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Chlorination by the Myeloperoxidase-H_2O_2-Cl^- Antimicrobial System at Acid and Neutral pH (39684)

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The enzyme, myeloperoxidase (MPO), has been widely studied. However, it was not until recently that information relative to its biological significance has become available. MPO, in the presence of H_2O_2 and a halide has been found to constitute a powerful antimicrobial system (1, 2). Since polymorphonuclear leukocytes (PMN) appear to be the only source of this enzyme, and since a primary function of these phagocytes is to destroy engulfed microbes, it is reasonable to postulate that the physiological function of MPO is its participation in host-defense mechanisms. With chloride as the halide, MPO has been found to catalyze the oxidation of Cl^- ion by H_2O_2 to yield the chlorinating agent, the chlorinium ion (Cl^+) (3, 4). The antimicrobial activity of the MPO-H_2O_2-Cl^- system is probably due to the formation of this chlorinium ion. The Cl^+ ion can react with substrates such as bacterial cell walls, (5), amines, (4), amino acids (3) and other substrates (5, 6) resulting in chlorination and antimicrobial activity.

It has been reported that the optimal pH for chlorination, decarboxylation and bactericidal activity is 5.5 (1). Although it has been suggested that the pH can drop to this level in the phagocytic vacuole, (7, 8), there is no definitive proof that this pH is actually reached in the human PMN. Interestingly, it has been found that chlorination and decarboxylation can occur optimally at a pH range of 3.6–5.0 providing the chloride concentration is changed from 10 to 400 m*M*, respectively. This observation indicates that a relationship between [Cl^-] and optimal pH exists (4). Similar to H_2O_2, Cl^- is also known to be bound to Fe-porphyrin groups of MPO (6). Thus, it would appear reasonable to postulate that H_2O_2 may also affect pH optimum.

In this study, we will attempt to show that MPO-mediated chlorination and bactericidal activity can occur over a wide range of pH, [Cl^-], and [H_2O_2]. We will also discuss the complex kinetic behavior of MPO towards pH, [Cl^-] and [H_2O_2] that accounts for this apparently high flexibility of the enzyme.

Materials and methods. MPO mediated chlorination was studied with a preparation of MPO obtained from human granulocytes. The enzyme was purified by a previously described method (9) and had an *RZ* value of 0.6 ($A_{430/280\ \mathrm{nm}}$). Diethanolamine was used as substrate (10 m*M*) and the velocity of diethanol chloramine formation at different pH values (4–6) was measured at 265 nm. This concentration of diethanolamine will saturate the enzyme since it is >10 times its K_m value. For any given pH, different Cl^- (2.5–400 m*M*) and H_2O_2 (0.1–2.5 m*M*) concentrations were employed.

Results. MPO-Cl^- affinity studies were carried out at three different H_2O_2 concentrations (2.5, 1.0 and 0.5 m*M*). The experiments were performed at seven different pH values ranging from 4.0 to 6.0 (100 m*M* citrate-phosphate buffer). Affinity was calculated from the initial velocity of diethanolamine (10 m*M*) chlorination. The MPO concentration was 10 μg/ml. Cl^- concentration was varied from 2.5 to 200 m*M*. The results are presented in Fig. 1. It may be noted that the apparent affinity between MPO and Cl^- varies with the pH and/or H_2O_2 concentration. Increasing H^+ concentration results in an increase in MPO-Cl^- affinity. Conversely, increasing the H_2O_2 concentration results in a decrease in MPO-Cl^- affinity.

If H_2O_2 can affect Cl^- binding to MPO,

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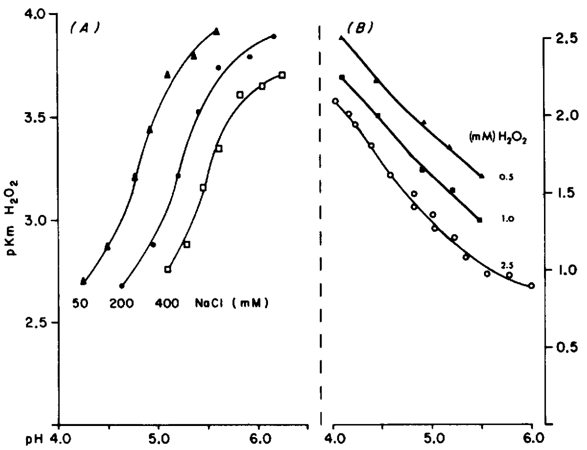

FIG. 1. (A) Influence of pH and Cl^- on apparent affinity between myeloperoxidase and H_2O_2. (B) Influence of pH and H_2O_2 on apparent affinity between myeloperoxidase and Cl^-. The velocity of diethanolchloramine formation at 20° was measured as increase in absorbance at 265 nm. Reaction mixture contained 10 m*M* citrate phosphate buffer at the indicated pH and different concentrations of H_2O_2 and Cl^-. K_m values for chloride and H_2O_2 were calculated using Lineweaver-Burk plots ($pkm = -\log K_m$). (A) Cl^- concentrations 50 (▲——▲), 200 (●——●), 400 (□——□) m*M*. Five different H_2O_2 concentrations from 0.1 to 5.0 m*M* were used for any experimental point. (B) H_2O_2 concentrations 0.5 (▲——▲), 1.0 (■——■), and 2.5 (○——○) m*M*. Five different NaCl concentrations from 2.5 to 200 m*M* were used for any experimental point.

different Cl^- concentrations can also be expected to affect MPO-H_2O_2 affinity. Experiments similar to those above were designed to determine the correctness of this hypothesis. The H_2O_2 concentration was varied from 0.1 to 5 m*M*. The Cl^- concentrations used were 50, 100, 200, 300, and 400 m*M*. For sake of clarity, only 50, 200, and 400 m*M* data were shown. As expected, Cl^- ion concentration affected the apparent affinity between MPO and H_2O_2. Increasing concentrations of Cl^- ion decreased MPO-H_2O_2 affinity. Similar to MPO-Cl^- affinity, MPO-H_2O_2 affinity is also pH dependent.

The observed influence of pH on affinity relationships between MPO and H_2O_2 and MPO and Cl^- led us to expect different optimum pH values for MPO mediated chlorination with different concentrations of H_2O_2 and Cl^-. An experiment to test this hypothesis was performed and the results may be seen in Fig. 2. It is evident that there is no fixed optimum pH for chlorination. Rather, optimal chlorination can occur over a wide pH range. For instance, one may obtain optimal chlorination between pH 4.0 and 6.0 by simply adjusting the Cl^- and H_2O_2 concentrations.

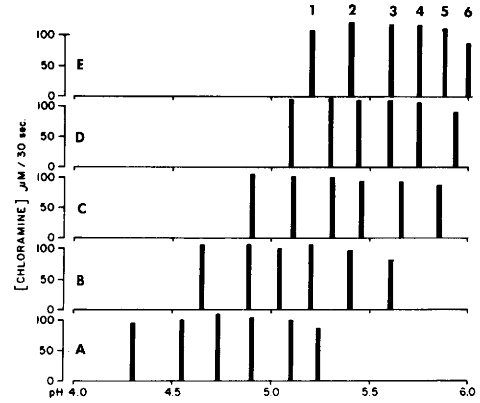

FIG. 2. Influence of H_2O_2 and Cl^- concentrations on optimal pH for myeloperoxidase mediated chlorination. Experimental details were as described for Fig. 1. Cl^- concentrations (m*M*): 50 (A), 100 (B), 200 (C), 300 (D), 400 (E). H_2O_2 concentrations (m*M*): 1.667 (1), 0.833 (2), 0.667 (3), 0.5 (4), 0.333 (5) and 0.167 (6).

From the above results, one can see that the $[H_2O_2]/[Cl^-]$ ratio is critical for the optimal velocity of chlorination for any given pH. The relationship between pH and the required $[H_2O_2]/[Cl^-]$ ratio for optimal chlorination may be seen in Fig. 3. It is evident that pH changes are proportional to changes of log $[H_2O_2]/[Cl^-]$. Since pH is a logarithmic function of H^+ concentration, the experimentally obtained curve may be expressed by a single equation: $K = [H_2O_2]/$

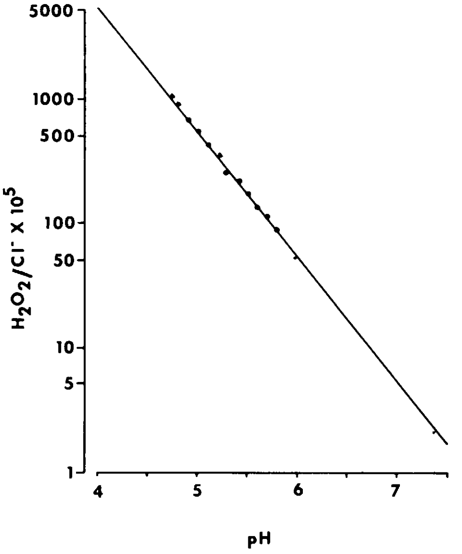

FIG. 3. Optimal pH for myeloperoxidase mediated chlorination at different H_2O_2/NaCl ratios. Data calculated from results presented in Fig. 2.

$[Cl^-]/[H^+]$, where K is the proportionally coefficient and is experimentally found to be equal to 560. A change in this value will result in a decrease of velocity. For example, if the $[H_2O_2]/[Cl^-][H^+]$ ratio is 250 or 1250, the velocity of the reaction was found to be 75% of optimal.

The relationship between Cl^- and H_2O_2 concentrations and pH was experimentally established for pH values between 4 and 6. Since the relationship of pH and log $[H_2O_2]/[Cl^-]$ ratio is a straight line, extrapolation of optimal condition for pH values higher than pH 6 is theoretically possible. It would be of interest to see if extrapolation of the curve to neutral pH can be experimentally confirmed.

From the above formula, for pH 7.4 and 150 mM Cl^- ion concentration, optimal concentration of H_2O_2 should be 3.4 μM. This amount of H_2O_2, even after complete conversion to chloramine, will have an absorbance of 0.0012 for a 1 cm light path. Such low values cannot be accurately measured. Hence, in chlorination experiments at pH 7.4, H_2O_2 was added to achieve 10 times higher than optimal concentration and in multiple steps; sodium chloride concentration was also increased to 400 mM. Under these experimental conditions, the velocity of the reaction should be 50–75% of the optimal velocity predicted by the formula. By doing this, sufficient accumulation of product for accurate measurement was obtained in spite of the fact that low amounts of H_2O_2 were used. The results of this experiment may be seen in Fig. 4. This indicates that chlorination occurs efficiently at pH 7.4. It may be noted that an immediate conversion of H_2O_2 to product occurs within seconds. Further, the conversion is stoichiometric. It is obvious from these data that extrapolation of the experimental data shown in Fig. 3 can be made correctly.

Discussion. There exist numerous reports that at acidic pH, MPO-H_2O_2-Cl^- system is bactericidal to a variety of bacteria, fungi, mycoplasma, and virus. It was of interest to ascertain, whether such a universal microbicidal system may also be able to function at a wide variety of physiological conditions such as varying pH, $[H_2O_2]$ and $[Cl^-]$ that may exist in the granulocyte. We have approached this problem by investigating the apparent affinity relationship between MPO-H_2O_2 and MPO-Cl^- at different Cl^- and H_2O_2 concentrations, respectively. Also, the influence of pH on these parameters was examined. Although multireactant enzyme kinetics are complex, Cleland (10) has shown that initial velocity analysis suffices for Michaelis constants and maximum velocities. Most of our kinetic data were obtained by measuring the initial velocity of the reaction.

Results presented in Fig. 1 show that the apparent affinity of MPO to Cl^- varies with pH and/or $[H_2O_2]$. Higher $[H^+]$ or lower $[H_2O_2]$ leads to an increase in the affinity of MPO to Cl^-. Somewhat similar to our findings, Hager *et al.* (11) have reported that the affinity of chloroperoxidase to Cl^- also varies with pH and changes in the same direction as in MPO. However, H_2O_2 does not affect chloroperoxidase-Cl^- affinity. Similarly, the apparent affinity of MPO to H_2O_2 also varies with pH and/or $[Cl^-]$. This property of MPO appears to be unique among all enzymes utilizing H_2O_2 substrate. Even chloroperoxidase, which is very similar to MPO in its mechanism of chlorination does not show variable affinity toward H_2O_2 (11).

In addition, by examining V_{max} values from Lineweaver-Burk plots (data not

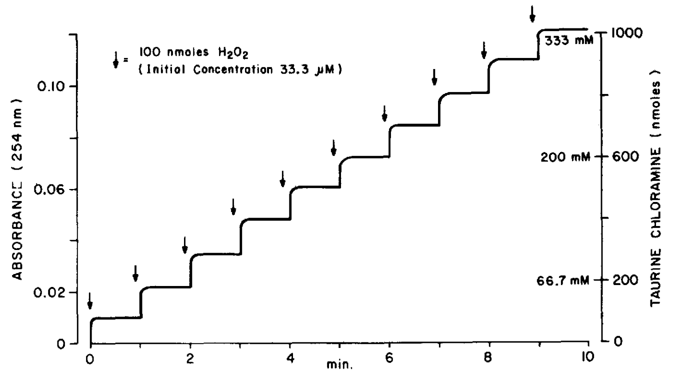

FIG. 4. Myeloperoxidase mediated taurine chlorination at pH 7.4. Reaction mixture contains: 20 m*M* taurine, 400 m*M* NaCl, 10 μg of MPO/ml, 100 m*M* citrate phosphate buffer at pH 7.4. Reaction was started by the addition of 5 μl of 20 m*M* H_2O_2 (arrows) to obtain temporal concentration of H_2O_2 in the sample (3 ml) equal to 33.3 *M*. Chlorination was measured as absorbance increase at 254 nm at 20°.

shown) one can note the competitive nature between H_2O_2 and Cl^- ion, H_2O_2 and H^+ and Cl^- and OH^-. This is, of course, true because the V_{max} in each instance is similar (Fig. 2). These results further indicate that Cl^-, H^+, and H_2O_2 bind to the same locus of MPO. Further, these bindings most probably occur in the Fe-porphyrin nucleus, since they cause spectral changes in the Soret region. Since varying, at constant ratio, the concentrations of two substrates (H_2O_2/Cl^-) results in optimal velocity of chlorination for any given pH, a Ping Pong mechanism (12) may be operating. From a physiological point of view, this may be of significance. Heretofore, the MPO-H_2O_2-Cl^- antimicrobial system was reported to be effective only at acid pH. Since controversy exists as to whether the pH drops intracellularly to 5.0 in human cells, a microbicidal system functioning at neutral pH values would be desirable.

It may be concluded from this study, that the MPO-H_2O_2-Cl^- antimicrobial system may be functional over a wide range of pH values (4.0–7.4), H_2O_2 and Cl^- ion concentrations. It is no longer necessary to associate the activity of this system strictly to the acidic pH. A subsequent publication will show that the MPO-H_2O_2-Cl^- system is bactericidal at pH 7.4.

From these and previous kinetic studies of MPO, it is apparent that this enzyme is unique. It has at least two different primary substrates (i.e., H_2O_2 and Cl^-), it has a broad pH optimum (4.0–7.4) depending on the relative concentrations of these substrates, and, finally these substrates compete for the same site of the enzyme. All these properties are compatible for MPO activity over a broad biological range.

Summary. The oxidation of Cl^- ion by H_2O_2 yielding Cl^+ is catalyzed by myeloperoxidase (MPO) prepared from human PMN. Both H_2O_2 and Cl^- in this reaction serve as substrates. Affinity (apparent) studies between MPO and Cl^- and MPO and H_2O_2 were carried out. Both (apparent) affinities vary according to pH. The higher the pH, the less the affinity for Cl^-. In contrast, the affinity between MPO and H_2O_2 increases with pH. The dissociation constant of the MPO-Cl^- enzyme substrate complex is affected by the second substrate, H_2O_2. The effect of H_2O_2 on the MPO-Cl^- affinity is competitive. Likewise, the MPO-H_2O_2 affinity is decreased with increasing Cl^- concentration. This influence is also competitive. From these interactions, it may be concluded that the optimal pH for chlorination is dependent on the $[H_2O_2]/[Cl^-]$ ratio. The relationship of pH, H_2O_2, and Cl^- concentrations may be expressed by the following formula: $pH = \log [Cl^-] \times 560/[H_2O_2]$. Using the formula, it can be shown that optimal chlorination may occur between pH 4.0 and 7.4.

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