Studies on the Chlorinating Activity of Myeloperoxidase*

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JOHN E. HARRISON AND JULIUS SCHULTZ

From the Papanicolaou Cancer Research Institute, Miami, Florida 33123

Two methods were utilized to demonstrate the peroxidation of chloride ion to a free species (HOCl or ClO) by myeloperoxidase. The peroxidase caused the volatilization of radioactivity from solutions containing hydrogen peroxide and [¹⁴C]NaCl, and catalyzed the formation of HOCl when solutions containing these components were passed through a Millipore filter to which the peroxidase was adsorbed. In this flow system, 90 μg of canine myeloperoxidase generated 80 μM HOCl in the presence of 200 μM H₂O₂ at a rate corresponding to a turnover of 100 min⁻¹. Under these conditions, o-tolidine, whose oxidation can be coupled to CI⁻ peroxidation in free solution, did not accelerate turnover.

In contrast to chloroperoxidase and horseradish peroxidase, myeloperoxidase does not utilize chlorite for chlorination reactions. This oxidant inactivates the enzyme. At low pH, chloride ion suppresses the oxidation of myeloperoxidase (to the stable compound II) by both hydrogen peroxide and hypochlorite.

Acceptor chlorination is therefore not a rate-controlling reaction in the myeloperoxidase mechanism, and the potential of the functional peroxidase couple is higher than the HOCl/Cl⁻ couple under chlorinating conditions. The product-forming step may be a reverse of compound I formation at the expense of HOCl, rather than the chlorination of Cl⁻ by a chloroperoxidase-like chlorinating intermediate.

The antimicrobial activity of myeloperoxidase has been related to the chloride peroxidase activity of the enzyme (1-3). Agner first brought attention to the oxidative potential of myeloperoxidase in the presence of Cl⁻ and proposed that the formation of free HOCl could account for the observed chemical transformations (2). Subsequently Zbigczynski and co-workers have described some of the properties of the myeloperoxidase-H₂O₂-Cl⁻ system and have shown that the decarboxylation of amino acids proceeds via the formation of amino acid chloramines (4, 5). In no case, however, has the formation of free HOCl been demonstrated.

The biosynthetic enzyme, chloroperoxidase, chlorinates "acceptor" molecules via the formation of a chlorinating intermediate, a complex of chloride ion and the peroxidase compound I (6). Rates of turnover dependent on the nature of the acceptor have clearly shown that the decomposition of the chlorinating intermediate can be the rate-controlling process (7).

In this paper we demonstrate that myeloperoxidase catalyzes the peroxidation of chloride ion to free HOCl, and that the mechanism of chlorination is distinct from that of chloroperoxidase to the extent that a product-forming step is not rate-controlling. The rate of HOCl formation can account for the rate of acceptor chlorination. Since the couple involved in chloride peroxidation must be of higher potential than the HOCl/Cl⁻ couple, HOCl formation may proceed via a reverse of peroxidase compound I formation at the expense of HOCl.

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EXPERIMENTAL PROCEDURES

Materials

Canine myeloperoxidase was solubilized from canine pus with cetyl trimethylammonium bromide and purified to homogeneity using ammonium sulfate fractionation, and ion exchange and adsorption chromatography. In these procedures, the digestion steps (8) and use of Trypsin (9) were eliminated. Details of this modification will be published elsewhere. The final preparations exhibited RZ values of 0.78 to 0.80. Horseradish peroxidase (RZ = 3.1) was obtained from Worthington and used without further treatment. Monochlorodimethine was synthesized by the chlorination of dimethine as described by Hager et al. (10). Sodium chloride was obtained from MC&B Manufacturing Chemists, and ortho-tolidine from Eastman. [²⁴Cl⁻] was obtained from New England Nuclear and diluted to give a stock solution (1 M NaCl) of specific activity 1 mCi/mmol.

Methods

Sodium chlorite solutions were standardized by thiosulfate (standard thiosulfate, Mallinckrodt) titration using KI. Chlorine solutions were prepared by dilution of sodium hypochlorite, and standardized by determining the discharge of I⁻ + I₂⁻ from KI solution (10 mm) using a value of 2.3 x 10⁷ M⁻¹ cm⁻² s⁻¹ for the relationship between A_t and the concentration of I⁻ + I₂⁻ (11). Standard chlorine solutions were then utilized to obtain a standard curve for Cl₂ using the o-tolidine reagent. Chlorine solutions (0.01 to 0.2 ml) were added to 0.5 ml of o-tolidine reagent (3 N phosphoric acid/0.1% o-tolidine), the volumes were made up to 1.0 ml, and the solutions were read at 445 nm on a Gilford spectrophotometer. The standard curve was linear in the range 0 to 50 nmol of Cl₂ (0 to 50 μM, final concentration). Difference spectra were measured using a Zeiss PMQ II scanning spectrophotometer. Myeloperoxidase was dialyzed against water and diluted with buffer to give the stated concentrations of enzyme and buffer. Details of assay experiments appear in the figure legends.

Flow System—A Buchler peristaltic pump fed substrate buffer through a Nucleopore membrane holder, containing a 13-mm Millipore
filter (HAWP, 0.1300) connected to a hypodermic needle. This was clamped into the drop counter of a Gilson microfractionator set in the drop mode. The Millipore membrane was charged with myeloperoxidase by passing 2.0 ml of myeloperoxidase solution (0.2 to 0.3 mg/ml of myeloperoxidase in water) through the membrane; the amount of myeloperoxidase absorbed was calculated from the change in absorbance at 430 nm, using an extinction coefficient of 180 μM⁻¹ cm⁻¹. The filter was rinsed with 2.0 ml of substrate buffer (minus H₂O₂) and shaken free of excess liquid. The pump was started, and 18-dps fractions were collected into tubes containing 1.0 ml of ortho-tolidine reagent. The time per fraction was checked using a stopwatch, and was found not to vary by more than 5% from run to run. The initial fraction collected was generally ignored, since a surge of effluent could not be avoided. The concentration of HOCl in each fraction was calculated from the absorbance at 445 nm and the volume of each fraction (18 dps = 0.32 ml).

**Trapping of °Cl₂**—The reaction mixture was contained in a small Thunberg tube equipped with a serum cap. Nitrogen was passed over the surface of the reaction mixture by means of a long hypodermic needle. The effluent from the tube side arm was passed through 2.0 ml of water-saturated toluene, and the effluent was sampled for 1-min intervals after the injection of H₂O₂ into the reaction mixture. The trapping medium was mixed with 10 ml of toluene-based scintillant and counted on an Intertechnique scintillation spectrometer. The efficiency was 96%.

**RESULTS**

The Formation of HOCl—When peroxide is added to a solution containing myeloperoxidase and chloride ion at pH values between 3.5 and 6.0, the odor of Cl₂ can be detected within a few seconds. This simple test is negative when applied to horseradish peroxidase and chlorite under conditions where this system catalyzes chlorination. This observation suggested that a quantitative difference exists between the myeloperoxidase and H₂O₂ system and their capacities to generate free Cl₂. Although free Cl₂ is far in favor of HOCl under experimental conditions, the odor of Cl₂ can be detected in the effluent from the system.

The measured activity of myeloperoxidase in this system is low, being only 100 min⁻¹ under the conditions, compared to apparent rates of chlorination and chloride-dependent oxidation of 2000 min⁻¹ in free solution. When ortho-tolidine was included in the substrate buffer, however, the same rates of turnover were obtained as in its absence (Fig. 3). In free solution, o-tolidine competes effectively with other chloride acceptors, and under identical conditions is oxidized at the same rate as monochlorodimedone is chlorinated. Thus an apparent chloride acceptor, or HOCl-reactive reagent, does not increase the rate of myeloperoxidase turnover. The low activity observed with Millipore-bound myeloperoxidase is probably due to the presence of the membrane.
FIG. 3. Comparison of the rate of chloride peroxidation by membrane-bound myeloperoxidase in the presence (■) and absence (●) of o-tolidine in the substrate buffer. The conditions were identical with those of Fig. 2 except when o-tolidine (0.1 mM) was present. In each experiment the membrane absorbed comparable amounts (90 µg ± 10 µg) of myeloperoxidase.

FIG. 4. Myeloperoxidase-catalyzed chlorination of monochlorodimedone. The cuvette contained, in a total volume of 1.0 ml: KH₂PO₄-NaOH, 100 µmol, pH 4.5; KCl, 50 µmol (when present); myeloperoxidase, 15 µg (0.12 nmol); monochlorodimedone, 0.1 µmol. The reaction was initiated with 0.2 µmol of H₂O₂ in 2 µ1 of H₂O.

The laboratory has shown that like chloroperoxidase, myeloperoxidase catalyzes a single chlorination of monochlorodimedone to yield dichlorodimedone in high yield.

The essential differences between myeloperoxidase and horseradish peroxidase in their respective capacities to utilize chlorite and peroxide in the chlorination of monochlorodimedone is shown in Fig. 5. The data on horseradish peroxidase has been previously published by Hager and co-workers (6) and is included here to underline the differences and to serve as a control. Myeloperoxidase exhibits very low apparent activity at the expense of chlorite (cf. H₂O₂) and undergoes inactivation, whereas horseradish peroxidase exhibits the converse pattern. Experiments with **Cl⁻ have shown that at the level of myeloperoxidase utilized in this assay, no chlorine incorporation into dichlorodimedone can be detected. Hence the low extent of loss of ultraviolet absorbance seen in the figure, which does not exceed the chloride-independent rate of destruction (Fig. 4) is not due to chlorination. Studies on the mechanism of inactivation of myeloperoxidase by ClO²⁻ using substrate enzyme levels have revealed that the heme moiety is rapidly oxidized to an oxyheme (12).

Oxidation of Myeloperoxidase by HOCl and H₂O₂—Both

Chlorite at Oxidant: Comparisons with Horseradish Peroxidase—In order to compare the properties of the chlorinating activity of myeloperoxidase with those of horseradish peroxidase, the assay system described by Hager et al. was adopted (10). Shown in Fig. 4 is the stimulation of the loss of ultraviolet absorbance of monochlorodimedone by chloride ion in the presence of myeloperoxidase and peroxide. Work in this

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horseradish peroxidase and chloroperoxidase have been shown to undergo oxidation at the expense of H$_2$O$_2$ and HOCl (6). However, an enzymatic couple capable of generating HOCl from Cl$^-$ should exhibit resistance to oxidation by the HOCl/Cl$^-$ couple. At pH 8.6 both H$_2$O$_2$ and HOCl in 10$^x$ excess over heme, generate myeloperoxidase compound II to similar extents (not shown). At pH 4.5, the extent of formation of compound II at the expense of peroxide is slightly increased, but decreased at the expense of HOCl. When the same determination is made in the presence of 50 mM Cl$^-$, the difference spectra obtained are not significantly different from the prerecorded base-lines (Fig. 6). Under these conditions, peroxide is decomposed with the formation of HOCl, as shown above. Following the addition of HOCl, the solution remains positive for an oxidizing agent (o-tolidine test) for several minutes.

**DISCUSSION**

Unlike the protoheme peroxidase chloroperoxidase, myeloperoxidase (a) can continuously peroxidase Cl$^-$ to HOCl, (b) does not require organic chloride acceptors for turnover or does not undergo rate acceleration in the presence of the latter, (c) does not utilize chlorite for chlorination reactions, and (d) is not oxidized by HOCl to a stable oxidation state (under chlorinating conditions).

There are two ways in which nonacceleration of turnover can be rationalized. The first of these is simply that organic acceptors do not interact with a chlorinating form of myeloperoxidase, either because such a form is inaccessible, or because it does not exist as an intermediate. Under this alternative, a free species (HOCl or Cl$_2^-$) would be an obligatory intermediate in acceptor chlorination. The second alternative is that a rate-determining step precedes the decomposition of a chlorinating intermediate. If, for example, our experimental setup were measuring a 95% rate-determining step, then the direct interaction of organic acceptors with a chlorinating intermediate could occur (at any rate), but turnover would be accelerated 5% at most. The data rule out a chlorinating intermediate whose decomposition is rate-determining.

A previous report from this laboratory has ruled out myeloperoxidase compound II as an intermediate in the catalytic cycle (13), and recent studies have confirmed the existence of an unstable primary peroxide compound (compound I). Two possible mechanisms for chloride peroxidation suggest themselves. The first of these is that generally accepted for halide peroxidations, in which a halogenating intermediate reacts with a second halide ion, to form free halogen (14). The second alternative is that oxygen transfer from compound I to bound hydrogen chloride (H$^+$, Cl$^-$) occurs. This scheme requires a reaction similar to the reverse of compound I formation at the expense of peroxides (15), peroxynitrobenzeno acids (16, 17), and HOCl itself. The latter reaction has been demonstrated in horseradish peroxidase (6) and presumably occurs in myeloperoxidase itself with the Cl$^-$ free form (Fig. 6).

Preliminary experiments utilizing the flow system have shown that the effluent is capable of killing Escherichia coli. It is therefore probable that the antibacterial activity of myeloperoxidase described by Agner (1) and Klebanoff (3) and the cytotoxic effect on normal and tumor cells (18) is due to the generation of HOCl. The involvement of singlet O$_2$ in killing (19) cannot yet be ascertained, but our data appear to indicate that the reaction between HOCl and H$_2$O$_2$ does not proceed at a significant rate compared with the rate of chloride peroxidation.

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