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Oxidative Peptide Cleavage and Decarboxylation by the MPO-H_2O_2-Cl^- Antimicrobial System

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The antimicrobial activities of the myeloperoxidase-H_2O_2-halide system have received considerable attention recently. The precise mechanism by which this system exerts its lethal activity is presently not clear. In an effort to learn more regarding a possible mechanism of action, the susceptibility of protein-bound amino acids to enzymatic attack by myeloperoxidase (MPO) in the presence of chloride ions was investigated. [1,7-^{14}C]diaminopimelic acid (DAP) was incorporated into *Escherichia coli* W-7 proteins with little randomization of the radioactivity. Under appropriate conditions, it was observed that the MPO-H_2O_2-halide system released approximately 94% of the radioactivity from labeled bacteria. This would indicate that, in addition to decarboxylation, peptide bonds are also split during this reaction. The oxidative decarboxylation of DAP-labeled bacteria by MPO (i) is Cl^- dependent, (ii) has an acid pH optimum, (iii) requires a specific concentration of H_2O_2 for activity, (iv) reaches a plateau by 25 min, and (v) is markedly inhibited by taurine. These properties are similar to those observed with free amino acids. It appears from these data that MPO can not only decarboxylate free and bound amino acids, yielding aldehydes, but also it can actively participate in oxidative peptide cleavage. Both of these activities may play a critical role in the microbicidal action of the leukocyte.

The ability of leukocytes to kill bacteria has been attributed to a wide variety of agents that are either present in the leukocyte or elaborated during phagocytosis (2, 6, 8, 10, 13, 18). Of these, the antimicrobial properties of the myeloperoxidase (MPO)-H_2O_2-halide system have attracted considerable interest recently. Malfunction of this antimicrobial system as observed, for example, in chronic granulomatous disease leads to a fatal breakdown of the host's defense against bacterial infection (6). The precise mechanism(s) by which the MPO-H_2O_2-halide system exerts its antimicrobial activities is presently under investigation (8, 9, 11, 14, 15). When the halide is iodide, iodination of bacteria has been suggested as the cause of bacterial death (8). When the halide is chloride, reports from our laboratory have shown that amino acids are oxidatively decarboxylated to form aldehydes with potential antimicrobial properties (15). Amino acid chloramines (19) and singlet oxygen (R. C. Allan and R. H. Steele, Fed. Proc. **32**:478, 1973) have been suggested as possible intermediates in aldehyde formation. Data have been presented indicating that the aldehydes are formed from amino acids of microbial origin (11). By labeling bacterial cell wall proteins with [1,7-^{14}C]diaminopimelic acid (DAP), we are now able to present direct evidence that this enzyme system can attack the free alpha-amino carboxyl groups of protein-bound DAP. In addition, and perhaps equally important, we have noted that the MPO system can also oxidatively cleave peptide bonds. Therefore, in addition to the formation of aldehydes with bactericidal potency, MPO has the capability of denaturing indispensable protein molecules. This activity may also play a critical role in the antimicrobial system(s) of leukocytes.

MATERIALS AND METHODS

All chemicals used in this investigation were of reagent grade. Purified human MPO was a generous gift from J. Schultz, Papanicolaou Institute, Miami, Fla. A DAP-requiring mutant of *Escherichia coli* strain W-7 was kindly supplied by J. T. Park, Tufts University School of Medicine, Boston, Mass. [1,7-^{14}C]DAP was purchased from Cal Atomic, Los Angeles, Calif. Glucose oxidase (type V) was obtained from Sigma Chemical Co., St. Louis, Mo. Decarboxylation

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studies were carried out in Krebs-Ringer phosphate medium (KRPM) essentially as described previously (14).

RESULTS

Previous reports have shown that the MPO-H_2O_2-Cl^- system deaminates and decarboxylates glycine, alanine, aspartic acid, cysteic acid, cystine, lysine, serine, phenylalanine, valine, leucine, and isoleucine (20). The results obtained in this investigation indicate that DAP can also be decarboxylated efficiently by MPO. In fact, the observed K_m for DAP, 5.9×10^{-5} M, is one of the lowest reported for an amino acid in this system (Fig. 1). Hence, attempts were made to label bacteria with [1,7-^{14}C]DAP.

Preliminary experiments (Fig. 2) showed that *E. coli* W-7 has an absolute growth requirement for DAP. Maximal growth occurred at a DAP concentration of 2.5 μg/ml in Trypticase soy broth. A batch of [1,7-^{14}C]DAP-labeled bacteria were prepared by growing *E. coli* W-7 in 750 ml of Trypticase soy broth supplemented with lysine (375 mg) and [1,7-^{14}C]DAP (1.9 mg; 50 μCi). Overnight cultures of the labeled bacteria were heat killed, harvested by centrifugation, and washed free from soluble radioactivity. Under these conditions, 60% of the added [1,7-^{14}C]DAP was found to be taken up by the bacteria. Most of the radioactivity that was incorporated in the bacteria was located in large molecules; 5% trichloroacetic acid at room temperature for 15 min extracted only 1.4% of the radioactivity from the labeled bacteria.

To explore the possibility that the radioactivity of the added [1,7-^{14}C]DAP might have been

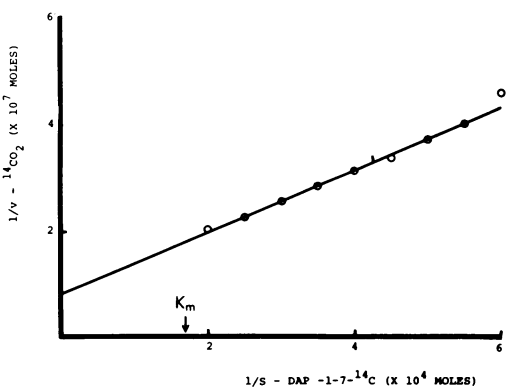

FIG. 1. *Michaelis constant for [1,7-^{14}C]DAP decarboxylation. Reaction mixture contained 10^{-4} M H_2O_2, 0.025 guaiacol units of MPO, Krebs-Ringer phosphate medium at pH 6.5, and varying concentrations of [1,7-^{14}C]DAP. Incubation period was 30 min at 37 C.*

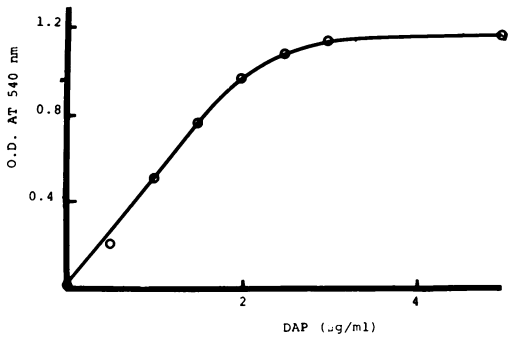

FIG. 2. *DAP requirement of E. coli W-7. Trypticase soy broth was supplemented with 0.5 mg of lysine per ml and DAP as indicated. Bacterial growth was determined by measuring the optical density after 18 h of incubation (37 C) at 540 nm in a Bausch and Lomb Spectronic 20 colorimeter.*

redistributed into other products by the growing bacteria, a sample of labeled bacteria (3×10^{10} bacteria, 6.7×10^5 dpm) was hydrolyzed with 10 ml of 6 N HCl at 115 C for 18 h. After repeated evaporation under reduced pressure to remove HCl, the hydrolysate was dissolved in 0.5 ml of distilled water. A series of 5- and 10-μliter volumes, along with authentic DAP and lysine, were chromatographed on Whatman 3 MM paper with *n*-butanol-acetic acid-water (3:1:1, vol/vol) for 15 h. One part of the chromatogram was sprayed with ninhydrin; another part of the chromatogram was exposed to X-ray film (GAF-HR-3000) for 70 h. Only one spot was visible in the radioautograph. This spot was identified as DAP by its R_f value. DAP and lysine, the closest ninhydrin-reactive spots with R_f values of 0.086 and 0.143, respectively, resolved sufficiently to permit removal of the DAP spot quantitatively without contamination by any other ninhydrin-reacting compound. Finely cut pieces of the radioactive spot and a sample of the hydrolysate spread over a similar area on Whatman 3 MM paper were assayed for radioactivity. All the radioactivity of the hydrolysate (>98%) was recovered in the DAP spot, thereby indicating that the label in *E. coli* W-7 was present almost exclusively in DAP.

Decarboxylation of DAP-labeled bacteria by MPO-H_2O_2-Cl^- system during a period of 45 min is shown in Fig. 3. The results show a continuously decreasing velocity of the reaction, with maximal $^{14}CO_2$ production occurring approximately at 25 min. Controls from which either MPO, H_2O_2, and/or Cl^- were omitted showed no significant $^{14}CO_2$ formation. The effect of pH on the oxidation of free DAP and DAP-labeled bacteria is shown in Fig. 4. The

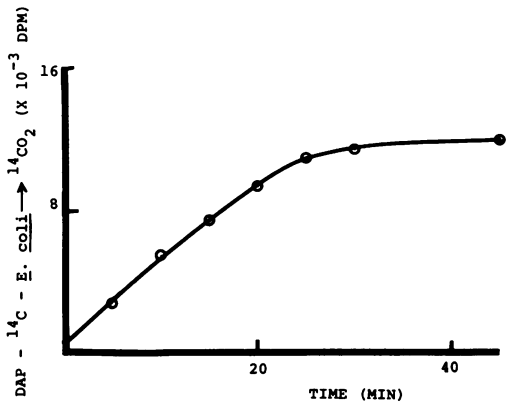

FIG. 3. *Oxidation of [1,7-^{14}C]DAP-labeled E. coli W-7. [1,7-^{14}C]DAP-labeled E. coli W-7 (67 × 10^3 dpm) was incubated for varying time periods under the conditions described in Fig. 1.*

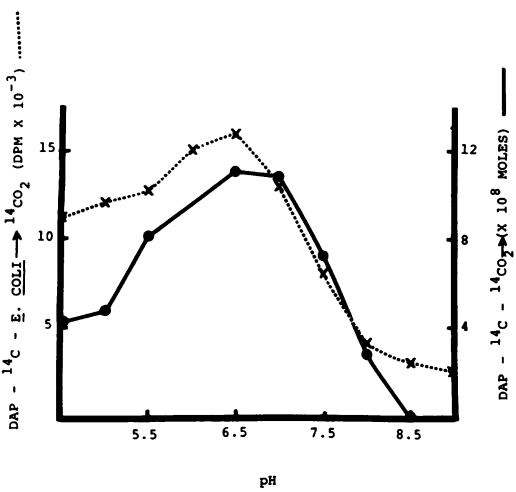

FIG. 4. *pH optima for the oxidation of free and protein-bound [1,7-^{14}C]DAP. [1,7-^{14}C]DAP (1.8 × 10^{-3} M) and [1,7-^{14}C]DAP-labeled E. coli W-7 (67 × 10^3 dpm) were incubated at varying pHs under conditions described in Fig. 1.*

results show that the optimal pH of this reaction is 6.5. The activity dropped sharply in the alkaline range. Similar to the results obtained with L-[1-^{14}C]alanine as substrate (15), the MPO-H_2O_2-Cl^- system showed a requirement for a specific concentration of H_2O_2 (10^{-4} M). There is a considerable decrease in the reaction rate at both high and low concentrations of H_2O_2 (Fig. 5). The velocity of the reaction as influenced by enzyme concentration is shown in Fig. 6. Decarboxylation of labeled bacteria is linear up to 0.01 guaiacol units of MPO per ml. The optimal enzyme concentration was 0.03 guaiacol units per ml, and increasing the enzyme concentration to 0.15 guaiacol units per ml did not result in an appreciable increase in $^{14}CO_2$ production. Taurine, a competitive inhibitor of L-alanine decarboxylation by this system (20), also effectively inhibits decarboxylation by DAP-labeled bacteria (Table 1). Oxidation of viable DAP-labeled bacteria was also observed to be catalyzed by MPO. However, CO_2 production from viable, labeled bacteria was only 32% when compared to heat-killed bacteria.

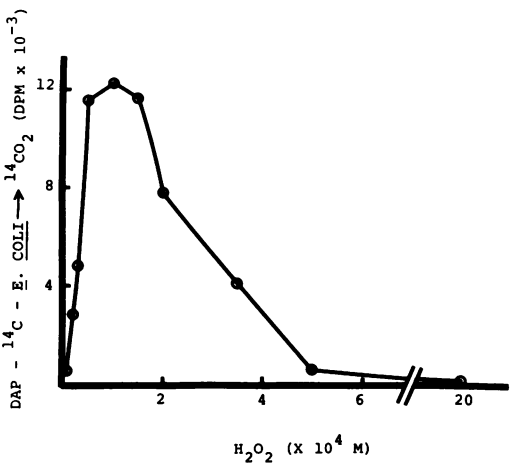

FIG. 5. *Effect of H_2O_2 concentration on the oxidation of [1,7-^{14}C]DAP-labeled E. coli W-7. [1,7-^{14}C]DAP-labeled E. coli W-7 (67 × 10^3 dpm) was incubated with varying concentrations of H_2O_2 under the conditions described in Fig. 1.*

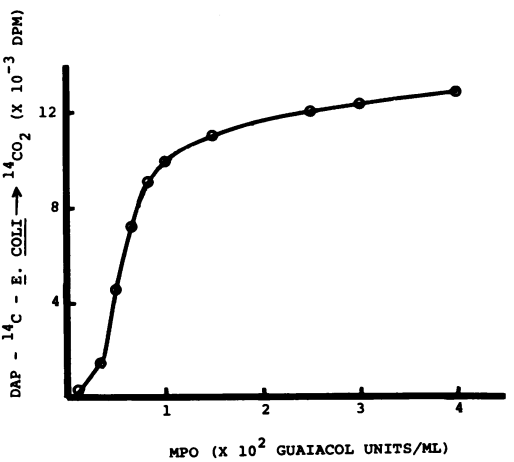

FIG. 6. *Effect of MPO concentration on the oxidation of [1,7-^{14}C]DAP-labeled E. coli W-7. [1,7-^{14}C]DAP-labeled E. coli W-7 (67 × 10^3 dpm) was incubated with varying concentrations of MPO under the conditions described in Fig. 1.*

TABLE 1. *Effect of taurine on the decarboxylation of [1,7-^{14}C]DAP-labeled E. coli W-7 by MPO*[a]

Taurine (10^{-4} M)	$^{14}CO_2$ (dpm × 10^{-3})	Inhibition (%)
0 (26)	13.45 ± 0.29	0
0.5 (3)	6.78 ± 0.51	49.6
5 (4)	1.77 ± 0.05	86.8
50 (3)	0.72 ± 0.02	94.6

[a] [1,7-^{14}C]DAP-labeled *E. coli W-7* (67×10^3 dpm) was incubated with 10^{-4} H_2O_2 and 0.025 guaiacol units per ml of MPO for 30 min with the indicated concentrations of taurine. Results are given as mean ± standard error with the number of observations shown in parentheses.

The comparative susceptibility of the free and protein-bound DAP to enzymatic attack by MPO was investigated by attempts to achieve maximal oxidation of the labeled bacteria. With 0.025 guaiacol units of MPO and 0.01 mM H_2O_2, approximately 21% of the radioactivity was recovered as $^{14}CO_2$, in 30 min, from labeled *E. coli* (Table 1). The release of radioactivity from [1,7-^{14}C]DAP-labeled *E. coli* ceased after 25 min (Fig. 3). Low H_2O_2 concentrations due to spontaneous decomposition are probably responsible for this observation. H_2O_2 concentrations could not be increased in the reaction mixture without an adverse effect on the enzyme system (Fig. 5). Thus, an H_2O_2-generating system involving 20 mM glucose and 10 U of glucose oxidase was used to maintain optimal H_2O_2 concentration. The concentration of MPO was also increased to 0.15 guaiacol units per ml. Under these conditions, $^{14}CO_2$ production from DAP-labeled *E. coli* could be increased to 84.2% in 3 h. Free [1,7-^{14}C]DAP was oxidized to the extent of 91.3% under similar conditions (Table 2).

DISCUSSION

The precise mechanism(s) responsible for the antimicrobial activity of the MPO-H_2O_2-halide system is under investigation in several laboratories. In an effort to learn more regarding the mode of action of the system, the susceptibility of bacterial protein-bound amino acids to enzymatic degradation by MPO-H_2O_2 in the presence of Cl^- was investigated and is being reported here.

DAP, a known bacterial cell wall constituent, was used to label *E. coli* W-7, a mutant that requires DAP for growth. Because of the diamino dicarboxyl groups in DAP, even protein-bound DAP will have free alpha-amino carboxyl groups. These are similar to free amino acids and thus may be susceptible to enzymatic attack. Free DAP is effectively decarboxylated by the MPO-H_2O_2-Cl^- system. It was observed that the K_m for DAP is the lowest reported for any amino acid in this system (15, 20). Thus, in spite of the low concentration of DAP in bacteria, it is likely that protein-bound DAP can be decarboxylated efficiently by MPO.

Studies carried out with DAP-labeled bacteria have demonstrated conclusively that free COOH groups containing adjacent free alpha-amino groups of the protein-bound DAP can also serve as well as free DAP as substrates for the MPO-H_2O_2-Cl^- system. Thus, MPO-catalyzed oxidative decarboxylation of DAP-labeled bacteria by H_2O_2 (i) is Cl^- dependent; (ii) reaches a plateau by 25 min; (iii) has an acid pH optimum with a marked reduction in activity at alkaline pH; (iv) has a sharp peak of activity at a specific concentration of H_2O_2; (v) has a low threshold for the enzyme, resulting in a very narrow range of linearity and rapidly attained enzyme saturation; and (vi) is markedly inhibited by taurine. All these properties are similar to those observed with free amino acids, such as alanine, as substrate (15, 16). The present finding that free COOH groups of the protein-bound cell wall amino acid (DAP) can serve as a substrate for the MPO-H_2O_2-Cl^- system is in agreement with the work of Cline and Lehrer (2). These authors have shown that the terminal free COOH groups of cell wall amino acids are attacked by the D-amino acid oxidase of leukocytes.

The apparent increased susceptibility to enzymatic attack of bound DAP from heat-killed bacteria as opposed to that of viable bacteria is of interest. The difference in decarboxylation activity between heat-killed and viable bacteria could be due to alterations in a variety of factors including permeability barriers and possible structural alterations. Also, in viable bacteria, components or reactions that may utilize and

TABLE 2. *Maximal decarboxylation of free and protein-bound [1,7-^{14}C]DAP by MPO*[a]

Substrate	$^{14}CO_2$ (%)
Free [1,7-^{14}C]DAP	91.3 ± 4.6 (6)
Bound [1,7-^{14}C]DAP	84.2 ± 3.1 (3)

[a] [1,7-^{14}C]DAP-labeled *E. coli W-7* (67×10^3 dpm) or free [1,7-^{14}C]DAP (70×10^3 dpm, 10^{-4} M) was incubated with 0.15 guaiacol units of MPO, 10 units of glucose oxidase, and 20 mM glucose for 3 h. Results are given as mean ± standard error with the number of observations shown in parentheses.

reduce the effective concentrations of H_2O_2 (i.e., catalase and/or peroxidase) may be operative.

According to the current concept of the structure of the DAP containing glycosaminopeptide in the cell wall, for every two DAP molecules only two of the four carboxyl groups are free; of these two carboxyl groups only one has a free alpha-amino group. So, the maximal $^{14}CO_2$ production from DAP in the labeled bacteria should be (i) <25%, if only free carboxyl groups containing adjacent free alpha-amino groups are attacked; (ii) 25 to 50%, if only free carboxyl groups are attacked; and (iii) >50%, if all the carboxyl groups including those in peptide linkage are attacked. Results obtained in this study show that under appropriate conditions at least 84.2% of the radioactivity can be recovered as $^{14}CO_2$ from DAP-labeled bacteria. Thus, the data probably illustrate a universal reaction that may occur with any protein in general, rather than a specific reaction confined to DAP containing proteins.

The precise mechanism(s) by which MPO-H_2O_2-Cl^- splits the peptide bonds for decarboxylation of amino acids is not known. Zgliczynski et al. (19) have demonstrated that MPO-H_2O_2-Cl^- and amino acids interact to form amino acid chloramines. These chloramines are unstable and, in the presence of water, spontaneously decompose to aldehydes, CO_2, and NH_4Cl. It is conceivable that MPO-H_2O_2-Cl^- similarly chlorinates peptide bonds with the formation of amino acid chloramines and results in peptide cleavage. The possible sequence of reactions occurring is illustrated in Fig. 7. Furthermore, Allen et al. (Fed. Proc. 32:478, 1973) have demonstrated the formation of singlet oxygen by MPO-H_2O_2 in the presence of Cl^- ions. Being a potent oxidant, singlet oxygen could also participate in oxidative cleavage of peptide bonds. Obviously this important area requires further work.

Recently Elsbach et al. (4) have demonstrated a rapid decrease in trichloroacetic acid-precipitable radioactivity of [1,7-^{14}C]DAP-labeled *E. coli* W-7 and [^{14}C]leucine-labeled *E. coli* by intact and disrupted rabbit granulocytes. The degradation of macromolecules in labeled *E. coli* observed by these authors is attributed to lysosomal hydrolytic enzymes. However, the contribution of lysosomal hydrolytic enzymes and/or MPO-H_2O_2-Cl^- system to the breakdown of macromolecules reported by Elsbach et al. is not clear. The neutral pH employed by these authors and the similar efficiency of both intact and disrupted granulocytes are compatible with their conclusions that

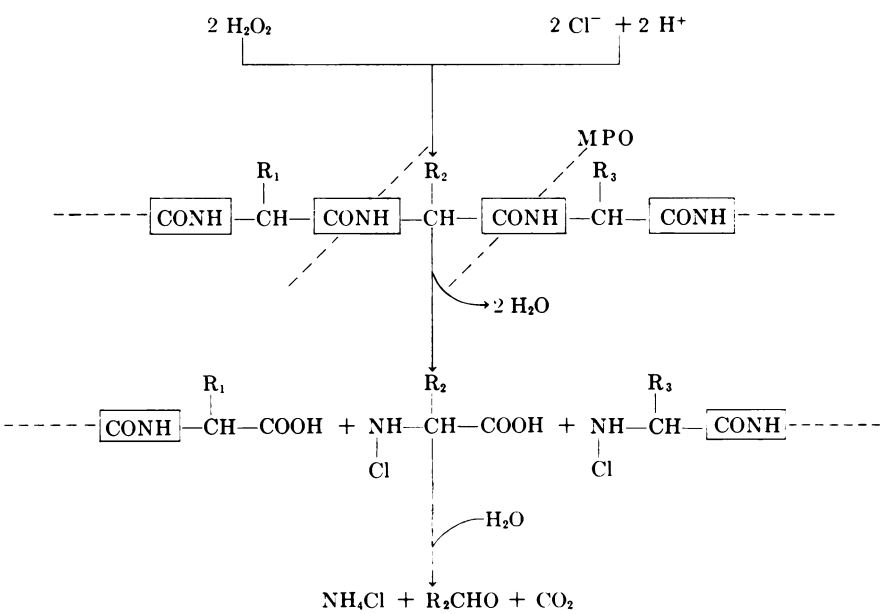

FIG. 7. *Proposed mechanism of oxidative peptide cleavage by MPO-H_2O_2-Cl system. Cleavage of the two peptide bonds adjacent to R_2 results in the formation of R_2 amino acid chloramine. This chloramine is hydrolyzed and spontaneously decarboxylates and deaminates, yielding aldehyde, CO_2, and NH_4Cl. Note that R_1 and R_3 end up with free carboxyl and chloramine groups, respectively. Further attack of these amino acids will require only one H_2O_2 molecule.*

lysosomal hydrolytic enzymes are involved.

Admittedly, decarboxylation of DAP resulting in aldehyde formation and possible structural alterations in the cell wall is not the sole mechanism by which the MPO system can inactivate microorganisms. The finding that mycoplasma species (8) and viruses (2) are susceptible to this system substantiates the thinking that bacterial cell wall material is not mandatory for antimicrobial activity. However, the results of the present study indicate a general protein-splitting capacity of the MPO-H_2O_2-Cl^- system that may be operative with any viable cell. The use of mono-amino mono-carboxyl amino acids as markers in protein molecules will permit us to obtain more direct evidence for peptide cleavage. We are currently investigating this possibility with the aid of [^{14}C]leucine-labeled bacteria. Further support for this theory is provided by the recent observations of Edelson and Cohn (3). These authors have demonstrated a highly efficient cytotoxic effect by MPO-H_2O_2-Cl^- system against mouse lymphoma cells. Also, a number of biologically active proteins are known to be inactivated by peroxidase without decarboxylation of amino acids and this might also lead to antimicrobial activity. For instance, tyrosyl groups (12, 17) or thio groups might undergo oxidation under the influence of peroxidase.

Decarboxylation of protein-bound amino acids and the cleavage of peptide bonds by the MPO-H_2O_2-Cl^- system in leukocytes is of extreme interest. Its possible significance as a defense mechanism in the leukocyte remains to be elucidated and must await further study.

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