(RS)-mTrp-OH, 95388-80-0; Boc-D-Trp(For)-OH, 64905-10-8; Boc-D-Trp(For)-NH_2, 95388-81-1; H-Phe-OH, 63-91-2; Nps-Thr-(Bu^t)-OSu, 32137-86-3; H-Thr(Bu^t)-Ser(Bu^t)-Cys(SBu^t)-OBu^t·HCl, 77946-22-6.

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Differential Effects of Oxidizing Agents on Human Plasma α_1-Proteinase Inhibitor and Human Neutrophil Myeloperoxidase†

Nancy R. Matheson and James Travis*

Department of Biochemistry, University of Georgia, Athens, Georgia 30602

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ABSTRACT: Human α_1-proteinase inhibitor is easily susceptible to inactivation because of the presence of a methionyl residue at its reactive site. Thus, oxidizing species derived from the myeloperoxidase system (enzyme, H_2O_2, and Cl^-), as well as hypochlorous acid, can inactivate this inhibitor, although H_2O_2 alone has no effect. Butylated hydroxytoluene, a radical scavenger, partially protects α_1-proteinase inhibitor from the myeloperoxidase system and completely protects it from hypochlorous acid. Each oxidant also reacts differently with the inhibitor, in that the myeloperoxidase system and hypochlorous acid can each oxidize as many as six methionyl residues, but hypochlorous acid can also oxidize a single tyrosine residue. Myeloperoxidase can be inactivated by hypochlorous acid, by autoxidation in the presence of H_2O_2 and Cl^-, as well as by H_2O_2 alone. Butylated hydroxytoluene completely protects this enzyme from hypochlorous acid inactivation, does not affect the action of H_2O_2, and enhances autoinactivation. As many as six methionyl residues and two tyrosine residues could be oxidized during autoxidation and six methionine residues by H_2O_2 alone. Eight methionine residues and one tyrosine residue could be oxidized by hypochlorous acid. The tyrosine residue in myeloperoxidase was oxidized only at a relatively high concentration (600 μM) of hypochlorous acid at which point the enzyme simultaneously and completely lost its enzymatic activity. Loss of activity of myeloperoxidase could also be correlated with the loss of the heme groups present in the enzyme when a relatively high concentration of hypochlorous acid (600 μM) was used and also during autoxidation. It appears that once there is sufficient oxidant to modify one of the tyrosine residues, the heme group itself becomes susceptible.

Human α_1-proteinase inhibitor (α_1-PI)[1] is a major circulating plasma protein which is also capable of passing through vascular membranes into tissues during inflammatory episodes. Thus, it is thought to play an important role in controlling tissue proteolysis caused by the massive quantities of proteinases released from polymorphonuclear leukocytes which are attracted to the lungs and joints during inflammation. These

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[1] Abbreviations: α_1-PI, α_1-proteinase inhibitor; MPO, myeloperoxidase; BHT, butylated hydroxytoluene; HOCl, hypochlorous acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Me_2SO, dimethyl sulfoxide.

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cells also release myeloperoxidase (MPO) and other degradative enzymes from azurophil and specific granules during inflammation-induced phagocytosis (Zurier et al., 1973; Klebanoff & Clark, 1978; Henson et al., 1978) and produce large amounts of superoxide, H_2O_2, and other oxidants during the respiratory burst (Root et al., 1975; Levine et al., 1976). In the presence of H_2O_2 and Cl^-, MPO is capable of causing the oxidation of two methionine residues in α_1-PI, one of which is at the reactive site, and this effect results in the inactivation of the inhibitor (Matheson et al, 1979). However, if α_1-PI or another oxidizable substrate is absent, MPO autoxidizes (Naskalski, 1977; Matheson et al., 1981a). Several investigators have also claimed that the MPO system (MPO, H_2O_2, and Cl^-) generates hypochlorous acid (HOCl) as the functional oxidative intermediate (Agner, 1972; Harrison et al., 1976). Thus, it is possible that some or all of these activated oxygen species might contribute to the oxidative inactivation of α_1-PI and MPO.

The aims of the present investigation were to explore the ability of H_2O_2, HOCl, and H_2O_2 and Cl^- (in the presence of MPO) to inactivate α_1-PI, as well as to cause the autoinactivation of MPO itself. Since such effects are usually associated with the destruction of susceptible amino acid residues, we also examined the amino acid composition of each protein before and after treatment with each individual oxidizing agent. Finally, the effect of oxidants on maintaining the integrity of the essential heme groups of MPO was examined.

Experimental Procedures

Materials

α_1-PI and MPO were prepared as described previously (Pannell et al., 1974; Matheson et al., 1981a). Porcine pancreatic elastase, *tert*-butoxycarbonyl-L-alanine *p*-nitrophenyl ester, and butylated hydroxytoluene were purchased from Sigma Chemical Co. Sodium hypochlorite was from Aldrich Chemical Co. and H_2O_2 (30%) from J. T. Baker Chemical Co. All chemicals were of analytical grade.

Methods

Oxidative Inactivation of α_1-Proteinase Inhibitor. Both HOCl and H_2O_2 were prepared from stock solutions twice daily into containers which had been rinsed with the same solution. With such precautions to ensure that the oxidants did not degrade in the time span of collecting data, the experiments were reproducible.

The inactivation of α_1-PI by HOCl was performed by mixing inhibitor (2.86 μM) with 1–1200 μM HOCl, both in 0.2 M sodium phosphate buffer, pH 6.2 (final volume 0.5 mL), at room temperature for 2.5 min, with aliquots removed at 30-s intervals to tubes containing porcine pancreatic elastase. After a 5-min incubation, residual α_1-PI activity was determined as the retention of elastase inhibitory activity (Matheson et al., 1979). The HOCl concentration was determined by the method of Ushijima & Nakano (1980). In some experiments, 8, 16, or 32 μM butylated hydroxytoluene (BHT) was included in the incubation mixture. BHT was dissolved in Me_2SO and buffer then added so that the final Me_2SO concentration was at 10%. For inactivation of α_1-PI by H_2O_2, the inhibitor was treated as described above except that 1 mM H_2O_2 replaced HOCl. The inactivation of α_1-PI by the MPO system was performed as described previously (Matheson et al., 1979).

Oxidative Inactivation of Myeloperoxidase. For the inactivation of MPO by HOCl, enzyme (46.8 nM) was incubated in 0.5 mL for 10 min with 20–3000 μM HOCl and 0.2 M sodium phosphate buffer, pH 6.2, at room temperature. Aliquots were removed at 2-min intervals, and each was assayed immediately by the guaiacol assay (Matheson et al., 1981a). In some experiments, 32 μM BHT was included in the incubation mixtures. To test for inactivation of MPO by H_2O_2 alone, enzyme was incubated as described above except that 340 μM H_2O_2 replaced HOCl in the reaction mixtures both in the presence and in the absence of 300 μM BHT. In order to follow the autoinactivation of MPO in the presence of H_2O_2 and Cl^-, enzyme was incubated as described above except that 340 μM H_2O_2 and 0.16 M NaCl were included in the reaction mixtures. In some experiments, 32 μM BHT was also included.

Amino Acid Analysis of Oxidized α_1-Proteinase Inhibitor and Oxidized Myeloperoxidase. α_1-PI (6.29 nmol) was incubated in 2.2 mL with 1–1220 μM HOCl in 0.2 M sodium phosphate buffer, pH 6.2, for 1 h or with 6.5–66.5 nM MPO, 0.16 M NaCl, and 340 μM H_2O_2 in buffer for 1–4 h. MPO (1.19 nmol) was incubated in 34 mL of buffer with 20–3000 μM HOCl, 340 μM H_2O_2, or 340 μM H_2O_2 and 0.16 M NaCl for 1 h. After incubation, the proteins were dialyzed overnight against distilled water with two changes and hydrolyzed in vacuo in constant boiling HCl for 24 h at 105 °C. Amino acid analysis was carried out with a Beckman Model 119CL amino acid analyzer. Methionine sulfoxide content was determined after cyanogen bromide cleavage of the oxidized or control protein followed by acid hydrolysis in the presence of dithiothreitol (Schechter et al., 1975). Free -SH groups were determined by reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and comparison was made between oxidized protein and controls. Tryptophan was determined by fluorometry and comparison to a control (Brand & Witholt, 1967).

Absorption Spectrum Measurements of Myeloperoxidase. MPO was incubated for 1 h with either 600 μM HOCl, 340 μM H_2O_2, or 340 μM H_2O_2 and 0.16 M NaCl, as described above. The spectra were then collected on a Cary Model 14 spectrophotometer and compared to that given by a control sample.

Results

Effect of Oxidants on the Activity of α_1-Proteinase Inhibitor. H_2O_2, alone, was found to have no effect on the activity of α_1-PI. However, with the complete MPO system (H_2O_2, enzyme, and Cl^-) inactivation was found to readily occur (Figure 1), as has been previously shown (Matheson et al., 1981b). BHT, a radical scavenger, partially prevented the inactivation with increasing concentrations of BHT resulting in increasing protection for α_1-PI. This would indicate that BHT and α_1-PI were competing for MPO or its reaction product.

HOCl also inactivated α_1-PI even at concentrations in the micromolar range. BHT totally prevented the inactivation of α_1-PI by HOCl, thus indicating that a free radical reaction was involved in this case. There was no competition between BHT and α_1-PI; however, the reaction mechanism may not be the same as that with the MPO system, or alternatively, the presence of a large protein, MPO, itself may influence the reaction.

Effect of Oxidants on the Amino Acid Residues in α_1-Proteinase Inhibitor. Since there is a methionine residue in the active site of α_1-PI, the oxidation of that single residue would be sufficient for inactivation. However, more than one methionine residue is oxidized depending on the oxidant used, the concentration of the oxidant, and the time of incubation. H_2O_2 caused no inactivation, and no amino acids were oxidized. HOCl caused inactivation even at very low concentrations. In fact, at the lowest concentration used (1 μM), three methionine residues of the eight present were oxidized

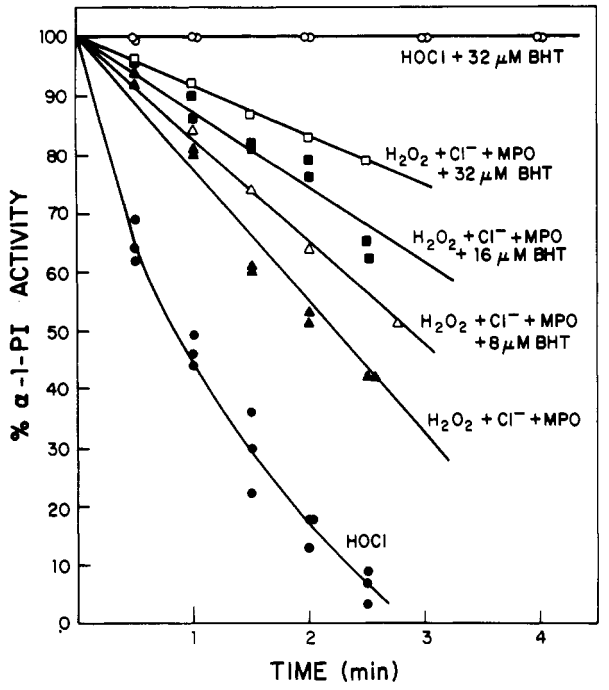

FIGURE 1: Time course for the inactivation of α_1-PI by HOCl or H_2O_2, Cl^-, and MPO and the effect of BHT. α_1--PI (2.86 μM) was incubated with 17 μM HOCl or with 340 μM H_2O_2, 0.16 M NaCl, and 5.42 nM MPO in 0.2 M sodium phosphate buffer, pH 6.2, in the presence and absence of 8, 16, or 32 μM BHT. Assays are as described under Methods. (●) HOCl; (○) HOCl + 32 μM BHT; (▲) H_2O_2 + Cl^- + MPO; (△) H_2O_2 + Cl^- + MPO + 8 μM BHT; (■) H_2O_2 + Cl^- + MPO + 16 μM BHT; (□) H_2O_2 + Cl^- + MPO + 32 μM BHT.

Table I: Amino Acids Oxidized in α_1-Proteinase Inhibitor by Various Oxidants[a]

oxidant	time of incubation (h)	no. of residues oxidized: methionine	no. of residues oxidized: tyrosine[b]
HOCl (μM)			
1	1	2.6 ± 0.3	0.3
7	1	4.9 ± 0.5	0.2
27	1	4.6 ± 0.4	1.0
67	1	4.8 ± 0.6	0.9
1220	1	5.9 ± 0.6	0.8
MPO (nM)[c]			
6.5	1	2.4 ± 0.2	0.2
	2	3.3 ± 0.3	0.1
	4	3.8 ± 0.3	0.2
13.0	1	2.9 ± 0.1	0.1
	2	3.8 ± 0.4	0.1
	4	5.1 ± 0.2	0.3
32.5	1	4.2 ± 0.5	0.1
	2	4.1 ± 0.2	0.2
	4	5.6 ± 0.4	0.1
66.5	1	4.1 ± 0.3	0.1
	2	4.7 ± 0.0	0.0
	4	6.1 ± 0.1	0.2

[a] Final concentration of α_1-PI = 2.9 μM. [b] Obtained by difference between control and oxidized samples. [c] Final MPO concentration.

(Table I). At higher concentrations (7–1220 μM), five to six methionines were oxidized, and above 27 μM, also one tyrosine, but no other amino acid residues, was affected.

With an incubation time of 1 h to ensure total inactivation of α_1-PI, the MPO system caused the oxidation of two methionine residues (Table I), in agreement with the original results (Matheson et al., 1979). However, if the concentration of MPO was increased 2-, 5-, or 10-fold and the time of incubation was also increased, up to six methionine residues could be oxidized. Even at the highest concentration of MPO and the longest time of incubation, however, no tyrosine residues were affected. Thus, the MPO system appears to be capable of oxidizing only methionine residues in α_1-PI.

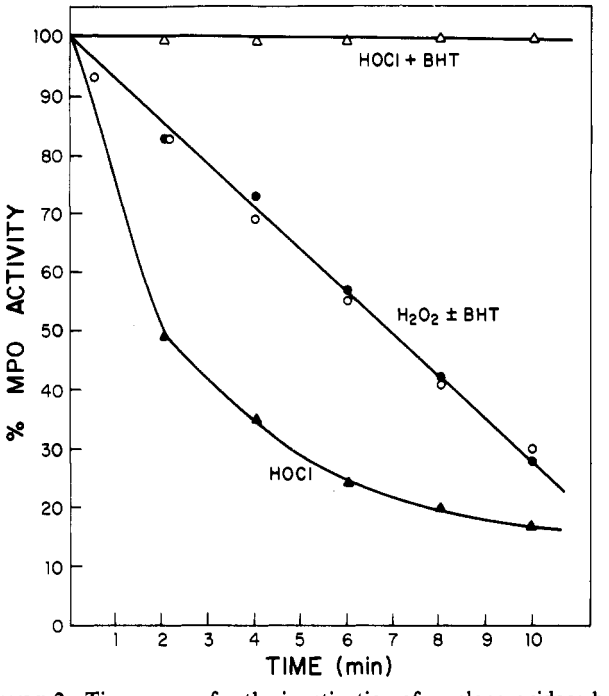

FIGURE 2: Time course for the inactivation of myeloperoxidase by HOCl or H_2O_2 and the effect of BHT. MPO (46.8 nM) was incubated with 20 μM HOCl or 340 μM H_2O_2 in 0.2 M sodium phosphate buffer, pH 6.2, in the presence and absence of 32 or 300 μM BHT and assayed as described under Methods. (●) H_2O_2; (○) H_2O_2 + 300 μM BHT; (▲) HOCl; (△) HOCl + 32 μM BHT.

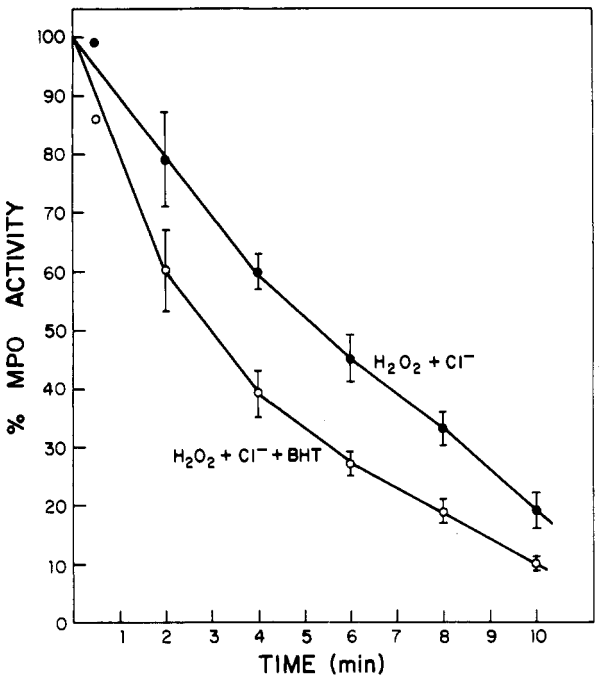

FIGURE 3: Time course for the inactivation of myeloperoxidase by H_2O_2 and Cl^- and the effect of BHT. MPO (46.8 nM) was incubated with 340 μM H_2O_2 and 0.16 M NaCl in 0.2 M sodium phosphate buffer in the presence and absence of 32 μM BHT. (●) H_2O_2 + Cl^-; (○) H_2O_2 + Cl^- + BHT.

Effect of Oxidants on the Activity of Myeloperoxidase. HOCl was also found to inactivate MPO (Figure 2), and as in the case of α_1-PI, this inactivation was completely prevented by the presence of BHT. However, unlike α_1-PI, MPO was inactivated by H_2O_2, and the presence of BHT had no effect (Figure 2), indicating no involvement of free radicals. In a previous report (Matheson et al., 1981a), MPO was shown

Table II: Amino Acids Oxidized in Myeloperoxidase by Various Oxidants[a]

oxidant	no. of residues oxidized: methionine	tyrosine[b]	(%)
HOCl (μM)[c]			
20	6.3 ± 0.5	0.0	91
50	7.3 ± 0.1	0.0	91
200	8.4 ± 0.3	0.2	68
400	7.9 ± 0.3	0.2	71
670	7.9 ± 0.2	0.8	0
1000	7.6 ± 0.3	0.7	0
3000	8.3 ± 0.2	1.0	0
H_2O_2[c] (750 μM)	5.7 ± 0.4	0.2	0
H_2O_2 (750 μM) + Cl^- (160 mM)[c]	6.3 ± 0.3	1.8	0

[a] Final MPO concentration = 35 nM. [b] Obtained by difference between control and oxidized samples. [c] Time of incubation was 1 h.

to inactivate itself in the presence of H_2O_2 and Cl^- and in the absence of an oxidizable substrate. That result is corroborated here (Figure 3) with a slightly faster rate of inactivation being found than with H_2O_2 alone. The effect of BHT, however, was unusual. Rather than preventing inactivation or having no effect, BHT enhanced the inactivation of MPO in the presence of H_2O_2 and Cl^-. Thus, various biological oxidants are capable of inactivating α_1-PI and MPO, but the mechanisms appear to be different as indicated by the results with BHT.

Effect of Oxidants on the Amino Acid Residues in Myeloperoxidase. When MPO was inactivated by HOCl, 6 of the 12 methionine residues present were oxidized, even at the lowest concentration used (20 μM), and with increasing concentrations (200–3000 μM), up to 8 methionines were converted (Table II). At 670 μM HOCl and higher, one tyrosine was oxidized as well, but no other amino acid residues were. The oxidation of the single tyrosine residue can be seen to approximately correlate with the loss of enzyme activity.

Unlike α_1-PI, MPO could be inactivated by H_2O_2 alone. Amino acid analyses showed that six methionine residues but no tyrosine residues were oxidized. The addition of Cl^- to the reaction mixture resulted in the oxidation of six methionine and two tyrosine residues and also total loss of activity. When this experiment had been performed previously (Matheson et al., 1981a), the concentration of MPO was extremely high because incubations were performed in small volumes but with large amounts of protein, which were needed for the amino acid analyses. Therefore, the reaction proceeded extremely rapidly and extensively. However, in the present experiments, the MPO concentration during the reaction was at assay concentration but in a large enough volume to contain sufficient protein for analysis. Thus, apparently only the most easily oxidized and accessible amino acids are altered under the dilute conditions. Cysteine residues are presumed not to be important since we could find no loss of enzyme activity during the titration with DTNB of the native protein.

Effect of Oxidation on the Absorption Spectra of Myeloperoxidase. The comparative spectra of native MPO and those samples treated with the various oxidants are shown in Figure 4. Untreated MPO has a peak at 426 nm and a smaller peak at 570 nm. When MPO was incubated with 340 μM H_2O_2 and 0.16M Cl^-, or 600 μM HOCl, each for 1 h, both peaks disappeared permanently, indicating that the heme groups had been oxidized and destroyed. At 300 μM HOCl, the peak at 426 nm became smaller but did not disappear (not shown). In the presence of H_2O_2 alone, however, a new peak appeared at 630 nm which eventually disappeared as the peak at 570 nm reappeared (not shown), indicating that activity may appear to be lost because MPO is maintained in a reduced state as long as H_2O_2 is present.

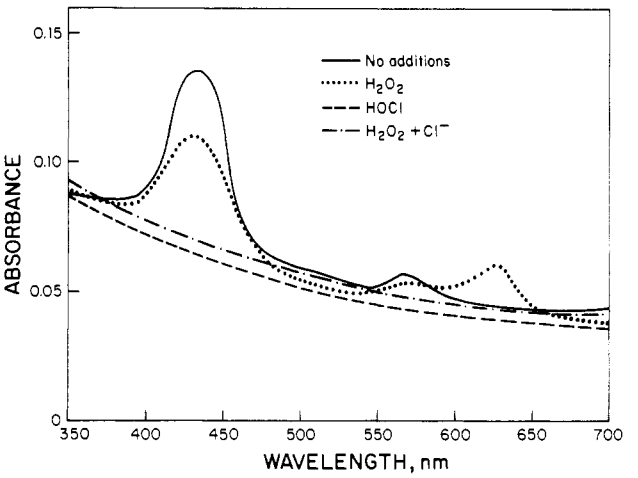

FIGURE 4: Absorption spectra of myeloperoxidase after treatment with HOCl, H_2O_2, or H_2O_2 and Cl^-. MPO (0.25 μM) was incubated with 600 μM HOCl, 340 μM H_2O_2, or 340 μM H_2O_2 and 0.16 M NaCl for 1 h before spectra were collected. (—) No additions; (---) HOCl; (-·-) H_2O_2 + Cl^-; (···) H_2O_2.

Discussion

Several years ago Agner (1972) proposed that the MPO system operates by generating HOCl which then becomes the actual oxidant. He showed that identical products were formed when either the MPO system or authentic HOCl reacted with sulfanilamide. Later, Harrison & Schultz (1976) showed that the MPO system could produce a diffusible oxidant, apparently chlorine, which was detected by using *o*-tolidine, a reagent for free chlorine in water. This substance could also chlorinate monochloromedone to dichloromedone. Other evidence has been that both the MPO system and HOCl convert amino acids to chloramines (Zgliczynski et al., 1971; Stelmaszynska & Zgliczynski, 1974). However, the present experiments suggest that either HOCl is not the functional oxidant produced by the MPO system or that other interactions modify the effect of the HOCl produced. The discrepancies between the MPO system and authentic HOCl are twofold in that (a) α_1-PI is not fully protected by BHT against the MPO system product but is fully protected from HOCl, and (b) HOCl can oxidize (or chlorinate) a tyrosine residue in α_1-PI while the MPO system cannot, even with a large enough amount of enzyme and sufficient time of incubation to cause oxidation of six methionine residues. A possible explanation for the latter discrepancy is that MPO and α_1-PI are both large proteins and in such close proximity (perhaps even momentarily bound) that an otherwise accessible tyrosine residue in α_1-PI cannot be reached by the oxidant produced by MPO. Another related possibility is that the HOCl or its radicals produced by the MPO system are, unlike authentic HOCl, not freely diffusible. Youngman et al. (Youngman & Elstner, 1981; Youngman, 1984) proposed that reactions can occur with homolytic cleavage of H_2O_2 and formation of a reactive species which they called "crypto-HO·". The existence of this species could not be proven directly, but the concept could explain how HO· could exist with its reactions restricted to its site of generation rather than being freely diffusible. They proposed that crypto-HO· is surrounded by a "cage" of solvent or other molecules or that it sits in an enzyme crevice. Therefore, potential substrate molecules are restricted in their access to the radical, yielding some degree of specificity. Thus, the tyrosine residue in α_1-PI which is modified by free HOCl may

not be accessible to crypto-HO· and therefore is not modified by the MPO system.

This hypothesis also states that, at low concentrations, scavengers apparently only react with the more free HO·, which escapes and which is an equilibrium with crypto-HO·. Thus, the effect should be an enhancement of the reaction of crypto-HO· since the scavenger is protecting the enzyme from gradual inactivation by the free HO· released while crypto-HO· reacts with the substrate. When the scavenger concentration is high, then both released HO· and crypto-HO· are inhibited. While there was no enhancement of the inactivation of α_1-PI, there was less than complete protection by BHT in comparison to the experimental results obtained when free authentic HOCl was the oxidant.

Previous investigations (Matheson et al., 1979) carried out with purified native α_1-PI and MPO indicated that only two methionines were oxidized in this inhibitor. However, when α_1-PI was purified from the synovial fluid of arthritic patients, four methionines were found to be oxidized, a puzzling inconsistency (Wong & Travis, 1980). The present experiments show that six methionines in α_1-PI are capable of being oxidized by the MPO system. Thus, it would appear that four are the number which are modified under in vivo conditions.

When MPO reacts with either HOCl or H_2O_2 and Cl^-, there is little difference in the amino acids oxidized by either oxidant. However, again there is a discrepancy between the two in their interaction with BHT in that the inactivation of MPO with H_2O_2 and Cl^- is enhanced by BHT, while with authentic HOCl the inactivation of MPO is eliminated by BHT. This also could be explained by the production of crypto-HO·. In the case of autoxidation of MPO, there is indeed enhancement of the inactivation of the enzyme in the presence of low concentrations of BHT. Apparently, BHT is protecting MPO from gradual inactivation by the small amount of free HO· released, but MPO is also the substrate which may react with crypto-HO·. Enhancement of a reaction by BHT is very unusual, but the hypothesis presented above serves as a possible explanation for its occurrence here.

The experiments with authentic HOCl and BHT are also interesting in that they indicate that, under the conditions used, HOCl splits into free radicals, a reaction of HOCl not previously documented. The radicals could be either H· and ·OCl or HO· and Cl·. The oxidation of three methionine residues in 4.3 nmol of α_1-PI by 1.0 nmol of HOCl also indicates that the reaction must have proceeded, in fact, by a propagated free radical chain reaction in which a larger number of radicals were generated than were in the original solution.

The heme moiety of MPO or any other peroxidase appears to be an integral part of the reaction mechanism. When MPO is inactivated with H_2O_2 and Cl^- or with HOCl, the heme moiety is destroyed, as shown by the permanent disappearance of the peak at 426 nm, thereby explaining the loss of activity. However, the total loss of activity and disappearance of the 426 nm peak did not occur until higher concentrations (600 μM) of HOCl were used. The total loss of activity of MPO was also correlated with the loss of one tyrosine residue by higher concentrations of HOCl. Thus, a single tyrosine residue may be important to the susceptibility of the heme group to oxidation by free HOCl by protecting it until the tyrosine itself is oxidized.

Registry No. α_1-PI, 9041-92-3; MPO, 9003-99-0; BHT, 128-37-0; HOCl, 7790-92-3; H_2O_2, 7722-84-1; Cl^-, 16887-00-6; tyrosine, 60-18-4; methionine, 63-68-3.

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