Substrates and products of eosinophil peroxidase

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Eosinophil peroxidase has been implicated in promoting oxidative tissue damage in a variety of inflammatory conditions, including asthma. It uses H_2O_2 to oxidize chloride, bromide and thiocyanate to their respective hypohalous acids. The aim of this study was to establish which oxidants eosinophil peroxidase produces under physiological conditions. By measuring rates of H_2O_2 utilization by the enzyme at neutral pH, we determined the catalytic rate constants for bromide and thiocyanate as 248 and 223 s^{-1} and the Michaelis constants as 0.5 and 0.15 mM respectively. On the basis of these values thiocyanate is preferred 2.8-fold over bromide as a substrate for eosinophil peroxidase. Eosinophil peroxidase catalysed substantive oxidation of chloride only below pH 6.5. We found that when eosinophil peroxidase or myeloperoxidase oxidized thiocyanate, another product besides hypothiocyanite was formed; it also converted methionine into methionine sulfoxide. During the oxidation of thiocyanate, the

peroxidases were present as their compound II forms. Compound II did not form when GSH was included to scavenge hypothiocyanite. We propose that the unidentified oxidant was derived from a radical species produced by the one-electron oxidation of hypothiocyanite. We conclude that at plasma concentrations of bromide (20–120 μM) and thiocyanate (20–100 μM), hypobromous acid and oxidation products of thiocyanate are produced by eosinophil peroxidase. Hypochlorous acid is likely to be produced only when substrates preferred over chloride are depleted. Thiocyanate should be considered to augment peroxidase-mediated toxicity because these enzymes can convert relatively benign hypothiocyanite into a stronger oxidant.

Key words: hypobromous acid, hypothiocyanite, myeloperoxidase, thiocyanate.

INTRODUCTION

Eosinophils are white blood cells that cause some of the tissue damage that occurs in asthma and other diseases [1,2]. They contain a potent arsenal of cytotoxic proteins that are released after activation. The most abundant of these is the haem enzyme eosinophil peroxidase. It uses H_2O_2 to generate reactive oxidants from halides and the pseudohalide thiocyanate [3,4]. H_2O_2 reacts with the ferric enzyme (EP^{3+}) to form compound I:

The iron in this redox intermediate has a formal oxidation state of 5+. Compound I oxidizes the halides (X^-), except fluoride, and thiocyanate to their respective hypohalous acids:

Compound I can also undergo a single one-electron reduction to form compound II, in which the iron has a formal oxidation state of 4+ [5]:

Bromide and thiocyanate have been variously reported to be the major substrate for this enzyme [3,4,6]. Chloride is not considered a major substrate, even though its concentration in plasma is approx. 100 mM and far exceeds those of bromide (20–100 μM) and thiocyanate (20–120 μM) [7–9]. The evidence for bromide as the major substrate comes from studies analysing halide oxidation in mixtures of chloride, bromide and iodide. By measuring the kinetics of 5-thio-2-nitrobenzoic acid (TNB) bleaching, Slungaard and Mahoney concluded that thiocyanate was the major and perhaps exclusive substrate for eosinophil peroxidase

[4]. However, with the same method of analysis, Wu et al. [10] found that the apparent specificity constant for thiocyanate was only 2.8-fold that for bromide. This indicates that at plasma concentrations of thiocyanate and bromide, oxidation of both would be expected. In agreement with this proposal, Obinger and co-workers determined rate constants for the reaction of substrates with compound I of eosinophil peroxidase and found that thiocyanate reacts only 5-fold faster than bromide with compound I [11].

Reports of chloride oxidation by eosinophil peroxidase are varied. Several studies suggest this that halide is oxidized only at acidic pH [12,13]. However, Thomas et al. [14] detected chloramine formation by eosinophil peroxidase in the presence of chloride and bromide at neutral pH, indicating that chloride was oxidized under their reaction conditions. The eosinophil peroxidase/chloride/ H_2O_2 system has been shown to be toxic to pneumocytes [15], respiratory epithelium [16], *Escherichia coli* [17] and *Trypanosoma cruzi* trypomastigotes [18]. In general these activities have been demonstrated at acidic pH levels. However, several studies have shown residual activity with chloride at pH 7.0 [17–19].

Although hypothiocyanite seems to be the major oxidant produced during the oxidation of thiocyanate, differing antibacterial effects of hypothiocyanite and the lactoperoxidase/ H_2O_2 /thiocyanate system have suggested that other products might be formed [20,21]. Pruitt et al. [22,23] have proposed that hypothiocyanous acid (HOSCN) might be oxidized by excess H_2O_2 or the enzyme to short-lived oxidants, namely cyanosulphurous acid (HO_2SCN) and cyanosulphuric acid (HO_3SCN). More recently, cyanate (OCN^-) has been shown to be a major oxidation product of thiocyanate [24], which is presumably derived from the breakdown of cyanosulphurous acid or cyanosulphuric acid. The formation of additional oxidants suggests

Abbreviations used: DTPA, diethylenetriaminepenta-acetic acid; TNB, 5-thio-2-nitrobenzoic acid.

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that the peroxidase/H₂O₂/thiocyanate system might be less benign than generally considered.

To establish the contribution that eosinophil peroxidase makes to the pathogenesis of inflammatory diseases, it is necessary to know which of the above oxidants are produced *in vivo*. These oxidants differ in their cytotoxic potentials. Hypochlorous acid and hypobromous acid are highly reactive and extremely toxic to almost all cell types [25,26]. Hypothiocyanite, in contrast, is a weak oxidizing agent that reacts primarily with protein thiol groups [27,28]. In the present study we determined the substrate preference of eosinophil peroxidase with a H₂O₂ electrode to measure enzyme activity. We found that chloride was oxidized by eosinophil peroxidase only below pH 6.5. We also show that although thiocyanate is the preferred substrate of eosinophil peroxidase, hypobromous acid is also formed at plasma concentrations of thiocyanate and bromide. We investigated the products formed during the oxidation of thiocyanate and propose that hypothiocyanite can also act as a substrate for peroxidases, with the formation of more reactive and potentially more damaging products.

MATERIALS AND METHODS

Materials

Myeloperoxidase was purified from human leucocytes as described previously and had a purity index (A_{430}/A_{280}) of more than 0.72 [29]. Its concentration was determined with an ϵ_{430} of 91 000 M⁻¹·cm⁻¹ per haem [30]. CM-Sepharose was from Pharmacia (Uppsala, Sweden). Hexadecyltrimethylammonium chloride was from Acros Organics (Fairlawn, NJ, U.S.A.). CompleteTM protease inhibitor mixture tablets, which are inhibitory to a large spectrum of serine proteases, were from Roche Molecular Biochemicals. 5,5'-Dithiobis-(2-nitrobenzoic acid), diethylenetriaminepenta-acetic acid (DTPA) and bovine liver catalase were from Sigma Chemical Co. (St Louis, MO, U.S.A.). D,L-Methionine was from BDH Chemicals (Poole, Dorset, U.K.). TNB was prepared from 5,5'-dithiobis-(2-nitrobenzoic acid) as described previously [31]. H₂O₂ solutions were prepared daily by diluting a 30% (v/v) stock; the concentration was calculated by measuring its A_{240} (ϵ_{240} 43.6 M⁻¹·cm⁻¹) [32]. *o*-Phthalaldehyde was from Fisher Scientific (Fairlawn, NJ, U.S.A.). HPLC-grade methanol was supplied by Mallinckrodt (Paris, KY, U.S.A.). All other HPLC-grade solvents were from BDH Chemicals.

Purification of eosinophil peroxidase

Eosinophil peroxidase was purified from eosinophils isolated from horse blood by the method of Jorg et al. [33]. Proteins were extracted from the eosinophils by sonicating them in 100 mM sodium acetate buffer, pH 6.0, containing 100 mM NaCl, 0.3% cetyltrimethylammonium bromide (cetrimide) and 1 mg/ml CompleteTM protease inhibitors. The cell debris was pelleted at 2000 g and the supernatant was dialysed against 100 mM sodium phosphate buffer, pH 6.0, containing 100 mM NaCl and 0.05% cetrimide. A single-step chromatographic purification procedure was used to purify the enzyme from the crude cell extract. The enzyme was eluted from the column with a 400 ml linear gradient from 0.1 to 2 M NaCl in 100 mM sodium phosphate buffer, pH 7.4. Fractions with a purity index (A_{415}/A_{280}) of more than 0.69 were stored in the elution buffer at -80 °C and used in experiments. Separation of eosinophil peroxidase from myeloperoxidase was confirmed with native PAGE, demonstrating minimal staining for myeloperoxidase [34,35]. For spectral studies, the enzyme preparation was dialysed against 50 mM

sodium phosphate buffer, pH 7.0, containing 100 mM NaCl, 0.02% cetyltrimethylammonium chloride and 2% (v/v) glycerol.

Measurement of H₂O₂ utilization

The utilization of H₂O₂ by eosinophil peroxidase 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.) [36]. The electrode was covered with a single layer of dialysis tubing and equilibrated against known concentrations of H₂O₂. GSH was included in the reaction mixtures to scavenge the hypohalous acids and prevent them from interfering with the electrode signal [36]. All reactions were started with the addition of eosinophil peroxidase. Initial rates of H₂O₂ loss were determined over the first 10 s of the reaction.

Measurement of formation of hypohalous acid

Hypochlorous acid formation by purified eosinophil peroxidase was determined by measuring accumulation of taurine chloramine [31]. Eosinophil peroxidase (5 nM) was incubated for 20 min at 21 °C in 100 mM sodium phosphate buffer at various pH values containing 100 mM chloride, 10 mM taurine and 20 μ M DTPA. Reactions were started with the addition of 30 μ M H₂O₂ and stopped with 20 μ g/ml catalase. The amount of taurine chloramine formed was assayed with TNB and quantified by measuring the decrease in A_{412} (ϵ_{412} 14 100 M⁻¹·cm⁻¹) [37].

The amount of hypothiocyanite that accumulated during the oxidation of thiocyanate by eosinophil peroxidase or myeloperoxidase was measured by its ability to oxidize TNB, which was added at the end of the reaction. The total amount of oxidant produced during this reaction was measured by including TNB in the reaction system from the start and monitoring the change in A_{412} continuously. The production of hypothiocyanite by eosinophil peroxidase in the presence of thiocyanate and bromide was measured by including methionine (1 mM) to scavenge hypobromous acid [35].

Formation of methionine sulphoxide

Hypothiocyanite does not oxidize methionine [28]. Therefore, to determine whether or not a stronger oxidant than hypothiocyanite was produced during the oxidation of thiocyanate, methionine (5 mM) was included in reactions with 5 nM eosinophil peroxidase or 10 nM myeloperoxidase and various concentrations of thiocyanate. Reactions were performed in 50 mM sodium phosphate buffer, pH 7.4, at 21 °C. They were started by the addition of 30 μ M H₂O₂ and stopped after 10 min with 20 μ g/ml catalase. Methionine sulphoxide content was measured by HPLC analysis with precolumn derivatization with *o*-phthalaldehyde [38]. Methionine sulphoxide was identified by comparison of its retention time with an authentic standard.

Spectral analysis of peroxidases

The absorbance spectra of eosinophil peroxidase and myeloperoxidase were recorded during the oxidation of substrates by using a Beckman 7500 diode-array spectrophotometer. H₂O₂ was generated with glucose oxidase. Reactions were started by the addition of glucose oxidase to 840 nM peroxidase in 100 mM sodium phosphate buffer, pH 7.4, containing 1 mg/ml glucose with thiocyanate (100 μ M), bromide (100 μ M) or chloride (100 mM) at room temperature. Methionine (5 mM) was included to scavenge hypochlorous acid and hypobromous acid and to prevent them from reacting with the enzyme [35].

RESULTS

Chloride, bromide and thiocyanate as substrates for eosinophil peroxidase

We used a H_2O_2 electrode to determine the substrate preference of eosinophil peroxidase because, unlike most chromogenic detector molecules that react with hypohalous acids, it does not interfere with enzyme activity [36]. Eosinophil peroxidase catalysed the loss of H_2O_2 in the presence of bromide or thiocyanate at neutral pH (Figure 1). However, chloride was not oxidized by the enzyme. The rate of oxidation of $100\ \mu\text{M}$ thiocyanate was greater than for $100\ \mu\text{M}$ bromide, and there was only a small increase in H_2O_2 loss when bromide was included with thiocyanate. The extent of either bromide or thiocyanate oxidation when both substrates are present is determined by their specificity constants. These values were calculated by dividing the catalytic

Figure 1 Loss of H_2O_2 during the oxidation of chloride, bromide and/or thiocyanate catalysed by eosinophil peroxidase

Reactions were started by the addition of 2 nM eosinophil peroxidase (EPO) to 100 μM phosphate buffer, pH 7.4, containing 30 μM H_2O_2 , 100 mM chloride, 100 μM bromide and/or 100 μM thiocyanate, 30 μM GSH and 20 μM DTPA at 21 °C. Loss of H_2O_2 was monitored continuously with a H_2O_2 electrode. Traces are representative of at least three experiments.

Figure 2 Effect of substrate concentration on the activity of eosinophil peroxidase

Reactions were started by the addition of 2 nM eosinophil peroxidase to 30 μM H_2O_2 and 100 μM GSH in 100 mM sodium phosphate buffer, pH 7.4, at 21 °C. The initial rates of H_2O_2 loss were measured over the first 10 s of each reaction at various concentrations of bromide (◆) or thiocyanate (●). Results are means and ranges of duplicate experiments.

Table 1 Kinetic parameters for substrates of eosinophil peroxidase

The V_{max} and K_m values for thiocyanate and bromide were determined by fitting rectangular hyperbolae to the corresponding curves in Figure 2. The catalytic rate constants (k_{cat}) were calculated by dividing V_{max} values by the concentration of eosinophil peroxidase. The specificity constants (k_x) were determined by dividing k_{cat} by K_m . Values in parentheses are from [10].

Substrate	V_{max} ($\mu\text{M}/\text{min}$)	k_{cat} (s^{-1})	K_m (mM)	k_x ($\text{M}^{-1} \cdot \text{s}^{-1}$)	k_x/k_{bromide}
Thiocyanate	27	223 (15.4)	0.15 (1.4)	1.5×10^6 (1.1×10^4)	2.8 (2.7)
Bromide	30	248 (3.6)	0.5 (0.9)	5.3×10^5 (4.0×10^3)	1

rate constant (k_{cat}) by the Michaelis constant (K_m) derived from data presented in Figure 2 [39]. As with myeloperoxidase and lactoperoxidase, eosinophil peroxidase activity is affected by pH and by the concentrations of thiocyanate and H_2O_2 [40]. These results were therefore obtained at neutral pH and low H_2O_2 concentrations to minimize inhibition by H_2O_2 (Figure 2). From these results it is apparent that thiocyanate is the preferred substrate of eosinophil peroxidase, with a 2.8-fold preference over bromide (Table 1). In solutions containing 100 μM thiocyanate and 100 μM bromide, eosinophil peroxidase would convert approx. 75 % of the H_2O_2 into hypothiocyanite and 25 % into hypobromous acid. Although the relative specificity constant that we obtained was the same as published previously, the kinetic constants were very different [10]. In the previous study the catalytic rate constants were 14–70-fold lower and the K_m values were 2–10-fold higher than we obtained (see Table 1).

To determine how the activity of eosinophil peroxidase was affected when both substrates were present together, the concentration of one substrate was kept constant while the other was varied. The rate of H_2O_2 loss in the presence of 100 μM thiocyanate was minimally affected by bromide, with only a 50 % increase in activity at 800 μM bromide (Figure 3). In contrast, thiocyanate had a marked effect on enzyme activity in the presence of 100 μM bromide. These results are in agreement with the K_m values that were obtained from Figure 2. With 100 μM thiocyanate, which is close to the K_m for this substrate, adding bromide would be expected to at most double the rate of loss of H_2O_2 . However, with 100 μM bromide, which is well below the K_m for this substrate, addition of thiocyanate would be expected to increase activity to a much greater extent.

To determine the extent to which thiocyanate is oxidized in the presence of bromide, the accumulation of hypothiocyanite was measured at various concentrations of bromide. Methionine (1 mM), which does not react with hypothiocyanite [28], was included in the reaction system to scavenge hypobromous acid and prevent it from reacting with eosinophil peroxidase and thiocyanate. In the absence of bromide, 5 nM eosinophil peroxidase produced 18 μM hypothiocyanite from 30 μM H_2O_2 and 100 μM thiocyanate. Bromide inhibited hypothiocyanite formation by 25 % at 100 μM and by 50 % at 1 mM (results not shown). Bromide must have acted by competing with thiocyanate for oxidation by compound I rather than inhibiting the enzyme, because it also increased the rate at which eosinophil peroxidase consumed H_2O_2 (Figure 3). Thus at physiological concentrations of bromide and thiocyanate both substrates are oxidized by eosinophil peroxidase.

To establish whether chloride is oxidized by eosinophil peroxidase at physiological pH, we measured H_2O_2 loss and hypochlorous acid formation throughout the reaction. At neutral pH there was negligible loss of H_2O_2 in the presence of 100 mM chloride (Figure 1). However, decreasing the pH below 6.5

Figure 3 Effect of competing substrates on the rate of H_2O_2 loss catalysed by eosinophil peroxidase

Reaction conditions were as described in the legend to Figure 2, except that 100 μM thiocyanate was present in all reactions in (A) and 100 μM bromide in all reactions in (B). Results are means and ranges of duplicate experiments.

resulted in increased utilization of H_2O_2 (not shown). In line with these results, the production of hypochlorous acid was apparent, but only below pH 6.5 (Figure 4). At pH 5.0 almost half of the H_2O_2 was converted into hypochlorous acid.

Detection of a short-lived oxidant formed from hypothiocyanite

When eosinophil peroxidase (10, 20 or 50 nM) was added to 100 μM thiocyanate and 30 μM H_2O_2 at pH 7.4, a maximum of only 16.5 ± 0.1 μM hypothiocyanite ($n = 8$) accumulated. This low stoichiometry could have occurred either because hypothiocyanite was unstable and decayed during the course of the reaction or because H_2O_2 reacted in other ways besides forming hypothiocyanite. As the concentration of thiocyanate was increased, there was a progressive increase in the recovery of hypothiocyanite. At 1 mM thiocyanate, all of the H_2O_2 was accounted for as hypothiocyanite (results not shown). Similar results were obtained with eosinophil peroxidase and myeloperoxidase. This result indicates that at low concentrations of thiocyanate, H_2O_2 was involved in other reactions besides the formation of hypothiocyanite.

When TNB was present during the oxidation of 100 μM thiocyanate to trap oxidants as they were formed, all the H_2O_2 was accounted for by the formation of 5,5'-dithiobis-(2-nitrobenzoic acid) (results not shown). Similar results were obtained with eosinophil peroxidase and myeloperoxidase and

Figure 4 Effect of pH on formation of taurine chloramine by eosinophil peroxidase

Reactions were started by the addition of 30 μM H_2O_2 to 5 nM eosinophil peroxidase, 100 mM chloride and 10 mM taurine in 100 mM phosphate buffer containing 20 μM DTPA. Reactions were performed at 21 °C and stopped after 20 min by the addition of catalase. The concentration of accumulated taurine chloramine was measured by determining its ability to oxidize TNB. Results are means $\pm$ S.D. for triplicate reactions.

Figure 5 HPLC analysis of oxidation products of methionine produced by peroxidase/thiocyanate/ H_2O_2

Reactions were started by the addition of 30 μM H_2O_2 to 50 mM phosphate buffer, pH 7.4, containing 5 mM methionine, 10 μM DTPA, 5 nM eosinophil peroxidase or 10 nM myeloperoxidase, and 150 μM thiocyanate. Reactions were carried out at 21 °C and stopped after 10 min by the addition of 20 μg/ml catalase. Trace a, methionine (5 mM) only, showing background content of methionine sulfoxide; trace b, representative trace of control experiments containing all except one of peroxidase, H_2O_2 , thiocyanate and methionine; trace c, the complete reaction system containing thiocyanate and either eosinophil peroxidase or myeloperoxidase; trace d, authentic methionine sulfoxide (30 μM) only.

were independent of enzyme concentration. Oxidation of TNB by peroxidase and H_2O_2 was minimal in the absence of thiocyanate. Thus all the H_2O_2 was used by the peroxidases to convert thiocyanate into oxidants that were able to oxidize TNB. One of these oxidants was hypothiocyanite, which accumulated; other less stable oxidants were also produced.

To confirm that a short-lived oxidant other than hypothiocyanite was formed in the peroxidase/thiocyanate/ H_2O_2 system, we included methionine in reactions to scavenge it. Methionine is not oxidized by hypothiocyanite [28]. The form-

Figure 6 Absorption spectra of ferric eosinophil peroxidase, compound II and compound III

(A) The spectrum of native eosinophil peroxidase (EP³⁺) was recorded before the addition of 100 μ M H₂O₂ to 600 nM enzyme in 100 mM phosphate buffer, pH 7.4, containing 0.02% hexadecyltrimethylammonium chloride. (B) Conditions as in (A) except that 1 mM H₂O₂ was added to the native enzyme. The spectral changes were recorded within 15 s of adding the H₂O₂. Arrows indicate changes in the native spectrum. Each spectrum was recorded over 2 s and is the average of 20 spectra. Spectra are representative of two separate experiments.

ation of methionine sulphoxide would therefore indicate the production of an additional and more powerful oxidizing agent. Methionine sulphoxide was detected in this reaction system (Figure 5). The removal of enzyme, thiocyanate or H₂O₂ prevented its formation. Thus a short-lived oxidant in addition to hypothiocyanite is produced when peroxidases catalyse the oxidation of thiocyanate. As the thiocyanate concentration was increased, the amount of methionine sulphoxide formed increased, with maximum formation at approx. 150–200 μ M thiocyanate. At higher concentrations of thiocyanate the production of methionine sulphoxide declined. These results indicate that although the unstable oxidant must be derived from thiocyanate, high concentrations of thiocyanate inhibit its formation.

Spectral changes of peroxidases during oxidation of thiocyanate

To investigate the mechanism by which peroxidases promote the formation of the short-lived oxidant, we monitored the absorption spectra of eosinophil peroxidase and myeloperoxidase during enzyme turnover. First, we characterized the absorption

Figure 7 Absorption spectra of eosinophil peroxidase during the oxidation of thiocyanate and bromide

Reactions were performed at room temperature in 100 mM phosphate buffer, pH 7.4, containing 5 mM methionine, 840 nM eosinophil peroxidase, 1 mg/ml glucose and 100 μ M thiocyanate (A) or 100 μ M bromide (B). The native spectrum of eosinophil peroxidase (EP³⁺) was recorded before the addition of glucose oxidase calculated to produce 2.6 μ M/min H₂O₂. Spectra were recorded at 0, 2.5 and 6 min for (A) and at 0 and 6 min for (B). The arrows indicate changes in the native spectrum. Each spectrum was recorded over 2 s and is an average of 20 spectra. Results are representative of at least duplicate experiments.

spectra of compound II and compound III of eosinophil peroxidase. To produce compound II, 100 μ M H₂O₂ was added to native eosinophil peroxidase. This caused an immediate shift in the Soret peak from 415 to 435 nm, with an isosbestic point at 425 nm (Figure 6A). There was also the formation of a minor peak at 575 nm. A similar spectrum for compound II of the human enzyme was reported recently [11]. Compound II slowly decayed back to the ferric enzyme (results not shown). Compound III was formed by adding 1 mM H₂O₂ to native eosinophil peroxidase. This resulted in an immediate spectral shift of the Soret peak to 427 nm, with an isosbestic point at 419 nm; three new peaks in the visible range appeared at 551, 588 and 641 nm (Figure 6B).

When eosinophil peroxidase oxidized thiocyanate there was a shift in its Soret peak from 415 to 435 nm and there was a distinct isosbestic point at 425 nm (Figure 7A). These changes are indicative of the conversion of the native enzyme into compound II. The addition of GSH to scavenge hypothiocyanite prevented these spectral changes (results not shown). During the oxidation of bromide there was no change in the absorbance spectrum of eosinophil peroxidase (Figure 7B). Myeloperoxidase was also

converted into its compound II form during the oxidation of thiocyanate, as indicated by a shift in its Soret peak from 430 to 455 nm, with the isosbestic point at 441 nm. As with eosinophil peroxidase, formation of compound II was blocked by GSH and did not occur when chloride was the reducing substrate (results not shown). GSH would not have contributed to the turnover of compound II because it did not reduce preformed compound II of either peroxidase (results not shown). These results demonstrate that hypothiocyanite must have reacted with the peroxidases to convert them into their compound II forms. When bromide or chloride was used as the substrate, the hypobromous acid or hypochlorous acid formed would not have reacted with the peroxidases because both these oxidants would have been scavenged by the methionine present in the buffer.

DISCUSSION

The recent identification of brominated proteins in bronchoalveolar lavage from asthmatics provides the most convincing evidence that eosinophils are activated and generate hypobromous acid in the airways [41]. This result strongly suggests that eosinophil peroxidase might have a pivotal role in the pathophysiology of asthma. It is therefore important to appreciate the cytotoxic potential of this comparatively little-studied enzyme. In the present study we investigated the substrate preference of eosinophil peroxidase to clarify what oxidants it is likely to produce *in vivo*. Two related studies have been undertaken previously, with very dissimilar results [4,10]. We used a different approach to determine the substrate preference of the enzyme and showed that thiocyanate is preferred only 2.8-fold over bromide. These results indicate that the enzyme can be expected to produce both hypothiocyanite and hypobromous acid in the airways of asthmatics.

In the present study fundamentally different results were obtained in comparison with earlier investigations [4,10]. These differences can be attributed to our use of a H_2O_2 electrode to measure enzyme activity; they have important implications for the action of eosinophil peroxidase *in vivo*. Slungaard and Mahoney [4] based their conclusion that thiocyanate is the major and possibly exclusive substrate of eosinophil peroxidase on results obtained for the initial rate of oxidation of TNB. They calculated a rate of oxidation with 100 μM thiocyanate 2.5-fold that with 100 μM bromide. This value is in good agreement with the 2.8-fold higher specificity constant for thiocyanate over bromide obtained in this study (Table 1) and with that reported by Wu et al. [10]. However, they also found that the rate of oxidation of TNB by eosinophil peroxidase/thiocyanate/ H_2O_2 was minimally affected by bromide and concluded that, when both substrates were present together, thiocyanate is oxidized in preference to bromide. In agreement with them, we showed that bromide had only a small effect on the rate of consumption of H_2O_2 by eosinophil peroxidase and thiocyanate. However, by measuring specificity constants as well as undertaking product analysis, we were able to show that thiocyanate and bromide are both oxidized when present together.

Although the relative specificity constant that we obtained was the same as that reported by Wu et al. [10], the kinetic constants in the two studies were very different. In the previous study, in which enzyme activity was measured with TNB, much lower values were obtained for the catalytic rate constants and the K_m values were considerably higher. This strongly suggests that in their assay eosinophil peroxidase was largely inhibited. This would have occurred, as it does with myeloperoxidase [35], because TNB is not an inert detector of hypohalous acids. It was shown to react directly with eosinophil peroxidase because it

was oxidized in the absence of reducing substrates [10]. As with other thiols, TNB would inhibit eosinophil peroxidase by converting it into compound II [42]. On the basis of the K_m values that they measured, Wu et al. concluded that at plasma concentrations of its substrates eosinophil peroxidase is far from saturated [10]. However, in the present study we found the K_m for thiocyanate to be 150 μM . This concentration could feasibly be reached *in vivo*. Thus in some situations the enzyme's activity might be close to maximal. This was evident in our study when the enzyme was present with 100 μM bromide and 100 μM thiocyanate together (Figure 1).

Given the fact that the preferences for thiocyanate and bromide are not markedly different, the precise mix of oxidants produced by eosinophil peroxidase is determined largely by the local substrate concentrations. For example, at 90 μM bromide and 30 μM thiocyanate, these substrates are oxidized equally and hypobromous acid is a major product. Concentrations of these substrates in the airways of asthmatics vary considerably between individuals. This has a major impact on the potential cytotoxicity of eosinophil peroxidase because hypobromous acid is extremely damaging to cells, whereas hypothiocyanite is much less toxic [43]. Paradoxically, smoking might have a beneficial effect on asthma because it elevates thiocyanate levels [8], but anti-asthma drugs that are administered as bromide salts (e.g. fenoterol and ipratropium bromide) could potentiate inflammatory tissue damage.

A novel finding in our study is that hypothiocyanite is not the only oxidant produced by peroxidases from thiocyanate. We also found that a short-lived oxidant was generated by eosinophil peroxidase and myeloperoxidase. It was more reactive than hypothiocyanite because it was able to oxidize methionine to methionine sulfoxide. In accord with our results, the existence of additional oxidation products besides hypothiocyanite in the eosinophil peroxidase/thiocyanate/ H_2O_2 system has recently been demonstrated by showing that hypothiocyanite and cyanate (OCN^-) are formed in roughly equimolar amounts [24]. It was suggested that cyanate might form through a sequence of reactions beginning with the oxidation of hypothiocyanite by H_2O_2 , as proposed previously by Pruitt et al. [23]. Our results suggest that this reaction is catalysed by peroxidases because a direct reaction between hypothiocyanite and H_2O_2 does not fit with our finding that the production of hypothiocyanite was independent of enzyme concentration. If a direct reaction occurred, it would have been in competition with the consumption of H_2O_2 by the peroxidases, so that more hypothiocyanite would have been produced at the higher enzyme concentrations. We propose that hypothiocyanite reduces compound I and compound II and is thereby converted into stronger oxidizing species. This proposal is based on the findings that the peroxidases were present as compound II during the oxidation of thiocyanate and that the removal of hypothiocyanite by GSH prevented the accumulation of compound II. It is also supported by the finding that more hypothiocyanite was detected at high concentrations of thiocyanate. This would occur because of the competition between thiocyanate and hypothiocyanite for compound I. The formation of higher oxidation products of hypothiocyanite extends the cytotoxic potential of peroxidase/thiocyanate/ H_2O_2 systems beyond mere damage to thiols. It also explains the results of Jong et al. [17], who showed that eosinophil peroxidase was bactericidal to *E. coli* at 100 μM thiocyanate, but not at higher or lower concentrations.

In agreement with other investigators [11,12,14] we showed that chloride is a very poor substrate for eosinophil peroxidase. However, we did find that the enzyme was capable of producing substantial quantities of hypochlorous acid below pH 6.5.

Eosinophil peroxidase could therefore produce hypochlorous acid *in vivo* at low pH, especially when the concentrations of thiocyanate and bromide are low, or after these substrates have been consumed.

In conclusion, we have shown that eosinophil peroxidase is capable of producing a variety of reactive oxidants at physiological concentrations of chloride, bromide and thiocyanate. The relative concentrations of these substrates determine the type and extent of inflammatory tissue damage caused by the enzyme. Our demonstration that a short-lived oxidant is formed from hypothyocyanite indicates that peroxidase/thiocyanate/H₂O₂ systems have multiple molecular targets besides thiols.

We thank the Health Research Council of New Zealand for financial support, and Professor Christine Winterbourn for helpful advice on this project.

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