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# The Mechanism of Peroxidase-Mediated Cytotoxicity. II. Role of the Heme Moiety[1] (42629)

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*Abstract.* Various peroxidases in the presence of hydrogen peroxide and a halide ion have been shown to exert a cytolytic activity against erythrocytes and other cells. However, few studies have been done to elucidate the active site on the enzymes that is responsible for the cytotoxic activity. In addressing this question we found that boiling of horseradish peroxidase only partially abolishes its cytotoxic activity, suggesting that an intact tertiary structure of the protein may not be essential for the cytotoxic activity. This conclusion was confirmed by demonstrating that microperoxidase, hemin, and hematoheme also exert cytotoxic activity in the presence of hydrogen peroxide and iodide, the kinetics of which were identical to those obtained with the peroxidases. Fluoride, bromide, and thiocyanate could not replace iodide in any of these systems. These results indicate that the active site for the cytotoxic activity of the peroxidases is located within the heme moiety, whereas the protein portions of the enzymes affect the cytotoxic activity of the enzymes only in an indirect manner. We also tested a variety of compounds for their ability to inhibit the cytolytic reaction toward erythrocytes. We found that compounds such as thiourea, thionicotinamide, and uric acid are much more potent inhibitors of the cytolytic reaction than tyrosine and histidine. These observations support the concept that oxidative reactions rather than halogenation reactions are the primary cause of the peroxidase-mediated lysis of erythrocytes. 

The mechanism by which the peroxidase-$H_2O_2$-halide system exerts its cytotoxic activity against various cell types is still not well characterized. In fact, only a few studies have been done to establish whether the cytotoxic activity promoted by myeloperoxidase, lactoperoxidase, horseradish peroxidase, and other peroxidases proceeds by the same mechanism, i.e., generate the same toxic product(s) (1).

Moreover, considerable uncertainty still exists as to the nature of the damage done to a target cell that results in its demise. Amino acid oxidation, lipid peroxidation, and halogenation of membrane proteins have at one time or another been suggested as the major cause of cell death (2–7), but convincing evidence for each of these mechanisms is lacking. This problem is complicated by the fact that peroxidases are able to catalyze a variety of different reactions, including dehydrogenations, oxidations, peroxidations, and halogenations. It is therefore not impossible that more than one reaction takes place at the cell membrane and that the major type of damage done to a target cell may, at least in part, depend on the type of target cell used.

Considering the complexity of the cytotoxic reaction one could conclude that the peroxidase-$H_2O_2$-halide system acting on living target cells is too complicated of a system to be used for the purposes of unravelling the chemical events that take place. However, when in order to simplify the system the target cells are replaced by simple target molecules, a serious question arises as to whether the system under study is an adequate model of that using whole cells.

In a previous communication (1) we described the kinetics of the lactoperoxidase and horseradish peroxidase-catalyzed cytolysis of erythrocytes in the presence of hydrogen peroxide and iodide. In this communication we intend to demonstrate that the active center of the peroxidases with respect to their cytotoxic activity appears to consist solely of

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the heme portion of the enzymes; none of the amino acid residues, except the heme-bound histidine, appears to be essential for the cytotoxic activity.

**Materials and Methods.** Horseradish peroxidase (type VI), lactoperoxidase, microperoxidase (MP-8), hemin, hematoporphyrin, 3-aminotyrosine, and mimosine were all obtained from Sigma Chemical Co. (St. Louis, MO). Hydrogen peroxide (30%) was obtained from Fischer Scientific (Pittsburgh, PA).

Cytolytic assays were performed as previously described (8). Erythrocyte suspensions were prepared daily in phosphate-buffered saline (9.5 m*M* phosphate, 140 m*M* NaCl, pH 7.2) from freshly drawn rabbit blood.

$Fe^{3+}$-hematoheme was prepared from hematoporphyrin with the method described by Inubushi and Yonetani (9). The obtained product was identified as hematoheme by its characteristic absorption spectrum (10) and was judged to be free of hematoporphyrin by two criteria, the lack of any hematoporphyrin fluorescence and the lack of any cytolytic activity following illumination (11).

Photooxidation of histidine residues was carried out with a 10 m*M* solution of microperoxidase in phosphate-buffered saline, containing 0.01% methylene blue (12). Solutions were illuminated for up to 30 min in a lightbox (Model 300 LS, Bio-Rad Laboratories).

Carbethoxylation of histidine residues was performed as described by Miles (13). To a solution of 10 m*M* microperoxidase in 0.1 *M* phosphate buffer, pH 6.5, was added diethylpyrocarbonate to a final concentration of 25 m*M*. The reaction was allowed to proceed in an ice bath. The reaction was monitored by removing aliquots at various time intervals and, after appropriate dilution, following the increase in absorbance at 240 nm until no further change occurred (approximately 60 min). The modified peroxidase was then separated from the excess reagent by chromatography over a Sephadex G-10 column.

**Results.** *Cytolytic activity of denatured peroxidases.* Table I shows the cytolytic activity of horseradish peroxidase and lactoperoxidase toward erythrocytes in the presence of hydrogen peroxide and iodide. No lytic activity is present when the peroxidase is omitted from the reaction mixture or in the absence of hydrogen peroxide or iodide. The reactions were strongly inhibited by the peroxidase inhibitors 3-aminotyrosine and mimosine. Most of the cytolytic activity of lactoperoxidase was lost when incubated at 100°C for 10 min prior to use. However, a small amount of cytolytic activity remained which could not be destroyed by a longer incubation at 100°C. Horseradish peroxidase, on the other hand, retained almost half of its cytolytic activity, even after boiling the solution for 30 min.

TABLE I. COMPARATIVE RATES OF ERYTHROCYTE CYTOLYSIS BY THE PEROXIDASE–HYDROGEN PEROXIDE–IODIDE SYSTEMS

| Reagents | Rate of erythrocyte lysis[a] (cells/min/$\mu M$) |
|---|---|
| Lactoperoxidase–$H_2O_2$–$I^-$ | $2.9 \times 10^7$ |
| $H_2O_2$ omitted | 0 |
| $I^-$ omitted | 0 |
| Peroxidase omitted | 0 |
| Peroxidase boiled (10 min) | $1 \times 10^4$ |
| 3-Aminotyrosine (10 $\mu M$) added | $6.4 \times 10^6$ |
| Mimosine (10 $\mu M$) added | $6.2 \times 10^6$ |
| Horseradish peroxidase–$H_2O_2$–$I^-$ | $1.4 \times 10^5$ |
| $H_2O_2$ omitted | 0 |
| $I^-$ omitted | 0 |
| Peroxidase omitted | 0 |
| Peroxidase boiled (10 min) | $6.4 \times 10^4$ |
| (30 min) | $5.9 \times 10^4$ |
| 3-Aminotyrosine (10 $\mu M$) added | $2.2 \times 10^4$ |
| Mimosine (10 $\mu M$) added | $1.9 \times 10^4$ |
| Microperoxidase–$H_2O_2$–$I^-$ | $1.4 \times 10^3$ |
| $H_2O_2$ omitted | 0 |
| $I^-$ omitted | 0 |
| Peroxidase boiled (30 min) | $1.2 \times 10^3$ |
| 3-Aminotyrosine (10 $\mu M$) | $1.2 \times 10^2$ |
| Mimosine (10 $\mu M$) added | $1.0 \times 10^2$ |

[a] Rates were calculated from the linear portion of the spectrophotometer recordings and are the average of at least three independent assays.

These results indicate that an intact protein structure may not be an absolute requirement for the expression of the cytolytic activity.

*Cytolytic activity of microperoxidase.* Microperoxidase is a compound obtained from cytochrome *C* after extensive tryptic digestion. It consists of the heme moiety with

amino acids 14 through 21 still covalently linked to the porphyrin ring. Included in these amino acids is the histidine that is coordinated to the heme iron (14, 15). Microperoxidase thus resembles the heme portion of horseradish peroxidase and lactoperoxidase, but lacks the bulk of the protein. Its structure is illustrated in Scheme I. The compound catalyzes the oxidation of suitable substrates in the presence of $H_2O_2$ and is therefore appropriately called a peroxidase.

In attempting to further define the active site generating the cytotoxic activity we tested microperoxidase for its ability to lyse erythrocytes. We found that microperoxidase promotes erythrocyte lysis under conditions that strongly resemble those observed with horseradish peroxidase and lactoperoxidase. The lysis profile obtained, and shown in Fig. 1, strongly resembles the profiles observed with lactoperoxidase, horseradish peroxidase, and myeloperoxidase under identical conditions (1, 8). The reaction was found to be dependent on the presence of $H_2O_2$ as well as iodide (Table I). Maximal activity was observed at concentrations of about 25 $\mu M$ KI and 40 $\mu M$ $H_2O_2$. Higher concentrations of $H_2O_2$ inhibited the rate of erythrocyte lysis. These conditions are identical to those found with horseradish peroxidase and lactoperoxidase (1).

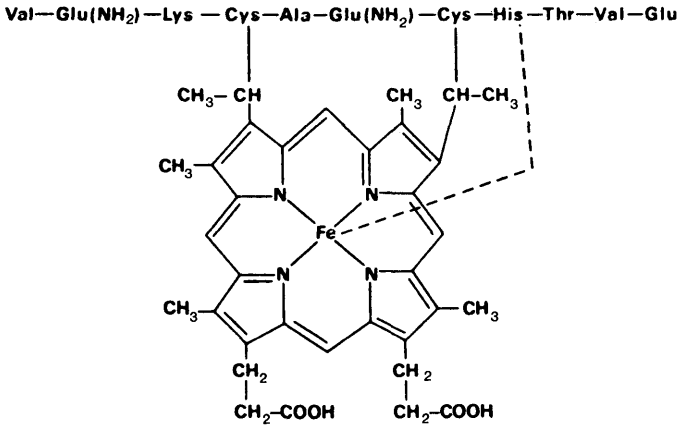
The specific activity of microperoxidase under these conditions is $2.0 \times 10^4$ cells lysed/min/$\mu$mole. This value compares to $1.7 \times 10^8$ and $5.0 \times 10^6$ cells lysed/min/$\mu$mole, which are the values determined for lactoperoxidase and horseradish peroxidase, respectively, under the same conditions. Fluoride, bromide, or thiocyanate at concentrations up to 250 $\mu M$ could not substitute for iodide in the reaction mixture. Furthermore, the cytolytic activity of microperoxidase was strongly inhibited by 3-aminotyrosine and mimosine, whereas it was not affected by boiling (Table I).

*Role of the iron-bound histidine moiety.* We subsequently addressed the question whether the histidine moiety that occupies the fifth ligand of the iron atom is essential for the expression of the cytolytic activity.

In a first approach we attempted to eliminate the histidine residue by the traditional method of photooxidation. Using amino acid analysis we found, however, that this residue is resistant to photooxidation and cannot be destroyed by this method, even with prolonged exposure to light.

Alternatively we altered the histidine moiety of microperoxidase by substituting the hydrogen atom at N-3 for a diethylcarbonyl group, as described under Materials and Methods. When we tested this compound for its cytolytic activity toward erythrocytes we found that its activity was about 210% of that of unaltered microperoxidase.

*Cytolytic activity of iron porphyrins.* To ascertain whether the presence of the liganded histidine residue (and/or any other amino acid residues) is absolutely essential for the expression of cytotoxicity we tested




SCHEME I

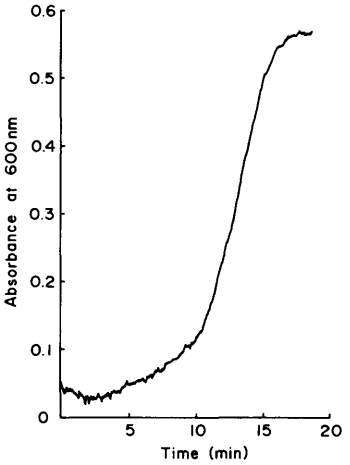



FIG. 1. Profile of erythrocyte lysis by microperoxidase. Conditions: 10 $\mu M$ microperoxidase, 40 $\mu M$ $H_2O_2$, 25 $\mu M$ KI, and $2 \times 10^6$ rabbit erythrocytes were present in 1 ml 9.5 m$M$ phosphate buffer, pH 7.2, containing 140 m$M$ NaCl.

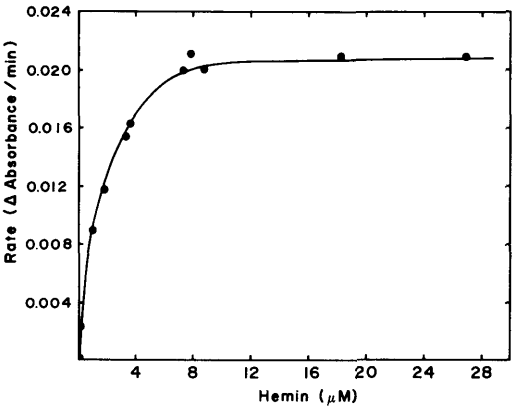



FIG. 2. Concentration dependence of the cytolytic activity of hemin. Conditions as in Fig. 1, except that various concentrations of hemin were used instead of microperoxidase.

whether hemin could replace microperoxidase in the cytotoxic assay. As shown in Table II, hemin at 1 $\mu M$ concentrations and in the presence of 40 $\mu M$ $H_2O_2$ and 25 $\mu M$ $I^-$ exerts cytolytic activity toward erythrocytes at a rate comparable to that of 13.6 $\mu M$ microperoxidase. The kinetics of the hemin-mediated cytolytic activity were again found to strongly resemble those of lactoperoxidase and horseradish peroxidase (1), including the fact that the compound shows saturation kinetics, as shown in Fig. 2.

Similar results were obtained with $Fe^{3+}$-hematoheme. Its specific cytolytic activity, however, was somewhat higher than that of hemin. Hematoporphyrin at concentrations as high as 1 m$M$ was found to be completely devoid of any cytolytic activity under our assay conditions, provided that care was taken to avoid photoactivation of the hematoporphyrin (11).

TABLE II. COMPARATIVE RATES OF ERYTHROCYTE LYSIS BY HEMIN AND HEMATOHEME IN THE PRESENCE OF $H_2O_2$ AND IODIDE

| Reagents | Rate of erythrocyte lysis (cells/min/$\mu M$) |
|---|---|
| Hemin–$H_2O_2$–$I^-$ | $1.9 \times 10^4$ |
| $H_2O_2$ omitted | 0 |
| $I^-$ omitted | $2.7 \times 10^3$ |
| Hematoheme–$H_2O_2$–$I^-$ | $1 \times 10^4$ |
| $H_2O_2$ omitted | 0 |
| $I^-$ omitted | $3 \times 10^3$ |

*Inhibition of the cytolytic activity.* To further compare the mechanisms by which the various peroxidases exert their cytolytic activity we tested the effects of several singlet oxygen quenchers, hydroxyl radical scavengers, and other compounds on the activities of the enzymes. The obtained results are tabulated in Table III. Most of the compounds tested were found to be potent inhibitors of the cytolytic reaction, yielding more than 50% inhibition at micromolar levels. Pyridine was without effect at concentrations up to 1% and, except for those listed, none of the natural amino acids affected the reaction at concentrations up to 1 m$M$.

The most potent inhibitor of the cytolytic systems tested was thiourea, which inhibited the lactoperoxidase-catalyzed reaction 50% at 2 $\mu M$, the horseradish peroxidase-catalyzed reaction 50% at 0.5 $\mu M$, and the microperoxidase-catalyzed reaction at 0.2 $\mu M$. This is of special interest since urea at 1 m$M$ did not inhibit any of the cytolytic reactions. Similarly, thionicotinamide showed inhibitory properties comparable to those of thiourea, whereas nicotinamide at 1 m$M$ was without effect.

**Discussion.** In previous communications (1, 8) we reported that lactoperoxidase,

TABLE III. INHIBITION OF THE CYTOLYTIC ACTIVITY OF HORSERADISH PEROXIDASE, LACTOPEROXIDASE, AND MICROPEROXIDASE BY VARIOUS AGENTS

| Inhibitor | Concentration ($\mu M$) | % Inhibition[a] | | |
|---|---|---|---|---|
| | | Lactoperoxidase | Horseradish peroxidase | Microperoxidase |
| Histidine | 100 | 51 | 80 | 100 |
| | 10 | 15 | 40 | 50 |
| Tyrosine | 10 | 45 | 96 | 100 |
| | 1 | N.S. | 60 | 74 |
| Tryptophan | 20 | 74 | 100 | — |
| | 10 | 40 | 84 | 100 |
| Cysteine | 20 | 49 | 100 | 100 |
| | 10 | 30 | 85 | 90 |
| Uric acid | 12 | 87 | — | — |
| | 1 | 15 | 40 | 64 |
| Pyridine (1%, v/v) | — | 0 | 0 | 0 |
| Thiourea | 2 | 49 | — | 100 |
| | 0.5 | 20 | 50 | 79 |
| Urea | 1000 | 0 | 0 | 0 |
| Thionicotinamide | 2 | 53 | — | 100 |
| | 0.5 | 19 | 47 | 73 |
| Nicotinamide | 1000 | 0 | 0 | 0 |

[a] Values are averages of at least three independent assays.

horseradish peroxidase, and myeloperoxidase exhibit similar reaction kinetics in their lytic activity toward erythrocytes. From these data we concluded that the mechanisms of these enzymes may be identical, or at least closely related, with respect to their cytolytic activity toward erythrocytes.

Part of the information needed in order to establish the chemical mechanism of an enzymatic reaction is knowledge of the various residues in the enzyme molecule that comprise the active site. Although a large number of studies have been devoted to establishing the active site of various peroxidases, these were aimed at the traditional peroxidative activity, usually the oxidation of an organic substrate. Since the cytotoxic activity of the peroxidases, unlike their peroxidative activity, is totally dependent on the presence of a halide ion (which may bind to some residue on the enzyme), there was a priori no reason to assume that the "cytotoxic" active site would be identical to the "peroxidative" active site. Our aim for the present study was therefore to establish the composition of the active site of the peroxidases as it relates to their cytotoxic activity.

Our results revealed that the active center of the lytic activity is exclusively located in the heme portion of the peroxidases. This is proven by the fact that hemin and hematoheme in the presence of $H_2O_2$ and iodide display a cytolytic activity toward erythrocytes that has the same characteristics as those of the peroxidases. Furthermore, with each of the compounds tested we found that optimal activity is obtained with a peroxide concentration of 40 $\mu M$ and an iodide concentration of 25 $\mu M$. The fact that these values do not change, whether protein residues are present or not, indicates that these substrates do not bind to any of the amino acid residues of the peroxidases and may interact exclusively with the heme moiety. This is not unexpected as far as hydrogen peroxide is concerned, since this is known to interact with the heme iron in all peroxidase reactions. The site of interaction of the iodide ion, however, has thus far not been elucidated, although it has been suggested that this may bind to the heme iron as well (22).

The fact that hemin is able to promote erythrocyte lysis was reported earlier by Chou and Fitch (23). Subsequently, the cytotoxic effect of hemin toward malaria parasites was demonstrated (24). More recently, Aft and Mueller (25, 26) investigated the degradative activity of hemin toward pro-

teins and DNA. In each case hemin displayed its toxic activity without an apparent requirement for $H_2O_2$; in fact, the degradative activity of hemin toward DNA and proteins was shown to be dependent on the presence of oxygen and a reducing agent, 2-mercaptoethanol. Thus, the mechanism by which hemin exerts its toxic activity in the instances cited could be different from the mechanism by which hemin promotes erythrocyte lysis under our conditions, the latter being dependent on the presence of $H_2O_2$ and iodide. In a separate study, the details of which were presented elsewhere, we demonstrated that hemin can indeed exert toxic activity by two separate mechanisms (27).

The increase in cytolytic activity observed with microperoxidase after modification of the histidine residue could indicate that the iron-liganded histidine residue in some manner facilitates the binding of $H_2O_2$ to the heme iron. However, its modification may also affect such parameters as the rate of the reaction or the binding of the compound to the cell membrane.

It is noteworthy that there is a considerable difference among the specific activities of the various catalysts. It is clear from the data presented in Tables I and II that lactoperoxidase is a much better catalyst than horseradish peroxidase and horseradish peroxidase in turn is about 100 times more efficient than microperoxidase. These differences could be due to differences in the rates by which toxic intermediates are generated, but they also could reflect differences in binding affinities to the erythrocyte membrane. Lactoperoxidase is known to have a high affinity for membranes as well as for other hydrophobic surfaces (28). Within this context it is of interest to note that the specific activity of the hemes is considerably higher than that of microperoxidase. This also could reflect differences in binding affinities, since the hemes are considerably more hydrophobic than microperoxidase. Thus, the increase in the specific activity of microperoxidase observed upon modification of the histidine residue may only reflect an increase in the hydrophobicity of the peroxidase. Binding constants for each of the various catalysts will have to be determined before a meaningful comparison of the specific activities can be made.

Tryptophan, tyrosine, and cysteine were found to be about equally potent inhibitors of the cytolytic activity, whereas histidine was considerably weaker. Assuming that these compounds inhibit the cytolytic reaction because they are themselves subject to attack by the toxic intermediate, we conclude that tryptophan, cysteine, and tyrosine are about equally sensitive to the action of the peroxidase systems, whereas histidine is considerably less sensitive and lysine is negligibly affected. These results are inconsistent with the concept that halogenation is a major component of the cytolytic activity of peroxidases, but they are consistent with an oxidative mechanism.

The most potent inhibitors of the cytolytic activity that we have found thus far are thiourea and thionicotinamide. This is of significant interest since their structurally closely related analogs urea and nicotinamide have no effect on the reaction. (A similar difference in activity was found between thiourea and other carboxyl-sulfur-containing compounds and their oxygen-containing analogs in studies on the stimulation of the hexosemonophosphate shunt activity during phagocytosis in human polymorphonuclear leukocytes (29).) Thus, the presence of a carbonyl-sulfur group appears to constitute a structure with potent inhibitory properties, whereas a carbonyl-oxygen is devoid of any inhibitory activity. These results may be related to the fact that the carbonyl-sulfur group can be further oxidized to a sulfoxide and a sulfone, whereas the carbonyl-oxygen group cannot be further oxidized. In addition, the carbonyl-sulfur group seems to be a considerably more potent inhibitor than the sulfhydryl group, since an approximately 10 times higher concentration of cysteine than thiourea is required to obtain a comparable degree of inhibition.

Further information concerning the chemical mechanism of the cytolytic reaction under study may be obtained from an identification of the products formed by the various inhibitors when these compounds are exposed to the peroxidase system under conditions that are identical to those used in our

lytic assay. Such studies are presently in progress.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