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Production of the superoxide adduct of myeloperoxidase (compound III) by stimulated human neutrophils and its reactivity with hydrogen peroxide and chloride

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Examination of the spectra of phagocytosing neutrophils and of myeloperoxidase present in the medium of neutrophils stimulated with phorbol myristate acetate has shown that superoxide generated by the cells converts both intravacuolar and exogenous myeloperoxidase into the superoxo-ferric or oxyferrous form (compound III or MPO_2). A similar product was observed with myeloperoxidase in the presence of hypoxanthine, xanthine oxidase and Cl^-. Both transformations were inhibited by superoxide dismutase. Thus it appears that myeloperoxidase in the neutrophil must function predominantly as this superoxide derivative. MPO_2 autoxidized slowly ($t_{\frac{1}{2}} = 12$ min at 25°C) to the ferric enzyme. It did not react directly with H_2O_2 or Cl^-, but did react with compound II ($MP^{2+}\cdot H_2O_2$). MPO_2 catalysed hypochlorite formation from H_2O_2 and Cl^- at approximately the same rate as the ferric enzyme, and both reactions showed the same H_2O_2-dependence. This suggests that MPO_2 can enter the main peroxidation pathway, possibly via its reaction with compound II. Both ferric myeloperoxidase and MPO_2 showed catalase activity, in the presence or absence of Cl^-, which predominated over chlorination at H_2O_2 concentrations above 200 μM. Thus, although the reaction of neutrophil myeloperoxidase with superoxide does not appear to impair its chlorinating ability, the H_2O_2 concentration in its environment will determine whether the enzyme acts primarily as a catalase or peroxidase.

Neutrophils and monocytes provide the main defence against microbial infection. The invading microbe is engulfed into a phagocytic vacuole, where the reduced oxygen products of an oxidase electron-transport chain and proteins contained within cytoplasmic granules are released, and the organism is killed. The importance of the electron-transport chain is highlighted by observations that many bacteria are killed inefficiently by neutrophils either under anaerobic conditions or when the oxidase is defective, as in chronic granulomatous disease (Holmes *et al.*, 1968). How this oxidase system facilitates killing is not entirely clear. It generates O_2^- and H_2O_2, and possibly also $OH^\cdot$ (Badwey & Karnovsky, 1980), which may be directly toxic, and elevates the pH within the phagocytic vacuole to what might be the optimum for the antimicrobial activity of the cationic granule proteins (Segal *et al.*, 1981). The H_2O_2 may also act as a substrate for myeloperoxidase, which is released into the vacuoles.

Myeloperoxidase, with Cl^- as an electron donor, can catalyse the production of a microbicidal species which is very similar to, if not identical with, HOCl (Stelmaszynska & Zgliczynski, 1974; Harrison & Schultz, 1976; Klebanoff & Clark, 1978; Albrich *et al.*, 1981; Winterbourn, 1985). The kinetics of the reaction are complex, with a pH optimum of 5 (Bakkenist *et al.*, 1980; Andrews & Krinsky, 1982). HOCl formation is thought to proceed via a classical peroxidase mechanism in which H_2O_2 reacts with ferric enzyme (MP^{3+}) to

Abbreviations used: MP^{3+}, ferric myeloperoxidase; MP^{2+}, ferrous myeloperoxidase; MPO_2, oxyferrous myeloperoxidase or compound III; MCD, monochlorodimedon; PMA, phorbol myristate acetate; Me_2SO, dimethyl sulphoxide; SOD, superoxide dismutase.

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give compound I ($MP^{3+}\cdot H_2O_2$), which reacts with Cl^- to give HOCl (reactions 1 and 2 in Table 1) (Harrison & Schultz, 1976; Odajima & Yamazaki, 1970; Harrison *et al.*, 1980). In the absence of Cl^- or an alternative electron donor, compound II ($MP^{2+}\cdot H_2O_2$), accumulates as an inactive by-product (reaction 3, Table 1). In addition, MP^{3+} can undergo reaction (4) (Table 1) with O_2^- to give compound III ($MP^{3+}O_2^-$ or $MP^{2+}O_2$), which can also be formed reversibly (reaction 5, Table 1) from MP^{2+} and O_2 (Odajima & Yamazaki, 1970, 1972). Reaction 6, between myeloperoxidase and H_2O_2 at high concentrations, also produces $MP^{2+}O_2$ (hereafter referred to as 'MPO_2') (Odajima & Yamazaki, 1970). With horseradish peroxidase, the superoxide adduct is not considered to be on the main reaction pathway (Yamazaki & Yokota, 1973; Halliwell *et al.*, 1982), but little is known of the reactivity of MPO_2.

Although recent studies have shown that HOCl can be detected in the extracellular fluid when neutrophils are stimulated with soluble stimuli (Weiss *et al.*, 1982; Foote *et al.*, 1983), the reactions that myeloperoxidase normally undertakes within the phagocytic vacuole have not been clearly established. In particular, how the O_2^- that is continuously generated by the cells affects the form and function of myeloperoxidase has not been considered. The present study addresses this question, and also considers how the intravacuolar H_2O_2 concentration could affect the function of the enzyme.

Methods

Neutrophils were isolated from buffy-coat residues from normal donors (Segal *et al.*, 1981). Myeloperoxidase was purified from human neutrophil granules (Matheson *et al.*, 1981). The A_{430}/A_{280} ratio was 0.80, which compared favourably with 0.83 for the crystalline enzyme (Schultz & Shmukler, 1964) and that of 0.71 for the preparation obtained by Matheson *et al.* (1981). For experiments performed in the absence of Cl^-, the latter was removed by dialysis of the enzyme against a suspension of Amberlite MB.1 resin (BDH) in 10mM-potassium phosphate buffer pH7.5. Superoxide dismutase, catalase, xanthine oxidase (type III), monochlorodimedon (MCD) and other biochemicals were obtained from Sigma.

MPO_2 was prepared by reducing myeloperoxidase with a few crystals of sodium dithionite and applying approx. 0.1ml of the solution to a 4cm × 1cm-diameter column of Sephadex G-10 at below 4°C. The ferrous protein separates from the dithionite and reacts with O_2 dissolved in the buffer. Spectra of eluates showed 75–90% MPO_2, the remainder MP^{3+}. MP^{3+} concentrations were determined by using an ε_{430} (mM with respect to Fe) value of 91 (Odajima & Yamazaki, 1970), and MPO_2 concentrations were calculated by reducing with dithionite and comparing the A_{470} with that of a similarly treated solution of MP^{3+} of known concentration.

Difference or absolute spectra were recorded on a Pye–Unicam SP.8-200 spectrophotometer. Experiments with neutrophils were carried out in colourless RPMI 1640 medium (Flow Laboratories) containing Hepes (10mM, pH7.4) and heparin (51i.u./ml). All other experiments were carried out in 0.05M-sodium phosphate buffer, pH7.5, in the presence or absence of 100mM-NaCl. The extent of conversion of MP^{3+} into MPO_2 was determined from difference spectra by measuring A_{620} and comparing it with A_{620} observed when MP^{3+} was completely converted into MPO_2 by 2mM-H_2O_2 in the absence of Cl^- and the spectrum immediately recorded. Alternatively, the extent of conversion was determined from absolute spectra from the absorbance ratio of the 625nm and 425–435nm peaks (0.06 for MP^{3+} and 0.33 for MPO_2).

Before recording neutrophil spectra (Segal & Coade, 1978), the cells were temperature- and gas-equilibrated by stirring for 3min in an oxygen-electrode chamber with the tip of the chamber off. The stimulus, either PMA (1.0μl/ml of a 1mg/ml solution in Me_2SO), latex particles (Segal & Coade, 1978) or staphylococci opsonized with human IgG (Segal *et al.*, 1981), or serum-opsonized zymosan (Hurst *et al.*, 1984), was then added. After incubation with the stimulus, samples were aspirated with pre-cooled glass pipettes and transferred to plastic cuvettes on ice. Reference samples

Table 1. *Reactions of myeloperoxidase*

MP^{2+} and MP^{3+} refer to ferrous and ferric myeloperoxidase respectively. $MP^{3+}\cdot H_2O_2$, compound I; $MP^{2+}\cdot H_2O_2$, compound II; $MP^{2+}O_2$ (=$MP^{3+}O_2^-$), compound III.

$MP^{3+}+H_2O_2$	$\rightarrow MP^{3+}\cdot H_2O_2$	(1)
$MP^{3+}\cdot H_2O_2+Cl^-$	$\rightarrow MP^{3+}+OH^-+HOCl$	(2)
$MP^{3+}+MP^{3+}\cdot H_2O_2+2OH^-$	$\rightarrow 2MP^{2+}\cdot H_2O_2$	(3)
$MP^{3+}+O_2^-$	$\rightleftharpoons MP^{2+}O_2$	(4)
$MP^{2+}+O_2$	$\rightleftharpoons MP^{2+}O_2$	(5)
$MP^{2+}H_2O_2+H_2O_2$	$\rightarrow MP^{2+}O_2+2H_2O$	(6)
$MP^{2+}O_2+MP^{2+}H_2O_2$	$\rightarrow 2MP^{3+}+O_2+2OH^-$	(7)

were treated like the test samples, except that the additions were made after the cells had been cooled to 4°C. In the case of latex, non-opsonized particles were used as reference. Spectra were run on the cooled samples at a rate of 5nm/s. In some experiments on phagocytosing neutrophils, either superoxide dismutase, catalase or albumin was covalently bound to carboxylated latex (Muller *et al.*, 1980). The particles were then opsonized with IgG in the usual way. The concentrations of SOD and catalase bound to the particles were the same as described by Segal *et al.* (1983). When purified myeloperoxidase was added, samples were maintained at 25°C, with 10^7 neutrophils and 2.4mM-myeloperoxidase. After adding PMA, either absolute spectra against stimulated cells only, or difference spectra against unstimulated cells plus myeloperoxidase, were recorded.

Spectral changes in the presence of hypoxanthine and xanthine oxidase were recorded at 25°C with 3 μM-myeloperoxidase, 125 μM-hypoxanthine and 0.018 unit of xanthine oxidase/ml, against solutions containing all reagents except hypoxanthine. The rate of O_2^- production was 10 μM/min determined independently as the rate of cytochrome *c* reduction [ε_{550} (reduced − oxidized) 21100 litre·mol^{-1}·cm^{-1}]. To increase the proportion of univalent reduction of O_2 from 29 to 76%, the reaction was carried out in O_2 with 60 μM-hypoxanthine (Fridovich, 1970). These proportions were determined from the independently measured rates of cytochrome *c* reduction and of urate production, measured by continuously recording A_{290} (ε 14000 litre·mol^{-1}·cm^{-1}).

Rates of myeloperoxidase-dependent chlorination of MCD (100 μM) were determined at 25°C in the presence of 100mM-NaCl by continuously monitoring A_{290} ($A_{max.}$ for MCD at pH7.5, ε = 17700 litre·mol^{-1}·cm^{-1}). In most experiments, H_2O_2 consumption and chlorination were measured simultaneously. H_2O_2 concentrations were determined before and after the reaction, and HOCl production (equivalent to the MCD loss) relative to H_2O_2 consumption was calculated. In the absence of Cl^-, rates of H_2O_2 consumption were measured by determining H_2O_2 concentrations at the start and at intervals after adding myeloperoxidase.

H_2O_2 (0.05–2mM) was measured by allowing 0.8ml of the solution to react with 0.25ml of 20% (w/v) H_2SO_4 and 0.15ml of 1M-$TiSO_4$ (BDH). A_{408} was determined (after dilution if necessary) and the H_2O_2 concentration calculated by using ε_{408} 750 litre·mol^{-1}·cm^{-1}. Appropriate myeloperoxidase blanks were subtracted. With high protein concentrations, solutions were centrifuged before analysis.

Chlorination of MCD by myeloperoxidase and xanthine oxidase (0.01 unit/ml) plus hypoxanthine (125 μM) was measured as described above. The baseline was ΔA_{290} due to urate oxidation, which was independent of the concentration of myeloperoxidase, superoxide dismutase or catalase. Reaction rates were determined over the first 2min, when they were linear. Subsequently, accumulation of urate, which competed with MCD for the HOCl, caused progressive inhibition.

Chlorination of MCD by neutrophils stimulated by PMA [10 μl of a 0.1mg/ml solution freshly prepared in aq. 1% (w/v) Me_2SO] was measured with 2×10^6–5×10^6 cells in NaCl (150mM) containing sodium phosphate (5mM, pH7.4), $CaCl_2$ (2mM), glucose (10mM), Hepes (pH7.4, 10mM), and heparin (5i.u./ml). Changes in A_{290} relative to A_{330} (to account for differential sedimentation of the cells) either were continuously monitored against an unstimulated cell blank (with 100 μM-MCD) or were determined after 12min (with 800 μM-MCD). Chlorinating efficiencies in each incubation mixture were determined by measuring ΔA_{290} on adding a known concentration of HOCl. Rates of O_2^- production were measured independently with 40 μM-cytochrome *c*.

Catalase activity of MP^{3+} was measured by the evolution of O_2 in an oxygen-electrode chamber (Rank Brothers, Bottisham, Cambridge, U.K.).

Results

Spectral changes of myeloperoxidase in stimulated neutrophils

For comparison, absolute and difference spectra of MP^{3+}, MPO_2 and $MP^{2+}\cdot H_2O_2$ are shown in Fig. 1.

Cells stimulated with opsonized latex, staphylococci and zymosan or PMA underwent marked spectral changes (Fig. 2, spectra a, c and d). Three main patterns could be distinguished. (i) The α-(559nm) and γ-(428nm) peaks of cytochrome b_{-245} developed when the system became anaerobic and disappeared on reoxygenation (Fig. 2, spectrum e). (ii) A deep trough, maximal at 415nm and superimposed on the γ-peak of cytochrome *b*. Its origin has not been identified, but it is most likely due to a conformational change in myeloperoxidase associated with degranulation and not with products of the respiratory burst, since it occurred in cells from patients with chronic granulomatous disease (Fig. 2, spectra g and h). (iii) Peaks at about 620 and 450nm (marked with arrows in Fig. 2), which could correspond to either MPO_2 or $MP^{2+}\cdot H_2O_2$. These two peaks were also produced when high concentrations of H_2O_2 were added to resting cells (Fig. 2, spectrum f).

With all the stimuli, the development of the 620 and 450nm peaks had the same kinetics as the

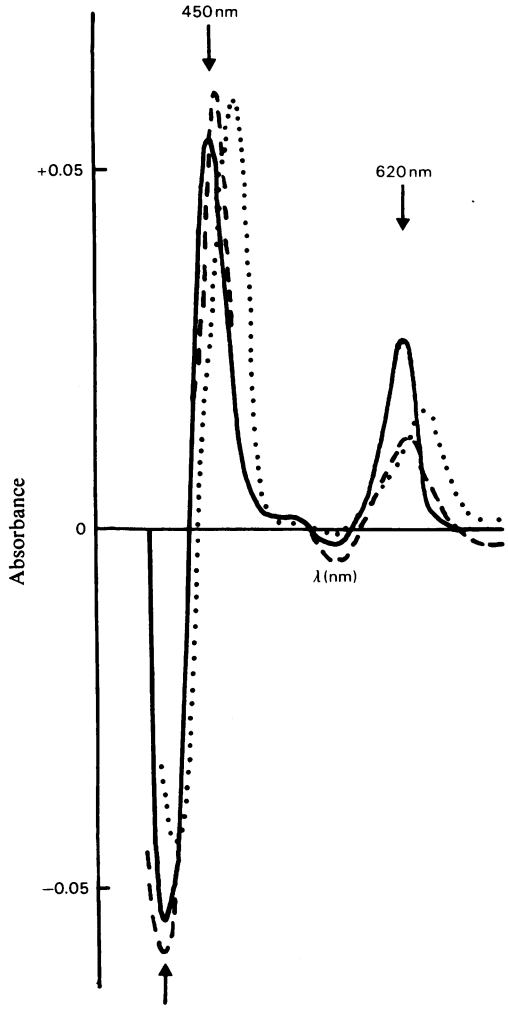

Fig. 1. *Difference spectra of oxyferrous myeloperoxidase (MPO_2), compound II ($MP{\cdot}H_2O_2$) and ferrous myeloperoxidase (MP^{2+}) against the ferric enzyme (MP^{3+})*
All concentrations were approx. 2 μM (with respect to iron): ····, MP^{2+}; ——, MPO_2; – – – –, $MP{\cdot}H_2O_2$.

respiratory burst. With latex these peaks first became apparent after about 30s, were maximal between about 1 and 3min and then disappeared over the next few minutes. With particulate stimuli, neither SOD nor catalase had a clear effect on the spectrum, whether added in solution (SOD, 50 μg/ml; catalase, 34×10^3 units/ml) or coupled to the latex. Azide greatly amplified the heights of the peaks (Fig. 2, spectrum b), possibly by allowing the accumulation of high concentrations of H_2O_2. Evidence for the formation of MPO_2 is a peak shift from 450 (MP^{2+}) to 470nm (MPO_2) in cells that become anaerobic through the consumption of available oxygen (identifiable by the presence of the reduced-cytochrome b_{-245} peak at 429nm), and reversal on reoxygenation. This is best seen with opsonized zymosan (Fig. 2, spectra d and e), but a mixture of the two species of myeloperoxidase (a peak at 450 and shoulder at about 470nm) can be seen with cells stimulated with latex and PMA (Fig. 2, spectra a and c). These changes were markedly diminished in cells stimulated under anaerobic conditions from the outset.

Spectral changes of purified myeloperoxidase induced by PMA-stimulated neutrophils or xanthine oxidase

Purified myeloperoxidase added to neutrophils stimulated with PMA also underwent spectral changes indicating conversion of MP^{3+} into MPO_2. The positions and relative heights of the peaks were the same as for MPO_2 (Fig. 1). The spectral changes indicated complete conversion of MP^{3+} into MPO_2, and occurred within a few minutes of adding PMA and remained stable for more than 10min. If the cell suspension became anaerobic, the spectrum changed to that of MP^{2+}. The spectral changes were prevented by superoxide dismutase (10 μg/ml) in the medium, but catalase had no effect.

Hypoxanthine and xanthine oxidase in the presence of 100mM-Cl^- also caused myeloperoxidase to undergo spectral changes indicative of conversion of MP^{3+} into MPO_2. These were completely inhibited by superoxide dismutase. Only 60–70% of the MP^{3+} was converted by MPO_2. This percentage was increased to 80% by adding catalase or to 88% by using less hypoxanthine and an O_2 atmosphere, thereby increasing the ratio of O_2^- to H_2O_2 produced directly by the xanthine oxidase. If 50 μM-H_2O_2 was present before adding MP^{3+}, there was only 50% conversion. Thus the amount of conversion of MP^{3+} into MPO_2 depended on the initial ratio of O_2^- to H_2O_2, and the complete conversion observed with neutrophils implies that, at least in the presence of myeloperoxidase, there is little if any direct production of H_2O_2 by the cells.

In the absence of Cl^-, MP^{3+} was converted by hypoxanthine and xanthine oxidase into compound II. Superoxide dismutase had no effect, but in the presence of catalase, the product was MPO_2.

Stability of MPO_2 and reactions with H_2O_2 and compound II

MPO_2, produced by reducing then re-oxygenating MP^{3+}, was stable for several hours at 0°C, but at 25°C serial spectra showed autoxidation to MP^{3+}, with a half-life ($t_{\frac{1}{2}}$) of 12min. This rate was the same in the presence or absence of Cl^-, or of catalase, but was faster in the presence of superoxide dismutase (Fig. 3), and substantially

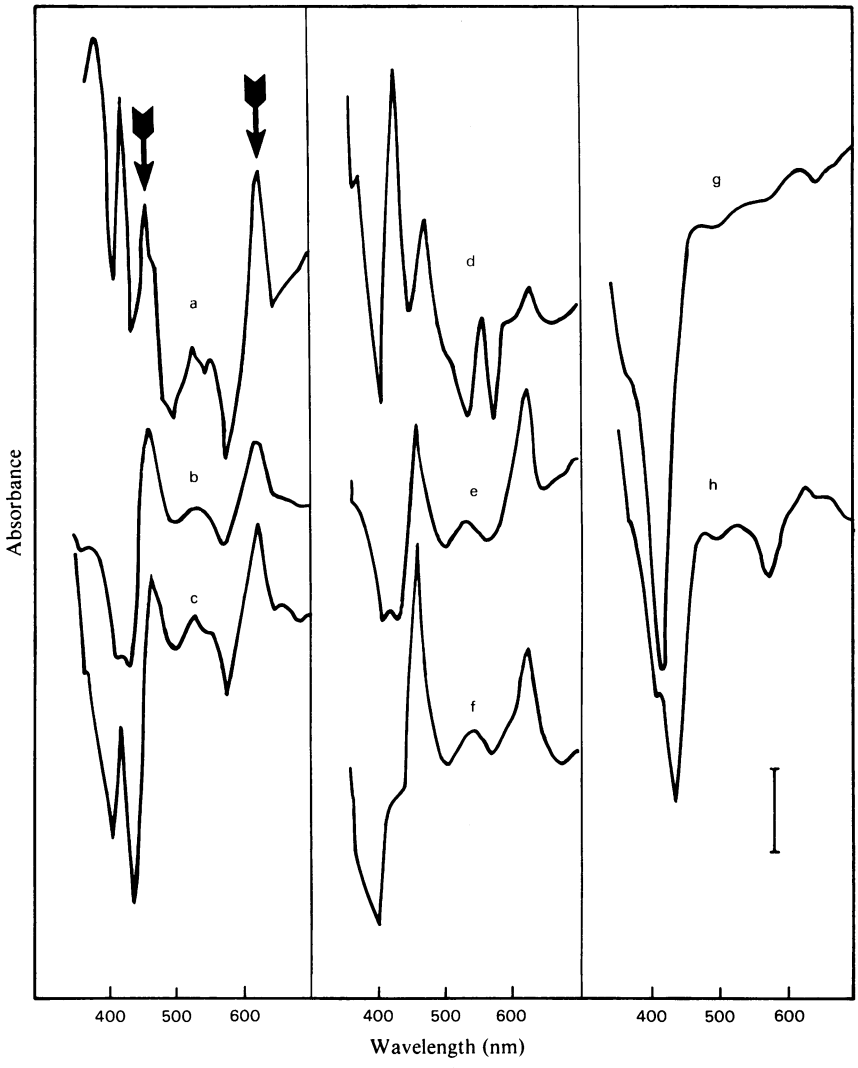

Fig. 2. *Difference spectra of intact neutrophils*

Spectra were run 90s after the addition of various materials listed below to the cells (1.7×10^8/ml) as described in the Methods section. a, Latex particles (1×10^9/ml) opsonized with IgG; b, as for a, except azide (1mM) added to the cells 1min before the latex; c, PMA (1 μg/ml); d, serum-opsonized zymosan (1mg/ml); e, reoxygenation of d by air; f, H_2O_2 (10mM); g, cells (3.3×10^7/ml) from a patient with chronic granulomatous disease after stimulation with PMA, compared with the same number of cells from a healthy subject, h. In d, 17% of the cytochrome b_{-245} and 3.5% of the myeloperoxidase were reduced. Absorbance scale marker = 0.005 absorbance unit for a, 0.05 for b, 0.01 for c–f and 0.02 for g and h. Arrows indicate the peaks of MPO_2 at 450 and 620nm.

decreased in the presence of O_2^- generated from xanthine oxidase and hypoxanthine.

The spectral changes observed on adding H_2O_2 to MPO_2 in the absence of Cl^- are shown in Fig. 4(*a*). MPO_2 solutions always contained 10–25% MP^{3+}, and the initial change, occurring in less than 1min, was the conversion of MP^{3+} into compound II. Compound II disappeared within a few minutes, and the spectrum then closely resembled the inverse of the MPO_2-versus-MP^{3+} difference spectrum. This indicates conversion of both compound II and MPO_2 into MP^{3+}, with the MPO_2 disappearing more rapidly than in the slowly autoxidizing reference solution. The initial increase in A_{625} (Fig. 4*b*) is due to the conversion of MP^{3+} into compound II, then, after 2–3min of rapid decrease, the curve is the same as for autoxidizing MPO_2. The spectral changes can be

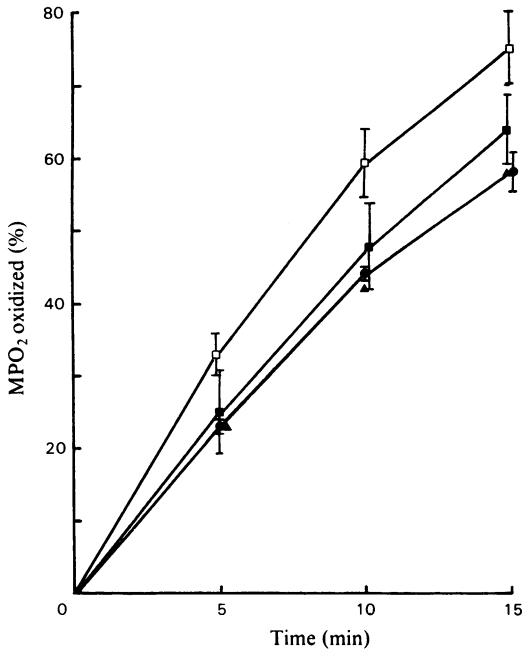

Fig. 3. *Rate of autoxidation of MPO_2*
MPO_2 was prepared as described in the Methods section and the reaction was monitored spectrally at 25°C and pH 7.5 in the absence (●) or presence (▲) of 100mM-NaCl. ■, Plus catalase; □, plus SOD. Means ± S.D. from three (no enzymes or plus catalase) or ten experiments (plus SOD) are shown.

explained by the continual formation of compound II via reactions (1) and (3) (Table 1) until all the H_2O_2 was consumed, and disappearance of compound II and MPO_2 via reaction (7), the net effect being breakdown of H_2O_2 to O_2 and H_2O. In the presence of 100mM-Cl^-, similar spectral changes were observed, but they occurred more rapidly and were of lesser magnitude. In this case, reaction (2) would be favoured over reaction (3), consuming H_2O_2 and resulting in less compound II being available for reaction (7).

There was no spectral evidence for a direct reaction between H_2O_2 and MPO_2 in the presence or absence of Cl^-.

Chlorination and H_2O_2 consumption by MP^{3+}

The myeloperoxidase-catalysed reaction between H_2O_2 and Cl^- was detected by its converting MCD, which absorbs at 290nm, into non-absorbing dichlorodimedon (Hager *et al.*, 1966; Harrison & Schultz, 1976; Bakkenist *et al.*, 1980). As shown in Fig. 5(*a*), at pH 7.5 and with 0.1M-Cl^-, the rate of HOCl production was maximal with 50–100 μM-H_2O_2, and then showed the expected steady decrease. Myeloperoxidase at this concentration of enzyme was fully converted into MPO_2 by 1mM-H_2O_2 and there was very little conversion with 0.4mM. With five-times-more-concentrated myeloperoxidase, the rate of HOCl production per unit of enzyme was similar to that in Fig. 5, but 1mM-H_2O_2 gave only about 30% conversion into MPO_2. Thus the decrease in chlorinating ability does not parallel the increase in proportion of MPO_2. Although HOCl formation fell dramatically with increasing H_2O_2 concentration, H_2O_2 consumption slightly increased (Fig. 5*b*). Thus the enzyme catalysed H_2O_2 breakdown at high H_2O_2 concentrations, and as a consequence, conversion of H_2O_2 into HOCl decreased from nearly 100% with 50–100 μM-H_2O_2 to less than 3% (Fig. 5*c*).

MP^{3+} also catalysed H_2O_2 breakdown in the absence of Cl^- or other electron donors. At low H_2O_2 concentrations, this reaction was slower than in the presence of Cl^-, but there was a greater increase in rate with increasing H_2O_2 concentration (Fig. 6). The reaction resulted in the evolution of O_2. The addition of 0.72, 1.43 and 2.86nmol of MP^{3+} to 1ml of H_2O_2 (2mM) at 37°C caused the evolution of O_2 at rates of 588, 1344 and 2268nmol/min respectively, compared with a basal rate of 246 ± 57nmol/min. O_2 evolution was not affected by the addition of NaCl (100mM). These findings were not due to contamination by catalase, since similar rates of O_2 evolution were obtained with myeloperoxidase that had been further purified by isoelectric focusing [pI > 11, compared with neutrophil catalase, about 5.5 (A. W. Segal & A. M. Harper, unpublished work).

Chlorination and H_2O_2 consumption by MPO_2

MPO_2 (containing 15–35% MP^{3+}) also catalysed chlorination of MCD by H_2O_2 and Cl^-. As with MP^{3+}, rates were linear for several minutes after adding the enzyme. In 13 paired assays, with H_2O_2 concentrations from 40 to 600 μM, the rate of chlorination by MPO_2 was 94 ± 9% of that with MP^{3+}. This marginal difference in rate is not nearly enough for MP^{3+} to be the only active form of the enzyme. In addition, SOD did not accelerate the reaction, as would be expected if autoxidation of MPO_2 were a necessary step. The H_2O_2-dependence of the reaction was similar to that with MP^{3+}, as was the ability of MPO_2 to catalyse H_2O_2 breakdown, both in the presence and absence of Cl^- (Figs. 5*b* and 6). Thus the chlorinating efficiency of MPO_2 (□, Fig. 5*c*) was indistinguishable from that with MP^{3+}.

Chlorination by myeloperoxidase, hypoxanthine and xanthine oxidase

We have already shown that hypoxanthine and xanthine oxidase in air cause 60–70% conversion of

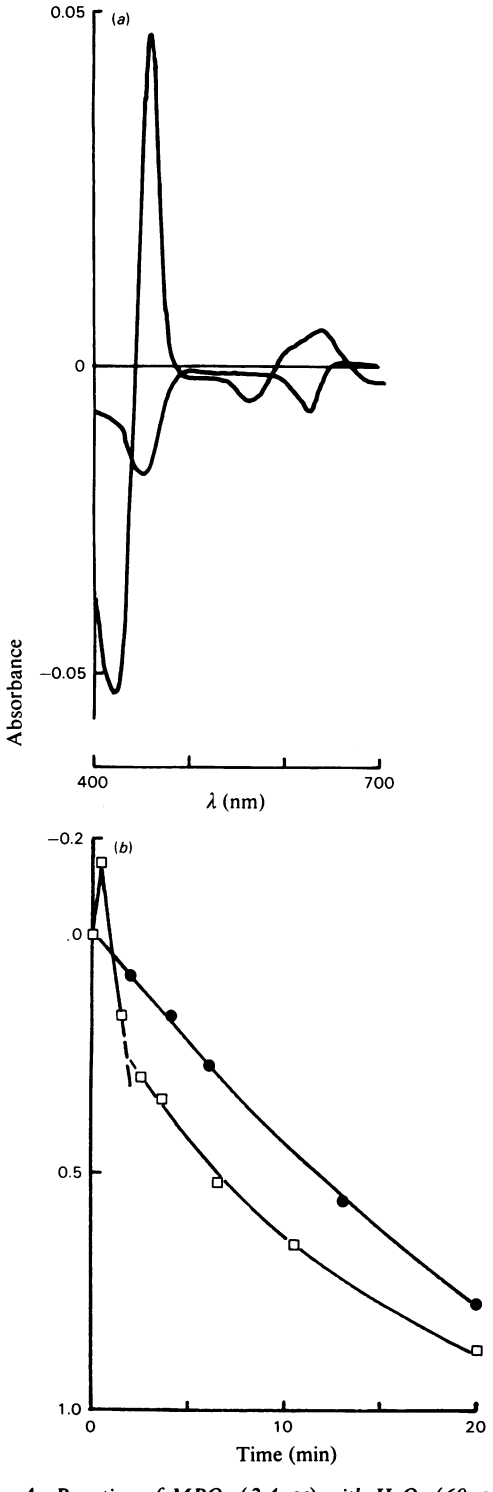

Fig. 4. *Reaction of MPO_2 (2.4 μM) with H_2O_2 (60 μM)* (*a*) Difference spectra (against MPO_2) recorded before adding H_2O_2 and after 0.5 and 3 min. (*b*) Progressive change in A_{625} recorded under the same conditions (□), compared with the change due to autoxidation (●). The MPO_2, prepared as described in the Methods section, contained 15% MP^{3+}. The reaction was at pH 7.5 in the absence of Cl^-.

MP^{3+} into MPO_2. The rate of chlorination of MCD by xanthine oxidase and hypoxanthine increased from zero with no myeloperoxidase to a maximum with 0.6 μM or higher concentrations, when 81–94% of the O_2 consumed (calculated from the rate of urate production) gave MCD chlorination. Addition of SOD prevents conversion of MP^{3+} into MPO_2, thereby increasing the concentration of MP^{3+} approx. 3-fold. Therefore, with

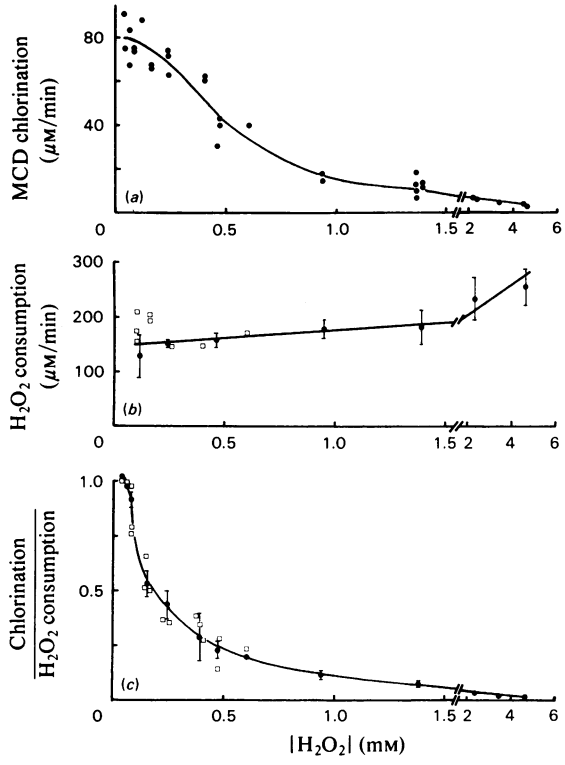

Fig. 5. *Comparison of HOCl production and H_2O_2 consumption by myeloperoxidase at different H_2O_2 concentrations*

(*a*) Rate of MCD chlorination from 1.8 μM-MP^{3+} in phosphate buffer, pH 7.5, containing 100 mM-NaCl. (*b*) Rate of H_2O_2 consumption under same conditions. ●, MP^{3+}; □, MPO_2. For MP^{3+}, means ± S.D. for three to six analyses at each H_2O_2 concentration, plus the calculated regression line are shown. For MPO_2, individual analyses are plotted. (*c*) Ratio of MCD chlorinated to H_2O_2 consumed. ●, MP^{3+} (means ± S.D. as for *b*); and □, MPO_2 (individual analyses). Methods of MPO_2 preparation and measuring MCD chlorination and H_2O_2 consumption are given in the Methods section.

Table 2. *Chlorination of MCD by PMA-stimulated neutrophils*

Cells (5×10^6) in 0.44ml of buffered medium at 25°C containing 0.8mM-MCD were stimulated with PMA. Samples (0.1ml) were removed at 0 and 12min, added to 0.7ml of ice-cold buffer, centrifuged for 2min in an MSE Micro Centaur microcentrifuge and the A_{290} and A_{330} were measured. ΔA_{330} values (<0.009) were subtracted as blanks. Results quoted are the means and range obtained in duplicate assays. Superoxide production rate was 40 μM/min, with an approx. 2min delay, so theoretically approx. 200 μM-H_2O_2 was produced during the experiment.

Conditions	ΔA_{290}/12min	HOCl produced* (μM)
No MCD	0.002	<2
MCD	0.071 ± 0.003	60 ± 2
+0.6 μM-MP^{3+}	0.096	81
+2.5 μM-MP^{3+}	0.102 ± 0.009	86 ± 7
+0.6 μM-MP^{3+} + 1mM-Azide	0.009 ± 0.014	8 ± 13 (4)
+0.6 μM-MP^{3+} + 10 μg of SOD/ml	0.095 ± 0.007	80 ± 6
+2.5 μM-MP^{3+} + 10 μg of SOD/ml	0.099 ± 0.012	82 ± 10

* Calculated by allowing for chlorinating efficiencies (approx. 50%) as described in the Methods section.

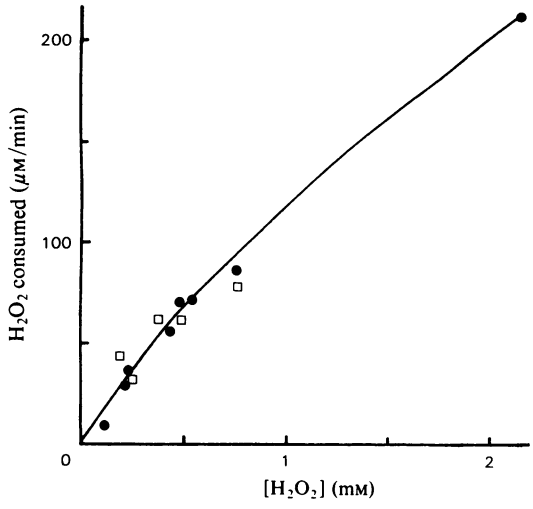

Fig. 6. *Rates of H_2O_2 consumption by 1.8 μM-MP^{3+} (●) and MPO_2 (□) in phosphate buffer, pH 7.5, in the absence of Cl^-*

H_2O_2 consumption was calculated from the H_2O_2 concentrations determined at the start of each reaction and at two points during the first 4–6min.

sub-maximal concentrations of myeloperoxidase, if MP^{3+} were much more reactive than MPO_2, it should also increase the rate of chlorination. However, with 0.15 or 0.3 μM-myeloperoxidase, SOD (10 μg/ml) had no measurable effect. Catalase, on the other hand, inhibited chlorination. With 0.5 μM-myeloperoxidase, 6 μg of catalase/ml gave 65% and 25 μg/ml gave 93% inhibition.

Chlorination of MCD by PMA-stimulated neutrophils

Myeloperoxidase plus neutrophils stimulated with PMA, after a lag of about 2min, caused a linear decrease in MCD absorbance. Typically, with 2×10^6 cells, producing 5.2 μM-O_2/min, the rate of chlorination of 100 μM-MCD was 0.70 μM/min. Chlorination was decreased to <8% by catalase, but SOD had no effect (103 ± 22%, $n = 5$). That the A_{290} changes were due to myeloperoxidase-dependent chlorination is clear from Table 2, showing no change in the absence of MCD, and almost complete inhibition by azide. Externally added myeloperoxidase was not necessary, although it increased the amount of chlorination. Under these conditions chlorination was not affected by SOD, and almost half the theoretically produced H_2O_2 gave rise to HOCl accessible to MCD.

Discussion

These studies show that neutrophils stimulated by phagocytosis or soluble stimuli induce the conversion of MP^{3+} into MPO_2 and that this compound is reactive, undertaking chlorination and/or catalase activity depending on the concentration of H_2O_2.

The production of MPO_2 is most clearly seen when MP^{3+} is present in the extracellular environment of neutrophils stimulated with a soluble stimulus and conversion is complete. The spectral changes observed with phagocytosing cells are more complicated, but the presence of MPO_2 is indicated by peaks at 450 and 620nm which convert reversibly to the spectrum of MP^{2+} upon deoxygenation. MPO_2 could be produced by the reaction of MP^{3+} either with O_2^- or with high concentrations of H_2O_2. With exogenous myeloperoxidase it is clearly the former. This is most likely to be the case also with intravacuolar myeloperoxidase and particulate stimuli, even though the change was not inhibited by soluble or latex-

coupled SOD. Catalase did not inhibit either, and it is probable that sufficiently high intravacuolar concentrations of the enzymes were not achieved with either method.

What then happens to the MPO_2? It is obviously not an end product, because it fails to accumulate. Our studies with purified MPO_2 enzyme have shown that, although it is more stable than the superoxide adduct of other peroxidases (Yokota & Yamazaki, 1965; Phelps *et al.*, 1974), it does gradually autoxidize. It does not react with H_2O_2 or Cl^- directly, but reacts with compound II to regenerate MP^{3+} (reaction 7, Table 1). Rather surprisingly, we found it to catalyse HOCl formation at practically the same rate as MP^{3+}. Experiments (*a*) comparing rates of chlorination of MCD by MP^{3+} and MPO_2, and (*b*) in which SOD was added to either stimulated neutrophils or hypoxanthine and xanthine oxidase to prevent conversion of MP^{3+} into MPO_2, support this conclusion. Regarding the mechanism, we found no evidence for a direct reaction between MPO_2 and Cl^-. This contrasts with horseradish peroxidase, which can react with some electron donors, but several orders of magnitude more slowly than does compound I (Yokota & Yamazaki, 1965; Tamura & Yamazaki, 1972). It appears that MPO_2 can enter the main chlorination pathway involving compound I (reactions 1 and 2), possibly via reaction (7).

Although it is well documented that H_2O_2 inhibits chlorination by myeloperoxidase (Bakkenist *et al.*, 1980; Andrews & Krinsky, 1982), catalase activity is not usually considered. Nevertheless, it is evident from our findings that, whereas at low H_2O_2 concentrations of MP^{3+} or MPO_2, chlorination is almost 100% efficient, as the H_2O_2 concentration increases, catalytic breakdown of H_2O_2 without HOCl formation becomes progressively more important.

How then can we relate these reactions to the normal biology of phagocytosing neutrophils? The most direct evidence for the chlorination reaction is in response to a soluble stimulus such as PMA, as in our experiments, or those of Weiss *et al.* (1982) and Foote *et al.* (1983). Under these circumstances, both H_2O_2 (Test & Weiss, 1984), and myeloperoxidase are present at low concentration in the extracellular environment, and the conditions would favour chlorination. These conditions are likely to pertain in the body when neutrophils are faced by a soluble stimulus, e.g. complement subcomponent C5a, or a surface that they cannot engulf, such as a fungal mycelium, in which case discharge of diffusable chlorinating species would be clearly advantageous.

The conditions within the phagocytic vacuole would be much less conducive to chlorination. Although the MPO_2 formed is catalytically active, our studies show that, at pH values of 7.4–7.8, which exist during the period when bacterial killing occurs (Segal *et al.*, 1981), myeloperoxidase at H_2O_2 concentrations above about 0.5 mM functions predominantly as a catalase rather than as a peroxidase. The H_2O_2 concentration could therefore be restrictive. We have estimated the intravacuolar H_2O_2 concentration that could be achieved on ingestion of bacteria from the rate of O_2 uptake and the vacuole volume. [Assuming saturation of phagocytosis at about 50 bacteria per cell and relatively homogeneous degranulation, approx. 2% of the cells' complement of myeloperoxidase (about 5% of the total protein) will enter each vacuole (4×10^{-19} mol/vacuole). The normal oxygen consumption is about 40 nmol/min per 10^7 cells with each cell taking up about 10 particles, giving a rate of about 4×10^{-16} mol of O_2 (and two to four times greater of O_2^-) in each vacuole per min. This would require the myeloperoxidase to turn over about a thousand cycles, which would take several minutes at this pH. The volume of the vacuole containing a bacterium is about 5 μm^3, giving a production rate of 100 mM-H_2O_2/min.] Provided there was little diffusion or breakdown, this could be in the region of 100 mM. Evidence suggesting that there may be very little intravacuolar chlorination comes from a study of normal and myeloperoxidase-deficient cells (Nauseef *et al.*, 1983), showing that both primarily break down H_2O_2 catalytically rather than peroxidatively. Also, although HOCl, and myeloperoxidase and H_2O_2 acting under conditions in which chlorination occurs, bleach both free fluorescein and fluorescein bound to bacteria or zymosan (Hurst *et al.*, 1984), this bleaching is not observed after the coated particles have been phagocytosed (Cech & Lehrer, 1984; Segal *et al.*, 1981).

A possible series of reactions within the phagocytic vacuole could be the following. The microbicidal electron-transport chain releases O_2^-, where a proportion of it dismutates to form H_2O_2. O_2^- and H_2O_2 combine with MP^{3+} to form MPO_2 and $MP^{2+}H_2O_2$ respectively, and these compounds then react to release O_2 and regenerate MP^{3+}. In this situation the myeloperoxidase is acting as an additional component, a terminal oxidase or catalase, of the electron-transport chain. This would allow the oxidase system to elevate the pH within the phagocytic vacuole without the excessive accumulation of O_2^-, H_2O_2 or HOCl and concomitant oxidative denaturation of cellular and granule constituents. As the respiratory burst terminates and the pH decreases, or under relatively anaerobic conditions, small amounts of chlorinating species could then be produced. It has recently been shown (Hamers *et al.*, 1984)

that myeloperoxidase may play a more important role in the secondary inactivation of bacterial enzymes rather than in the actual killing process, which would be compatible with these proposals.

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