Mechanism of inactivation of myeloperoxidase by 4-aminobenzoic acid hydrazide

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Hypochlorous acid is the most powerful oxidant generated by neutrophils and is likely to contribute to the damage mediated by these inflammatory cells. The haem enzyme myeloperoxidase catalyses its production from hydrogen peroxide and chloride. 4-Aminobenzoic acid hydrazide (ABAH) is a potent inhibitor of hypochlorous acid production. In this investigation we show that, in the presence of hydrogen peroxide, ABAH irreversibly inactivates myeloperoxidase. ABAH was oxidized by myeloperoxidase, and kinetic analysis of the inactivation conformed to that for a mechanism-based inhibitor. Inactivation was exacerbated by concentrations of hydrogen peroxide greater than 50 μM and by the absence of oxygen. Hydrogen peroxide alone caused minimal inactivation. Reduced glutathione inhibited the oxidation of ABAH as well as the irreversible inhibition of myeloperoxidase. In the presence of oxygen, ABAH and hydrogen peroxide initially converted myeloperoxidase into compound III, which susequently lost haem absorbance. In the absence of oxygen, the enzyme was converted into ferrous myeloperoxidase and its haem groups were rapidly destroyed. We propose that myeloperoxidase oxidizes ABAH to a radical that reduces the enzyme to its ferrous intermediate. Ferrous myeloperoxidase reacts either with oxygen to allow enzyme turnover, or with hydrogen peroxide to give irreversible inactivation.

INTRODUCTION

Hypochlorous acid is the most powerful oxidant produced by neutrophils in appreciable amounts [1,2]. It reacts with many biomolecules and therefore has the potential to cause considerable tissue damage in diseases characterized by an infiltration of neutrophils [3,4]. The haem enzyme myeloperoxidase catalyses the production of hypochlorous acid from hydrogen peroxide and chloride [5]. Hydrogen peroxide is formed from the dismutation of superoxide, which is generated by an NADPH oxidase in the plasma membrane of neutrophils [6]. Numerous compounds inhibit myeloperoxidase [7,8]. These inhibitors are useful for probing the role of hypochlorous acid in inflammatory tissue damage and microbial killing, and provide valuable information on the mechanism of action of the enzyme. Also, inhibitors directed against myeloperoxidase may eventually be useful in attenuating oxidant-dependent injury in inflammatory diseases.

Phenols and anilines prevent myeloperoxidase from producing hypochlorous acid by trapping the enzyme as inactive compound II [8]. This type of inhibition is reversible and limited because superoxide reduces compound II back to the active ferric enzyme [2]. Apart from producing hypochlorous acid, myeloperoxidase displays considerable peroxidation activity and oxidizes phenols, anilines and β-diketones to reactive free radicals [9]. Generally these radicals either dimerize or dismutate to give stable end products. However, it is possible that they could react with the enzyme and inactivate it. This type of suicide inactivation occurs with other peroxidases [10,11] and is potentially the best route to preventing production of hypochlorous acid during inflammation. Isoniazid [12] and propylthiouracil [13] have been identified as suicide substrates of myeloperoxidase. However, the mechanisms by which they act have not been established. 4-Aminobenzoic acid hydrazide (ABAH) is one of the best inhibitors of hypochlorous acid production that we have identified [14]. It is considerably more potent than salicylhydroxamic acid, which is used as a specific inhibitor of myeloperoxidase [15,16]. In contrast to most phenols and anilines, ABAH does not act by converting the enzyme into compound II, since inhibition is not reversed by ascorbate [14]. In the present study we have investigated the mechanism by which ABAH inhibits myeloperoxidase. We have found that it is a suicide substrate of myeloperoxidase and promotes inactivation through a reaction that involves the ferrous enzyme.

MATERIALS AND METHODS

Materials

ABAH was Purum grade from Fluka. It was made up as a 10 mM stock solution in 10 mM HCl with 100 μM diethylenetriaminepenta-acetic acid (DTPA). Tetramethylbenzidine, GSH, taurine, 5,5′-dithiobis-(2-nitrobenzoic acid), DTPA, glucose oxidase from *Aspergillus niger*, bovine liver catalase and bovine erythocyte superoxide dismutase were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 5-Thio-2-nitrobenzoic acid was prepared from 5,5′-dithiobis-(2-nitrobenzoic acid) [17]. Myeloperoxidase was purified from human neutrophils to a purity index (A_{430}/A_{280}) of at least 0.72 as described previously [18]. Its concentration was calculated using $\epsilon_{430} = 91\,000\ M^{-1}\cdot cm^{-1}$ per haem [19]. Hydrogen peroxide solutions were prepared daily by diluting a stock solution, and concentrations were determined using $\epsilon_{240} = 43.6\ M^{-1}\cdot cm^{-1}$ [20].

Oxidation of ABAH

Oxidation of ABAH by myeloperoxidase and hydrogen peroxide was measured by recording difference spectra against unoxidized substrate or by monitoring the increase in A_{325}. Oxidation products were separated by HPLC using a 250 mm × 4.6 mm Nucleosil 5 μm C18 column with an isocratic mobile phase of 76% water, 24% acetonitrile and 0.1% acetic acid. The flow

Abbreviations used: ABAH, 4-aminobenzoic acid hydrazide; DTPA, diethylenetriaminepenta-acetic acid.

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rate was 0.8 ml/min, and ABAH was quantified by measuring its peak area at 280 nm using a Philips PU 4120 diode array detector. To determine $\Delta\epsilon_{325}$ for oxidized ABAH, the loss in ABAH catalysed by myeloperoxidase was determined by HPLC and then plotted against the increase in absorbance at 325 nm. From the slope of this plot, $\Delta\epsilon_{325}$ was calculated to be 10300 $M^{-1}\cdot cm^{-1}$ ($n = 10$, $r^2 = 0.99$).

All reactions were carried out at 20 ± 2 °C in 100 mM phosphate buffer (pH 7.4) containing 100 μM DTPA. Oxygen consumption during the oxidation of ABAH was monitored continuously using a YSI model 53 oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.). Glucose oxidase was also used as a source of hydrogen peroxide, and its activity was measured using a YSI model 25 oxidase meter fitted with a YSI 2510 oxidase probe [17]. When required, buffers were made anaerobic by bubbling with nitrogen for 5 min in a sealed cuvette and maintaining its flow in the headspace during reactions.

Measurement of the activity of myeloperoxidase

ABAH was incubated with myeloperoxidase in 100 mM phosphate buffer, pH 7.4, containing 100 μM DTPA and hydrogen peroxide. The amount of peroxidase activity remaining after 30 min was determined by measuring oxidation of tetramethylbenzidine [21]. The enzyme was diluted 100-fold into 0.5 ml of 50 mM sodium acetate buffer, pH 5.4, containing 1.4 mM tetramethybenzidine and 8 % dimethylformamide. Reactions were started by adding 300 μM hydrogen peroxide and stopped after 5 min at 37 °C by adding 20 μg/ml catalase and 2.25 ml of ice-cold 200 mM acetic acid. Peroxidase activity was measured by recording the increase in A_{655}. Residual activites were corrected to account for inhibition of the oxidation of tetramethylbenzidine by the 0.5 μM ABAH that was present in these assays [14].

The concentration of hypochlorous acid produced by myeloperoxidase in the presence of hydrogen peroxide and chloride was determined by measuring the conversion of taurine into taurine chloramine, which was assayed using 2-nitro-5-thiobenzoate ($\epsilon_{412} = 14100$ $M^{-1}\cdot cm^{-1}$) [17]. Reactions were carried out in 100 mM phosphate buffer, pH 7.4, containing 100 μM DTPA, 100 mM chloride and 15 mM taurine.

Formation of ferrous myeloperoxidase

Ferrous myeloperoxidase was formed by making enzyme solutions anaerobic, then adding one or two grains of sodium dithionite. All absorption measurements were carried out on a Beckman DU 7500 diode array spectrophotometer.

Kinetic analysis of progress curves

Progress curves for the formation of the product from oxidation of ABAH by myeloperoxidase were used to monitor inactivation of the enzyme [22]. Double-exponential rise equations were fitted to the progress curves using the iterative non-linear regression program in Sigmaplot (Jandel Scientific, San Rafael, CA, U.S.A.). The parameters for these equations were used to calculate the final concentration of product formed (p_f). The half-time for inactivation of the enzyme ($t_{\frac{1}{2}}$) was calculated from the rate constant for the first exponential. From these values we determined the partition ratio, the inactivation rate constant (k_{in}) and the dissociation constant for ABAH (K'). The partition ratio, r, is defined by eqn. (1), where e_0 is the initial concentration of enzyme:

$$r = p_f/e_0 \quad (1)$$

K' and k_{in} are obtained from eqn. (2), where $[I]_0$ is the concentration of inhibitor:

$$[I]_0\cdot t_{\frac{1}{2}} = \left[\frac{\ln(2-M)}{1-M}\right]\frac{K'}{k_{in}} + \frac{\ln 2}{k_{in}}\cdot[I]_0 \quad (2)$$

where:

$$M = (1+r)\cdot e_0/[I]_0$$

$[I]_0\cdot t_{\frac{1}{2}}$ was plotted against $[I]_0$ for a series of experiments in which $[I]_0$ was varied but $e_0/[I]_0$ was kept constant; K' and k_{in} were obtained from the intercept and slope respectively [22] by linear regression using SigmaStat (Jandel Scientific).

RESULTS

Irreversible inactivation of myeloperoxidase by ABAH plus hydrogen peroxide

To determine whether ABAH irreversibly inhibits myeloperoxidase, 150 nM enzyme was incubated alone, with 50 μM ABAH, with 50 μM hydrogen peroxide or with ABAH plus hydrogen peroxide. After 30 min the residual peroxidase activity was measured. Hydrogen peroxide and ABAH in combination inactivated myeloperoxidase by 85 ± 3 % ($n = 3$) compared with enzyme incubated alone. In contrast, when myeloperoxidase was incubated with either ABAH or hydrogen peroxide the enzyme lost only 23 ± 3 % ($n = 3$) or 16 ± 10 % ($n = 3$) respectively of its activity. The same results were obtained when the peroxidase activity was measured immediately, or when the diluted samples were left a further 30 min to allow any compound III to decay back to the ferric enzyme. Since there was no recovery of activity, we conclude that myeloperoxidase had been irreversibly inactivated.

Oxidation of ABAH by myeloperoxidase and hydrogen peroxide

When ABAH was incubated with myeloperoxidase and hydrogen peroxide, it was oxidized to a single stable product with an absorption maximum at 325 nm (Figure 1). This is shown by the

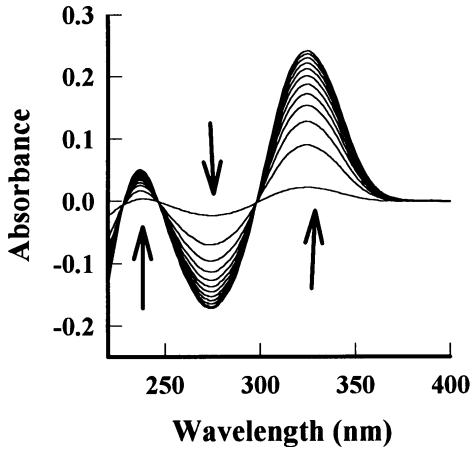

Figure 1 Serial spectra of the oxidation of ABAH by myeloperoxidase

ABAH (50 μM) and myeloperoxidase (300 nM) were incubated in 100 mM phosphate buffer, pH 7.4, containing 100 μM DTPA. The reaction was started by adding 50 μM hydrogen peroxide, and spectra against unoxidized ABAH were recorded every 1 min. Each spectrum is an average of 10 scans taken in 1 s. Arrows indicate the direction of spectral changes.

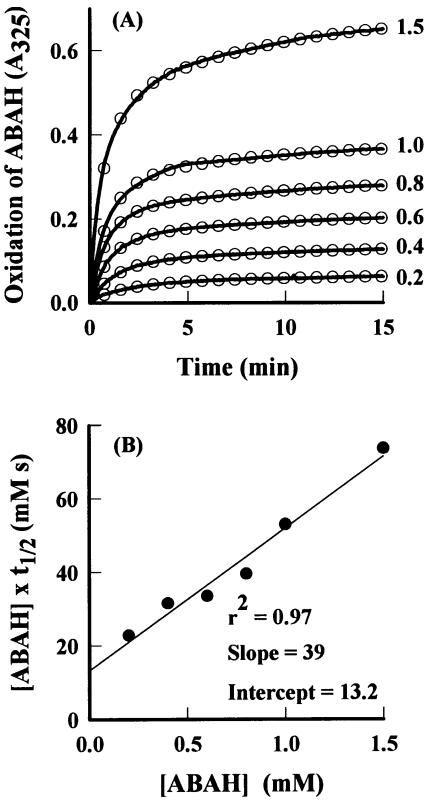

Figure 2 Determination of kinetic parameters for inactivation of myeloperoxidase by ABAH

(**A**) Myeloperoxidase was incubated with various concentrations of ABAH in 100 mM phosphate buffer, pH 7.4, containing 100 μM DTPA. The ratio of enzyme concentration to inhibitor concentration was 1:7400, and reactions were started by adding 100 μM hydrogen peroxide. Numbers on the right represent the millimolar concentration of ABAH. The solid lines represent the double-exponential rise equations fitted to the experimental data points (○). (**B**) Half-time plot for mechanism-based inhibition by ABAH. See the text for details.

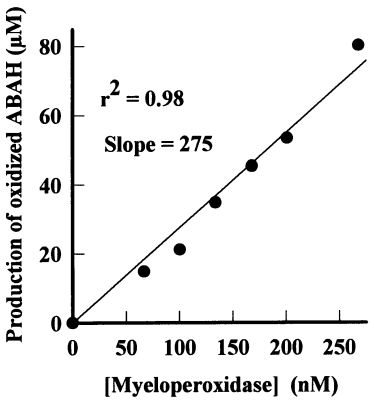

Figure 3 Partition ratio for inactivation of myeloperoxidase by ABAH

ABAH (1 mM) was incubated with various concentrations of myeloperoxidase, and reactions were started by adding 100 μM hydrogen peroxide. Other conditions were as described in the legend to Figure 1. The partition ratio for inactivation of myeloperoxidase was determined from the secondary plot of enzyme concentration versus the final extent of oxidation.

isosbestic points at 228, 247 and 300 nm for the difference spectra of the oxidation product against unoxidized ABAH. There was minimal oxidation in the absence of enzyme or hydrogen peroxide. By measuring initial rates of product formation at 325 nm, under the same conditions as described in Figure 4, the k_{cat} and K_m values for ABAH were 7.7 s^{-1} and 1.4 mM respectively. When the concentration of ABAH was 1 mM the K_m for hydrogen peroxide was 12 μM, and initial rates of oxidation became independent of hydrogen peroxide concentration above 50 μM.

Determination of kinetic parameters for inactivation of myeloperoxidase by ABAH

It is apparent that ABAH inactivates myeloperoxidase but it is also oxidized by the enzyme. Thus it is possible that ABAH is a suicide substrate for myeloperoxidase. If so, the inactivation should conform to the kinetic equations developed for mechanism-based inhibitors [22]. The course of inactivation was followed by monitoring the formation of product (Figure 2A) [22]. Under the conditions shown in Figure 2(A) for 1 mM ABAH, addition of a second equivalent concentration of myeloperoxidase after oxidation of ABAH had ceased caused a further increase in A_{325} of 0.21 unit over 5 min. In contrast, oxidation of ABAH could not be restored by adding more hydrogen peroxide or ABAH. This indicates that myeloperoxidase was inactivated and that the stable product of ABAH oxidation was not responsible for inactivation.

Kinetic analysis was performed under pseudo-first-order conditions where the concentration of ABAH underwent minimal change. Hydrogen peroxide was used at a concentration of 100 μM because at each concentration of ABAH it was saturating and remained so during the course of the reaction. To obtain k_{in} and K' values, the concentration of ABAH was varied while the ratio of myeloperoxidase to ABAH was kept constant (see the Materials and methods section). Double-exponential rise equations were fitted to the progress curves generated under these conditions (Figure 2A). The two components of the progress curves are attributed to the dominant oxidation by myeloperoxidase and the minor non-enzymic oxidation by hydrogen peroxide. The half-times for inactivation ($t_{\frac{1}{2}}$ values) were taken from the first exponential and plotted according to eqn. (2) (Figure 2B). From the slope of this secondary plot, k_{in} was determined to be 0.0180 ± 0.002 s^{-1} (mean $\pm$ S.E.M.; $n = 6$), while K' was calculated from the intercept as 375 ± 80 μM ($n = 6$).

We determined the partition ratio for inactivation of myeloperoxidase by ABAH by generating progress curves at various concentrations of enzyme and fixed concentrations of ABAH and hydrogen peroxide (results not shown). The final concentration of product formed was corrected for non-enzymic oxidation and plotted according to eqn. (1) (Figure 3). The slope of this secondary plot corresponds to a partition ratio of 275 ± 17 (mean $\pm$ S.E.M.; $n = 7$). It is evident from this kinetic analysis that the inactivation of myeloperoxidase conforms to the model proposed for mechanism-based inhibitors [22].

Effects of hydrogen peroxide on inactivation of myeloperoxidase

The final extent of oxidation of ABAH increased with increasing concentrations of hydrogen peroxide up to a maximum at 40 μM (Figure 4). Above this concentration there was a progessive decline in the oxidation of ABAH. At concentrations of hydrogen peroxide greater than 100 μM, enzyme inactivation was complete within 5 min. Inhibition was not solely due to hydrogen peroxide, because when myeloperoxidase was incubated with 200 μM hydrogen peroxide for 5 min in the absence of ABAH it lost only 17% of its activity.

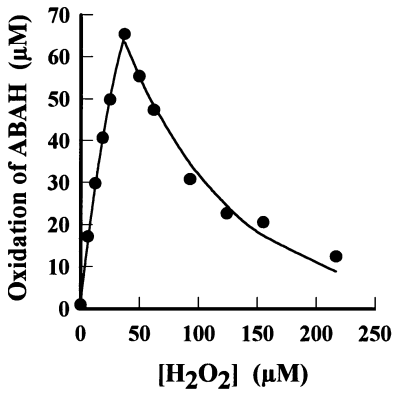

Figure 4 Effect of hydrogen peroxide on inactivation of myeloperoxidase by ABAH

Myeloperoxidase (135 nM) was incubated with 1 mM ABAH in 100 mM phosphate buffer, pH 7.4, containing 100 μM DTPA. Reactions were started by adding hydrogen peroxide, and the total extent of enzymic oxidation of ABAH was determined.

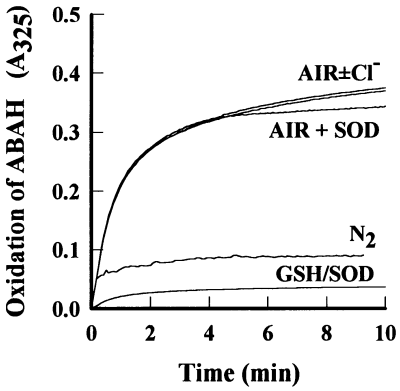

Figure 5 Effects of superoxide dismutase, chloride, oxygen and glutathione on the oxidation of ABAH by myeloperoxidase

Conditions were as described in the legend to Figure 3, except that the concentrations of myeloperoxidase, chloride, GSH and superoxide dismutase (SOD) were 135 nM, 100 mM, 100 μM and 20 μg/ml respectively. Cuvettes were made anaerobic by bubbling with nitrogen for 5 min before adding hydrogen peroxide and maintaining its flow in the headspace during the reaction.

We further investigated the oxidation of ABAH under the same conditions as in Figure 4, except that a low flux of hydrogen peroxide (1.5 μM/min) was generated with glucose oxidase and glucose. There was a steady increase in A_{325} until it plateaued at 1.4 units after 40 min. This corresponds to the oxidation of 135 μM ABAH and a partition ratio of 1000. Thus, at low fluxes of hydrogen peroxide, ABAH is extremely inefficient at inactivating myeloperoxidase.

Modulation of the inactivation of myeloperoxidase by ABAH

We determined the influence of chloride, superoxide, oxygen and glutathione on the oxidation of ABAH (Figure 5). At 100 mM, chloride affected neither the rate nor the extent of oxidation of ABAH. Similarly, superoxide dismutase was without effect, which excludes the involvement of superoxide in the inactivation of myeloperoxidase. In the absence of oxygen, oxidation of ABAH was substantially decreased and myeloperoxidase was completely inactivated, as measured by its ability to oxidize tetramethylbenzidine. Thus oxygen must protect myeloperoxidase from being inactivated. Glutathione almost completely blocked the oxidation of ABAH. Since superoxide is formed when glutathione scavenges radicals [23], 20 μg/ml superoxide dismutase was included to prevent superoxide from reacting with myeloperoxidase. Glutathione did not reduce the preformed oxidation product of ABAH and it is not oxidized directly by myeloperoxidase [24]. Thus glutathione most probably acted by scavenging the ABAH radicals formed in the one-electron oxidation of ABAH by myeloperoxidase. Inactivation of 150 nM myeloperoxidase by 50 μM ABAH and 50 μM hydrogen peroxide was decreased from 81 % to 43 % in the presence of 100 μM glutathione.

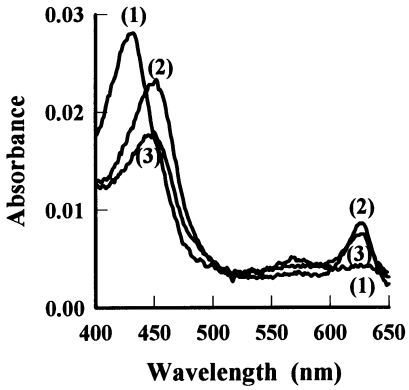

Figure 6 Spectral changes of myeloperoxidase during the oxidation of ABAH

Myeloperoxidase (300 nM) was incubated in 100 mM phosphate buffer, pH 7.4, containing 100 μM DTPA (scan 1). Scans were taken 15 s after adding 100 μM each of ABAH and hydrogen peroxide (scan 2) and then after another 5 min (scan 3). Other conditions were as described in the legend to Figure 5. Each scan is an average of 10 spectra taken in 1 s.

Spectral changes of myeloperoxidase during oxidation of ABAH

Upon addition of 100 μM hydrogen peroxide and ABAH, myeloperoxidase was converted into a redox intermediate with absorbance maxima at approx. 456 and 625 nm, which are characteristic of compound III (Figure 6, scan 2) [25]. After 5 min there was a loss in absorbance at 456 nm without a corresponding increase at 430 nm (Figure 6, scan 3). This indicates modification of the haem. When ABAH was added to myeloperoxidase in the absence of hydrogen peroxide, the spectral changes were similar to those presented in Figure 6 scan 2, except the changes in absorbance at 430 nm, 456 nm and 625 nm were smaller and indicated partial conversion of the enzyme into compound III. Also, the spectral changes were stable for at least 30 min. In the absence of added hydrogen peroxide, formation of compound III was inhibited by 40 μg/ml catalase, but 20 μg/ml superoxide dismutase had no effect. Adding only hydrogen peroxide to myeloperoxidase converts the enzyme into compound II [18].

Difference spectra of myeloperoxidase were recorded when ABAH was oxidized under an atmosphere of nitrogen. At low concentrations of hydrogen peroxide, ABAH initially converted myeloperoxidase into a species with absorbance maxima at approx. 475 and 643 nm (Figure 7A, scan 1). These spectral changes are characteristic of ferrous myeloperoxidase [26]. After 1 min the enzyme reverted to its native form (Figure 7A, scan 2).

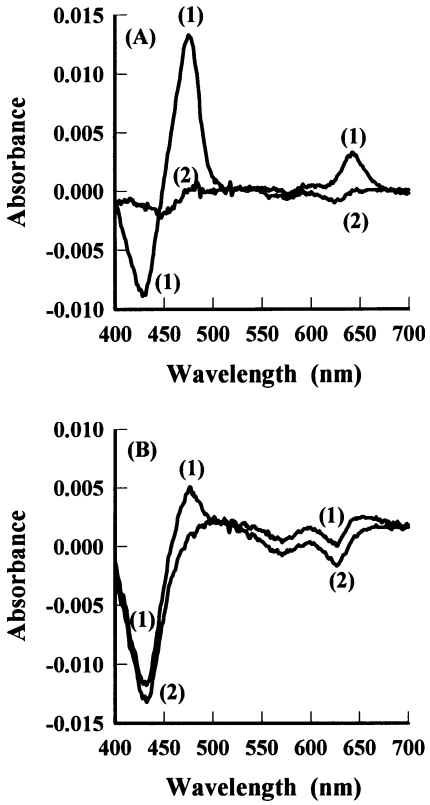

Figure 7 Spectral changes of myeloperoxidase during oxidation of ABAH in the absence of oxygen

(**A**) Myeloperoxidase was incubated with 100 μM ABAH, and within 10 s of adding 5 μM hydrogen peroxide the difference spectra against the native enzyme were recorded (scan 1). The second scan was taken 1 min after adding the hydrogen peroxide. (**B**) The conditions were the same as in (**A**), but 100 μM hydrogen peroxide was added to myeloperoxidase and ABAH. Cuvettes containing buffer, enzyme and ABAH were kept anaerobic by bubbling with nitrogen for 5 min before adding hydrogen peroxide and maintaining its flow in the headspace during the reaction. Other conditions were as described in the legend to Figure 6.

With 100 μM hydrogen peroxide, there was an immediate and substantial loss in absorbance at 430 nm and a small increase in absorbance at 475 nm (Figure 7B, scan 1). These changes indicate that some of the enzyme was converted into ferrous myeloperoxidase, but that the majority of the haem had been destroyed. After an additional 30 s, the ferrous intermediate was no longer apparent and there was further loss of haem absorbance (Figure 7B, scan 2).

When ferrous myeloperoxidase was preformed by adding a few grains of dithionite to an anaerobic solution of myeloperoxidase, adding either ABAH or hydrogen peroxide alone had no effect on its spectrum. However, when these compounds were added together, the absorbance associated with the haem groups was completely abolished and the spectral changes were essentially the same as those presented in Figure 7(B).

DISCUSSION

ABAH is an excellent inhibitor of myeloperoxidase [14]. In the present investigation we have shown that it acts by irreversibly inactivating the enzyme. The evidence for inactivation is that activity was not recovered by dilution of the enzyme and inhibitor, and that the haem groups of myeloperoxidase were considerably modified. Inactivation of myeloperoxidase by ABAH also conformed to the kinetic analysis developed for mechanism-based inhibitors [22]. At 100 μM hydrogen peroxide and an ambient oxygen tension, the partition ratio, K' and k_{in} were 275, 375 μM and 0.018 s^{-1} respectively. The partition ratio r defines the commitment to turnover, and $1+r$ is the number of turnovers that inactivates the enzyme. The constant K' is an analogue of a dissociation constant, and the catalytic rate constant for inactivation, k_{in}, can represent the rate constant for conversion of an enzyme intermediate into the inactive complex. Since inactivation was affected by hydrogen peroxide and oxygen, these values will vary with the relative concentrations of hydrogen peroxide and oxygen. For example, at low fluxes of hydrogen peroxide the partition ratio increased to 1000. However, the values indicate that oxidation of ABAH is favoured over inactivation of the enzyme, that ABAH has only moderate affinity for myeloperoxidase, and that the inactivation step is relatively slow. Similar values for these kinetic parameters have been obtained for inhibition of horseradish peroxidase by azide and alkylhydrazides [11,27].

Peroxidases oxidize hydrazides and hydrazines via compound I and compound II to free-radical intermediates that react further to form diazenes [28]. Reactions (1)–(4) below are the corresponding reactions for myeloperoxidase (MP^{3+}). The diazenes are then thought to undergo further oxidation. However, from our spectral data (Figure 1) it is apparent that ABAH is converted into a single stable product ($ABAH_{ox}$):

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

$$\text{Compound I} + \text{R-NH-NH}_2 \rightarrow \text{compound II} + \text{R-NH-NH}^{\bullet} + H^+ \quad (2)$$

$$\text{Compound II} + \text{R-NH-NH}_2 + H^+ \rightarrow MP^{3+} + \text{R-NH-NH}^{\bullet} + H_2O \quad (3)$$

$$2\text{R-NH-NH}^{\bullet} + H^+ \rightarrow \text{R-NH} = \text{NH} + \text{R-NH-NH}_2 \quad (4)$$

The classical peroxidase mechanism does not adequately describe the oxidation of ABAH, because during the reaction the enzyme was converted into compound III in the presence of oxygen, but formed ferrous myeloperoxidase in the absence of oxygen. Inhibition by reduced glutathione implies that oxidation proceeds via a radical intermediate (reactions 2 and 3). We propose that these radicals then reduce the native enzyme to ferrous myeloperoxidase (MP^{2+}) (reaction 5), which rapidly binds oxygen to form compound III ($MP^{2+}O_2$) (reaction 6). Since compound III is the dominant form of the enzyme present at steady state, its turnover will represent the rate-limiting step in the oxidation of ABAH.

$$MP^{3+} + ABAH^{\bullet} \rightarrow MP^{2+} + ABAH_{ox} \quad (5)$$

$$MP^{2+} + O_2 \rightarrow MP^{2+}O_2 \quad (6)$$

This mechanism is supported by our finding that catalase prevented the conversion of ferric myeloperoxidase into compound III. We have proposed a similar mechanism for the formation of compound III during oxidation of hydroquinone and amsacrine [29,30]. It has also been shown that, during oxidation of isoniazid, myeloperoxidase exists predominantly as compound III and that superoxide is not produced during the reaction [31]. Compound III is also formed by reaction of the ferric enzyme with superoxide or high concentrations of hydrogen peroxide. However, these routes can be excluded, since oxidation of ABAH and formation of compound III were unaffected by superoxide dismutase, and the concentrations of hydrogen peroxide used were too low to convert myeloperoxidase into compound III [25].

The mechanism of inactivation of myeloperoxidase is different from that established for inactivation of horseradish peroxidase

by hydrazines. Horseradish peroxidase is not converted into compound III, and its inactivation is enhanced by oxygen [10,32]. It has been proposed that horseradish peroxidase oxidizes phenylhydrazine and alkylhydrazines to diazenes, which are subsequently oxidized to the phenyl radical and alkyl radicals respectively [10,11]. These radicals then add to the *δ-meso* carbon of the haem prosthetic group, causing loss of activity of the enzyme. With ABAH and myeloperoxidase, inactivation and haem loss were enhanced in the absence of oxygen. Inactivation was greatest at high concentrations of hydrogen peroxide, while at low concentrations oxidation of ABAH was favoured. Since oxygen binds rapidly to the ferrous enzyme [33], we propose that formation of ferrous myeloperoxidase is essential for inactivation. Hydrogen peroxide did not cause destruction of the haem in the ferrous enzyme unless ABAH was present. Therefore it cannot react directly with ferrous myeloperoxidase to produce hydroxyl radicals that promote cleavage of the porphyrin ring, as proposed for lactoperoxidase and cytochrome *P*-450 [34,35]. Instead, we propose that hydrogen peroxide and ABAH react with ferrous myeloperoxidase (MP^{2+}) to inactivate the enzyme (reaction 7). Thus oxygen and hydrogen peroxide compete for ferrous myeloperoxidase and, depending on their relative concentrations, ABAH is oxidized or the enzyme is inactivated (MP_{inact}):

$$MP^{2+} + H_2O_2 + ABAH \rightarrow MP_{inact} \quad (7)$$

An alternative explanation is that radicals derived from ABAH inactivate myeloperoxidase, and oxygen protects it by scavenging these radicals. In this mechanism, inactivation would increase with the initial rate of radical production. However, under the conditions we used to investigate enzyme inactivation, the initial rate of ABAH oxidation was independent of hydrogen peroxide concentration. Thus this mechanism does not explain why inactivation was dependent on the concentration of hydrogen peroxide. Enhancement of inactivation in the absence of oxygen and the lack of effect of superoxide dismutase rules out the involvement of oxygen radicals.

Under the conditions of our experiments, ABAH proved to be only a modest suicide substrate for myeloperoxidase. However, in situations where oxygen tension is low and the hydrogen peroxide concentration is high, it should be a potent irreversible inhibitor. These constraints should be met within the phagocytic compartments of neutrophils, where oxygen is rapidly consumed by the NADPH oxidase [6] and hydrogen peroxide is likely to accumulate [26]. ABAH is still a useful inhibitor of the extracellular production of hypochlorous acid by neutrophils where the concentration of hydrogen peroxide is low [14]. This inhibition is best explained by ABAH competing with chloride and acting as a competitive substrate.

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