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Myeloperoxidase-Mediated Oxidation of Methionine and Amino Acid Decarboxylation

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The myeloperoxidase (MPO)-mediated decarboxylation of amino acids and the MPO-mediated oxidation of methionine, two potential bactericidal mechanisms, were compared. In the presence of the MPO system (MPO, 50 mU/ml; H_2O_2, 0.1 mM; Cl^-, 75 mM), 50% of alanine (0.1 mM) was decarboxylated, whereas only 5% of methionine (0.1 mM) was decarboxylated. In contrast, under similar conditions, 80% of methionine was oxidized to methionine sulfoxide. Once methionine was oxidized to methionine sulfoxide, it was decarboxylated (75%) by the MPO system. Methionine at 0.1 mM completely inhibited the decarboxylation of alanine, whereas alanine at a concentration 200 times that of methionine had no effect on the MPO-mediated oxidation of methionine. Sodium azide, an MPO inhibitor, inhibited the decarboxylation of alanine and the oxidation of methionine to the same extent. Tryptophan markedly inhibited the oxidation of methionine, whereas histidine stimulated it. Alanine, glycine, and taurine had no effect. In contrast, all of these amino acids and taurine markedly inhibited the MPO-mediated decarboxylation of alanine. NaN_3, tryptophan, and methionine, which inhibited the MPO-mediated oxidation of methionine, also inhibited the killing of *Staphylococcus aureus* or *Klebsiella pneumoniae* by the MPO system; whereas histidine, alanine, and glycine, which did not inhibit the oxidation of methionine, had less or no effect on the killing of these two bacteria by the MPO system. Results suggest that methionine is preferentially oxidized to methionine sulfoxide by the MPO system. Once methionine is oxidized to methionine sulfoxide, it is then readily decarboxylated by the MPO system. The agent responsible for the oxidation of methionine may play an important role in the MPO-mediated killing of bacteria.

The myeloperoxidase (MPO) system (MPO-H_2O_2-halide) is a potent antimicrobial system operative in neutrophils (9). The exact mechanism by which this system kills bacteria is not clear. Several possibilities have been proposed; these include MPO-mediated halogenation (8, 14), decarboxylation of amino acids with the generation of aldehydes (11, 13, 22), and MPO-mediated production of singlet oxygen ($^1O_2$) (9, 12). However, whether the MPO system produces $^1O_2$ remains controversial (6, 7).

Previous studies (17) from this laboratory have demonstrated that during phagocytosis, neutrophils oxidize methionine to methionine sulfoxide and that the oxidation of methionine is dependent on the MPO system. Evidence has also accumulated to indicate that MPO-mediated oxidation of methionine is responsible for the inactivation of α_1-proteinase inhibitor (α_1-antitrypsin) (2, 10), *n*-formylmethionyl chemotactic peptides (3, 18), or C5a (3) by human neutrophils or by the MPO system. The MPO-mediated inactivation of α_1-proteinase inhibitor may contribute to the pathogenesis of emphysema (2, 10). The MPO-mediated inactivation of chemotactic peptides may be one mechanism by which neutrophils modulate the inflammatory process (3, 18). If the MPO system also oxidizes methionine at the active site of bacterial enzymes, this may be one mechanism by which this system damages microorganisms.

In this study, the MPO-mediated oxidation of methionine and decarboxylation of alanine were compared. Evidence that the agent responsible for the MPO-mediated oxidation of methionine might play an important role in the killing of bacteria is also provided.

MATERIALS AND METHODS

Chemicals. L-[*U*-^{14}C]methionine and L-[*U*-^{14}C]alanine were obtained from New England Nuclear Corp., Boston, Mass. L-[1-^{14}C]alanine was obtained from ICN Pharmaceuticals, Inc., Irvine, Calif. L-methionine-DL-sulfoxide and L-methionine sulfone were from Sigma Chemical Co., St. Louis, Mo. L-[*U*-^{14}C]methionine sulfoxide was obtained through chemical oxidation of L-[*U*-^{14}C]methionine by adding 0.1 ml of 3%

H_2O_2 to 5 μCi of L-[*U*-^{14}C]methionine (293 mCi/mM) in a final volume of 0.5 ml in water. The mixture was incubated at 4°C for 3 h. It was then evaporated to dryness in a vacuum dessicator. The reaction product was dissolved in 5 ml of water. Using thin-layer chromatography (silica gel without fluorescence indicator; Eastman Kodak, Rochester, N.Y.) with three different solvent systems, we found that under this condition, all [^{14}C]methionine was oxidized to [^{14}C]methionine sulfoxide, and no [^{14}C]methionine sulfone was obtained. The solvent systems used were *tert*-butanol:methylethylketone:water:ammonium hydroxide, 40:30:20:10; *n*-butanol:glacial acetic acid:water, 50:25:25; and a lower-phase solvent from a mixture of phenol and water (5).

Isolation of MPO. Canine MPO was isolated as described previously (M. F. Tsan, J. Cell. Physiol., in press). Briefly, dogs were given an intramuscular injection of terpentine (1 ml) to induce leukocytosis. One week later, leukocytes were isolated from 2 liters of heparinized blood. MPO was then isolated from canine leukocytes through the end of step 6 (the step before crystallization) by the method of Agner (1). The final preparation exhibited a Reinheitszahl value (absorbancy at 430 nm/absorbancy at 280 nm) of 0.78. The activity of MPO was determined by the *ortho*-dianisidine method (20). One unit of peroxidase is the amount of enzyme which decomposes 1 μmol of H_2O_2 per min at 25°C.

MPO-mediated oxidation of methionine. Oxidation of methionine was performed as described previously (Tsan, in press). The complete system included MPO, 50 mU/ml; H_2O_2, 0.1 mM; Cl^-, 75 mM; and 0.1 μCi of [^{14}C]methionine, 0.1 mM in a final volume of 1 ml in 0.02 M phosphate buffer, pH 6.0. H_2O_2 was added last to initiate the reaction. After being incubated for desired intervals at 37°C in a water bath with constant shaking, the test tubes were immersed in ice, and 0.1 ml of 50 mM methionine was added to prevent further oxidation of [^{14}C]methionine. [^{14}C]methionine and [^{14}C]methionine sulfoxide were separated by using thin-layer chromatography and quantified with a liquid scintillation counter as described before (17). The results were expressed as the percentage of methionine oxidized to methionine sulfoxide. Control experiments were always done in the absence of the MPO system to assess the spontaneous oxidation of methionine, which was negligible.

MPO-mediated decarboxylation of amino acids. Measurement of [^{14}C]carbon dioxide production was done as described previously (19). The complete system included MPO, 50 mU/ml; H_2O_2, 0.1 mM; Cl^-, 75 mM; and 0.1 mM ^{14}C-amino acid in a final volume of 1 ml in 0.02 M phosphate buffer, pH 6.0. The amounts of radioactivity used were 0.1 μCi for ^{14}C uniformly labeled alanine, methionine, or methionine sulfoxide and 0.03 μCi for [1-^{14}C]alanine. Similar results were obtained whether [*U*-^{14}C]alanine or [1-^{14}C]alanine was used. H_2O_2 was added last to initiate the reaction. After incubation for desired intervals at 37°C in a water bath with constant shaking, the reaction was stopped, and $^{14}CO_2$ was quantified as described previously (19). The results were expressed as the percentage of amino acid decarboxylated. Control experiments were always performed in the absence of the MPO system to assess the spontaneous release of $^{14}CO_2$, which was negligible.

MPO-mediated bacterial killing. MPO-mediated bacterial killing was performed as described previously (19). The complete system included MPO, 50 mU/ml; H_2O_2, 0.1 mM; Cl^-, 75 mM; and 5×10^6 *Staphylococcus aureus* (ATCC 25923) or 3×10^7 *Klebsiella pneumoniae* (a clinical isolate from The Johns Hopkins Hospital) in a final volume of 1 ml in 0.02 M phosphate buffer, pH 6.0. H_2O_2 was added last to initiate the reaction. After being incubated for 1 h at 37°C in a water bath with constant shaking, serial dilutions were made, and the number of bacteria was determined by the pour plate technique as described previously (19).

Statistical analysis. Statistical differences were determined by using Student's *t* test for independent means (4).

RESULTS

MPO-mediated decarboxylation of alanine and methionine. Zgliczynski et al. (22) demonstrated that the MPO system decarboxylates a variety of amino acids. However, in previous studies (17; Tsan, in press) it was shown that methionine is oxidized to methionine sulfoxide by the MPO system. Therefore, the MPO-mediated decarboxylation of alanine and methionine was compared. As shown in Fig. 1a, 52% of alanine was decarboxylated after a 30-min incubation, whereas only 5% of methionine was decarboxylated. Increasing the concentration of H_2O_2 from 0.1 to 1 mM did not increase the decarboxylation of methionine by the MPO system (three experiments, data not shown). In contrast, under similar conditions, 83% of methionine was oxidized to methionine sulfoxide (Fig. 1b). The decarboxylation of alanine or methionine and the oxidation of methionine required the complete MPO system; elimination of MPO, H_2O_2, or Cl^- abol-

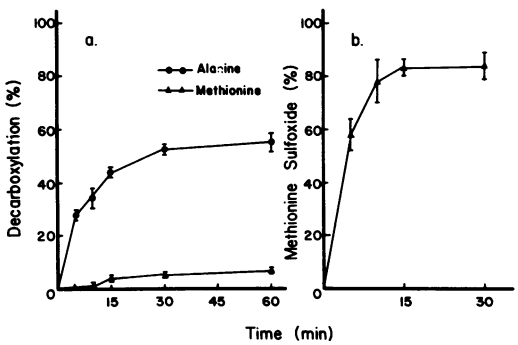

FIG. 1. MPO-mediated decarboxylation of alanine and methionine (a) and oxidation of methionine (b). The complete system included MPO, 50 mU/ml; H_2O_2, 0.1 mM; Cl^-, 75 mM; and 0.1 mM [^{14}C]alanine or [^{14}C]methionine in a final volume of 1 ml in 0.02 M phosphate buffer, pH 6.0. After being incubated for various intervals at 37°C, the production of $^{14}CO_2$ or oxidation of methionine to methionine sulfoxide was determined. Each point was the mean ± the standard error of the mean of three experiments.

ished the reactions completely (three experiments, data not shown).

Thus, methionine was preferentially oxidized to methionine sulfoxide by the MPO system. It is possible that the thioether group of methionine prevents its carboxylic group from being decarboxylated. To test this possibility, methionine was oxidized to methionine sulfoxide by incubating methionine with the MPO system for 30 min. Another sample of H_2O_2 (0.1 ml, 1 mM) was then added to the reaction mixtures, and $^{14}CO_2$ production was determined. The addition of the second sample of H_2O_2 caused a marked output of $^{14}CO_2$ (Fig. 2a). Decarboxylation of methionine sulfoxide by the MPO system was further confirmed by using [^{14}C]methionine sulfoxide. Addition of the MPO system to [^{14}C]methionine sulfoxide caused a marked $^{14}CO_2$ output (Fig. 2b). Chromatographic analysis revealed that a new product was formed, presumably the decarboxylated product of methionine sulfoxide (Fig. 3). No methionine sulfone was noted, suggesting that methionine sulfoxide was not oxidized to methionine sulfone by the MPO system.

To determine whether the agent responsible for the MPO-mediated decarboxylation of alanine is the same as that for the oxidation of methionine, I studied the effect of methionine on the MPO-mediated decarboxylation of alanine and the effect of alanine on the MPO-mediated oxidation of methionine. Methionine markedly inhibited the decarboxylation of alanine; at the same concentration as alanine (0.1 mM), methionine inhibited the decarboxylation of alanine completely (Fig. 4a). In contrast, alanine at a concentration of 20 mM, which was 200 times that of methionine, had no effect on the MPO-mediated oxidation of methionine (Fig. 4b). It should be pointed out that methionine also markedly inhibited the MPO-mediated decarboxylation of methionine sulfoxide (68% inhibition at 0.1 mM methionine and 100% inhibition at 1 mM methionine, mean of two experiments, methionine sulfoxide concentration was 0.1 mM).

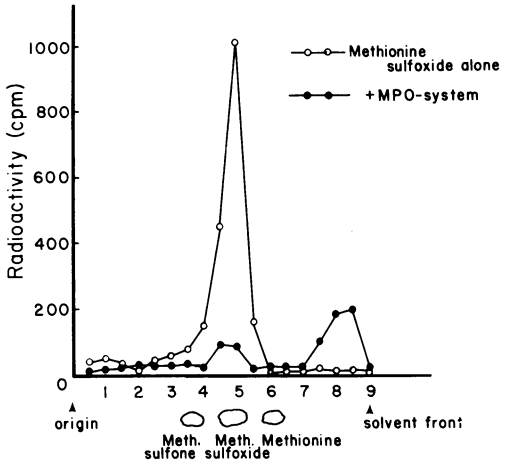

FIG. 3. Chromatographic separation of methionine sulfoxide and its decarboxylated product. [^{14}C]methionine sulfoxide (0.1 mM) was incubated in the presence or absence of the MPO system for 30 min at 37°C. The final media were then chromatographed by ascending thin-layer chromatography, using water-saturated phenol as the solvent system.

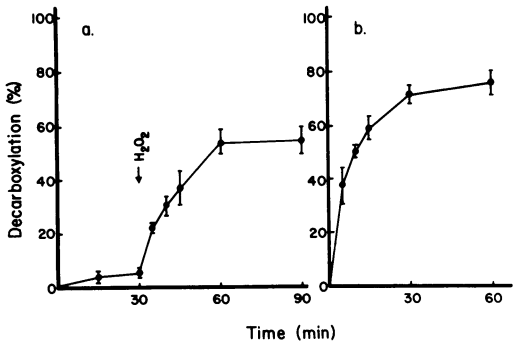

FIG. 2. MPO-mediated decarboxylation of methionine (a) and methionine sulfoxide (b). Experimental conditions were as in Fig. 1, except that methionine was first incubated with the MPO system for 30 min at 37°C; another sample of H_2O_2 (0.1 ml, 1 mM) was then added, and $^{14}CO_2$ production was monitored. Each point was the mean ± the standard error of the mean of three experiments.

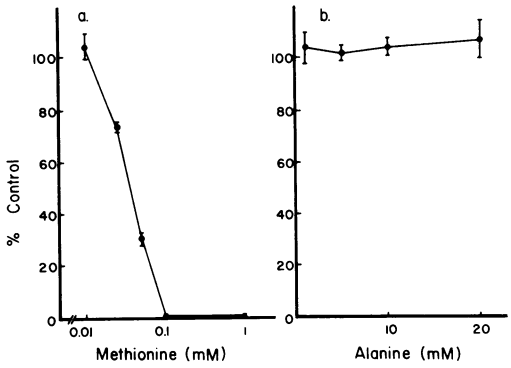

FIG. 4. Effect of methionine on the MPO-mediated decarboxylation of alanine (a). Effect of alanine on the MPO-mediated oxidation of methionine (b). Experimental conditions were as in Fig. 1, except that various amounts of inhibitors were added. Each point was the mean ± the standard error of the mean of three experiments.

TABLE 1. Effect of various inhibitors on the MPO-mediated oxidation of methionine and decarboxylation of alanine[a]

Inhibitor (mM)	Control value (%)	
	Methionine oxidation (no. of expt)	Alanine decarboxylation (no. of expt)
NaN (0.1)	5.1 ± 1.9 (4)[b]	3.9 ± 1.4 (3)[b]
Methionine (1)	12.2 ± 0.6 (4)[b]	0.0 ± 0.0 (3)[b]
Alanine (1)	111.5 ± 6.9 (5)	10.5 ± 1.3 (3)[b]
Tryptophan (1)	7.5 ± 1.7 (5)[b]	0.6 ± 0.2 (3)[b]
Histidine (1)	131.6 ± 13.2 (5)[b]	1.7 ± 0.5 (3)[b]
Glycine (1)	105.8 ± 3.2 (5)	15.1 ± 1.3 (3)[b]
Taurine (1)	100.3 ± 4.4 (4)	3.3 ± 0.6 (3)[b]

[a] The experiments were performed in 1 ml of phosphate buffer (pH 6.0) containing MPO, 50 mU/ml; H_2O_2, 0.1 mM; Cl^-, 75 mM; and methionine, 0.1 mM or alanine, 0.1 mM. The incubation time was 15 min at 37°C for methionine oxidation and 30 min at 37°C for alanine decarboxylation. The control value for the MPO-mediated oxidation of methionine was 77.2 ± 9.7% (7) of methionine oxidized to methionine sulfoxide and 42.6 ± 4.6% for alanine decarboxylation (3). The numbers in parentheses indicate the number of experiments.

[b] $P < 0.01$.

Table 1 shows a comparison of the effect of NaN_3 and various amino acids on the MPO-mediated decarboxylation of alanine and oxidation of methionine. NaN_3, a myeloperoxidase inhibitor, inhibited the decarboxylation of alanine and the oxidation of methionine to the same extent. Tryptophan markedly inhibited the oxidation of methionine, whereas histidine stimulated it. Alanine, glycine, and taurine had no effect. These results are similar to those observed previously (Tsan, in press), which were obtained at a different pH (pH 7.0). In contrast, all of these amino acids and taurine markedly inhibited the MPO-mediated decarboxylation of alanine. Previous studies (17; Tsan, in press) demonstrated that these amino acids have no effect on the peroxidase activity of MPO.

MPO-mediated bacterial killing. The MPO-mediated amino acid decarboxylation has been proposed as a possible bacterial killing mechanism (11, 13, 22). It has been shown that MPO-mediated oxidation of methionine is responsible for the inactivation of α_1-proteinase inhibitor (2, 10) or methionine-containing chemotactic peptides (3, 18) by human neutrophils or the MPO system. Thus, if the MPO system also oxidizes methionine at the active site of bacterial enzymes, this may be one mechanism by which this system damages microorganisms. As shown in Table 2, NaN_3, methionine, and tryptophan, which inhibited the MPO-mediated oxidation of methionine (see Table 1), also inhibited the killing of *S. aureus* or *K. pneumoniae* by the MPO system; whereas histidine, alanine, and glycine, which did not inhibit the oxidation of methionine, had less or no effect on the killing of these two bacteria by the MPO system.

DISCUSSION

In this study, the MPO-mediated oxidation of methionine and the MPO-mediated decarboxylation of amino acids were compared. Since previous work on the MPO-mediated decarboxylation of amino acids has been primarily done with alanine (11, 15, 16, 22) it was also used in this study. Both MPO-mediated oxidation of methionine and decarboxylation of alanine have an acid pH optimum (15; Tsan, in press). Consequently, the experiments were carried out at pH 6. As demonstrated in this study, the MPO system decarboxylated 50% of alanine, whereas

TABLE 2. Effect of various inhibitors on the MPO-mediated bacterial killing[a]

Inhibitor	Viable cell count	
	S. aureus × 10^{-6} (no. of expt)	*K. pneumoniae* × 10^{-7} (no. of expt)
None	5.51 ± 0.64 (9)	3.17 ± 0.29 (7)
Complete system (MPO-H_2O_2-Cl^-)	0.00000 ± 0.00000 (9)	0.00000 ± 0.00000 (7)
MPO omitted	5.66 ± 1.52 (5)	2.10 ± 0.32 (3)
H_2O_2 omitted	6.12 ± 1.33 (4)	1.80 ± 0.15 (3)
+ 0.1 mM NaN_3	2.79 ± 0.56 (4)	
+ 1 mM methionine	5.98 ± 1.06 (5)	2.70 ± 0.56 (3)
+ 1 mM alanine	0.00000 ± 0.00000 (3)	0.00000 ± 0.00000 (4)
+ 1 mM tryptophan	3.60 ± 0.61 (4)	2.33 ± 0.22 (3)
+ 1 mM histidine	0.014 ± 0.008 (4)	0.00003 ± 0.00003 (3)
+ 1 mM glycine	0.003 ± 0.003 (3)	0.00000 ± 0.00000 (3)

[a] The bacteria (*S. aureus*, 5 × 10^6 or *K. pneumoniae*, 3 × 10^7) were incubated in the presence or absence of the MPO system (MPO, 50 mU/ml; H_2O_2, 0.1 mM; Cl^-, 75 mM) in 1 ml of phosphate buffer (pH 6.0) for 60 min at 37°C. Additions were as indicated in the table. After incubation, the viable bacterial count was determined by the pour plate technique. The numbers in parentheses indicate the number of experiments.

under the same experimental conditions, it only decarboxylated 5% of methionine. In contrast, it oxidized 80% of methionine to methionine sulfoxide. Once methionine was oxidized to methionine sulfoxide, it was decarboxylated by the MPO system. Thus, the thioether group of methionine prevented its carboxylic group from being decarboxylated by the MPO system. Methionine at the same concentration as alanine completely inhibited the decarboxylation of alanine. In contrast, alanine at a concentration 200 times that of methionine had no effect on the MPO-mediated oxidation of methionine. Furthermore, histidine stimulated the oxidation of methionine, whereas it markedly inhibited the decarboxylation of alanine. The decarboxylation of alanine is thought to be mediated by OCl^- produced by the MPO-H_2O_2-Cl^- system (21). Although OCl^- can oxidize methionine to methionine sulfoxide, our previous study (Tsan, in press) has shown that the MPO-mediated oxidation of methionine is not mediated by OCl^-. Thus, the agent responsible for the oxidation of methionine is different from the agent responsible for the decarboxylation of alanine by the MPO system. This is consistent with the previous observation that the MPO-H_2O_2-I^- system does not decarboxylate alanine (11), whereas it is able to oxidize methionine to methionine sulfoxide (Tsan, in press).

MPO-mediated decarboxylation of amino acids has been suggested as one mechanism by which the MPO system kills microorganisms. Zgliczynski et al. (21) propose that in the presence of MPO and H_2O_2, Cl^- is oxidized to hypochlorous acid. Hypochlorous acid reacts with amino acids to form chloramine, which in turn decomposes spontaneously to ammonia, CO_2, Cl^-, and a corresponding aldehyde. The bacteria are killed either by the formation of soluble aldehydes with microbicidal activity (11, 15, 16) or by the formation of structural aldehydes on the surfaces of the organisms (13). However, it has been shown that free aldehydes are either not toxic enough (11) or are not produced in sufficient quantity (9) to account for the observed bacterial killing. Thus, if the MPO system kills bacteria by the decarboxylation of amino acids, it would presumably be through the disruption of amino acid-containing macromolecules (13).

Recently, the MPO system has been found to inactivate a number of enzymes or bioactive peptides through the oxidation of methionine to methionine sulfoxide. These include α_1-proteinase inhibitor (2, 10), C5a (3) and *n*-formylmethionyl synthetic chemotactic peptides (3, 18). In the cases of MPO-mediated oxidation and inactivation of α_1-proteinase inhibitor (2) or *n*-formylmethionylleucylphenylalanine (18), no decarboxylation of amino acids has been noted. Thus, in the presence of methionine, decarboxylation of amino acids is unlikely to occur.

In this study, it was demonstrated that there was a better correlation between MPO-mediated oxidation of methionine and bacterial killing than between MPO-mediated decarboxylation of alanine and bacterial killing. Methionine and tryptophan, which inhibited MPO-mediated oxidation of methionine, inhibited the MPO-mediated killing of *S. aureus* and *K. pneumoniae*; whereas histidine, alanine, and glycine did not inhibit the MPO-mediated oxidation of methionine and had less or no effect on the killing of these two microorganisms. In contrast, all of these amino acids inhibited the decarboxylation of alanine. Paul et al. (11) have shown that glycine and alanine inhibit the killing of *Escherichia coli* by the MPO system. However, although the final concentrations of alanine and glycine in their study are the same as those in mine, they used only 10^4 *E. coli*, whereas I used 5×10^6 *S. aureus* and 3×10^7 *K. pneumoniae*. Increasing the concentration of these amino acids to 100 mM, e.g., 100-fold higher, alanine, but not glycine, completely inhibited the killing of *S. aureus* or *K. pneumoniae* by the MPO system (three experiments, data not shown). Thus, there is competition between bacterial components and these amino acids for the agent(s) responsible for the MPO-mediated bacterial killing. In addition, there is also some species difference.

The results presented in this study suggest that the agent(s) responsible for the oxidation of methionine may play an important role in the bacterial killing. The agent (or agents) responsible for the MPO-mediated oxidation of methionine is not clear. However, my previous study (Tsan, in press) suggests that it is neither $^1O_2$ nor hypochlorite. Further studies are necessary to clarify this point.

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