Superoxide modulates the activity of myeloperoxidase and optimizes the production of hypochlorous acid

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Myeloperoxidase catalyses the conversion of H_2O_2 and Cl^- to hypochlorous acid (HOCl). It also reacts with O_2^- to form the oxy adduct (compound III). To determine how O_2^- affects the formation of HOCl, chlorination of monochlorodimedon by myeloperoxidase was investigated using xanthine oxidase and hypoxanthine as a source of O_2^- and H_2O_2 . Myeloperoxidase was mostly converted to compound III, and H_2O_2 was essential for chlorination. At pH 5.4, superoxide dismutase (SOD) enhanced chlorination and prevented formation of compound III. However, at pH 7.8, SOD inhibited chlorination and promoted formation of the ferrous peroxide adduct (compound II) instead of compound III. We present spectral evidence for a direct reaction between compound III and H_2O_2 to form compound II, and for the reduction of compound II by O_2^- to regenerate native myeloperoxidase. These reactions enable compound III and compound II to participate in the chlorination reaction. Myeloperoxidase catalytically inhibited O_2^- -dependent reduction of Nitro Blue Tetrazolium. This inhibition is explained by myeloperoxidase undergoing a cycle of reactions with O_2^- , H_2O_2 and O_2^- , with compounds III and II as intermediates, i.e., by myeloperoxidase acting as a combined SOD/catalase enzyme. By preventing the accumulation of inactive compound II, O_2^- enhances the activity of myeloperoxidase. We propose that, under physiological conditions, this optimizes the production of HOCl and may potentiate oxidant damage by stimulated neutrophils.

INTRODUCTION

Neutrophils respond to phagocytic and inflammatory stimuli by producing superoxide (O_2^-) and H_2O_2 , and by releasing granule proteins either into the phagosome or extracellularly. Myeloperoxidase, the most abundant granule enzyme, catalyses the conversion of H_2O_2 and Cl^- to HOCl [1]. Although HOCl is potently microbicidal [2] and cytotoxic [3,4], it is unclear how efficiently it is formed within the phagosome and how much it contributes to bacterial killing and other inflammatory reactions of neutrophils.

It is generally considered that myeloperoxidase produces HOCl by a classical peroxidase mechanism [reactions (1) and (2) of Table 1]. Little attention, however, has been focused on the reaction of myeloperoxidase with O_2^- . Odajima & Yamazaki [5] first showed that O_2^- reacts with ferric myeloperoxidase (MP^{3+}) to form the oxy derivative or compound III [reaction (3) of Table 1] and Winterbourn *et al.* [6] showed that O_2^- produced by stimulated neutrophils converts co-released MP^{3+} to compound III. Compound III, like compound II, is thought to be inactive with respect to halogenation [7]. In support of this, Cuperus *et al.* [8] observed concurrent inhibition of myeloperoxidase-dependent chlorination of monochlorodimedon (MCD) and formation of compound III, in the presence of D-penicillamine. However, in the presence of Cl^- and H_2O_2 , MP^{3+} and compound III chlorinate MCD at the same rate [6]. Also, chlorination by xanthine oxidase, hypoxanthine and myeloperoxidase is no faster when compound III formation is prevented by superoxide

Table 1. Reactions of myeloperoxidase

The different redox forms of myeloperoxidase are ferric myeloperoxidase (MP^{3+}), compound I ($\text{MP}^{3+}\text{H}_2\text{O}_2$), compound II ($\text{MP}^{2+}\text{H}_2\text{O}_2$) and compound III ($\text{MP}^{3+}\text{O}_2^- = \text{MP}^{2+}\text{O}_2$).

dismutase (SOD) [6]. These findings suggest that under some conditions compound III may be able to enter the chlorination pathway. Two routes have been suggested. One is by reacting with compound II to regenerate MP^{3+} [reaction (4) of Table 1] [6], the other by reacting with O_2^- to form compound I [reaction (5) of Table 1] [9]. The latter would imply that myeloperoxidase has SOD activity and that chlorination can occur without H_2O_2 . However, as we shall show, neither of these explanations is satisfactory.

Rapid reaction of MCD with HOCl makes it convenient for following chlorination by peroxidases. We have recently shown, however, that such studies are complicated because MCD is also a peroxidase substrate of myeloperoxidase (A. J. Kettle & C. C. Winterbourn, unpublished work). At pH 5.4, it can react with both compounds I and II, but at pH 7.8, only the compound

Abbreviations used: SOD, superoxide dismutase; O_2^- , superoxide anion; XO, xanthine oxidase; HX, hypoxanthine; MP^{3+} , ferric myeloperoxidase; MCD, monochlorodimedon; NBT, Nitro Blue Tetrazolium.

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I reaction is significant. This results in the build-up of compound II, so that during chlorination almost all the enzyme is in this form. Therefore, any mechanism of MCD chlorination must consider reactions of compound II.

We have further investigated how O_2^- affects chlorination by myeloperoxidase, both near its acid pH optimum, and at neutral pH such as would be expected extracellularly or during the early stages of phagocytosis [10]. We demonstrate a reaction between O_2^- and compound II that can enhance activity, and a reaction with H_2O_2 that enables compound III to participate in the catalytic cycle.

MATERIALS AND METHODS

Purification of myeloperoxidase from human neutrophils was based on the method of Pember *et al.* [11]. Neutrophils were sonicated in 1 M-NaCl. The supernatant was dialysed against 50 mM-Tris/HCl, pH 7.8, and applied to a DEAE-Sephadex column equilibrated in the same buffer. Unbound protein was concentrated, dialysed against 25 mM-sodium acetate, pH 4.7, containing 0.2 M-NaCl and applied to a CM-Sephadex column. Fractions containing myeloperoxidase with a purity index (A_{430}/A_{280}) of greater than 0.5 were applied to an Ultrogel Ac 34 gel filtration column. Those fractions that eluted with a purity index between 0.7 and 0.79 were used in this investigation. All purification steps were performed at 4 °C.

SOD, catalase, cytochrome *c* (type III), MCD, Nitro Blue Tetrazolium (NBT) and xanthine oxidase (XO) (type III) were purchased from Sigma. All other chemicals were of the highest grade available. The concentration of catalase was calculated by using ϵ_{406} 115 $\text{mm}^{-1}\cdot\text{cm}^{-1}$ per haem [12] and that of H_2O_2 using ϵ_{240} 43.6 $\text{M}^{-1}\cdot\text{cm}^{-1}$ [13]. Reactions were carried out at 25 °C in 50 mM-phosphate buffer containing 0.1 mM-EDTA and, when present, 100 mM- Cl^- and 100 μM -MCD. All solutions were maintained at a constant ionic strength when pH, or the concentration of Cl^- , was altered by adding Na_2SO_4 .

Compound III was prepared by reducing deoxygenated MP^{3+} with a few crystals of sodium dithionite and passing it through a column of Sephadex G-50 (0.3 cm $\times$ 8 cm) at 4 °C. The ferrous myeloperoxidase separates from the dithionite and reacts with oxygen to form compound III. Solutions of compound III prepared in this way contained up to 40 % MP^{3+} . Concentrations of MP^{3+} , compound II and compound III in mixtures were calculated using eqns. (1), (2) and (3) below, where the corresponding absorption coefficients were based on A_{430} 91 $\text{mm}^{-1}\cdot\text{cm}^{-1}$ per haem for MP^{3+} [14] and determined from the absorption spectra in Fig. 4 (a).

$$A_{430} = 91[\text{MP}^{3+}] + 50[\text{compound II}] + 50[\text{compound III}] \quad (1)$$

$$A_{456} = 35[\text{MP}^{3+}] + 84[\text{compound II}] + 65[\text{compound III}] \quad (2)$$

$$A_{625} = 10[\text{MP}^{3+}] + 20[\text{compound II}] + 27[\text{compound III}] \quad (3)$$

Spectra and rate measurements were recorded on a Pye Unicam PU 8800 spectrophotometer.

Rates of myeloperoxidase-dependent chlorination were determined by continuously monitoring the decrease in MCD concentration at 290 nm (ϵ_{290} 19000 $\text{M}^{-1}\cdot\text{cm}^{-1}$).

With the XO oxidase system the baseline was taken as the increase in A_{290} due to urate (ϵ_{290} 11000 $\text{M}^{-1}\cdot\text{cm}^{-1}$) production and this was used as a measure of XO activity. Cytochrome *c* reduction was measured by continuously monitoring the increase in A_{550} [reduced – oxidized] 21000 $\text{M}^{-1}\cdot\text{cm}^{-1}$. NBT reduction was measured by continuously monitoring A_{560} (ϵ_{560} 15000 $\text{M}^{-1}\cdot\text{cm}^{-1}$).

RESULTS

Chlorination by myeloperoxidase and an $XO\ O_2^-/H_2O_2$ -generating system

The effect of O_2^- on chlorination of MCD by myeloperoxidase was investigated using XO and hypoxanthine (HX) (the XO system) to generate O_2^- and H_2O_2 . At pH 7.8, the rate of chlorination was proportional to the concentration of XO, i.e., the rate of O_2^- and H_2O_2 production (Fig. 1). SOD had no effect at XO concentrations producing less than 1.5 μM -urate min^{-1} but as the concentration of XO was increased, SOD prevented further increase in the rate of chlorination. This inhibition was transitory and had disappeared after 5 min due to the accumulation of urate (see below). Rates in Fig. 1 were determined over the first minute of reaction when inhibition by SOD was greatest.

Absorption spectra, recorded under similar conditions to those described in Fig. 1, showed, in agreement with Winterbourn *et al.* [6], that the XO system converted 60–70 % of MP^{3+} into compound III. SOD prevented this conversion. Myeloperoxidase species present in the

Fig. 1. Effect of XO on myeloperoxidase-dependent chlorination of MCD

Chlorination was initiated by adding 50 μM -HX to varying concentrations of XO, 200 nM-myeloperoxidase, 100 μM -MCD and 100 mM- Cl^- at pH 7.8, in the absence or presence of 20 μg of SOD/ml. Of the 4 reducing equivalents derived from HX, 30 % were directly converted to O_2^- , as measured by the reduction of cytochrome *c*, and the remainder was converted to H_2O_2 ; i.e. for 1 μM -urate min^{-1} , O_2^- production was 1.2 $\mu\text{M}\cdot\text{min}^{-1}$ and H_2O_2 production was 1.4 $\mu\text{M}\cdot\text{min}^{-1}$. In the presence of SOD, all the reducing equivalents were converted to H_2O_2 .

Fig. 2. Effect of SOD on myeloperoxidase-dependent chlorination of MCD by XO at pH 5.4

The change in A_{290} over time was measured after $50 \mu\text{M}$ -HX was added to XO, 20 nM -myeloperoxidase, $100 \mu\text{M}$ -MCD, 100 mM -Cl⁻ and when present $20 \mu\text{g}$ of SOD/ml. Urate production was $5.8 \mu\text{M} \cdot \text{min}^{-1}$ and O_2^- production was $5 \mu\text{M} \cdot \text{min}^{-1}$.

presence of SOD depended on the concentration of XO. At low concentrations, where SOD did not affect chlorination (Fig. 1), 20% of MP^{3+} was converted to compound II. When SOD inhibited chlorination, MP^{3+} was converted initially to 100% compound II, but coincident with the increase in the rate of chlorination, there was gradual conversion back to MP^{3+} until only 20% compound II remained. As shown later, this disappearance of compound II and associated loss of inhibition was due to reaction with urate produced from HX by XO.

At pH 5.4, SOD enhanced myeloperoxidase-dependent chlorination by the XO system (Fig. 2) from a steady-state rate of $5.0 \mu\text{M} \cdot \text{min}^{-1}$ to $8.5 \mu\text{M} \cdot \text{min}^{-1}$, i.e., O_2^- inhibited chlorination. In the presence of SOD, 100% MP^{3+} was maintained. In its absence, 65% of MP^{3+} was converted to compound III at steady state.

We have shown, therefore, that O_2^- promotes the formation of compound III, and prevents the accumulation of compound II. At pH 7.8, an increase in compound II, but not compound III, correlated with a decrease in chlorination. At pH 5.4, however, where compound II did not accumulate, formation of compound III was associated with inhibition of chlorination.

Effect of O_2^- on chlorination by myeloperoxidase/ H_2O_2 /Cl⁻

The rate of chlorination of MCD by myeloperoxidase and H_2O_2 , at pH 7.8, is maximal with about $100 \mu\text{M}$ - H_2O_2 [6]. Under these conditions essentially all the myeloperoxidase is converted to compound II. As the concentration of H_2O_2 is increased, there is a gradual inhibition of chlorination and compound II is converted

Table 2. Myeloperoxidase-dependent chlorination of MCD by the XO system and/or H_2O_2

Reaction mixtures contained 600 nM -myeloperoxidase, XO, $50 \mu\text{M}$ -HX and when present 0.5 mM - H_2O_2 . Urate production was $2.5 \mu\text{M} \cdot \text{min}^{-1}$ and O_2^- production was $3.0 \mu\text{M} \cdot \text{min}^{-1}$. Other conditions are described in Fig. 1. Results are means and ranges of duplicate experiments.

Reaction mixture	MCD chlorination ($\mu\text{M} \cdot \text{min}^{-1}$)
H_2O_2	4.0 ± 0.3
XO	5.0 ± 0.3
XO + SOD	5.5 ± 0.2
XO + H_2O_2	11.6 ± 0.6
XO + H_2O_2 + SOD	5.0 ± 0.2

Fig. 3. Effect of catalase on myeloperoxidase-dependent chlorination of MCD by the XO system

Chlorination was initiated by adding $100 \mu\text{M}$ -HX to XO and $3 \mu\text{M}$ - MP^{3+} (▲) or $1.5 \mu\text{M}$ - MP^{3+} (●). Urate production was $2.7 \mu\text{M} \cdot \text{min}^{-1}$ and O_2^- production was $3.2 \mu\text{M} \cdot \text{min}^{-1}$. The concentration of catalase is expressed per haem. Other conditions are described in Fig. 1.

to compound III. We found that with $500 \mu\text{M}$ - H_2O_2 , the XO system gave a 3-fold increase in rate, which was abolished by SOD (Table 2). XO, in the presence or absence of SOD, did not affect the amount of compound III present at steady state, which was virtually 100% of the myeloperoxidase present.

Viewed in another way, the results in Table 2 show that H_2O_2 doubled the rate of chlorination by myeloperoxidase and the XO system, but only in the absence of SOD. H_2O_2 , therefore, must be involved in the rate-determining step in chlorination only in the presence of O_2^- . Since compound III is the predominant species present under these conditions, this implies that the reaction of compound III with H_2O_2 is limiting.

Catalase-inhibited chlorination by myeloperoxidase and the XO system, demonstrating a requirement for H_2O_2 (Fig. 3). A concentration of catalase approximately equal to that of myeloperoxidase was needed to give

Fig. 4. Absorption spectra of reactions of myeloperoxidase with H_2O_2 and O_2^-

(a) $1.2 \mu\text{M}$ - MP^{3+} , compound II formed by adding $50 \mu\text{M}$ - H_2O_2 to MP^{3+} and compound III formed by adding 2 mM - H_2O_2 to MP^{3+} . (b) The reaction between $50 \mu\text{M}$ - H_2O_2 and a solution of $1.2 \mu\text{M}$ -compound III and $0.8 \mu\text{M}$ - MP^{3+} . (c) The decay of compound II formed by adding $50 \mu\text{M}$ - H_2O_2 to MP^{3+} and adding catalase to scavenge excess H_2O_2 . (d) The reaction between O_2^- and compound II. XO was added to preformed compound II with 10 mM -acetaldehyde. O_2^- production was $2.2 \mu\text{M} \cdot \text{min}^{-1}$ as measured by the rate of cytochrome *c* reduction. Numbers in parentheses indicate the time (min) after beginning the reaction. All reactions were carried out in the absence of Cl^- at pH 7.8.

greater than 90 % inhibition. This implies that compound I, the active chlorinating form of myeloperoxidase, is only formed by reaction of MP^{3+} with H_2O_2 , and not from compound III and O_2^- .

Reaction of compound III with H_2O_2

For compound III to participate in chlorination it must be turned over to active compound I so that HOCl

can be produced by reaction (2) (Table 1). Reaction with O_2^- is excluded because chlorination was inhibited by catalase. However, our finding that H_2O_2 enhances chlorination by myeloperoxidase and the XO system suggests that compound III is turned over by reacting with H_2O_2 . This reaction was demonstrated directly by adding H_2O_2 to preformed compound III, in the absence of Cl^- , and following changes in the absorption spectrum.

Fig. 4(a) shows the spectra of MP^{3+} , compound II and compound III, and Fig. 4(b) shows the spectral changes associated with the reaction of compound III with H_2O_2 . On addition of H_2O_2 there were immediate increases in A_{456} and A_{630} , attributable to conversion of the MP^{3+} present (up to 40% of total myeloperoxidase) to compound II. After 1 min there was a marked decrease in A_{630} , indicating a loss of compound III. Since there was little change in A_{430} or A_{456} , the product of this reaction was not MP^{3+} . This excludes a reaction between compounds II and III [reaction (4), Table 1]. Subsequent serial spectra passed through an isosbestic point at 440 nm and resembled the decay spectra of compound II to MP^{3+} (Fig. 4c). We conclude, therefore, that H_2O_2 reacted rapidly with MP^{3+} and more slowly with compound III, in each case producing compound II.

Reaction of compound II with O_2^-

The ability of O_2^- to decrease the steady-state concentration of compound II during chlorination of MCD suggests a direct reaction of O_2^- with compound II. This reaction was shown to occur by forming compound II from H_2O_2 and MP^{3+} , adding catalase to scavenge excess H_2O_2 , and then adding the XO O_2^- -generating system. (Acetaldehyde was used instead of HX, because HX slowly reduced compound II.) This caused rapid decreases in A_{456} and A_{630} and a rapid increase in A_{430} , indicating that MP^{3+} had formed from compound II (Fig. 4d). These changes were followed by a slight shift of the Soret peak to higher wavelengths and an increase in A_{625} , indicating conversion of MP^{3+} to compound II. SOD prevented all these changes and allowed compound II to decay as in Fig. 4(c). From these results we conclude that O_2^- reduced compound II to MP^{3+} which then reacted with more O_2^- to form compound III.

Fig. 5. Effect of catalase on the inhibition of cytochrome *c* reduction by myeloperoxidase

Solutions contained 50 μM -HX, XO, 30 μM -ferric cytochrome *c* and 1.1 μM - MP^{3+} , with (●) or without (▲) 100 mM- Cl^- . Urate production was 2.5 $\mu\text{M}\cdot\text{min}^{-1}$ and O_2^- production was 3.0 $\mu\text{M}\cdot\text{min}^{-1}$. Other conditions are described in Fig. 1.

Superoxide breakdown by myeloperoxidase

In agreement with Cuperus *et al.* [9], we found that myeloperoxidase gave concentration-dependent inhibition of reduction of cytochrome *c* by the XO system. This indicates that myeloperoxidase catalytically breaks down O_2^- . Inhibition can be explained by myeloperoxidase scavenging O_2^- , and compound I oxidizing reduced cytochrome *c* [9]. In keeping with this mechanism, inhibition was much less in the presence of Cl^- (Fig. 5) which can compete for compound I. Cuperus *et al.* [9] proposed that compound I was formed from MP^{3+} reacting with two molecules of O_2^- [reactions (3) and (5) of Table 1], i.e., by myeloperoxidase acting as a SOD. However, we have shown that catalase prevents chlorination by myeloperoxidase and the XO system, i.e., O_2^- cannot convert MP^{3+} to compound I. Furthermore, as shown in Fig. 5, catalase almost completely prevented myeloperoxidase from inhibiting the reduction of cytochrome *c*. There was still slight inhibition, indicating that even in the absence of H_2O_2 compound III was turned over to MP^{3+} . A possible mechanism is the reaction of compound III of myeloperoxidase with catalase compound I. This reaction would generate MP^{3+} and catalase compound II, and occurs with horseradish peroxidase and catalase [15]. Our findings suggest that reactions of O_2^- and H_2O_2 are involved in the inhibition of cytochrome *c* reduction by myeloperoxidase, and they are not evidence of SOD activity.

The cytochrome *c* system is complicated because reduced cytochrome *c* can react with H_2O_2 [16] and myeloperoxidase, and adding catalase introduces further possible haem interactions. Since the products of NBT reduction are unaffected by H_2O_2 or myeloperoxidase, we examined the effect of myeloperoxidase on NBT reduction by the XO system. Myeloperoxidase gave concentration-dependent inhibition (Fig. 6a). Since O_2^- was produced in excess of the myeloperoxidase present, the reaction of MP^{3+} with O_2^- cannot alone account for the inhibition and the compound III formed must be turned over. As discussed above, our evidence does not favour O_2^- reacting with compound III. However, we have shown that compound III reacts with H_2O_2 to form compound II, which is reduced by O_2^- to regenerate MP^{3+} . The sum of these two reactions plus reaction (3) (Table 1) would account for the observed inhibition.

By plotting the ratio of the rate of reduction of NBT in the absence of myeloperoxidase (V_{max}) to that in its presence (V_1), against the concentration of MP^{3+} according to equation (4) (Fig. 6b), the rate constant for the turnover of myeloperoxidase (k_{II}) can be determined [17]. Since compound III accumulates, this rate constant will reflect the rate of reaction between compound III and H_2O_2 .

$$V_{\text{max}}/V_1 = 1 + k_{\text{II}} \cdot [\text{myeloperoxidase}]/k_1 \cdot [\text{NBT}] \quad (4)$$

where k_1 is the rate constant for the reaction of O_2^- with NBT. Using a value of $5.88 \times 10^4 \text{ M}^{-1}\cdot\text{s}^{-1}$ for k_1 [18], k_{II} was calculated to be $1.8 \times 10^6 \text{ M}^{-1}\cdot\text{s}^{-1}$.

Effect of uric acid on chlorination

The transient inhibitory effect of SOD on chlorination by myeloperoxidase and the XO system, coupled with the gradual decline of compound II, suggests that the urate produced was reacting with compound II and accelerating chlorination. In the absence of SOD, where

Fig. 6. Effect of myeloperoxidase on reduction of NBT by the XO system

(a) Solutions contained $50 \mu M$ -HX, XO, $100 \mu M$ -NBT and $100 \mu M$ -methionine to scavenge HOCl with (i) no MP^{3+} (ii) $2.2 \mu M$ - MP^{3+} or (iii) $4.4 \mu M$ - MP^{3+} . Urate production was $2.3 \mu M \cdot min^{-1}$ and O_2^- production was $2.8 \mu M \cdot min^{-1}$. Other conditions are described in Fig. 1. (b) The relationship between V_{max}/V_1 and the concentration of myeloperoxidase for the steady-state inhibition of NBT reduction. The maximum rate of NBT reduction (V_{max}) was measured in the absence of myeloperoxidase and the inhibition of NBT reduction (V_1) was measured at various concentrations of myeloperoxidase.

O_2^- reduces compound II, addition of $10 \mu M$ -urate had no effect on chlorination (Table 3) or on compound III formation. However, when added in the presence of SOD, urate decreased compound II formation to 20% of the myeloperoxidase present, and the maximum

Table 3. Effect of urate on myeloperoxidase-dependent chlorination of MCD by XO or H_2O_2

With XO reactions were initiated by adding $50 \mu M$ -HX to 200 nm -myeloperoxidase in the presence or absence of $20 \mu g$ of SOD/ml and $10 \mu M$ -urate. Urate production was $3.7 \mu M \cdot min^{-1}$ and O_2^- production was $4.4 \mu M \cdot min^{-1}$. For H_2O_2 -dependent chlorination, $100 \mu M$ - H_2O_2 was added to 200 nm -myeloperoxidase to initiate chlorination. All other conditions are described in Fig. 1. Results are means and ranges of duplicate experiments.

Reaction mixture	Rate of MCD chlorination ($\mu M \cdot min^{-1}$)	
	No Urate	Urate ($10 \mu M$)
XO/HX	6.5 ± 0.2	6.6 ± 0.2
XO/HX + SOD	3.3 ± 0.1	6.5 ± 0.2
H_2O_2	1.2 ± 0.2	10.1 ± 0.2

chlorination rate was attained without a lag. Further, urate rapidly reduced preformed compound II to MP^{3+} (not shown), and increased the rate of chlorination by myeloperoxidase and H_2O_2 8-fold. Xanthine and HX also reduced compound II (not shown) with an order of reaction of urate > xanthine $\gg$ HX. From these results we conclude that urate (and to a lesser extent xanthine) behaves like O_2^- in preventing the accumulation of compound II and inhibition of chlorination. This accounts for the transience of SOD inhibition of chlorination by myeloperoxidase and the XO system.

DISCUSSION

Superoxide has previously been shown to react with MP^{3+} to give compound III [5,6]. The present study has shown that O_2^- also reacts with compound II to give MP^{3+} . We have demonstrated that this reaction enables O_2^- to increase the rate of chlorination of MCD by myeloperoxidase, H_2O_2 and Cl^- , and that SOD, by preventing O_2^- from reacting with compound II, inhibits chlorination by myeloperoxidase and an XO O_2^-/H_2O_2 -generating system.

We have also shown spectrally that compound III reacts with H_2O_2 to generate compound II. These two newly recognized reactions, in combination with other reactions of myeloperoxidase depicted in Fig. 7, explain how compound III, in the presence of H_2O_2 and O_2^- , participates in chlorination and other catalytic functions of myeloperoxidase. A number of properties of myeloperoxidase which have been puzzling can now be explained.

Previous studies have shown that with increasing concentrations of H_2O_2 , myeloperoxidase catalytically breaks down H_2O_2 [6] and the efficiency of chlorination declines [6,19]. Our demonstration that H_2O_2 reacts with compound III as well as compound II explains this catalase-like activity. Thus, in the absence of substrates that can reduce compound II to MP^{3+} , these two reactions will result in interconversion of compounds II and III giving the net reaction:

We propose that the rate-determining step in the

Fig. 7. Proposed reaction mechanism for myeloperoxidase-dependent production of HOCl by O₂⁻ and H₂O₂-generating systems

Abbreviations: MPO, myeloperoxidase; e⁻, electron.

turnover of myeloperoxidase in an O₂⁻ generating system is the reaction between compound III and H₂O₂, and have measured its rate constant as $1.8 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$. This is an order of magnitude slower than the reaction between MP³⁺ and H₂O₂ to form compound I ($k_1 = 2.3 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ [20]) and agrees with the slower rate of disappearance of compound III when H₂O₂ was added to a mixture of compound III and MP³⁺ (Fig. 4b). The analogous reaction has recently been shown to occur with chloroperoxidase [21] and myoglobin [22]. To account for the turnover of compound III during chlorination Winterbourn *et al.* [6] proposed a reaction between compounds II and III to generate MP³⁺ [reaction (4) of Table 1]. However, this is incompatible with the spectral changes in Fig. 4b which indicate that H₂O₂ converts both MP³⁺ and compound III to compound II.

Our results explain how myeloperoxidase catalytically inhibits reduction of cytochrome *c* or NBT by O₂⁻. Cuperus *et al.* [9] first described inhibition of cytochrome *c* reduction and proposed that this was due to O₂⁻ reacting with MP³⁺ and compound III in succession to produce compound I. This proposal was based on their findings that, in the presence of catalase, myeloperoxidase inhibited cytochrome *c* reduction by an XO system, and also promoted chlorination of MCD. We have shown, however, that when approximately equimolar catalase and myeloperoxidase are used, O₂⁻ is unable to form compound I, chlorination is inhibited, and myeloperoxidase only slightly inhibits cytochrome *c* reduction. This indicates that compound I was only formed by reaction 1. Cuperus *et al.* [9] used considerably less catalase, and it would appear that this was insufficient to suppress reactions of myeloperoxidase with H₂O₂. Although our results do not support myeloperoxidase having true SOD activity, we have shown that with H₂O₂ present, myeloperoxidase catalyses the breakdown of O₂⁻ and inhibits NBT reduction. The sum of reactions (3), (7) and (9) (of Fig. 7) provide a mechanism for this process. They constitute a cycle between MP³⁺,

compound III and compound II, with myeloperoxidase acting as a combined SOD/catalase enzyme, i.e.,

Superoxide enhanced chlorination of MCD when it prevented accumulation of compound II, but when compound II did not accumulate, O₂⁻ inhibited chlorination by forming compound III at the expense of MP³⁺. The rate of any enzymic reaction is determined by the rate of turnover of the enzyme species present at steady state. Thus, at pH 7.8, O₂⁻ enhanced chlorination by reducing compound II and correspondingly increasing its rate of turnover. Chlorination was enhanced even though O₂⁻ promoted the formation of compound III. This implies that reaction of compound III with H₂O₂ is faster than turnover of compound II in the absence of O₂⁻. At low XO concentrations (where SOD had no effect on chlorination) generation of H₂O₂ was rate-limiting. At pH 5.4, O₂⁻ promoted the formation of compound III at the expense of MP³⁺. This changed the rate-limiting step from the reaction of H₂O₂ with MP³⁺ to its reaction with compound III, which we have measured to be 10-fold slower. In this way O₂⁻ inhibited chlorination.

The mechanism by which O₂⁻ enhances myeloperoxidase-dependent chlorination is the same as that previously proposed for ascorbate [23]. We have shown that urate, and to a lesser extent xanthine, also increase chlorination by reducing compound II. Other one-electron donors may act similarly. However, in view of the concurrent release of myeloperoxidase and generation of O₂⁻ by stimulated neutrophils, the reaction with O₂⁻ is likely to be physiologically most significant. The effect of urate was apparent when XO and HX were used to generate O₂⁻, with the result that SOD had only a transient effect on chlorination of MCD. This raises the point that when XO is used in conjunction with myeloperoxidase, reactions of urate or xanthine with myeloperoxidase must be considered.

Our mechanism also explains why chlorination of MCD in the presence of ascorbate is inhibited by D-penicillamine, which promotes the formation of compound III [8]. Compound III is formed from O₂⁻ which is produced during autoxidation of D-penicillamine. In the absence of D-penicillamine, ascorbate would prevent the accumulation of compound II and the rate of chlorination would depend on the reaction between MP³⁺ and H₂O₂. This is analogous to chlorination at pH 5.4 in the absence of ascorbate. Conversion of MP³⁺ to compound III by O₂⁻ formed in the presence of D-penicillamine, therefore, would be expected to inhibit chlorination.

In our studies, O₂⁻ enhanced chlorination by myeloperoxidase primarily because MCD, used to trap HOCl, converts the enzyme to compound II (A. J. Kettle & C. C. Winterbourn, unpublished work). It could be argued, therefore, that our observations are peculiar to the MCD assay. However, other possible HOCl detectors also convert MP³⁺ to compound II (A. J. Kettle & C. C. Winterbourn, unpublished work), and it is likely that constituents of the environment in which myeloperoxidase normally functions also cause this reduction. An important physiological function of O₂⁻ may be, therefore, to maintain myeloperoxidase in its active form, preventing inhibition by high concentrations of H₂O₂,

and optimizing HOCl formation. From this it follows that HOCl formation by neutrophils could be inhibited by SOD, and this may be a factor in producing its anti-inflammatory properties [24,25].

The precise effect O_2^- will have on the function of myeloperoxidase will depend on the concentration of H_2O_2 . In the neutrophil, myeloperoxidase functions in conjunction with a flux of O_2^- , with H_2O_2 as a secondary product. If myeloperoxidase can trap most of the O_2^- , H_2O_2 may never build up, and the SOD/catalase function of myeloperoxidase may predominate. Doubts have been expressed about the efficiency of HOCl formation in the phagosome [26,27], and this mechanism provides an alternative pathway for removing O_2^- and H_2O_2 . On the other hand, if high concentrations of H_2O_2 accumulate, O_2^- will enhance the efficiency of myeloperoxidase-dependent chlorination by increasing the turnover of compound II and preventing H_2O_2 from interconverting it with compound III.

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