Influence of superoxide on myeloperoxidase kinetics measured with a hydrogen peroxide electrode

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Stimulated neutrophils discharge large quantities of superoxide ($O_2^{\cdot-}$), which dismutates to form H_2O_2 . In combination with Cl^- , H_2O_2 is converted into the potent oxidant hypochlorous acid (HOCl) by the haem enzyme myeloperoxidase. We have used an H_2O_2 electrode to monitor H_2O_2 uptake by myeloperoxidase, and have shown that in the presence of Cl^- this accurately represents production of HOCl. Monochlorodimedon, which is routinely used to assay production of HOCl, inhibited H_2O_2 uptake by 95%. This result confirms that monochlorodimedon inhibits myeloperoxidase, and that the monochlorodimedon assay grossly underestimates the activity of myeloperoxidase. With $10\ \mu M$ - H_2O_2 and $100\ mM$ - Cl^- , myeloperoxidase had a neutral pH optimum. Increasing the H_2O_2 concentration to $100\ \mu M$ lowered the pH optimum to pH 6.5. Above the pH optimum there was a burst of H_2O_2 uptake that rapidly declined due to accumulation of Compound II. High concentrations of H_2O_2 inhibited myeloperoxidase and promoted the formation of Compound II. These effects of H_2O_2 were decreased at higher concentrations of Cl^- . We propose that H_2O_2 competes with Cl^- for Compound I and reduces it to Compound II, thereby inhibiting myeloperoxidase. Above pH 6.5, $O_2^{\cdot-}$ generated by xanthine oxidase and acetaldehyde prevented H_2O_2 from inhibiting myeloperoxidase, increasing the initial rate of H_2O_2 uptake. $O_2^{\cdot-}$ allowed myeloperoxidase to function optimally with $100\ \mu M$ - H_2O_2 at pH 7.0. This occurred because, as previously demonstrated, $O_2^{\cdot-}$ prevents Compound II from accumulating by reducing it to ferric myeloperoxidase. In contrast, at pH 6.0, where Compound II did not accumulate, $O_2^{\cdot-}$ retarded the uptake of H_2O_2 . We propose that by generating $O_2^{\cdot-}$ neutrophils prevent H_2O_2 and other one-electron donors from inhibiting myeloperoxidase, and ensure that this enzyme functions optimally at neutral pH.

INTRODUCTION

Myeloperoxidase (MP) is the most abundant protein in human neutrophils and is involved in microbial killing and inflammatory tissue damage [1,2]. This enzyme catalyses the conversion of H_2O_2 and Cl^- into the potent oxidant hypochlorous acid (HOCl) via reactions (1) and (2) [3,4], where MP^{3+} is ferric myeloperoxidase:

H_2O_2 is supplied by the NADPH oxidase of neutrophils through dismutation of $O_2^{\cdot-}$ [5]. Recently we proposed that, apart from providing a source of H_2O_2 , $O_2^{\cdot-}$ may have a role in boosting the activity of MP by preventing the accumulation of inactive Compound II [6]. This proposal was based on studies using monochlorodimedon (MCD) to measure production of HOCl. We found that MCD also reacts directly with Compound I to promote accumulation of Compound II [7], and that the activity of MP is enhanced by $O_2^{\cdot-}$ and other reducing agents that convert Compound II back into the native enzyme [6].

Previous studies have used either MCD [6–10] or peroxidase substrates that also react with HOCl [11–14] to determine the kinetics of MP activity. However, since peroxidase substrates, including MCD [7,15], can interfere with HOCl production, these previous studies

may not have accounted fully for the kinetics of MP-dependent production of HOCl. We considered it appropriate, therefore, to re-investigate the kinetics of MP activity with the use of a detector that does not interfere with HOCl production. To achieve this we have used methionine to scavenge HOCl and an H_2O_2 electrode to monitor substrate disappearance. Using this assay, we have determined the effects that H_2O_2 and $O_2^{\cdot-}$ have on MP-dependent production of HOCl. We show that at high concentrations H_2O_2 inhibits MP and that this inhibition can be overcome by $O_2^{\cdot-}$, which allows MP to function maximally at neutral pH.

METHODS

MP was purified to a purity index of 0.70 as described previously [6]. Xanthine oxidase (XO) was purchased from Sigma Chemical Co. and separated from low- M_r constituents in the preparation on a $1.5\ cm \times 1\ cm$ Sephadex G-25 column. Acetaldehyde was redistilled before use. H_2O_2 solutions were prepared daily by dilution of a stock solution and concentrations were determined by using $\epsilon_{240}\ 43.6\ M^{-1}\ cm^{-1}$ [16]. Cytochrome c (type III), MCD and bovine erythrocyte superoxide dismutase (SOD) were purchased from Sigma Chemical Co. Catalase was purchased from Boehringer. Other

Abbreviations used: MP, myeloperoxidase; MP^{3+} , ferric myeloperoxidase; SOD, superoxide dismutase; XO, xanthine oxidase; MCD, monochlorodimedon.

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chemicals used were of analytical grade. Reactions were carried out at $23 \pm 2^\circ\text{C}$.

H_2O_2 uptake was measured continuously with a YSI model 25 oxidase meter fitted with a YSI 2510 oxidase probe (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.). The electrode was covered with an inner membrane of cellulose acetate and an outer membrane of collagen film. The cellulose acetate membrane was necessary to prevent interference from low- M_r compounds such as methionine and MCD [17]. None of the chemicals used in this study interfered with H_2O_2 detection by the electrode. The electrode gave a linear response between 0 μM - and 100 μM - H_2O_2 . Methionine was included in the reaction mixtures to scavenge HOCl and prevent it from inactivating MP [18]. We have previously shown that methionine does not react with the redox intermediates of MP [7]. Methionine sulfoxide, the product of the reaction between HOCl and methionine, did not affect the activity of MP when it was added at concentrations from 25 μM to 100 μM . The stoichiometry of H_2O_2 conversion into HOCl by MP was determined by stopping the reaction with 200 μg of catalase/ml after a measured amount of H_2O_2 had been consumed, and then measuring the amount of methionine sulfoxide produced from the reaction of HOCl with excess methionine [18] by amino acid analysis. When XO was used to generate $\text{O}_2^{\cdot-}$, the reaction was stopped with 200 μg of catalase/ml and 20 μg of SOD/ml plus 200 μM -allopurinol. Initial rates of H_2O_2 consumption were determined by drawing a tangent to the initial part of the curve for uptake of H_2O_2 . When XO was used to generate $\text{O}_2^{\cdot-}$, the increase in H_2O_2 production due to XO activity was taken as the baseline for H_2O_2 uptake. Reactions were carried out in a total volume of 3 ml and started by the addition of MP.

Rates of MP-dependent chlorination of MCD (100 μM) were determined by continuously monitoring the decrease in A_{290} (ϵ_{290} 19000 $\text{M}^{-1}\cdot\text{cm}^{-1}$) [10]. $\text{O}_2^{\cdot-}$ production by XO/acetaldehyde was determined by continuously monitoring the SOD-inhibitable reduction of cytochrome *c* at 550 nm ($\epsilon_{550}^{\text{(red.-ox.)}}$ 21 100 $\text{M}^{-1}\cdot\text{cm}^{-1}$) [19]. Spectra of MP were recorded on a Pye-Unicam PU 8800 spectrophotometer and the percentage of Compound II was determined by measuring the A_{452}/A_{625} ratio [10].

RESULTS

Measurement of MP activity with an H_2O_2 electrode

Addition of MP (20 nM) to a solution of 100 μM - H_2O_2 and 100 mM- Cl^- caused H_2O_2 to be consumed at an initial rate of 66 $\mu\text{M}/\text{min}$ (Fig. 1). Omission of Cl^- decreased the uptake of H_2O_2 to 1 $\mu\text{M}/\text{min}$. Determination of HOCl production by measuring conversion of methionine into methionine sulfoxide, after 20 μM - H_2O_2 had been consumed, revealed that 92% (mean of two determinations) of the H_2O_2 was converted into HOCl. From these results we conclude that measurement of H_2O_2 uptake by an H_2O_2 electrode is an accurate representation of HOCl production by MP.

After an initial short burst of activity, H_2O_2 uptake in buffer containing 100 mM- Cl^- was 95% inhibited by 100 μM -MCD (Fig. 1). The rate of H_2O_2 uptake in the presence of MCD approximated the rate of HOCl production measured with MCD under comparable

Fig. 1. Effects of Cl^- and MCD on the uptake of H_2O_2 by MP

H_2O_2 uptake was determined in the absence of Cl^- (---), in the presence of 100 mM- Cl^- (—), or in the presence of 100 mM- Cl^- and 100 μM -MCD (-·-). Reactions were started by adding MP (20 nM), indicated by the arrow, to 100 μM - H_2O_2 and 300 μM -methionine in 50 mM-phosphate buffer, pH 7.1. Ionic strength was maintained constant with Na_2SO_4 .

conditions (results not shown). These results confirm our previous finding that MCD inhibits MP [7], and show that the MCD assay grossly underestimates the true chlorinating activity of MP.

Effect of pH on MP activity

H_2O_2 uptake was proportional to the concentration of MP over the range of pH values investigated in this study (results not shown). At pH 4.5, 6.0 and 7.8 the

Fig. 2. Effect of H_2O_2 on the pH-activity profile of MP

Initial rates of H_2O_2 uptake were determined over the first 30 s of the reaction for 10 μM - H_2O_2 (■) and 100 μM - H_2O_2 (▲). Reactions were started by adding MP (10 nM) to H_2O_2 and 300 μM -methionine in buffer containing 50 mM-citrate, 50 mM-phosphate, 100 mM- NaCl and 200 mM- Na_2SO_4 . The pH was adjusted with 5 M- NaOH . Values are means of determinations in duplicate experiments with ranges being within the size of the symbols.

Fig. 3. Effect of pH on the time course of H_2O_2 uptake by MP

Conditions were as for Fig. 2 except that the concentration of MP was 50 nM.

regression coefficient for the correlation between H_2O_2 uptake and MP concentration was 0.99 and the maximum error for the regression constant was $\pm 7\%$. Using the H_2O_2 electrode, we found that with a concentration of $10 \mu M$ H_2O_2 MP had a pH optimum of 7.0 (Fig. 2). Increasing the concentration of H_2O_2 to $100 \mu M$ lowered the pH optimum to 6.5. These values are in relatively good agreement with those of pH 6.8 and 5.8 respectively predicted by Zgliczynski *et al.* [13].

As shown in Fig. 3, the profile for H_2O_2 uptake was dependent on pH. At and below the pH optimum, H_2O_2 uptake was monophasic. With 50 nM-MP it was demonstrated that above the pH optimum there was a rapid burst of H_2O_2 uptake followed by a much slower steady-state activity. At lower concentrations of MP the burst phase was correspondingly shorter, as we have found previously [7]. At pH 7.5 with $100 \mu M$ H_2O_2 as little as 5% of the H_2O_2 was consumed during the burst phase. This kinetic behaviour demonstrates that above the pH optimum, an inactive enzyme form accumulates until its turnover becomes limiting [20]. Previous studies have shown this to be Compound II [9,21].

Effect of H_2O_2 on MP activity and formation of Compound II

Since H_2O_2 can reduce Compound I to Compound II [22], we investigated the effect that H_2O_2 concentration has on the activity of MP. As the concentration of H_2O_2 was increased the initial rate of H_2O_2 uptake reached a maximum and then progressively declined (Fig. 4a). All the H_2O_2 was consumed in a single first-order reaction up to a threshold H_2O_2 concentration, above which the percentage consumed in the burst phase progressively declined (Fig. 5). When the concentration of Cl^- was increased, the initial rate of H_2O_2 uptake was enhanced at higher H_2O_2 concentrations. Increasing Cl^- concentration also extended the threshold of H_2O_2 concentration before the burst phase was inhibited, and limited the extent to which H_2O_2 inhibited the burst phase (Fig. 5). In parallel with the decline in enzyme activity seen

Fig. 4. Effects of H_2O_2 and Cl^- on the uptake of H_2O_2 by MP

(a) The effect of H_2O_2 on the initial rate of H_2O_2 uptake over the first 30 s of the reaction was determined at 50 mM- Cl^- (■), 100 mM- Cl^- (□) and 250 mM- Cl^- (▲) in 50 mM-phosphate buffer, pH 7.4, with 32 nM-MP and 300 μM -methionine. Constant ionic strength was maintained with Na_2SO_4 . (b) Double-reciprocal plots of the data in (a).

Fig. 5. Effect of H_2O_2 and Cl^- on the amount of H_2O_2 consumed during the burst phase of MP activity

Reactions were carried out in 50 mM-phosphate buffer, pH 7.4, with 50 mM- Cl^- (■) or 250 mM- Cl^- (▲). Other conditions were as described for Fig. 4. The logarithm of H_2O_2 concentration was plotted against time to determine at what H_2O_2 concentration the burst phase ceased and another second-order reaction began. The difference between this concentration and the initial H_2O_2 concentration was taken as the amount of H_2O_2 consumed in the burst phase.

Fig. 6. Effects of H₂O₂ and Cl⁻ on Compound II formation

Absorption spectra of MP were recorded 30 s after adding H₂O₂ to 180 nM-MP, 1 mM-methionine and 50 mM-Cl⁻ (■) or 250 mM-Cl⁻ (▲). The percentage of Compound II was calculated from the absorption spectra by determining the A_{452}/A_{625} ratio. Other conditions were as given for Fig. 4.

with increasing H₂O₂ concentration or decreasing Cl⁻ concentration, there was an increase in the proportion of MP present as Compound II (Fig. 6). Double-reciprocal plots of the data in Fig. 4(a) give the characteristic pattern for double competitive substrate inhibition in a ping-pong mechanism (Fig. 4b) [23]. We therefore propose that H₂O₂ and Cl⁻ compete at two steps in the mechanism for MP-dependent production of HOCl. One of the steps involves MP³⁺, as previously documented by other investigators [8,12,24]. In the second step Cl⁻ and H₂O₂ compete for Compound I to produce HOCl and Compound II respectively. This proposal is based on the above findings that Cl⁻, the substrate for Compound I, prevents H₂O₂ from decreasing the activity of MP and from promoting the formation of Compound II.

Effects of O₂^{•-} on MP activity

To test the effect of O₂^{•-} on the uptake of H₂O₂ by MP, O₂^{•-} (5 μM/min) was generated by the XO/ acetaldehyde system. Hypoxanthine was not used as the substrate for XO because it is oxidized to uric acid, which reduces Compound II and masks the effect of O₂^{•-} [6]. Neither XO nor acetaldehyde alone affected the rate of H₂O₂ uptake by MP and Cl⁻ (results not shown). Reaction mixtures of XO, acetaldehyde and H₂O₂ gave a steady increase in measured H₂O₂ concentration due to the activity of XO. The rate of H₂O₂ formation by XO/acetaldehyde in the absence of SOD was 3.5 μM/min whereas it was 4.0 μM/min in the presence of SOD. The additional H₂O₂ generated by XO/acetaldehyde in the presence or in the absence of SOD would not be expected to affect the activity of MP, because it would form only a small percentage of the 100 μM-H₂O₂ present. Adding MP at pH 7.5 gave rapid consumption of H₂O₂ that was substantially inhibited by SOD (Fig. 7a). Both the initial and the steady-state rates of H₂O₂ uptake in the presence of SOD were the same as those for MP and H₂O₂ alone (results not shown). Thus we conclude that at pH 7.5 O₂^{•-} increased the initial rate of H₂O₂ uptake and allowed

Fig. 7. Effects of O₂^{•-} on H₂O₂ uptake by MP

Sufficient XO was added to approx. 90 μM-H₂O₂ and 20 mM-acetaldehyde to generate 5 μM-O₂^{•-}/min at (a) pH 7.5 or (b) pH 6.0 in the presence (----) or absence (—) of 20 μg of SOD/ml. When the concentration of H₂O₂ reached 100 μM, MP (10 nM) was added to the reaction system as indicated by the arrow. Buffers were as described for Fig. 2 except for the addition of 100 μM-diethylenetriaminepenta-acetic acid. O₂^{•-} generation was measured independently by the cytochrome *c* assay.

MP to maintain a fast and relatively constant rate of H₂O₂ uptake. In contrast, at pH 6.0 O₂^{•-} decreased both the initial and the steady-state rates of H₂O₂ uptake (Fig. 7b).

The effect of O₂^{•-} on the initial rate of H₂O₂ uptake at different pH values is shown in Fig. 8. Above pH 6.5 increasing O₂^{•-} production to 10 μM/min further increased the initial rate of H₂O₂ uptake, and broadened the pH optimum to include pH 7.0. To determine the stoichiometry of this reaction at pH 7.0, the reaction was stopped by adding catalase, SOD and allopurinol. The loss of H₂O₂ measured by the H₂O₂ electrode was added to the H₂O₂ produced by XO/acetaldehyde to give the total H₂O₂ consumed. HOCl production was then measured as the conversion of methionine into methionine sulfoxide and accounted for 103% (mean of two determinations) of the 40 μM-H₂O₂ consumed. This indicates that the extra H₂O₂ consumed in the presence of O₂^{•-} was all converted into HOCl. Increasing the rate of O₂^{•-} production to 20 μM/min at pH 7.5 increased the initial rate of H₂O₂ uptake to 20 μM/min. Thus increasing the rate of O₂^{•-} generation extended the pH range for efficient activity of MP.

Fig. 8. Effect of $O_2^{\cdot-}$ on the initial rate of H_2O_2 uptake by MP at various pH values

Initial rates of H_2O_2 uptake were measured over the first 30 s of the reaction after adding MP (10 nM) to 100 μM - H_2O_2 and sufficient XO to generate 10 μM - $O_2^{\cdot-}$ /min. Assays were carried out in the presence (▲) or in the absence (■) of 20 μg of SOD/ml. Other conditions were as described for Fig. 7. Values represent means and ranges obtained for duplicate experiments.

DISCUSSION

In this investigation we have shown that H_2O_2 uptake by MP can be measured with an H_2O_2 electrode. H_2O_2 uptake by MP was greatly enhanced by Cl^- , and in the presence of Cl^- the H_2O_2 consumed was converted totally into HOCl. Thus the H_2O_2 electrode can be used to monitor HOCl production by MP, H_2O_2 and Cl^- . Using this system, we have confirmed that MCD inhibits MP and have shown that at neutral pH the chlorinating activity of MP is considerably greater than that measured by the MCD assay. We have also shown that, as predicted by Zgliczynski *et al.* [13], MP has a neutral pH optimum for HOCl production at 10 μM - H_2O_2 , and that this optimum declines with increasing concentration of H_2O_2 .

In spite of the above prediction, it has commonly been assumed that MP has an acidic pH optimum for HOCl production. This assumption has been based largely on the work of Klebanoff [25], who showed that the antimicrobial activity of MP was optimal at pH 4.5–5. In addition, subsequent investigations showed that MP activity measured with peroxidase substrates and/or high $[H_2O_2]/[Cl^-]$ ratios has an acidic pH optimum [9,11,12]. We contend that all these assays measured the pH optimum for peroxidase activity and not HOCl formation. As with all peroxidases, the pH optimum in these assays reflects the turnover of Compound II by the peroxidase substrate [26]. This also holds for HOCl formation when the $[H_2O_2]/[Cl^-]$ ratio is high, as we explain below.

We found that, as the $[H_2O_2]/[Cl^-]$ ratio was increased, HOCl formation declined, Compound II accumulated and the pH optimum decreased. These results indicate that at elevated pH, where the affinity of Cl^- for Compound I is lowered [8], H_2O_2 competed with Cl^- so that at each catalytic cycle a small fraction of MP was converted into Compound II, which accumulated. Thus, apart from undergoing a single two-electron reduction to generate HOCl (reaction 2), a small proportion of

Compound I underwent two successive one-electron reductions, with Compound II as the rate-limiting intermediate. Presumably Compound II would have been turned over to MP^{3+} by H_2O_2 , as proposed by Harrison [21]. The slow turnover of Compound II would then determine the activity of MP. Therefore at high $[H_2O_2]/[Cl^-]$ ratios the pH optimum for MP will reflect the turnover of Compound II, and not HOCl production.

In vivo, MP functions in concert with a flux of $O_2^{\cdot-}$. Therefore it was of interest to determine how $O_2^{\cdot-}$ affects the production of HOCl. In our previous study we showed that, when HOCl production is measured with MCD, $O_2^{\cdot-}$ enhances the activity of MP [6]. This occurs because $O_2^{\cdot-}$ reduces Compound II to MP^{3+} , and prevents Compound II from accumulating. We now show that $O_2^{\cdot-}$ also enhances the activity of MP in the absence of one-electron donors such as MCD, when H_2O_2 promotes the formation of Compound II. This allowed MP to function optimally from pH 6.5 to pH 7.5, under conditions where H_2O_2 would otherwise have promoted formation of Compound II and inhibited production of HOCl. In contrast, $O_2^{\cdot-}$ inhibited HOCl production at acidic pH, where there was no accumulation of Compound II. Under these conditions, where the rate-limiting step in the production of HOCl is reaction (1), $O_2^{\cdot-}$ should react with MP^{3+} to form Compound III. This would explain the inhibitory effect of $O_2^{\cdot-}$ at acidic pH.

From the results presented in this study and previous work by ourselves [6,7,10] and others [8,9,11–14,21,22], we propose that the reaction mechanism for MP-dependent production of HOCl is as outlined in Scheme 1. This is basically a ping-pong mechanism where H_2O_2 and Cl^- are competitive substrates for both MP^{3+} and Compound I. $O_2^{\cdot-}$ acts both as an inhibitor, through formation of Compound III, and as an activator, by reducing Compound II back to active MP^{3+} . Compound III is not a dead-end complex, but is turned over to Compound II by H_2O_2 [6]. $O_2^{\cdot-}$ enhances the activity of MP by reducing Compound II to MP^{3+} , even though it promotes formation of Compound III. Therefore we conclude that the turnover of Compound II in the absence of $O_2^{\cdot-}$, presumably by a slow reaction with H_2O_2 , must be slower than the turnover of Compound III by H_2O_2 [21].

This elaborate mechanism for HOCl production may be advantageous for neutrophils because of the high reduction potential of Compound I. Since Compound I can oxidize Cl^- , it has the capacity to oxidize many substrates with higher oxidation potentials than Cl^- . This is evidenced by the ability of H_2O_2 , serum components, tryptophan and MCD to promote accumulation of Compound II [7] and to inhibit MP. Therefore, if neutrophils produced only H_2O_2 , there would be a short burst of activity producing only a small amount of HOCl, after which MP would rapidly collapse to Compound II. This would allow the H_2O_2 concentration to become elevated, promoting formation of Compound III, which would be degraded at millimolar concentrations of H_2O_2 [6]. Ascorbate also reduces Compound II and enhances production of HOCl [9]. Although neutrophils have a high content of ascorbate, they do not release significant amounts when they are stimulated [27]. Thus $O_2^{\cdot-}$ discharged by neutrophils should have the dominant effect on the activity of co-released MP.

Thus, by generating $O_2^{\cdot-}$ and preventing accumulation

Scheme 1. Proposed mechanism for MP-dependent production of HOCl in the presence of $\text{O}_2^{\cdot-}$

of Compound II, neutrophils would ensure that MP can maintain continuous production of HOCl. The precise effect of $\text{O}_2^{\cdot-}$ on HOCl production by neutrophils, however, will depend on pH. When the pH is neutral or slightly alkaline, $\text{O}_2^{\cdot-}$ will allow MP to function optimally. In contrast, when the pH becomes acidic, $\text{O}_2^{\cdot-}$ will inhibit production of HOCl. On the basis of the results presented in this study, we conclude that MP can function optimally during microbial killing when the phagocytic pH is approximately neutral.

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