Transient and Steady-state Kinetics of the Oxidation of Substituted Benzoic Acid Hydrazides by Myeloperoxidase*

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Myeloperoxidase is the most abundant protein in neutrophils and catalyzes the production of hypochlorous acid. This potent oxidant plays a central role in microbial killing and inflammatory tissue damage. 4-Aminobenzoic acid hydrazide (ABAH) is a mechanism-based inhibitor of myeloperoxidase that is oxidized to radical intermediates that cause enzyme inactivation. We have investigated the mechanism by which benzoic acid hydrazides (BAH) are oxidized by myeloperoxidase, and we have determined the features that enable them to inactivate the enzyme. BAHs readily reduced compound I of myeloperoxidase. The rate constants for these reactions ranged from 1 to 3 × 10^6 M^{-1} s^{-1} (15 °C, pH 7.0) and were relatively insensitive to the substituents on the aromatic ring. Rate constants for reduction of compound II varied between 6.5 × 10^5 M^{-1} s^{-1} for ABAH and 1.3 × 10^6 M^{-1} s^{-1} for 4-nitrobenzoic acid hydrazide (15 °C, pH 7.0). Reduction of both compound I and compound II by BAHs adhered to the Hammett rule, and there were significant correlations with Brown-Okamoto substituent constants. This indicates that the rates of these reactions were simply determined by the ease of oxidation of the substrates and that the incipient free radical carried a positive charge. ABAH was oxidized by myeloperoxidase without added hydrogen peroxide because it underwent auto-oxidation. Although BAHs generally reacted rapidly with compound II, they should be poor peroxidase substrates because the free radicals formed during peroxidation converted myeloperoxidase to compound III. We found that the reduction of ferric myeloperoxidase by BAH radicals was strongly influenced by Hansch’s hydrophobicity constants. BAHs containing more hydrophilic substituents were more effective at converting the enzyme to compound III. This implies that BAH radicals must hydrogen bond to residues in the distal heme pocket before they can reduce the ferric enzyme. Inactivation of myeloperoxidase by BAHs was related to how readily they were oxidized, but there was no correlation with their rate constants for reduction of compounds I or II. We propose that BAHs destroy the heme prosthetic groups of the enzyme by reducing a ferrous myeloperoxidase-hydrogen peroxide complex.

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As yet there is no specific and potent inhibitor of myeloperoxidase that can be used to unambiguously identify the role of the enzyme in microbial killing and inflammation. Such an inhibitor would also be useful in attenuating myeloperoxidase-dependent inflammatory tissue damage. A number of strategies have been used to inhibit the enzyme. Its activity has been blocked by trapping it as inactive compound II (11), preventing the binding of chloride and hydrogen peroxide (12, 13), and destroying its heme prosthetic groups (14, 15). The most promising inhibitors identified to date are substituted benzoic acid hydrazides, which inhibited the peroxidation activity by 50% at concentrations less than 10 μM (16). 4-Aminobenzoic acid hydrazide (ABAH) was the best inhibitor of both peroxidation and production of hypochlorous acid. It had no effect on other neutrophil enzymes or on their production of superoxide (16). Thus, ABAH has considerable potential for identifying reactions of neutrophils that are dependent on myeloperoxidase.

ABAH was shown to be a suicide substrate of myeloperoxidase (15). The enzyme oxidizes it to free radical intermediates that reduce ferric myeloperoxidase to the ferrous enzyme (MP2-; Reaction 5). In the presence of hydrogen peroxide and ABAH, the heme groups of myeloperoxidase are destroyed. Oxygen protects the enzyme by converting ferrous myeloperoxidase to oxymyeloperoxidase or compound III (Reaction 6). To appreciate fully how benzoic acid hydrazides inactivate myeloperoxidase, it will be necessary to understand how these substrates are oxidized by the enzyme. In this investigation we have carried out kinetic studies to determine what features of benzoic acid hydrazides make them good substrates for compound I and compound II of myeloperoxidase. We have also investigated the mechanism by which myeloperoxidase oxidizes ABAH, and we have assessed the structural aspects of benzoic acid hydrazides that enable them to act as suicide substrates.

**EXPERIMENTAL PROCEDURES**

**Materials**

4-Aminobenzoic acid hydrazide (ABAH), 4-hydroxybenzoic acid hydrazide, homovanillic acid, diethylenetriaminepentacetic acid (DTPA), Cu,Zn-superoxide dismutase, and bovine liver catalase were purchased from Sigma. Fluka Chemicals supplied benzoic acid hydrazide, 3-nitrobenzoic acid hydrazide, and 4-chlorobenzoic acid hydrazide, and Aldrich supplied 3-methoxybenzoic acid hydrazide and 4-nitrobenzoic acid hydrazide. 4-Methoxybenzoic acid hydrazide was obtained from Cambrian Chemicals (Croydon, Surrey, UK). Stock solutions of these hydrazides were prepared daily in distilled water. Hydrogen peroxide solutions were prepared by dilution of a 30% stock solution (Merck), and their concentrations were determined by measuring their absorbance at 240 nm (ε240 39.4 M−1 cm−1) (17). CM-Sepharose CL-6B was purchased from Amersham Pharmacia Biotech.

**Methods**

**Purification of Myeloperoxidase**—Myeloperoxidase was purified from human neutrophils to a purity index of about 0.80 as described previously (18). Its concentration was calculated by measuring its absorbance at 270 nm (ε270 9100 M−1 cm−1 per heme) (19).

**Transient-state Kinetics**—Sequential stopped-flow measurements were performed with an Applied Photophysics (UK) instrument (model SX-18MV). When 100 μl was shot into a flow cell having a 1-cm light path, the fastest time for mixing two solutions and recording the first data point was approximately 1.5 ms. Reactions were carried out in 100 mM sodium/potassium phosphate buffer. Sequential stopped-flow (multi-mixing) analysis was used for kinetics measurements of the conversion of compound I to compound II because compound I is inherently unstable (20). With 0.5 μM myeloperoxidase, 5 μM was the minimum hydrogen peroxide concentration required for complete formation of compound I (characterized by a 50% hypochromicity in the Soret band). Under these conditions, compound I was completely formed within 40 ms and was stable for at least a further 10 ms. For reduction of compound I by the hydrazides, 2 μM ferric myeloperoxidase was premixed in the aging loop with 20 μM hydrogen peroxide for 40 s. The compound I was then allowed to react with varying concentrations of hydrazides. To ensure first order kinetics, their final concentrations were at least five times that of the enzyme. Formation of compound II was monitored at 456 nm, which is the wavelength where compound II absorbs maximally and the isosbestic point for the ferric enzyme and compound I (21). At least three determinations (1000–4000 data points) of kcat were performed for each substrate concentration. Second order rate constants were calculated from the slope of the plot of the mean kcat values versus substrate concentration.

Two methods were evaluated for measuring the rate of reduction of compound II by the hydrazides. In one method a large excess of hydrogen peroxide was used to drive compound II (21). In the other method, compound II was formed by using a 10-fold excess of hydrogen peroxide in the presence of a sub-stoichiometric concentration of homovanillic acid. We found that homovanillic acid readily reduces compound I (θ3 = 1.7 ± 0.15 × 106 M−1 s−1) but reacts slowly with compound II (θ3 = 230 ± 1.9 M−1 s−1, data not shown). Both methods gave identical kcat values for reaction of compound II with electron donors. However, we chose to use the latter method because a greater change in absorbance was achieved when compound II was reduced, and the lower concentrations of hydrogen peroxide in the incubation mixture guaranteed pre-steady-state conditions. For reduction of compound II, 2 μM ferric myeloperoxidase was premixed with 20 μM hydrogen peroxide and 1.8 μM homovanillic acid in the aging loop. Under these conditions, compound II was stable for at least 40 s. Twenty seconds after the initial mixing, compound II was allowed to react with a substrate. To ensure first order kinetics, the final concentrations of the hydrazides was at least five times that of the enzyme (2.5–50 μM). Reduction of compound II was monitored by recording the loss in absorbance at 456 nm (2000–4000 data points). At least three determinations of the pseudo-first order rate constants, kcat, were performed for each substrate concentration, and the mean value was used to calculate kcat as described above.

Formation of compound III during oxidation of the benzoic acid hydrazides was recorded using conventional stopped-flow. The native enzyme (2 μM) was mixed with 20 μM hydrogen peroxide in the presence of either 20 or 100 μM hydrazide, and the kinetics of compound III formation were followed at 625 nm. Compound III and compound II were distinguished by the ratios of their absorbances at 625 and 456 nm, which are 0.52 and 0.2, respectively (20).

**Steady-state Kinetics**—The oxidation of ABAH and the spectral changes of myeloperoxidase at steady state were recorded using either a Zeiss Specord S-10 or a Beckman DU 7500 diode array spectrophotometer. Oxidation of ABAH was determined by measuring the increase in absorbance at 325 nm (15). It was also measured by using HPLC with electrochemical detection. An aliquot of the reaction system (10 μl) was injected into a Waters 600E HPLC equipped with a Phenomenex Lunar C18 column (250 × 1.4 mm). ABAH was eluted at a flow rate of 0.9 ml/min with 50 mM phosphate buffer, pH 3.0, containing 11% methanol and detected with an ESA Coulachem detector (E1 100 mV and E2 500 mV).

**Measurement of Myeloperoxidase Activity and Its Inhibition by Benzoic Acid Hydrazides**—The activity of myeloperoxidase was determined by measuring the rate at which it oxidized 3,5,3′,5′-tetrathymethylbenzidine (TMB) at 25 °C over the 1st min of the reaction (22). The reaction was carried out in 100 mM acetate buffer, pH 5.4, containing 8% dimethylformamide and started by adding 300 μM hydrogen peroxide. The concentration of hydrazide that inhibited myeloperoxidase-dependent oxidation of TMB by 50% (IC50) was determined by fitting a rectangular hyperbola to the dose-response curve using nonlinear regression.

To measure residual activity of myeloperoxidase, the enzyme was extracted from reaction systems under conditions that would ensure that any compound III present would decay back to the active enzyme (23). After incubating the hydrazides with myeloperoxidase and hydrogen peroxide, reactions were stopped with 20 μM catalase, and the
Oxidation by Myeloperoxidase

Reduction of Compound I by Benzoic Acid Hydrazides—The instability of the redox intermediates of myeloperoxidase made it necessary to use the sequential mixing stopped-flow technique to determine the rate constants for their reactions with reducing substrates. Premixing of myeloperoxidase and hydrogen peroxide led to compound I formation within several milliseconds (not shown) and allowed the measurement of the rate of its subsequent reaction with an electron donor after a defined delay time (40 ms). The reaction of ferric myeloperoxidase with hydrogen peroxide to form compound I was measured at 426 nm and its bimolecular rate constant \( k_1 \) was derived from a plot of \( k_{\text{obs}} \) versus varied peroxide concentrations (not shown). It was calculated to be \((1.4 \pm 0.2) \times 10^7 \, \text{M}^{-1} \, \text{s}^{-1} \) at 15 °C (the temperature used in the presteady-state experiments), which is in accordance with previously published values (20, 21).

The kinetics of the reduction of compound I were followed at 456 nm, which is the isosbestic point between the ferric enzyme and compound I and the absorbance maximum for compound II formation (21). Kinetic traces for the reduction of compound I by substituted benzoic acid hydrazides displayed single exponential character. A typical trace for ABAH is shown in the inset of Fig. 1A. The pseudo-first order rate constants at pH 7.0 \( (k_{\text{obs}}) \) were obtained from these traces and plotted against the concentration of the benzoic acid hydrazide (Fig. 1A). Slopes of these secondary plots yielded the apparent second order rate constants \( k_3 \) (see Table I). The magnitudes of these rate constants varied by less than a factor of 3 and are some of the largest ever measured for reduction of compound I by organic substrates.

Reaction of compound I with the substituted benzoic acid hydrazides conform to the Hammett equation (Equation 1), where \( k_3 \) is the rate constant for the unsubstituted derivative; \( \rho \) is the constant for the reaction; and \( \sigma \) is a constant characteristic of each substituent (24).

\[
\log(k/k_3) = \rho \sigma \quad \text{(Eq. 1)}
\]

A positive value for \( \sigma \) indicates an electron-withdrawing group, and a negative value indicates an electron-donating group. We have plotted Brown-Okamoto \( \sigma^+ \) values, which were devised for electron-donating groups that can interact with a developing positive charge in the transition state. These constants gave a better correlation than the original \( \sigma \) values (Fig. 1B). The correlation coefficient \( r = -0.92 \) \((p = 0.001; n = 8)\) for \( \sigma^+ \) and \(-0.84 \) \((p = 0.009)\) for \( \sigma \). The value of \( \rho \), obtained from the slope of Fig. 1B, was \(-0.21\), which indicates that the electronic character of the substituents has only small effect on the oxidation of reducing substrates by compound I.

There was a finite intercept for the plot of \( k_{\text{obs}} \) versus the concentration of ABAH (Fig. 1A). This intercept was seen with all the hydrazides and was independent of the nature of the substrate (not shown). It indicates the rate of spontaneous reduction of compound I by excess hydrogen peroxide. The value we obtained \((4.5 \pm 1.7 \, \text{s}^{-1} \, (n = 8))\) is in good agreement with that determined previously \((2.2 \pm 1.2 \, \text{s}^{-1})\) (25).

**RESULTS**

**Reduction of Compound I by Benzoic Acid Hydrazides**—The stability of the redox intermediates of myeloperoxidase made it necessary to use the sequential mixing stopped-flow technique to determine the rate constants for their reactions with reducing substrates. Premixing of myeloperoxidase and hydrogen peroxide led to compound I formation within several milliseconds (not shown) and allowed the measurement of the rate of its subsequent reaction with an electron donor after a defined delay time (40 ms). The reaction of ferric myeloperoxidase with hydrogen peroxide to form compound I was measured at 426 nm and its bimolecular rate constant \( k_1 \) was derived from a plot of \( k_{\text{obs}} \) versus varied peroxide concentrations (not shown). It was calculated to be (1.4 ± 0.2) × 10^7 M⁻¹ s⁻¹ at 15 °C (the temperature used in the presteady-state experiments), which is in accordance with previously published values (20, 21).

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**FIG. 1. Reduction of compound I by substituted benzoic acid hydrazides.** A, the secondary plot of the pseudo-first order rate constants for the reduction of compound I \( (k_{\text{obs}}) \) versus the concentration of ABAH. Inset, typical sequential stopped-flow time trace of the reaction of compound I with ABAH. The incubation mixture contained 0.5 μM myeloperoxidase and 5.0 μM ABAH in 100 mM phosphate buffer, pH 7.0, at 15 °C. B, the correlation between the Brown-Okamoto substituent constants \( (\sigma^+) \) and the second order rate constants for reduction of compound I \( (k_3) \). The rate constant for benzoic acid hydrazide is \( k_{\text{BAH}} \).

**TABLE I**

| Substituted BAH | \( k_3 \) | \( k_4 \) | IC_{50} |
|-----------------|-----------|-----------|--------|
| 4-Amino-BAH     | 3.34 ± 0.06 | 645.8 ± 22.5 | 2      |
| 4-Hydroxy-BAH   | 3.34 ± 0.05 | 149.35 ± 0.89 | 18     |
| 4-Methoxy-BAH   | 2.70 ± 0.11 | 27.10 ± 0.54 | 18     |
| BAH             | 1.32 ± 0.09 | 17.40 ± 1.50 | 42     |
| 3-Methoxy-BAH   | 1.68 ± 0.01 | 1.18 ± 0.71 | 25     |
| 4-Chloro-BAH    | 1.80 ± 0.06 | 9.51 ± 0.06 | 250    |
| 3-Nitro-BAH     | 1.51 ± 0.12 | 1.80 ± 0.61 | ND     |
| 4-Nitro-BAH     | 1.33 ± 0.03 | 1.27 ± 0.57 | ND     |
have been measured using transient state conditions whereby compound II is formed by adding a 50-fold excess of hydrogen peroxide to myeloperoxidase (25). However, with excess hydrogen peroxide the enzyme is likely to cycle when the reducing substrate is added. Therefore, steady-state methods have been employed to measure the rate of reduction of compound II (22). This latter approach is not possible with the benzoic acid hydrazides because they inactivate myeloperoxidase during their oxidation. Therefore, we compared two pre-steady-state strategies for investigating the reduction of compound II. Either a 50-fold excess of compound II is formed by adding a 50-fold excess of hydrogen peroxide to the native enzyme (25) or a 10-fold excess of hydrogen peroxide plus a sub-stoichiometric concentration of homovanillic acid was added. Both approaches gave approximately the same absorbance amplitudes at 456 nm for the formation of compound II within 20 s of mixing. Using the sequential mixing mode, similar bimolecular rate constants \( k \) were calculated using these two strategies. However, the amplitude of the decrease in absorbance at 456 nm was much smaller when a 50-fold excess of hydrogen peroxide was used. This demonstrates that a large excess of hydrogen peroxide was less useful in guaranteeing pre-steady-state conditions.

Consequently, we premixed a 10-fold excess of hydrogen peroxide and sub-stoichiometric concentrations of homovanillic acid to generate compound II, and we then followed its reaction with the various hydrazides. In each case, the loss of absorbance at 456 nm displayed single exponential character. A typical time trace for the reaction of ABAH with compound II is shown in the inset of Fig. 2A. The apparent second order rate constants for the reduction of compound II \( (k_r) \) were obtained from secondary plots of the \( k_{obs} \) values versus the concentration of the hydrazides (Table I). The secondary plot for ABAH is shown in Fig. 2A. The magnitudes of the rate constants varied by as much as 600-fold. They differed from those obtained for compound I by as little as a factor of 5 for ABAH and as much as 1000-fold for 4-nitrobenzoic acid hydrazide. The Hammett plot, in which \( \sigma^+ \) values were used, demonstrated that there is a larger substituent effect for reduction of compound II by the hydrazides than for compound I (Fig. 2B). The value of \( p \) was \(-1.21 \) \( (r = -0.92; p = 0.001) \).

With the exception of ABAH, the intercepts on the ordinate for the secondary plots were small \( (0.13 \pm 0.09 \text{ s}^{-1}) \), not shown). However, \( k_{obs} \) for ABAH was \( 1.67 \pm 0.67 \text{ s}^{-1} \). This would normally suggest a reversible reaction (25). However, it most probably reflects formation of compound III, which was demonstrated to occur during the same time scale as compound II reduction (see Fig. 9). Oxidation of ABAH by compound II displayed a broad pH optimum between 5 and 7 (Fig. 3).

Oxidation of ABAH by Myeloperoxidase—Previously it was reported that myeloperoxidase requires hydrogen peroxide to catalyze oxidation of ABAH. We confirmed this result by showing that the initial rate of oxidation of 1 mM ABAH by 100 nM myeloperoxidase in the absence of hydrogen peroxide was only 3% of that in the presence of 50 \( \mu \text{M} \) hydrogen peroxide (results not shown). Oxidation of ABAH was most favored at pH 4 but also had a broad pH optimum between 7 and 8.5 (Fig. 4). In accordance with the earlier proposal that oxygen protects the enzyme from inactivation, we found that there was progressively more oxidation of ABAH and a longer time until the enzyme was inactivated when the atmosphere was changed from nitrogen to air to oxygen (not shown).

In the absence of added hydrogen peroxide, ABAH was still oxidized by myeloperoxidase and, even though the reaction was slow, more ABAH was eventually oxidized than with hydrogen peroxide present (Fig. 5). This situation arose because there was virtually no enzyme inactivation in the absence of exogenous hydrogen peroxide. In contrast, with 100 \( \mu \text{M} \) hydrogen peroxide, the enzyme was inactivated within 5 min. The spectral changes of the oxidation products of ABAH that were observed in the absence of hydrogen peroxide were identical to those recorded in its presence (not shown). This result indicates...
that the product of ABAH oxidation was the same regardless of whether or not hydrogen peroxide was added to the reaction system.

Based on the following findings, it is evident that ABAH did not react directly with ferric myeloperoxidase. Rather it initially underwent auto-oxidation to produce hydrogen peroxide, which was used by the enzyme for subsequent oxidation of ABAH. First, oxidation of ABAH did not occur under an atmosphere of nitrogen and was independent of the concentration of ABAH between 100 μM and 1 mM (not shown). The rate of oxidation was not linearly related to the concentration of myeloperoxidase but reached a plateau above 200 nM enzyme (Fig. 5). The length of the lag phase for oxidation of ABAH (Fig. 5). The length of the lag phase increased with the concentration of superoxide dismutase, whereas the steady-state rate of ABAH oxidation decreased as the concentration of superoxide dismutase was increased (not shown). At least 100 ng/ml (1.6 μM/heme) catalase was required to block oxidation of ABAH by 100 nM myeloperoxidase. Adding catalase after oxidation of ABAH had commenced inhibited to the same extent as when it was present from the start of the reaction. Thus hydrogen peroxide was required for the continual oxidation of ABAH. There was minimal oxidation of ABAH below pH 7 but at higher pH values the rate of oxidation increased markedly (Fig. 4).

Formation of Compound III during Oxidation of Benzoic Acid Hydrazides—When myeloperoxidase oxidizes ABAH, it is converted to its compound III form (15). It was proposed that an ABAH radical, formed in the classical peroxidase cycle (Reactions 1, 3, and 4), reduces the ferric enzyme to ferrous myeloperoxidase which subsequently binds oxygen to form compound III (Reactions 5 and 6). To verify this sequence of reactions, we monitored the inter-conversion of the redox intermediates of myeloperoxidase during the oxidation of ABAH (Fig. 7A). Hydrogen peroxide and ABAH were rapidly mixed with myeloperoxidase, and the absorption spectra of the enzyme were recorded during the first 2 s of the reaction (Fig. 7A). Within 200 ms there was a decrease in absorbance at 430 nm and an increase in absorbance at 451 and 625 nm. These changes are indicative of partial conversion of the enzyme from its native form to either compound II or compound III. The contribution of these redox intermediates make to the spectrum of myeloperoxidase can be deduced by calculating the ratio of absorbances at 625 and 456 nm (20, 26). Pure compound II has a ratio of 0.19 and that for pure compound III is 0.52. At 200 ms the absorbance ratio at 625/456 was 0.21 and at 2000 ms it had increased to 0.38. When the reaction was monitored continuously it was apparent that there was a biphasic increase in absorbance at 625 nm over time (Fig. 7B, trace 1). This characteristic pattern was also apparent for the corresponding reactions with p-hydroxybenzoic acid hydrazide and p-nitrobenzoic acid hydrazide (Fig. 7B, traces 2 and 3). The first increase in absorbance obeyed pseudo-first order kinetics with observed pseudo-first order rate constants being the same order of magnitude as demonstrated for the reduction of compound I by the hydrazides. Thus, the initial rapid increase in A625/A456 can be unequivocally attributed to compound II formation. The further increase at 625 nm coupled with the increase in A625/A456 illustrates that the enzyme is subsequently converted from compound II to compound III. This temporal analysis demonstrates that production of ABAH radicals must precede formation of compound III and therefore supports the earlier proposal that ABAH radicals promote the conversion of ferric myeloperoxidase to compound III.

In control experiments we found that hydrogen peroxide (10 μM) alone converted myeloperoxidase to compound II within 20 s, but there was no subsequent accumulation of compound.
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III. Superoxide dismutase (50 μg/ml) had no effect on production of compound III by any of the hydrazides. These results exclude the direct involvement of either hydrogen peroxide or superoxide in the formation of compound III.

Inhibition of Peroxidase Activity by Benzoic Acid Hydrazides—The capacity of benzoic acid hydrazides to inhibit the peroxidation activity of myeloperoxidase has previously been determined by measuring their ability to block oxidation of TMB (16). In this study we wanted to assess whether or not the inhibitory capacity of the hydrazides was related to the ability of myeloperoxidase to oxidize them. However, it has recently been shown that the method previously used to measure peroxidation activity of myeloperoxidase is flawed (22). We therefore adopted a modified method proposed by Marquez and Dunford (22) to re-evaluate the IC50 values. 3-Nitro- and 4-nitrobenzoic acid hydrazides were excluded from the analysis because they promoted the breakdown of the oxidation product of TMB. For each compound, the IC50 was determined from its dose-response curve, such as that shown for ABAH (Fig. 8A). The IC50 for ABAH was unchanged from the previous estimate, but those for the other hydrazides increased considerably. The revised values are given in Table I.

There were no significant correlations between IC50 or log (1/IC50) and either σ, σ′, or k. Thus, the ability of benzoic acid hydrazides to inhibit myeloperoxidase in the TMB assay is not related to the rate at which they are oxidized by the enzyme. In contrast, there was a very strong correlation between log (1/IC50) and Hansch's hydrophobicity constants (Fig. 8B; r = −0.94; p = 0.002) (27). The best inhibitors had substituents that make the benzoic acid hydrazide more hydrophilic (e.g. p-NH2), and the worst inhibitors had very hydrophobic substituents (e.g. p-Cl). Linear regression applied to the data in Fig. 8B yielded Equation 2 that can be used to predict the IC50 of a substituted benzoic acid hydrazide. II, is Hansch's hydrophobicity constant for substituent X.

\[ \log(1/\text{IC}_{50}) = 4.41 - 0.95 \times \Pi \]

(Eq. 2)

To determine why the IC50 values are related to Hansch's constants rather than Hammett's constants, we probed the mechanism by which ABAH inhibits myeloperoxidase in the TMB assay. When ABAH was present at about its IC50, the maximal inhibitory effect had occurred within the mixing time, and there was no observable lag phase (not shown). Thus, ABAH could not act as a competitive substrate because when fully oxidized its inhibitory effect would cease. This conclusion is reinforced by the fact that the rate constants for reduction of compound I and compound II by TMB (22) are about the same as those for ABAH.

Fig. 7. The time course for formation of compound III during oxidation of ABAH. A, sequential formation of compounds II and III upon mixing of myeloperoxidase (500 nM per heme) with ABAH (5 μM) and hydrogen peroxide (5 μM). Reactions were carried out in 100 mM phosphate buffer at pH 7.0, and serial spectra were recorded within 2 s after starting the reaction by adding hydrogen peroxide. Each spectrum is an average of two scans taken in 140 ms. Trace 1, 0 ms; trace 2, 200 ms; trace 3, 400 ms; and trace 4, 2000 ms. Arrows indicate the direction of maximum spectral changes. B, conventional stopped-flow time traces of the reaction between myeloperoxidase (1 μM per heme) and a mixture of 10 μM hydrogen peroxide and 10 μM substrate in 100 mM phosphate buffer, pH 7.0. Reactions were monitored at 625 nm as an indicator of compound II and compound III formation. Trace 1, p-aminobenzoic acid hydrazide; trace 2, p-hydroxybenzoic acid hydrazide; and trace 3, p-nitrobenzoic acid hydrazide.

Fig. 8. The effect of benzoic acid hydrazides on the oxidation of TMB by myeloperoxidase and hydrogen peroxide. A, the effect of ABAH on the rate of oxidation of 1.4 mM TMB by 1 mM myeloperoxidase and 300 μM hydrogen peroxide. Reactions were carried out in 100 mM acetate buffer, pH 5.4, at 20 °C, and the rate of increase at 655 nm due to the oxidation of TMB was determined over the 1st min of reaction. Data are means of triplicate experiments. B, the inhibitory effect of each substituted benzoic acid was measured by determining the concentration at which it inhibited oxidation of TMB by 50% (IC50) under the same conditions as described in A. These values were plotted against Hansch's constants (II) for the various substituents.
TMB in the assay than ABAH, only minimal oxidation of ABAH by myeloperoxidase would be expected. Alternative explanations for the effect of ABAH are that it either prevents hydrogen peroxide from reacting with ferric myeloperoxidase or it inactivates the enzyme. To check these possibilities, we incubated myeloperoxidase (2.7 nM) with TMB (1.5 mM) and hydrogen peroxide (280 μM), plus or minus ABAH (2 μM) in 100 mM acetate buffer, pH 5.4, containing 8% dimethylformamide. Under these conditions, oxidation of TMB was slowed by 66% over the first 5 min of the reaction. After 5 min, the enzyme was diluted 10-fold and its residual activity was measured. In the absence of ABAH, myeloperoxidase lost 27 ± 2% (n = 3) of its activity, whereas 73 ± 7% (n = 3) was lost in the presence of ABAH. If ABAH was simply binding to the enzyme and blocking its reaction with hydrogen peroxide, there would be no loss in activity after dilution of the enzyme. Thus, ABAH must inactivate myeloperoxidase. As reported previously, ABAH inactivated myeloperoxidase only in the presence of hydrogen peroxide. This demonstrates that an oxidation product of ABAH inactivates the enzyme.

To assess whether ABAH reversibly or irreversibly inactivated myeloperoxidase during the TMB assay, the reaction was stopped after 2 min by adding catalase (20 μg/ml), and myeloperoxidase (5 nM) was extracted with CM-Sepharose. The residual activity of the isolated enzyme was then measured. Extraction of myeloperoxidase was carried out at pH 7.4 to ensure that if any compound III was present it would decay back to the active ferric enzyme (see “Experimental Procedures”). When the TMB assay was run in the presence of ABAH (2 μM), 87 ± 12% (n = 4) of the peroxidase activity was recovered after extraction. This result demonstrates that ABAH reversibly inactivates myeloperoxidase during the TMB assay. Presumably, inactivation occurs through the formation of compound III.

It has been shown that ABAH inactivates myeloperoxidase by being oxidized to radical intermediates (15). As discussed above, the direct oxidation of ABAH by myeloperoxidase in the TMB assay is unlikely to occur. However, myeloperoxidase may oxidize ABAH indirectly by generating TMB radicals that interexchange with ABAH. We therefore investigated the ability of TMB to promote the oxidation of ABAH by myeloperoxidase (Table II). Under the reaction conditions, myeloperoxidase and hydrogen peroxide oxidized ABAH poorly. However, in the presence of TMB, 64% of ABAH was consumed. From these results we conclude that in the TMB assay myeloperoxidase oxidizes ABAH indirectly through the generation of TMB radicals. Once formed ABAH radicals would react with the ferric enzyme to promote formation of compound III. The extent to which benzoic acid hydrazides inhibit myeloperoxidase in the TMB assay will depend on how readily they reduce the ferric enzyme to compound III. The strong dependence of their IC50 values on Hansch’s substituent constants (α) indicates that binding of radical intermediates to the enzyme is the rate-determining step in this process.

Irreversible Inactivation of Myeloperoxidase by Benzoic Acid Hydrazides—Myeloperoxidase was incubated with each of the benzoic acid hydrazides under standard conditions at pH 7.4, and their ability to irreversibly inactivate the enzyme was determined (see Fig. 9). Three minutes after adding hydrogen peroxide to myeloperoxidase and the benzoic acid hydrazide, the reaction was stopped with catalase. Myeloperoxidase was extracted with CM-Sepharose to ensure it was free of inhibitor and to allow compound III to decay back to the native enzyme. Residual peroxidase activity was measured in the TMB assay. All the benzoic acid hydrazides tested promoted irreversible inactivation of myeloperoxidase. ABAH, p-hydroxybenzoic acid hydrazide, and p-methoxybenzoic acid hydrazide destroyed more than 90% of the activity of the enzyme. There were good correlations between Hammett’s substituent constants (σ) (r = 0.90; p = 0.003) or the Brown-Okamoto constants (σ*) (r = 0.88; p = 0.004) and percentage of residual enzyme activity (Fig. 9). The benzoic acid hydrazides that contained substituents that were strongly electron-donating were the better suicide substrates. This result indicates that oxidation of the benzoic acid hydrazides by an intermediate of myeloperoxidase determines how effective they are at irreversibly inactivating the enzyme. However, the correlations with k3 (r = -0.74; p = 0.04) and k4 (r = -0.75; p = 0.033) were poor. There was no significant correlation with Hansch’s constants. Thus binding of an oxidized form of the inhibitors by the enzyme cannot be involved in the rate-determining step for inactivation.

**DISCUSSION**

4-Aminobenzoic acid hydrazide is the most potent inhibitor of myeloperoxidase that has been identified to date. It is a mechanism-based inhibitor and must be oxidized to radical intermediates to promote irreversible inactivation (15). In this study we have investigated the mechanism by which myeloperoxidase oxidizes substituted benzoic acid hydrazides, and we have determined the features that make them good inhibitors of the enzyme. Our findings are pertinent to understanding...
how ABAH and related compounds are metabolized by myeloperoxidase and advance the knowledge of how this enzyme acts as a classical peroxidase. They also highlight potential strategies that may be useful in developing more effective inhibitors of myeloperoxidase.

All the benzoic acid hydrazides reacted rapidly with compound I and compound II. They react faster than tyrosine with compound I, and only the nitro derivatives are worse substrates than tyrosine for compound II (25). The adherence of these reactions to the Hammett rule (Figs. 1B and 2B) indicates that the hydrazide substituent is oxidized in preference to either the amino or hydroxyl groups in ABAH or 4-hydroxybenzoic acid hydrazide. Furthermore, it demonstrates that the rates of these reactions are simply related to the ease at which the hydrazides are oxidized. The small negative value of $\rho (-0.21)$ obtained for compound I (Fig. 1B) implies that substituents that donate electrons into the ring favor oxidation of benzoic acid hydrazides, but oxidation is relatively insensitive to electronic effects. For comparison, reduction of compound I of horseradish peroxidase by phenols, anilines, and indoleacetic acids have $\rho$ values of $-6.9$, $-7.0$, and $-5.6$, respectively (28, 29). There was a far greater variation in the rate constants for reduction of compound II by the hydrazides, but $\rho (-1.21)$ was still relatively small. The value of $\rho$ for reduction of horseradish peroxidase compound II by phenols is $-4.6$ (30). A likely explanation for the lesser reliance on electronic effects for oxidation by myeloperoxidase compared with horseradish peroxidase is that reduction potentials of the redox intermediates of the mammalian enzyme are considerably higher than for the plant enzyme (8).

Our results indicate that compound I of myeloperoxidase should be able to oxidize a wide range of organic substrates with similar efficiency. By contrast, reduction of compound II will be far more constrained by the oxidation potentials of substrates. Thus, substrates that react poorly with compound II would not be expected to be oxidized by the enzyme. However, in the presence of suitable co-substrates that readily reduce compound II, such as superoxide, tyrosine, and ascorbate (18, 25, 31), the high reduction potential of compound I could be exploited to catalyze oxidation of a variety of substrates.

We found that rate constants for reduction of compound I and compound II correlated better with Brown-Okamoto ($\sigma^*$) constants than with Hammett constants ($\sigma$). This result indicates that the incipient free radical formed in the oxidation of benzoic acid hydrazides must carry a positive charge. The positive charge will be delocalized via resonance between the substituent on the aromatic ring and the hydrazide group. In analogous reactions of horseradish peroxidase by phenols and anilines the correlation with $\sigma$, but not $\sigma^*$, was used as evidence for electron transfer between the substrate and the enzyme with simultaneous loss of a proton (28). This mechanism cannot apply to oxidation of benzoic acid hydrazides by myeloperoxidase. Rather electron transfer from the hydrazide to the enzyme must occur without transfer of a proton. In an earlier study it was found that the mechanism by which myeloperoxidase oxidizes anilines is quite different to that for horseradish peroxidase (11). With myeloperoxidase, peroxidation was strongly influenced by resonance effects. Thus, it is conceivable that electron transfer from substrate to compound I or compound II without simultaneous loss of a proton is a general mechanism for myeloperoxidase.

Myeloperoxidase did not need an exogenous source of hydrogen peroxide to catalyze the oxidation of ABAH. Rather, ABAH underwent metal-catalyzed auto-oxidation to produce hydrogen peroxide that the enzyme used for further oxidation of ABAH. The ability of DTPA, catalase, and superoxide dismutase to inhibit oxidation of ABAH by myeloperoxidase indicates that auto-oxidation of ABAH is likely to follow a mechanism similar to that for phenylhydrazine (Reactions 7–11) (32).

\begin{align*}
\text{Ph–CO–NH–NH}_2 + \text{Me}^{n+} & \rightarrow \text{Ph–CO–NH–NH} + \text{Me}^{(n-1)+} + \text{H}^+ \\
\text{REACTION 7} \\
\text{Ph–CO–NH–NH} + \text{O}_2 & \rightarrow \text{Ph–CO–N} = \text{NH} + \text{O}_2^+ + \text{H}^+ \\
\text{REACTION 8} \\
\text{Ph–CO–NH–NH}_2 + \text{O}_2 + \text{H}_2\text{O}_2 & \rightarrow \text{Ph–CO–NH–NH} + \text{H}_2\text{O}_2 \\
\text{REACTION 9} \\
2\text{Ph–CO–NH–NH}_2 & \rightarrow \text{Ph–CO–NH–NH}_2 + \text{Ph–CO–N} = \text{NH} \\
\text{REACTION 10} \\
\text{O}_2 + \text{HO}_2^+ + \text{H}_2\text{O}_2 & \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \\
\text{REACTION 11}
\end{align*}

The transitory effect of superoxide dismutase is explained by it initially retarding Reaction 9, which is likely to be rate-determining in the auto-oxidation of ABAH. However, once sufficient hydrogen peroxide is produced its subsequent formation would be reliant on Reactions 3, 4, 8, and 11 and thus independent of superoxide. Generation of hydrogen peroxide via this sequence of reactions must be essential for the continual oxidation of ABAH because catalase inhibited even when it was added during the reaction. Presumably, ABAH is resistant to oxidation below pH 7 (Fig. 4) because its protonated form does not undergo Reactions 7–9. The lack of oxidation at acidic pH cannot reflect a slow reaction with myeloperoxidase because, in the presence of hydrogen peroxide, ABAH was readily oxidized by the enzyme below pH 7 (Fig. 4), and it reacted most rapidly with compound II between pH 5 and 7 (Fig. 3). The rate of oxidation of ABAH was dependent on myeloperoxidase only at low concentrations of the enzyme. This phenomenon most likely reflects a change in the rate-determining step from Reaction 1 to the generation of hydrogen peroxide as the concentration of myeloperoxidase was increased. Other substrates, including cysteine, NADPH, dihydroxyfumaric acid, and indol-3-yI acetic acid (33), are also oxidized by myeloperoxidase in the absence of added hydrogen peroxide. This oxidase activity should allow myeloperoxidase to function at inflammatory sites long after the respiratory burst of neutrophils has stopped. It is likely to be involved in the metabolism of the anti-tuberculosis drug isoniazid (34) and other xenobiotics (35).

Although benzoic acid hydrazides react rapidly with compound II, they are poor peroxidase substrates because they convert the enzyme to compound III. This explains why the pH optimum for the oxidation of ABAH in the presence of hydrogen peroxide (Fig. 4) was markedly different to that for reduction of compound II (Fig. 3). Thus, the pH profile in Fig. 4 most likely reflects the turnover of compound III. In this study we have confirmed that compound III is formed from the reduction of the ferric enzyme by ABAH radicals (Reaction 5) generated in the classical peroxidation cycle (Reactions 1, 3, and 4). Formation of compound III hinders oxidation of reducing substrates and explains how the benzoic acid hydrazides reversibly inhibited oxidation of TMB. We found that inhibition of TMB by substituted benzoic acid hydrazides was related to Hansch’s hydrophobicity constants (Fig. 8B). From this result we conclude that binding of radicals to the ferric enzyme determines how effective they are at reducing it. The more hydrophilic substituents must favor binding by hydrogen bonding with amino acid side chains in the distal heme cavity of myeloperoxidase as proposed for salicylhydroxamic acid (36). Reduction
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of ferric myeloperoxidase by hydrazide radicals is a crucial step in its inactivation (15). Therefore, the strong influence of hydrophobicity on this reaction could be exploited to destroy the enzyme and prevent neutrophils from producing hypochlorous acid.

We were unable to identify the reaction responsible for turnover of compound III. It could react with superoxide, hydrogen peroxide, ABAH, or a reducing radical formed from the oxidation of ABAH. Superoxide can be excluded because oxidation of ABAH occurred in the presence of superoxide dismutase. The reaction of compound III with hydrogen peroxide is likely to be too slow. ABAH or an ABAH radical may reduce compound III to compound I in a similar fashion to ascorbate (37). This reaction is analogous to that proposed for the reduction of oxyhemoglobin by phenylhydrazine (32). We did not observe any spectral changes when ABAH was added to preformed compound III (not shown), but this may have been due to the fact that compound III was reformed by the reduction of ferric myeloperoxidase (Reactions 5 and 6).

Benzoic acid hydrazides inhibit myeloperoxidase reversibly by promoting the formation of compound III and irreversibly by destroying the heme prosthetic group (15). They inhibited myeloperoxidase in the TMB assay because TMB promoted their oxidation to radicals that subsequently converted the enzyme to compound III. The ability of benzoic acid hydrazides to inhibit myeloperoxidase was not related to how easily the enzyme oxidized them. This finding demonstrates that the TMB assay was unsuitable for identifying their potential as mechanism-based inhibitors. Thus, results obtained using the TMB assays should be interpreted with caution, and additional assays should be performed to determine how effectively a particular compound inhibits myeloperoxidase.

We determined how readily benzoic acid hydrazides irreversibly inhibit myeloperoxidase, and we found that inactivation was strongly related to Hammett’s substituent constants (Fig. 9). However, inactivation was poorly related to the rate at which they were oxidized by compound I or compound II. These results indicate that oxidation of the benzoic acid hydrazides is fundamental to inactivation, but the critical oxidation reaction does not involve compound I or compound II. Reaction with compound III cannot be involved either because oxygen protects the enzyme by binding to ferrous myeloperoxidase (Reaction 6) (15). Reaction of ferrous myeloperoxidase with hydrogen peroxide and ABAH causes destruction of the heme prosthetic group (15). Thus, a plausible reaction that precipitates inactivation is reduction of a ferrous myeloperoxidase-hydrogen peroxide complex by ABAH. We plan to investigate this possibility by determining the structural modifications of myeloperoxidase that occur when it is inactivated by ABAH.

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