. J. 228*, 583−592.

16. Iwamoto, H., Kobayashi, T., Hasegawa, E., and Morita, Y. (1987) *J. Biochem. 101*, 1407−1412.

17. Marquez, L. A., Huang, J. T., and Dunford, H. B. (1994) *Biochemistry 33*, 1447−1454.

18. Hoogland, H., Dekker, H. L., van Riel, C., van Kuilenburg, A., Muijsers, A. O., and Wever, R. (1988) *Biochim. Biophys. Acta 955*, 337−345.

19. Kettle, A. J. (1999) *Methods Enzymol. 300 Part B*, 111−120.

20. Zuurbier, K. W., van den Berg, J. D., van Gelder, B. F., and Muijsers, A. O. (1992) *Eur. J. Biochem. 205*, 737−742.

21. Beers, R. J., and Sizer, I. W. (1952) *J. Biol. Chem. 195*, 133−140.

22. Kettle, A. J., and Winterbourn, C. C. (1994) *Methods Enzymol. 233*, 502−512.

23. Fridovich, I. (1985) in *Handbook of Methods for Oxygen Radical Research* (Greenwald, R. A., Ed.) pp 213−215, CRC Press, Boca Raton.

24. Bolscher, B. G. J. M., and Wever, R. (1984) *Biochim. Biophys. Acta 788*, 1−10.

25. Fürtmuller, P. G., Burner, U., and Obinger, C. (1998) *Biochemistry 37*, 17923−17930.

26. Kettle, A. J., Sangster, D. F., Gebicki, J. M., and Winterbourn, C. C. (1988) *Biochim. Biophys. Acta 956*, 58−62.

27. Fürtmuller, P. G., Obinger, C., Hsuanyu, Y., and Dunford, H. B. (2000) *Eur. J. Biochem. 267*, 5858−5864.

28. Fürtmuller, P. G., Burner, U., Jantschko, W., Regelsberger, G., and Obinger, C. (2000) *Redox Rep. 5*, 173−178.

29. Magnusson, R. P., Taurog, A., and Dorris, M. L. (1984) *J. Biol. Chem. 259*, 197−205.

30. Kanofsky, J. R. (1984) *J. Biol. Chem. 259*, 5596−5600.

31. Kanofsky, J. R. (1989) *Arch. Biochem. Biophys. 274*, 229−234.

32. Andrews, P. C., and Krinsky, N. I. (1981) *J. Biol. Chem. 256*, 4211−4218.

33. Taylor, K. L., Guzman, G. S., Pohl, J., and Kinkade, J. M. (1990) *J. Biol. Chem. 265*, 15938−15946.

34. Marquez, L. A., and Dunford, H. B. (1996) *J. Biol. Chem. 270*, 30434−30440.

35. Kettle, A. J., and Candaeis, L. P. (2000) *Redox Rep. 5*, 179−184.

36. Odajima, T., and Yamazaki, I. (1972) *Biochim. Biophys. Acta 284*, 355−359.

37. Kettle, A. J., and Winterbourn, C. C. (1988) *Biochem. J. 252*, 529−536.

38. Maehly, A. C. (1955) *Methods Enzymol. 2*, 794−801.

39. Hoogland, H., Van Kuilengurg, A., van Reil, C., Muijers, A. O., and Wever, R. (1987) *Biochim. Biophys. Acta 916*, 76−82.

40. Waley, S. G. (1991) *Biochem. J. 279*, 87−94.

41. Dunford, H. B. (1999) *Heme Peroxidases*, Wiley-VCH, New York.

42. Hurst, J. K. (1991) in *Peroxidases in Chemistry and Biology* (Everse, J., Everse, K. E., and Grisham, M. B., Eds.) pp 37−62, CRC Press, Boca Raton.

43. Thomas, J. A., Morris, D. R., and Hager, L. P. (1970) *J. Biol. Chem. 245*, 3129−3134.

44. Nakajima, R., and Yamazaki, I. (1987) *J. Biol. Chem. 262*, 2576−2581.

45. Chance, B., and Herbert, D. (1950) *Biochem. J. 46*, 402-

46. Chance, B. (1950) *Biochem. J. 46*, 387−402.

47. Kirkman, H. N., Rolfo, M., Ferraris, A. M., and Gaetani, G. F. (1999) *J. Biol. Chem. 274*, 13908−13914.

48. Burner, U., Fürtmuller, P. G., Kettle, A. J., Koppenol, W. H., and Obinger, C. (2000) *J. Biol. Chem. 275*, 20597−20601.