Thiocyanate and chloride as competing substrates for myeloperoxidase

Christine J. van DALEN, Michael W. WHITEHOUSE, Christine C. WINTERBOURN and Anthony J. KETTLE[1]

Free Radical Research Group, Department of Pathology, Christchurch School of Medicine, P.O. Box 4345, Christchurch, New Zealand

The neutrophil enzyme myeloperoxidase uses H_2O_2 to oxidize chloride, bromide, iodide and thiocyanate to their respective hypohalous acids. Chloride is considered to be the physiological substrate. However, a detailed kinetic study of its substrate preference has not been undertaken. Our aim was to establish whether myeloperoxidase oxidizes thiocyanate in the presence of chloride at physiological concentrations of these substrates. We determined this by measuring the rate of H_2O_2 loss in reactions catalysed by the enzyme at various concentrations of each substrate. The relative specificity constants for chloride, bromide and thiocyanate were 1:60:730 respectively, indicating that thiocyanate is by far the most favoured substrate for myeloperoxidase. In the presence of 100 mM chloride, myeloperoxidase catalysed the production of hypothiocyanite at concentrations of thiocyanate as low as 25 μM. With 100 μM thiocyanate, about 50 % of the H_2O_2 present was converted into hypothiocyanite, and the rate of hypohalous acid production equalled the sum of the individual rates obtained when each of these anions was present alone. The rate of H_2O_2 loss catalysed by myeloperoxidase in the presence of 100 mM chloride doubled when 100 μM thiocyanate was added, and was maximal with 1 mM thiocyanate. This indicates that at plasma concentrations of thiocyanate and chloride, myeloperoxidase is far from saturated. We conclude that thiocyanate is a major physiological substrate of myeloperoxidase, regardless of where the enzyme acts. As a consequence, more consideration should be given to the oxidation products of thiocyanate and to the role they play in host defence and inflammation.

INTRODUCTION

The haem enzyme myeloperoxidase uses H_2O_2 to oxidize chloride to hypochlorous acid [1]. This is the most reactive oxidant produced by neutrophils in appreciable amounts [2–4]. Because of its broad reactivity, it is a prime candidate for promoting the oxidative killing of micro-organisms by neutrophils and the inflammatory tissue damage that these cells cause [5]. However, myeoperoxidase is relatively non-specific with respect to its reducing substrate and has the potential to catalyse the production of a variety of reactive species that could contribute to oxidative reactions of neutrophils [6].

Myeloperoxidase oxidizes bromide, iodide and thiocyanate as well as chloride [7]. Chloride is generally accepted to be its physiological substrate because it is present at high concentrations in plasma (100–140 mM) [8,9]. In contrast, iodide (< 1 μM), bromide (20–100 μM) and thiocyanate (20–120 μM) are present in relatively low concentrations [10,11]. Furthermore, myeloperoxidase promotes bacterial killing most effectively in solutions containing physiological concentrations of chloride as opposed to bromide, iodide or thiocyanate [8]. Myeloperoxidase oxidizes bromide and iodide to hypobromous acid and hypoiodous acid respectively [7]. By analogy with other peroxidases, it is considered to oxidize thiocyanate to hypothiocyanite either directly or via initial formation of thiocyanogen which rapidly hydrolyses to hypothiocyanite [12]. The pK_a of hypothiocyanous acid is 5.3 so that at physiological pH it is present predominantly as hypothiocyanite [13].

Little attention has been paid to the substrate preference of myeloperoxidase when a mixture of halides or the pseudohalide thiocyanate are present with the enzyme. Thomas and Fishman [14] investigated the oxidants produced by neutrophils in the presence of chloride and thiocyanate. They concluded that, under conditions similar to those in plasma, hypochlorous acid was the major product of myeloperoxidase, with little or no accumulation of hypothiocyanite. In contrast, under conditions similar to those of saliva, where thiocyanate is present at 1–5 mM [15], hypothiocyanite was the dominant oxidant formed. In this investigation we show that thiocyanate is by far the most preferred substrate for myeloperoxidase. Furthermore, in contrast with the results of Thomas and Fishman [14], at plasma concentrations of chloride and thiocyanate, up to half of the H_2O_2 used by myeloperoxidase is converted into hypothiocyanite. Thus thiocyanate is a major physiological substrate of myeloperoxidase.

MATERIALS AND METHODS

Materials

Myeloperoxidase was purified from human leucocytes as described previously [16]. Its purity index (A_{430}/A_{280}) was greater than 0.72 and its concentration was determined using ϵ_{430} 91000 $M^{-1}\cdot cm^{-1}$/haem [17]. Bovine liver catalase, 5,5′-dithiobis-(2-nitrobenzoic acid) and monochlorodimedon were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). 5-Thio-2-nitrobenzoic acid (TNB) was prepared from 5,5′-dithiobis-(2-nitrobenzoic acid) as described previously [18]. H_2O_2 solutions were prepared daily by diluting a 30 % stock and calculating its concentration by measuring its A_{240} (ϵ_{240} 43.6 $M^{-1}\cdot cm^{-1}$) [19]. Hypochlorous acid was purchased from Reckitt and Colman (NZ) Ltd. (Auckland, New Zealand). Its concentration was determined by measuring the decrease in A_{290} after allowing it to react with monochlorodimedon (ϵ_{290} 19000 $M^{-1}\cdot cm^{-1}$) [20]. All other chemicals were of the highest grade available.

Neutrophil extracts

Neutrophils were isolated from the blood of normal individuals and a myeloperoxidase-deficient individual as described previously [21]. Proteins were extracted from the neutrophil preparations by sonicating them in 35 mM β-alanine buffer, pH 4.5, with acetic acid, containing 0.3 % cetyltrimethylammonium

Abbreviation used: TNB, 5-thio-2-nitrobenzoic acid.

[1] To whom correspondence should be addressed.

bromide (cetrimide). The lysed cells were centrifuged at 9600 ***g*** (10000 rev/min) to pellet debris, and the supernatant was retained for analysis.

Gel electrophoresis

The purity of the myeloperoxidase preparations was determined by SDS/PAGE and native PAGE. The SDS/polyacrylamide gels contained 12 % acrylamide with a 3 % stacking gel. Samples were prepared and separated under conditions described previously [22].

Native PAGE was carried out essentially as described by Maurer [23] for his gel system number 8. Protein samples were separated on a 10 % polyacrylamide gel containing cetrimide (0.05 %, w/v) and glycerol (25 %, w/v). The electrode buffer contained the same concentrations of cetrimide and glycerol. The gel was photopolymerized using riboflavin (0.04 %, w/v) as the catalyst and by shining a 75 W UV B light source on it for 15 min. The gel was then pre-electrophoresed for 45 min at 100 V. Protein samples were mixed with equal volumes of sample buffer containing 25 % glycerol and Crystal Violet as the tracking dye before loading on to the gel. After the gel had been run to completion, it was washed in distilled water and incubated for 10 min in buffer A (10 mM sodium citrate, 10 mM disodium EDTA, pH 5.0), then 15 min in buffer B (buffer A with 1 % dextran sulphate), washed again with distilled water and then a further three times with buffer A. The gel was stained for peroxidase activity by adding 20 ml of buffer A containing 400 μM tetramethylbenzidine, 400 μl of DMSO, 5 mM NaBr and 200 μM H_2O_2. Development was stopped by washing several times with distilled water.

Measurement of hypohalous acid production by myeloperoxidase

Production of hypochlorous acid and hypothiocyanite by myeloperoxidase was determined by measuring the extent to which they bleached TNB [24]. Oxidants were allowed to accumulate, added to TNB and then the decrease in A_{412} was measured (ϵ_{412} 14100 $M^{-1} \cdot cm^{-1}$) [25].

Alternatively, TNB was included in the reaction mixture and A_{412} was monitored continuously. Under these conditions the rate of TNB oxidation was independent of its concentration, and therefore represents the rate at which hypohalous acids are produced by myeloperoxidase.

Hypochlorous acid was also determined by scavenging it with taurine (10 mM), which is converted into taurine chloramine and assayed by its ability to oxidize TNB [18].

Measurement of H_2O_2 utilization by myeloperoxidase

The utilization of H_2O_2 by myeloperoxidase in the presence of chloride, bromide or thiocyanate was measured continuously with a YSI 2510 oxidase probe fitted to a YSI model 25 oxidase meter (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.) [26]. The electrode was covered with a single membrane of collagen film. Glutathione was included in the reaction mixtures to scavenge the hypohalous acids and prevent them from interfering with the electrode signal [12,24]. Initial rates of H_2O_2 loss were determined by calculating the rate of loss over the first 10 s of the reaction.

Determination of the relative rate of reaction of hypochlorous acid with thiocyanate

The rate of reaction of thiocyanate with hypochlorous acid relative to monochlorodimedon was determined as described previously [27]. Hypochlorous acid (20 μM) was added to a vortexing solution of 10 mM phosphate buffer, pH 7.4, containing 140 mM NaCl, 100 μM monochlorodimedon and variable concentrations of thiocyanate. The extent of chlorination of monochlorodimedon was measured by determining its decrease in A_{290}.

Spectral analysis of myeloperoxidase

The visible absorbance spectrum of myeloperoxidase was recorded during the oxidation of thiocyanate using a Beckman 7500 diode array spectrophotometer. Reactions were started by adding 100 μM H_2O_2 to 500 nM myeloperoxidase and 100 μM thiocyanate in 100 mM phosphate buffer, pH 7.4, at room temperature. The reaction of thiocyanate with compound II was also investigated. Compound II was formed by adding 100 μM H_2O_2 to 500 nM myeloperoxidase in 100 mM phosphate buffer, pH 7.4, followed by 20 μg/ml catalase to scavenge excess H_2O_2. Thiocyanate (100 μM) was then added to the enzyme.

RESULTS

Purity of myeloperoxidase

Before investigating the substrate preference of myeloperoxidase, it was essential to ensure that our enzyme preparation was free of eosinophil peroxidase. This enzyme of eosinophils readily oxidizes thiocyanate to hypothiocyanite [28] and could co-purify with myeloperoxidase. The myeloperoxidase used in these studies had RZ values (A_{430}/A_{280}) of at least 0.7, which indicates that it was greater than 85 % pure. There was no shoulder at 412 nm in its absorption spectrum, which would indicate the presence of eosinophil peroxidase [29]. SDS/PAGE under reducing conditions gave three bands with molecular masses of 57, 39 and 15.5 kDa (result not shown), as reported by others [30–32]. We also ran native gels of our enzyme preparations and stained for peroxidase activity (Figure 1). An extract from granulocytes consisting of predominantly neutrophils had two major bands of activity. The upper band in lane 2 (Figure 1) was due to myeloperoxidase since the corresponding band in an extract from myeloperoxidase-deficient cells (lane 3) was very faint. The lower band in lanes 2 and 3 has the position and intensity expected for eosinophil peroxidase which is more basic than myeloperoxidase and accounts for about 15 % of the peroxidase activity in neutrophil preparations [14]. In lane 2, activities of the two peroxidases appear equal because the staining was enhanced

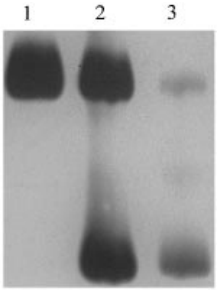

Figure 1 Assessment of the purity of myeloperoxidase

Proteins in preparations of purified myeloperoxidase (lane 1) and neutrophil extracts from a normal individual (lane 2) and a myeloperoxidase-deficient individual (lane 3) were separated by PAGE. Gels were stained for peroxidase activity. Conditions for the gel and stain are described in the Materials and methods section.

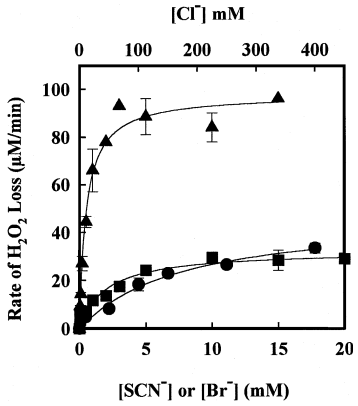

Figure 2 Effect of substrate concentration on the activity of myeloperoxidase

Reactions were started by adding 10 nM myeloperoxidase to 30 μM H_2O_2 and 100 μM glutathione in 100 mM phosphate buffer, pH 7.4, at room temperature. The initial rates of H_2O_2 loss were measured at various concentrations of chloride (●), bromide (■) or thiocyanate (▲). Data are means and ranges of duplicate experiments. The origin of the curves indicates insignificant H_2O_2 loss in the absence of any of the halides.

Table 1 Kinetic parameters for substrates of myeloperoxidase

The V_{max} and K_m values for chloride, bromide and thiocyanate were determined by fitting rectangular hyperbolae to the corresponding curves in Figure 2. The catalytic rate constants (k_0) were calculated by dividing V_{max} values by the concentration of myeloperoxidase. The specificity constants (k_{X^-}) were determined by dividing k_0 by k_m.

	V_{max} (μM/min)	k_0 (s^{-1})	K_m (mM)	k_{X^-} ($M^{-1} \cdot s^{-1}$)	k_{X^-}/k_{Cl^-}
Cl^-	47	79	175	4.5×10^2	1
Br^-	32	54	2	2.7×10^4	60
SCN^-	98	163	0.5	3.3×10^5	730

for eosinophil peroxidase by including bromide in the developer [33]. There was no band corresponding to eosinophil peroxidase in the purified myeloperoxidase (lane 1) and we conclude that it was free of eosinophil peroxidase.

Estimation of specificity constants for substrates of myeloperoxidase

The ability of an enzyme to discriminate in favour of a particular substrate in the presence of a mixture of competing substrates is determined by the specificity constants for each substrate. The specificity constant (k) is calculated by dividing the catalytic rate constant (k_0) by the Michaelis constant (K_m) [34]. These parameters are derived from data obtained with each substrate alone. With myeloperoxidase, determination of k_0 and K_m is hindered because, depending on pH, chloride, thiocyanate and H_2O_2 inhibit hypohalous acid production [35,36]. We therefore estimated the kinetic parameters for chloride, bromide and thiocyanate under conditions that minimized inhibition, i.e. neutral pH and a low concentration of H_2O_2. The plots of the rate of H_2O_2 loss against halide concentration are shown in Figure 2, and the kinetic parameters obtained from these plots are given in Table 1. From these data it is apparent that thiocyanate is by far the most specific substrate for myeloperoxidase. On the basis of these specificity constants, myeloperoxidase would convert about 40 % of H_2O_2 into hypothiocyanite and 60 % into hypochlorous acid in solutions containing 100 μM thiocyanate and 100 mM chloride.

Table 2 Formation of oxidants by myeloperoxidase in the presence of chloride and/or thiocyanate

Reactions were started by adding 30 μM H_2O_2 to 100 mM phosphate buffer, pH 7.4, containing 20 nM myeloperoxidase, 100 mM chloride and/or 100 μM thiocyanate, and where indicated 5 mM methionine or 10 mM taurine. Reactions were run at room temperature and stopped after 2 min by adding 20 μg/ml catalase. The concentration of oxidant present in the reaction system was then measured by determining its ability to bleach TNB. Data are means ± S.D. for triplicate experiments.

	Concentration of accumulated oxidant (μM)
Cl^-	5.1 ± 1.5
Cl^- + taurine	16.4 ± 0.6
Cl^- + methionine	2.5 ± 0.3
SCN^-	18.4 ± 0.3
SCN^- + methionine	19.4 ± 1.1
Cl^- + SCN^-	11.0 ± 0.8
Cl^- + SCN^- + methionine	16.2 ± 1.0

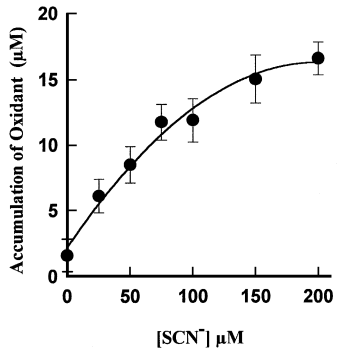

Figure 3 Effect of thiocyanate on the production of hypothiocyanite by myeloperoxidase in the presence of chloride

Reactions were started by adding 30 μM H_2O_2 to 20 nM myeloperoxidase, 100 mM chloride, 5 mM methionine and variable concentrations of thiocyanate. Reactions were stopped after 2 min by adding catalase, and the concentration of hypothiocyanite that had accumulated was measured by determining its ability to oxidize TNB. Other conditions were described in Figure 2. Data are means ± S.D. for triplicate experiments. The origin of the curve indicates the amount of oxidant produced in the absence of thiocyanate.

Effect of thiocyanate on oxidant production by myeloperoxidase

We wanted to establish that the specificity constants for chloride and thiocyanate accurately predict the amount of hypothiocyanite produced when both substrates are present with the enzyme. To determine this, we let hypothiocyanite accumulate over the course of the reaction, then added TNB to detect it (Table 2). Methionine was included in the assays because it reacts with hypochlorous acid but not hypothiocyanite [25]. It was present in a 50-fold excess over thiocyanate. In competition experiments with the chlorination of monochlorodimedon [28], we found that it reacts eight times faster with hypochlorous acid than does thiocyanate. Thus there was a sufficient excess of methionine to scavenge all the hypochlorous acid. We measured hypochlorous acid by trapping it with taurine and detecting taurine chloramine with TNB.

When myeloperoxidase was incubated with H_2O_2 and 100 mM chloride alone in the presence of taurine, about half the H_2O_2

Table 3 Rate of oxidant production by myeloperoxidase in the presence of chloride and/or thiocyanate

Reactions were started by adding 25 μM H_2O_2 to 100 mM phosphate buffer, pH 7.4, containing 20 nM myeloperoxidase, 50 μM TNB, 100 mM chloride and/or 100 μM thiocyanate, and where indicated 2 mM methionine. The rate of oxidant production was taken as the decrease in A_{412} over the first 30 s of the reaction. Reactions were carried out at room temperature. Data are means and ranges of duplicate experiments.

	Rate of oxidation of TNB ($-\Delta A_{412}$/min)
No X^-	0.01 ± 0.00
Cl^-	0.42 ± 0.03
Cl^- + methionine	0.07 ± 0.02
SCN^-	0.18 ± 0.01
SCN^- + methionine	0.20 ± 0.01
Cl^- + SCN^-	0.70 ± 0.01
Cl^- + SCN^- + methionine	0.28 ± 0.01

was recovered as taurine chloramine (Table 2). Similarly, about half the H_2O_2 was converted into hypothiocyanite when myeloperoxidase was incubated with 100 μM thiocyanate. Methionine substantially reduced the detection of hypochlorous acid but had no effect on the accumulation of hypothiocyanite. When chloride and thiocyanate were present together with methionine, almost as much hypothiocyanite was produced as with thiocyanate alone. Since methionine prevents detection of hypochlorous acid, the oxidant measured in the presence of chloride and thiocyanate must be hypothiocyanite. Accumulation of hypothiocyanite was dependent on the concentration of thiocyanate, and was detectable even at the lower end of its physiological concentration (Figure 3).

We investigated the rate of production of hypothiocyanite and hypochlorous acid by including TNB in the reaction mixture and monitoring the decrease in A_{412} (Table 3). Oxidation of TNB with chloride alone was inhibited 86% by methionine. In contrast, methionine did not affect the rate of oxidation of TNB with thiocyanate alone. When chloride and thiocyanate were present together, the rate of oxidation of TNB was greater than the sum of the individual rates. Methionine decreased this rate of oxidation to approximately that with thiocyanate alone. With both substrates together, thiocyanate accounted for about 36% of the rate of oxidation of TNB. This is in good agreement with 40% predicted by the specificity constants. The apparent synergistic effect when both substrates were present together occurs because TNB is a competitive inhibitor of myeloperoxidase (results not shown). At the higher concentration of competing substrate, TNB is less able to inhibit myeloperoxidase and the enzyme produces hypohalous acids more efficiently. Inhibition could result from a direct reaction of TNB with the enzyme, or through the formation of superoxide as occurs with other thiols [37].

Effects of chloride and/or thiocyanate on the utilization of H_2O_2 by myeloperoxidase

From the above result, it is apparent that myeloperoxidase is more active in the presence of thiocyanate and chloride than with either of these substrates alone. Therefore its rate of utilization of H_2O_2 should be greater with both anions present than with them individually. To verify this conclusion we used an H_2O_2 electrode to monitor the loss of H_2O_2 catalysed by myeloperoxidase (Figure 4). The loss of H_2O_2 had similar kinetics with 100 mM chloride or 100 μM thiocyanate. When thiocyanate and chloride were present together, the loss of H_2O_2 in the first minute increased from 8 μM with chloride or thiocyanate to 15 μM with both substrates present together. With 100 mM chloride and increasing concentrations of thiocyanate, there was a corresponding increase in the activity of myeloperoxidase until the enzyme was saturated at approx. 1 mM thiocyanate (Figure 5). From these results it is apparent that at plasma concentrations of thiocyanate and chloride, these substrates are oxidized at approximately equal rates and that myeloperoxidase is not fully saturated.

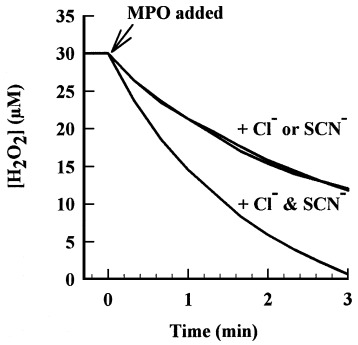

Figure 4 Effect of chloride and/or thiocyanate on the loss of H_2O_2 catalysed by myeloperoxidase

Reactions were started by adding myeloperoxidase (MPO) to 100 mM phosphate buffer (pH 7.4) containing 30 μM glutathione, 100 mM chloride and/or 100 μm thiocyanate. Loss of H_2O_2 was monitored continuously with a H_2O_2 electrode. Reactions were carried out at room temperature. Traces are representative of three experiments.

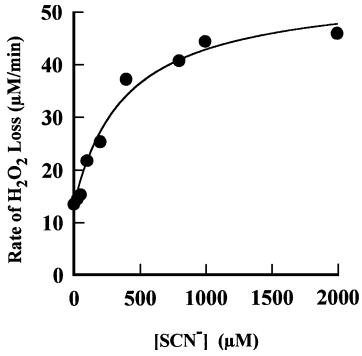

Figure 5 Effect of thiocyanate on the rate of loss of H_2O_2 catalysed by myeloperoxidase in the presence of chloride

Reaction conditions were as described in Figure 4, except chloride was always present at 100 mM. Rates of H_2O_2 loss were determined over the first 10 s of the reaction. Data are representative of three experiments. The origin of the curve indicates H_2O_2 loss in the presence of chloride alone.

When the data in Table 3 and Figure 4 are compared, it is apparent that the turnover of myeloperoxidase is considerably less for the rate of TNB oxidation than for the rate of loss of H_2O_2. This occurs because TNB inhibits myeloperoxidase (see above).

Reaction of hypothiocyanite with taurine chloramine

In contrast with our findings, Thomas and Fishman [14] concluded that there is little production of hypothiocyanite at plasma concentrations of chloride and thiocyanate. In a system containing both chloride and thiocyanate, they used taurine to

Table 4 Reactions of taurine chloramine with thiocyanate and hypothiocyanite

Taurine chloramine was produced by adding 100 μM hypochlorous acid to 1 mM taurine. Hypothiocyanite (60 μM) was formed when 100 μM H_2O_2 was added to 70 nM myeloperoxidase and 200 μM thiocyanate in 100 mM phosphate buffer, pH 7.4, and the reaction allowed to proceed for 5 mins. The amount of hypothiocyanite did not increase after this time and remained stable for several minutes. Equal volumes of taurine chloramine (100 μM) and hypothiocyanite (60 μM) were either added together or to thiocyanate (150 μM) or buffer, and the concentration of oxidant remaining after 1 min was measured with TNB. Data are means $\pm$ S.D. for triplicate experiments.

	[Oxidant] (μM)	Oxidant remaining (%)
SCN^-	0 ± 0	–
TauCl	49 ± 2	100
$OSCN^-$	29 ± 1	100
TauCl + SCN^-	45 ± 2	92
TauCl + $OSCN^-$	56 ± 4	72

trap hypochlorous acid. TNB was employed to measure formation of taurine chloramine and accumulation of hypothiocyanite. They reasoned that, if thiocyanate was oxidized in the presence of chloride, there should be an increase in TNB oxidation compared with a system containing chloride only. However, they found that, at less than 100 μM, thiocyanate decreased oxidation of TNB and concluded that it was not oxidized by myeloperoxidase but scavenged taurine chloramine. We repeated Thomas and Fishman's experiment and obtained essentially the same results (not shown). To reconcile their findings with our conclusion that thiocyanate at low concentrations is readily oxidized in the presence of chloride, we investigated the reaction of taurine chloramine with thiocyanate and hypothiocyanite. Taurine chloramine was added to solutions of thiocyanate or hypothiocyanite, and, after 1 min, the amount of oxidant remaining was measured with TNB (Table 4). Taurine chloramine and hypothiocyanite incubated alone were stable over this period. The reaction of thiocyanate with taurine chloramine was slow, and could therefore only partially explain the results of Thomas and Fishman. When taurine chloramine and hypothiocyanite were mixed, the amount of oxidant remaining after 1 min was 28 % less than the sum of the two, indicating a more rapid reaction. In view of the observed 3:1 stoichiometry of the reaction between taurine chloramine and thiocyanate [14], it is not surprising that taurine chloramine also reacts with hypothiocyanite. The effect of this reaction is that when taurine chloramine and hypothiocyanite are formed simultaneously, hypothiocyanite would not accumulate and total oxidant production would be underestimated.

Spectral investigations

To confirm that myeloperoxidase catalyses hypothiocyanite production via two-electron oxidation of thiocyanate by compound I, we recorded the absorbance spectrum of myeloperoxidase during enzyme turnover. When H_2O_2 was added to myeloperoxidase and thiocyanate, the enzyme remained predominantly in its ferric form (result not shown). In the absence of thiocyanate the enzyme was converted into compound II as has been shown previously [38,39]. Subsequent addition of thiocyanate had no effect on the spectrum of compound II (result not shown). These results indicate that thiocyanate neither converts the enzyme into compound II nor reduces compound II to ferric myeloperoxidase.

DISCUSSION

We have shown that thiocyanate is a far more favoured substrate for myeloperoxidase than chloride, and that it is readily oxidized by the enzyme in the presence of physiological concentrations of chloride. Based on the values obtained for the specificity constants for chloride, bromide and thiocyanate (Table 1), we predict that when the plasma concentrations of thiocyanate and chloride are 100 μM and 100 mM respectively, about 40 % of the H_2O_2 used by myeloperoxidase should be converted into hypothiocyanite and the remainder to hypochlorous acid. These estimates are in good agreement with the rates of oxidation of TNB by hypochlorous acid and hypothiocyanite when chloride and thiocyanate were present together (Table 3). At 25 μM thiocyanate, its minimum normal plasma concentration [11], about 15 % of the H_2O_2 used by myeloperoxidase should produce hypothiocyanite. In the mouth, where thiocyanate reaches 5 mM and chloride is as low as 25 mM [40], myeloperoxidase will produce only hypothiocyanite. Therefore, in contrast with the earlier study by Thomas and Fishman [14], we conclude that hypothiocyanite is a major physiological product of myeloperoxidase that will be produced over the entire physiological range of thiocyanate concentration. There is unlikely to be appreciable oxidation of bromide by myeloperoxidase *in vivo*. On the basis of the specificity constants and plasma concentrations of chloride, bromide and thiocyanate, less than 5 % of the H_2O_2 would be accounted for by the production of hypobromous acid.

The value of k_{SCN^-} is in good agreement with that obtained by steady-state kinetics for the reaction of thiocyanate with compound I of myeloperoxidase [35]. However, the value of k_{Cl^-} is considerably less than the rate constant of $(4.7 \pm 0.1) \times 10^6$ $M^{-1} \cdot s^{-1}$ for the reaction of chloride with compound I, which was obtained by pre-steady-state stopped-flow measurements [41]. The reason for this discrepancy is that our measurements were recorded over the first 10 s of the reaction where myeloperoxidase has lost considerable activity compared with that in the first few milliseconds [42]. Our value for the catalytic rate constant for chloride is similar to that of 25 s^{-1} measured previously after 1 s of reaction [42]. Thus the specificity constants in Table 1 do not represent the true rate constants for the reactions of compound I. Rather, they are apparent second-order rate constants that accurately predict the relative rates at which myeloperoxidase oxidizes chloride and thiocyanate.

Thiocyanate and chloride both bind to the haem iron at the active sites of myeloperoxidase [43]. At physiological concentrations of chloride and below 1 mM thiocyanate, our results imply that these anions do not compete for binding and subsequent oxidation by the enzyme. This proposal is based on our findings that thiocyanate increased the rate at which myeloperoxidase, in the presence of H_2O_2 and chloride, catalysed the oxidation of TNB (Table 3) and increased the rate of loss of H_2O_2 (Figure 4). Therefore, at physiological concentrations of chloride, myeloperoxidase is far from saturated. Addition of thiocyanate to myeloperoxidase and chloride increases the turnover of the enzyme, so that it uses H_2O_2 at a greater rate, producing both hypochlorous acid and hypothiocyanite.

Myeloperoxidase oxidizes substrates by either a single two-electron oxidation involving compound I or two successive one-electron oxidations involving compound I and compound II [6,7]. During the oxidation of thiocyanate, we found that myeloperoxidase was present in its ferric form and that there was no accumulation of compound II. We also found that thiocyanate was unable to reduce compound II to ferric myeloperoxidase. On the basis of these findings, it is likely that thiocyanate undergoes two-electron oxidation by compound I, as occurs with chloride

[reactions (1) and (2)] [6]. Although hypothiocyanite has been proposed to be the product of oxidation of thiocyanate [44], it may not be the initial oxidant formed. It is possible that thiocyanogen [$(SCN)_2$] is initially produced, which then rapidly hydrolyses to hypothiocyanite [reactions (3) and (4)] [13]. There is good evidence that thiocyanogen is produced by lactoperoxidase, since it incorporates thiocyanate into aromatic amino acids in reactions analogous to reagent thiocyanogen [12]. With horseradish peroxidase, oxidation of thiocyanate occurs optimally at pH 4 and thiocyanogen is the principal product [45].

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

$$\text{Compound I} + 2SCN^- \rightarrow MP^{3+} + (SCN)_2 \quad (2)$$

$$(SCN)_2 + H_2O \rightleftharpoons HOSCN + SCN^- + H^+ \quad (3)$$

$$HOSCN \rightleftharpoons {}^-OSCN + H^+ \quad (4)$$

where MP^{3+} is myeloperoxidase.

Our findings have important consequences for the involvement of neutrophil oxidants in host defence and inflammation. Hypothiocyanite is considerably less reactive than hypochlorous acid, and reacts mainly with thiols to form disulphides and regenerate thiocyanate [12]. Hypothiocyanite is also regarded as innocuous to mammalian cells [28], and, in contrast with hypochlorite [46], it is considerably less effective at lysing red blood cells [15]. Thus it is conceivable that thiocyanate may limit bacterial killing and neutrophil-mediated tissue damage by diverting H_2O_2 away from the production of hypochlorous acid.

In conclusion, we have shown that thiocyanate is likely to be a major substrate of myeloperoxidase in most environments in which this enzyme acts. As a consequence, more consideration should be given to the oxidation products of thiocyanate and to the role they play in host defence and inflammation.

We thank the Health Research Council, the New Zealand Asthma Foundation and the Canterbury Medical Research Foundation for financial support. We would also like to acknowledge the expert assistance of Mr. Karl Sluis in the development of the peroxidase activity stain.

REFERENCES

1 Harrison, J. E. and Shultz, J. (1976) J. Biol. Chem. **251**, 1371–1374
2 Foote, C. S., Goyne, T. E. and Lehler, R. I. (1983) Nature (London) **301**, 715–716
3 Weiss, S. J., Klein, R., Slivka, A. and Wei, M. (1982) J. Clin. Invest. **70**, 598–607
4 Kettle, A. J., Gedye, C. A. and Winterbourn, C. C. (1993) Biochem. Pharmacol. **45**, 2003–2010
5 Weiss, S. J. (1989) N. Engl. J. Med. **320**, 365–376
6 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
7 Klebanoff, S. J. (1991) in Peroxidases in Chemistry and Biology (Everse, J., Everse, K. E. and Grisham, M. B., eds.), pp. 1–35, CRC Press, Boca Raton
8 Klebanoff, S. J. and Clark, R. A. (1978) The Neutrophil: Function and Clinical Disorders, North-Holland Publishing Company, Amsterdam
9 Oka, S., Sibazaki, Y. and Tahara, S. (1981) Anal. Chem. **53**, 588–593
10 Holzbecher, J. and Ryan, D. E. (1980) Clin. Biochem. **13**, 277–278
11 Wood, J. L. (1975) in Chemistry and Biochemistry of Thiocyanic Acid and its Derivatives (Newman, A. A., ed.), pp. 156–221, Academic Press, Orlando
12 Thomas, E. L. (1985) in The Lactoperoxidase System: Chemistry and Biological Significance (Pruitt, K. M. and Tenovuo, J. V., eds.), pp. 31–53, Marcel Dekker Inc., New York
13 Thomas, E. L. (1981) Biochemistry **20**, 3273–3280
14 Thomas, E. L. and Fishman, M. (1986) J. Biol. Chem. **261**, 9694–9702
15 Grisham, M. B. and Ryan, E. M. (1990) Am. J. Physiol. Cell Physiol. **258**, C115–C121
16 Kettle, A. J. and Winterbourn, C. C. (1988) Biochem. J. **252**, 529–536
17 Odajima, T. and Yamazaki, I. (1970) Biochim. Biophys. Acta **206**, 71–77
18 Kettle, A. J. and Winterbourn, C. C. (1994) Methods Enzymol. **233**, 502–512
19 Beers, R. J. and Sizer, I. W. (1952) J. Biol. Chem. **195**, 133–140
20 Kettle, A. J. and Winterbourn, C. C. (1988) Biochim. Biophys. Acta **957**, 185–191
21 Kettle, A. J. and Winterbourn, C. C. (1994) J. Biol. Chem. **269**, 17146–17151
22 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
23 Maurer, H. R. (1971) Disc Electrophoresis and Related Techniques of Polyacrylamide Gel Electrophoresis, W. de Gruyter, Berlin and New York
24 Thomas, E. L., Grisham, M. B. and Jefferson, M. M. (1986) Methods Enzymol. **132**, 569–593
25 Riddles, P. W., Blakely, R. L. and Zerner, B. (1983) Methods Enzymol. **91**, 49–60
26 Kettle, A. J. and Winterbourn, C. C. (1989) Biochem. J. **263**, 823–828
27 Winterbourn, C. C. (1985) Biochim. Biophys. Acta **840**, 204–210
28 Slungaard, A. and Mahoney, J. R. (1991) J. Biol. Chem. **266**, 4903–4910
29 Wever, R., Plat, H. and Hamers, M. N. (1981) FEBS Lett. **123**, 327–331
30 Olsen, R. L. and Little, C. (1983) Biochem. J. **209**, 781–787
31 Pember, S. O., Shapira, R. and Kinkade, J. M. (1983) Arch. Biochem. Biophys. **221**, 391–403
32 Matheson, N. R., Wong, P. S. and Travis, J. (1981) Biochemistry **20**, 325–330
33 Bozeman, P. M., Learn, D. B. and Thomas, E. L. (1990) J. Immunol. Methods **126**, 125–133
34 Cornish-Bowden, A. (1995) Fundamentals of Enzyme Kinetics, Portland Press, London
35 Wever, R., Kast, W. M., Kasinoedin, J. H. and Boelens, R. (1982) Biochim. Biophys. Acta **709**, 212–219
36 Bakkenist, A. R. J., De Boer, J. E. D., Plat, H. and Wever, R. (1980) Biochim. Biophys. Acta **613**, 349–358
37 Pichorner, H., Metodiewa, D. and Winterbourn, C. C. (1995) Arch. Biochem. Biophys. **323**, 429–437
38 Winterbourn, C. C., Garcia, R. and Segal, A. W. (1985) Biochem. J. **228**, 583–592
39 Hoogland, H., Van Kuilengurg, A., van Reil, C., Muijers, A. O. and Wever, R. (1987) Biochim. Biophys. Acta **916**, 76–82
40 Tenovuo, J. and Makinen, K. K. (1976) J. Dent. Res. **55**, 661–663
41 Marquez, L. A. and Dunford, H. B. (1995) J. Biol. Chem. **270**, 30434–30440
42 Zuurbier, K. W. M., Bakkenist, A. R. J., Wever, R. and Muijsers, A. O. (1990) Biochim. Biophys. Acta **1037**, 140–146
43 Bolscher, B. G. J. M. and Wever, R. (1984) Biochim. Biophys. Acta **788**, 1–10
44 Aune, T. M. and Thomas, E. L. (1977) Eur. J. Biochem. **80**, 209–214
45 Modi, S., Behere, D. V. and Mitra, S. (1991) Biochim. Biophys. Acta **1080**, 45–50
46 Vissers, M. C. M., Stern, A., Kuypers, F., Van Den Berg, J. and Winterbourn, C. C. (1994) Free Radic. Biol. Med. **16**, 703–712

Received 14 January 1997/12 May 1997; accepted 4 June 1997