Inhibition of myeloperoxidase by benzoic acid hydrazides

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Myeloperoxidase is the most abundant protein in neutrophils and catalyses the conversion of H_2O_2 and chloride into HOCl. To help clarify the role of this enzyme in bacterial killing and inflammation, a specific and potent inhibitor needs to be identified. We have studied a series of benzoic acid hydrazides and found that in general they inhibit the peroxidation activity of myeloperoxidase with an IC_{50} value of less than 10 μM. The IC_{50} values of derivatives with substituents containing oxygen or nitrogen were related to their Hammett substituent constants. This indicates that myeloperoxidase oxidizes the hydrazide group of these compounds, and the degree to which they inhibit the enzyme is dependent on the ease of their oxidation. Unsubstituted benzoic acid hydrazide and its 4-chloro derivative were poor inhibitors of peroxidation. Thus it is likely that hydrogen-bonding of the enzyme to substituents containing oxygen or nitrogen increases the binding affinity of the hydrazides and enhances their oxidation by myeloperoxidase. 4-Aminobenzoic acid hydrazide (ABAH) was the most potent inhibitor of peroxidation. It irreversibly inhibited HOCl production by the purified enzyme, having an IC_{50} value of 0.3 μM. With neutrophils stimulated with opsonized zymosan or phorbol myristate acetate, ABAH inhibited HOCl production by up to 90% and the IC_{50} values were 16 μM and 2.2 μM respectively. In the presence of superoxide dismutase, these values decreased to 6.4 μM and 0.6 μM respectively. ABAH had no effect on superoxide radical ($O_2^{-\cdot}$) production and degranulation by neutrophils, nor did it inhibit catalase or glutathione peroxidase. Thus ABAH is an effective and selective inhibitor that should be useful for determining the contribution of myeloperoxidase to oxidant-mediated reactions of neutrophils.

INTRODUCTION

Neutrophils provide a first line of defence against invading pathogens and they are also important mediators of tissue injury in a variety of inflammatory pathologies [1]. When stimulated, they undergo a respiratory burst, generating superoxide radical ($O_2^{-\cdot}$) which is converted into the more reactive secondary oxidants H_2O_2 and HOCl [2]. Myeloperoxidase (donor: H_2O_2 oxidoreductase; EC 1.11.1.7) produces HOCl from H_2O_2 and chloride [3]. This haem enzyme is the most abundant protein in neutrophils, comprising 5% of their dry weight [2]. It initially reacts with H_2O_2 to form the redox intermediate compound I, which in turn reacts with chloride to liberate HOCl and regenerate the native enzyme (reactions 1 and 2) [4]. In addition to its chlorination activity, myeloperoxidase peroxidizes numerous phenols, anilines and β-diketones (AH) via the classical peroxidation cycle (reactions 3 and 4).

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

$$\text{Compound I} + Cl^- + H^+ \rightarrow MP^{3+} + HOCl \quad (2)$$

$$\text{Compound I} + AH \rightarrow \text{Compound II} + A^\cdot + H^+ \quad (3)$$

$$\text{Compound II} + AH \rightarrow MP^{3+} + A^\cdot + H^+ \quad (4)$$

$$\text{Compound II} + O_2^- \rightarrow MP^{3+} + O_2 \quad (5)$$

HOCl is the most powerful oxidant generated by neutrophils in appreciable amounts [5,6]. It oxidizes thiols and reacts with unsaturated fatty acids to form chlorohydrins which may destabilize lipid membranes [7], with α_1-proteinase inhibitor to nullify its ability to inhibit elastase, and with amino acid residues to disrupt the tertiary structure and function of proteins [8]. HOCl should play a major role in bacterial killing and has considerable potential to promote the tissue damage caused by errant neutrophils.

To help clarify the role of myeloperoxidase in bacterial killing and inflammation, a specific and potent inhibitor of this enzyme needs to be found. A number of clinically useful non-steroidal anti-inflammatory drugs inhibit myeloperoxidase [9,10]. Drugs such as dapsone and mefenamic acid are potent inhibitors because they are poor peroxidase substrates and trap the enzyme as compound II (reaction 3), which is inactive in chlorination [9]. However, $O_2^{-\cdot}$ limits the ability of these drugs to inhibit myeloperoxidase by reducing compound II back to the ferric enzyme (reaction 5) [11]. Thus other strategies are needed to inhibit myeloperoxidase. These include irreversible inactivation of the enzyme, competitive inhibition of chloride oxidation and conversion of the enzyme into a stable redox intermediate that does not oxidize chloride and is not recycled by $O_2^{-\cdot}$.

Recently, it has been reported that salicylhydroxamic acid is a specific inhibitor of myeloperoxidase [12,13]. It is oxidized in preference to chloride and prevents the binding of chloride to the haem groups of the enzyme. Hydrazines ($RNHNH_2$) and hydrazides ($RCONHNH_2$) are also likely inhibitors of myeloperoxidase, as they are suicide substrates of other peroxidases [14]. The antituberculous agent isonicotinic acid hydrazide (isoniazid) inhibits myeloperoxidase, causing irreversible loss of haem absorbance [15]. In this investigation we have studied a series of benzoic acid hydrazides to determine what properties make them effective inhibitors of myeloperoxidase, and assessed the ability of the most potent inhibitor to prevent production of HOCl by human neutrophils.

MATERIALS AND METHODS

Materials

4-Aminobenzoic acid hydrazide (ABAH), 4-hydroxybenzoic acid hydrazide, 4-chlorobenzoic acid hydrazide and 3-nitrobenzoic

Abbreviations used: ABAH, 4-aminobenzoic acid hydrazide; $O_2^{-\cdot}$, superoxide radical.
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acid hydrazide were from Fluka Chemicals (Buchs, Germany). 4-Methoxybenzoic acid hydrazide and 3-methoxybenzoic acid hydrazide were from Cambrian Chemicals (Croydon, Surrey, U.K.). Benzoic hydrazide was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Stock solutions of these compounds were prepared daily in 50 mM sodium acetate buffer (pH 5.4) or 10 mM sodium phosphate buffer (pH 7.4) containing 138 mM NaCl and 10 mM KCl (PBS), to which 50 μM diethylenetriaminepenta-acetic acid was added. Bovine erythrocyte superoxide dismutase, phorbol myristate acetate, taurine, cytochrome *c* (type III), bovine liver catalase, zymosan, diethylenetriaminepenta-acetic acid, salicylhydroxamic acid and xanthine oxidase were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). H_2O_2 was prepared daily by dilution of a stock solution (BDH Chemicals New Zealand Ltd., Palmerston North, New Zealand) and concentrations were determined using ϵ_{240} 43.6 $M^{-1} \cdot cm^{-1}$ [16]. Myeloperoxidase was isolated from human neutrophils and had a purity index of greater than 0.72 [17]. Its concentration was determined using ϵ_{430} 91 000 $M^{-1} \cdot cm^{-1}$ [18].

Purification of human neutrophils

Neutrophils were isolated from the blood of healthy donors by Ficoll/Hypaque centrifugation, dextran sedimentation and hypotonic lysis of contaminating erythrocytes [19]. Zymosan was boiled for 20 min and washed once in PBS. It was then incubated at 5 mg/ml in 30% human plasma with end-over-end rotation for 20 min at 37 °C. The opsonized zymosan was washed and resuspended at 50 mg/ml in PBS.

Determination of the activities of purified myeloperoxidase

As a measure of the chlorination activity of myeloperoxidase, H_2O_2 consumption by the purified enzyme in the presence of chloride was monitored continuously with a YSI model 25 oxidase meter fitted with a YSI 2510 oxidase probe (Yellow Springs Instruments, Yellow Springs, OH, U.S.A.) as described previously [9,20]. Reactions were performed at 25 °C and started by adding myeloperoxidase. Taurine (10 mM) was added to reaction systems to scavenge HOCl and prevent it from inactivating myeloperoxidase. Initial rates were determined by drawing a tangent to the initial linear part of the curve for H_2O_2 loss. The peroxidase activity of myeloperoxidase was assayed by measuring the oxidation of 1.4 mM tetramethylbenzidine at 655 nm in 3.5 ml of sodium acetate buffer with 0.3 mM H_2O_2 [21]. Dimethylformamide was included in the reaction buffer at 8% to keep tetramethylbenzidine in solution. Reactions were performed at 37 °C and stopped after 3 min by adding 10 μg/ml catalase and 3.4 ml of 0.2 M acetic acid. A rectangular hyperbola was fitted to the dose–response curve for each inhibitor using non-linear regression (SigmaPlot, Jandel Scientific, San Rafael, CA, U.S.A.). From the equation that was obtained, the concentration of inhibitor that decreased HOCl production by 50% (IC_{50}) was calculated.

Production of HOCl by human neutrophils

Neutrophils were incubated in PBS containing 15 mM taurine, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 1 mg/ml glucose. The cells were stimulated with either 100 ng/ml phorbol myristate acetate or 1 mg/ml opsonized zymosan, and at the end of the incubation period chlorination of taurine was stopped by adding 10 μg/ml catalase and placing tubes in melting ice. Neutrophils were then pelleted by centrifugation, and the supernatant was assayed for taurine chloramine using 2-nitro-5-thiobenzoate (ϵ_{412} 14 100 $M^{-1} \cdot cm^{-1}$) [22]. At a concentration of 100 μM, ABAH did not affect the reaction of HOCl with taurine or with the detection of taurine chloramine.

Determination of the effects of ABAH on enzyme activities

The effects of 100 μM ABAH on $O_2^{-\cdot}$ production by neutrophils, and the activities of catalase and glutathione peroxidase were also determined. $O_2^{-\cdot}$ production by neutrophils stimulated with 5 mg/ml opsonized zymosan was measured as superoxide dismutase-inhibitable cytochrome *c* reduction [23]. The activity of purified catalase was determined by adding the enzyme to a solution of H_2O_2 with an initial absorbance at 240 nm of approx. 0.5, and the time required for the absorbance to decrease from 0.450 to 0.400 was recorded [24]. The activity of glutathione peroxidase from erythrocyte lysates was determined by using *t*-butyl hydroperoxide as its substrate. Production of oxidized glutathione was coupled to its reduction by glutathione reductase, which was followed by measuring the formation of $NADP^+$ at 340 nm [25].

Degranulation of neutrophils

Neutrophils (1×10^7/ml) were incubated at 37 °C, treated with 10 μg/ml cytochalasin B for 10 min, then stimulated with 10 μm formylmethionyl-leucylphenylalanine for a further 10 min. The amounts of lysozyme [26] and β-glucuronidase [27] in the supernatants from stimulated cells in the presence or absence of 100 μM ABAH were expressed as percentages of the total amounts of each enzyme released from cells by lysis with 0.1% Triton X-100.

RESULTS

Inhibition of the peroxidation activity of purified myeloperoxidase

We determined the ability of a series of benzoic acid hydrazides (R-ϕ-$CONHNH_2$) to inhibit myeloperoxidase-dependent oxidation of tetramethylbenzidine. For each compound, the IC_{50} was determined from its dose–response curve, such as that shown for ABAH (Figure 1). The compounds tested were excellent

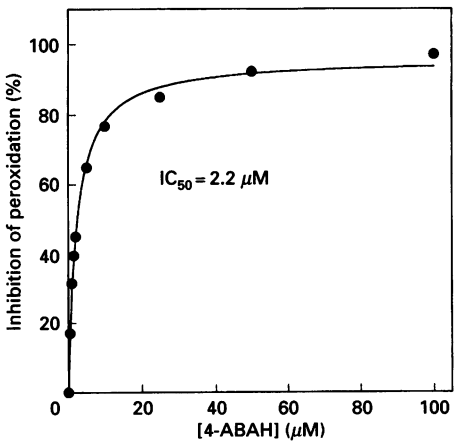

Figure 1 Effect of ABAH on the peroxidation activity of myeloperoxidase

Myeloperoxidase (2 nM) was incubated at 37 °C with 1.4 mM tetramethybenzidine in 50 mM acetate buffer, pH 5.4, containing 8% dimethylformamide and various concentrations of ABAH. The reaction was started by adding 300 μM H_2O_2. After 3 min, 10 μg/ml catalase was added and the increase in A_{655} was measured.

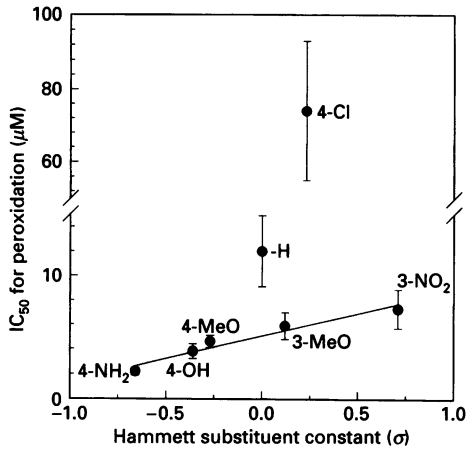

Figure 2 Relationship between the Hammett substituent constant and the ability of benzoic acid hydrazides to inhibit myeloperoxidase

The IC_{50} value of each benzoic acid hydrazide was determined under the same conditions as described in Figure 1. Hammett substituent constants were obtained from ref. [28]. IC_{50} values are the means ± S.D. of at least three experiments. Linear regression analysis was applied to all data points except for those for benzoic acid hydrazide and chlorobenzoic acid hydrazide.

inhibitors of myeloperoxidase, with the exception of 4-chlorobenzoic acid hydrazide. Their IC_{50} values, which are plotted against their Hammett substituent constants in Figure 2, were typically less than about 10 μM. For comparison, the IC_{50} for salicylhydroxamic acid in this assay was 32 μM.

Reactivity of aromatic compounds is often determined by the electron-donating or -withdrawing capacity of their substituents. The magnitude of these effects is related to the Hammett substituent constant σ [28]. A positive value of σ indicates that the substituent is electron-withdrawing, whereas a negative value indicates that it is electron-donating. If benzoic acid hydrazide and 4-chlorobenzoic acid hydrazide are excluded from the analysis, there is a very good correlation ($r^2 = 0.95$) between the IC_{50} values for peroxidation and σ.

Inhibition of the chlorination activity of purified myeloperoxidase

Inhibition of the peroxidation activity of myeloperoxidase by a compound does not necessarily indicate that the compound will also inhibit the chlorination activity of the enzyme [20]. We therefore determined the ability of ABAH, which was the most potent inhibitor of peroxidation, to inhibit the production of HOCl. Formation of this compound from purified myeloperoxidase, H_2O_2 and chloride was measured by continuously monitoring the loss of H_2O_2 in the presence of taurine (Figure 3a). We have shown previously that all the H_2O_2 is converted into HOCl which reacts with taurine to form taurine chloramine [29]. At 5 μM or greater, ABAH prevented the loss of H_2O_2 (Figure 3b). Ascorbate, which reduces compound II to active enzyme, did not reverse the inhibition [9]. The dose–response for inhibition of the initial rate of H_2O_2 loss gives an IC_{50} of 0.3 μM for ABAH (Figure 3b). For comparison, the IC_{50} for salicylhydroxamic acid was approx. 25 μM.

Inhibition of HOCl production by stimulated human neutrophils

When neutrophils were stimulated with opsonized zymosan, ABAH inhibited production of HOCl by up to about 90 %, with an IC_{50} of 16 μM (Figure 4). As $O_2^{-\cdot}$ decreases the ability of some

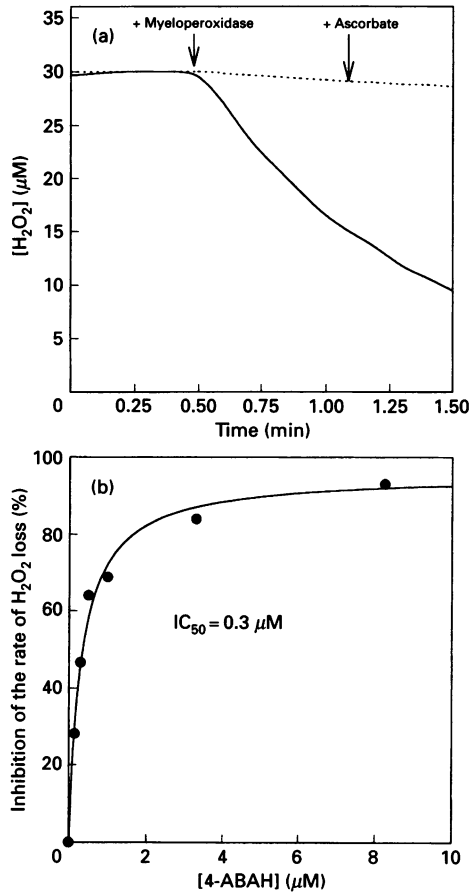

Figure 3 Effect of ABAH on the chlorination activity of myeloperoxidase

(**a**) Chlorination activity was measured by monitoring the loss of 30 μM H_2O_2 catalysed by 15 nM myeloperoxidase in 50 mM phosphate buffer, pH 7.4, containing 150 mM NaCl plus (---) or minus (——) 10 μM ABAH. The reaction temperature was 25 °C. Ascorbate (30 μM) was added to the reaction mixture containing ABAH at the time indicated by the arrow. (**b**) The initial rate of loss of H_2O_2 was measured and the percentage inhibition of myeloperoxidase was calculated at various concentrations of ABAH.

non-steroidal anti-inflammatory drugs to inhibit myeloperoxidase by reducing inactive compound II back to the active enzyme [11], we investigated the effect of superoxide dismutase on inhibition of HOCl production by ABAH. Maximum inhibition was still about 90 %, but the IC_{50} was decreased to 6.4 μM. Similar results were obtained when cells were stimulated with phorbol myristate acetate. The IC_{50} values were 2.2 μM and 0.6 μM in the absence and presence of superoxide dismutase respectively (results not shown). Salicylhydroxamic acid was a much poorer inhibitor of HOCl production with neutrophils stimulated with opsonized zymosan. Its IC_{50} was 60 μM, and 200 μM was required for complete inhibition (not shown).

Effects of ABAH on neutrophil function

To establish the specificity of ABAH for myeloperoxidase, we also determined its effect on other neutrophil enzymes involved in H_2O_2 metabolism, and on degranulation of these cells (Table 1). At 100 μM, it had no effect on $O_2^{-\cdot}$ production, and did not inhibit purified catalase or glutathione peroxidase from erythrocyte lysates. The conditions for the catalase assay were the same as those used to show that this enzyme is inhibited by the related compound phenylhydrazine [30]. ABAH also had no

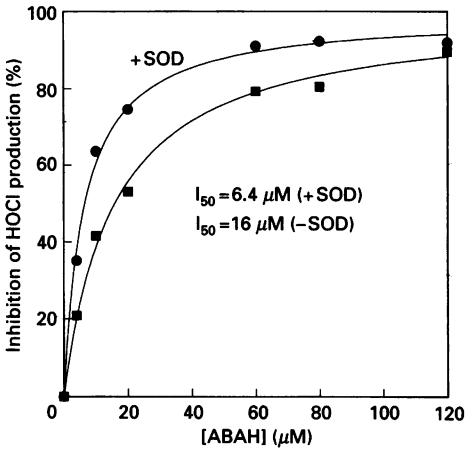

Figure 4 Effect of ABAH on the production of HOCl by human neutrophils

Cells (2×10^6/ml) were stimulated with 1 mg/ml opsonized zymosan in 10 mM phosphate buffer containing 140 mM NaCl, 15 mM taurine and various concentrations of ABAH. Reactions were performed at 37 °C in the presence (●) or absence (■) of 20 μg/ml superoxide dismutase and stopped after 20 min by adding 10 μg/ml catalase and placing cells on melting ice. Cells produced 37 μM HOCl in the presence of superoxide dismutase and 32 μM HOCl in its absence. Data are representative of three experiments. Other conditions are described in the Materials and methods section.

Table 1 Effect of ABAH on neutrophil function and enzyme activity

$O_2^{-\bullet}$ production by neutrophils stimulated with opsonized zymosan was determined by measuring cytochrome *c* reduction. Lysozyme and β-glucuronidase were measured after stimulating cells with formylmethionyl-leucylphenylalanine and cytochalasin B. The ability of 100 ng/ml catalase to degrade 12 mM H_2O_2 was determined by monitoring A_{240}. Erythrocyte glutathione peroxidase activity was measured by coupling it to the oxidation of NADPH. Details of these assays are described in the Materials and methods section. The mean and range of duplicate experiments are given.

	Control	+ABAH (100 μM)
Function		
$O_2^{-\bullet}$ production (μmol/min per 10^6 cells)	7.9 (0.1)	7.8 (0.2)
Lysozyme release (% of total cell content)	59 (2)	63 (2)
β-Glucuronidase release (% of total cell content)	27 (1)	27 (0)
Enzyme activity		
Catalase ($-\Delta A_{240}$/min)	0.028 (0.003)	0.028 (0.001)
Glutathione peroxidase ($-\Delta A_{340}$/min)	0.051 (0.004)	0.054 (0.004)

effect on degranulation of neutrophils as measured by the release of lysozyme and β-glucuronidase. At 200 μM, salicylhydroxamic acid inhibited $O_2^{-\bullet}$ production by cells stimulated with opsonized zymosan by 60%.

DISCUSSION

In this investigation we have shown that benzoic acid hydrazides are excellent inhibitors of myeloperoxidase. The 4-amino derivative (ABAH) was the most effective inhibitor of the peroxidation activity. It was also a potent inhibitor of HOCl production by the purified enzyme and stimulated neutrophils. In contrast with its effect on myeloperoxidase, ABAH did not inhibit other enzymes involved in H_2O_2 metabolism, nor did it alter degranulation of neutrophils. Therefore we conclude that, in neutrophils, ABAH reacts selectively with myeloperoxidase and should be useful for investigating the effects of myeloperoxidase on neutrophil-mediated processes.

Compared with a range of phenols and anilines investigated under the same experimental conditions, ABAH is one of the best inhibitors of the chlorination activity of purified myeloperoxidase that we have found [9]. Although other workers have used salicylhydroxamic acid to inhibit myeloperoxidase [12,13], we have found it to be a poor inhibitor of both the peroxidation and chlorination activities of myeloperoxidase in comparison with ABAH. Furthermore, at concentrations needed to inhibit HOCl production by neutrophils, salicylhydroxamic acid impaired their generation of $O_2^{-\bullet}$. Thus salicylhydroxamic acid is of limited value in determining the contribution of myeloperoxidase to oxidant-mediated reactions of neutrophils. ABAH should also be more useful than azide or cyanide in dissecting out a role for HOCl in oxidant-dependent reactions of neutrophils, as it inhibits myeloperoxidase but not catalase. In contrast, azide and cyanide inhibit both these haem enzymes so that reactions of HOCl are blocked whereas reactions of H_2O_2 are enhanced.

In general, the ability of benzoic acid hydrazides to inhibit the peroxidation activity of myeloperoxidase increased with the electron-donating capacity of the substituents on the aromatic ring. From this result we conclude that inhibition of myeloperoxidase is dependent on the ease of oxidation of these compounds, and that the hydrazide group is oxidized by the enzyme. Spectral changes in ABAH in the presence of myeloperoxidase and H_2O_2 indicate that oxidation does occur under these conditions (not shown). It is likely that an oxidation product, probably the radical, irreversibly inactivates the enzyme. Unsubstituted benzoic acid hydrazide and its 4-chloro derivative were poor inhibitors compared with the other compounds, and the degree to which they inhibited was not related to their Hammett substituent constants. A possible explanation is that they bind less strongly to myeloperoxidase than the other hydrazides. Salicylhydroxamic acid binds to myeloperoxidase about three orders of magnitude more strongly than benzohydroxamic acid [13]. It has been suggested that the aromatic ring of these compounds binds to a hydrophobic pocket at the distal haem cavity of myeloperoxidase with the hydroxamic side chain placed between the imidazole of the distal histidine and the haem iron [31]. The greater affinity of salicylhydroxamic acid was explained by an extra interaction of its hydroxy group with an amino acid side chain. By analogy, hydrazides with substituents containing oxygen or nitrogen could have a higher affinity for the enzyme through hydrogen bond formation. This would not occur with 4-chlorobenzoic acid hydrazide and benzoic acid hydrazide, and consequently they would be less readily oxidized.

ABAH was more effective at inhibiting purified myeloperoxidase than with neutrophils (compare Figures 3b and 4). In addition, its effect on cellular production of HOCl depended on the type of stimulus used and on the presence of $O_2^{-\bullet}$. As shown under other conditions [11], the $O_2^{-\bullet}$-independent differences in IC_{50} probably arise because the enzyme is variably saturated with H_2O_2 in the different situations. At high H_2O_2/myeloperoxidase ratios, when the enzyme is saturated, partial inactivation will decrease the rate of chlorination. However, when the ratio is lower, the H_2O_2-generation rate is more influential than the myeloperoxidase concentration, so that more of the enzyme will need to be inhibited to see an effect. The purified enzyme was

studied under saturating conditions. This is also the condition in cells stimulated with phorbol myristate acetate, but not with opsonized zymosan which causes the release of much more myeloperoxidase and less H_2O_2 [32]. The increasing trend in IC_{50} for the purified enzyme (0.3 μM) to that for cells stimulated with phorbol myristate acetate (0.6 μM) and opsonized zymosan (6.4 μM) is consistent with this explanation.

The more effective inhibition of HOCl formation by ABAH in the presence of superoxide dismutase cannot be explained by $O_2^{-\bullet}$ overcoming inhibition due to compound II formation [9,11]. This is because ascorbate, which also reduces compound II back to the active enzyme, did not reverse inactivation by ABAH (Figure 3). It is more likely that $O_2^{-\bullet}$ influences production or reactions of the ABAH radical, which appears to be responsible for inactivating the enzyme (A. J. Kettle, C. A. Gedye and C. C. Winterbourn, unpublished work).

In summary, we have shown that benzoic acid hydrazides are excellent inhibitors of myeloperoxidase. ABAH is particularly effective and should be useful in determining the extent to which myeloperoxidase and HOCl contribute to oxidant-mediated reactions of neutrophils. We have used it to establish that myeloperoxidase plays a dominant role in the killing of *Staphylococcus aureus* (M. B. Hampton, C. C. Winterbourn and A. J. Kettle, unpublished work), and catalyses the hydroxylation of salicylate by human neutrophils [33]. As far as we are aware, ABAH is the most potent and selective inhibitor currently available for preventing production of HOCl by neutrophils.

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