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The Mechanism of Peroxidase-Mediated Cytotoxicity. I. Comparison of Horseradish Peroxidase and Lactoperoxidase (42413)

STEVE J. MCFAUL, HUA LIN, AND JOHANNES EVERSE

Department of Biochemistry, Texas Tech University Health Sciences Center, Lubbock, Texas 79430

Abstract. The kinetics of the cytolytic activity expressed by lactoperoxidase and horseradish peroxidase toward erythrocytes in the presence of H_2O_2 and iodide have been investigated at physiological pH. The action of both enzymes was found to be very similar with respect to their kinetic mechanisms. Both enzymes showed saturation kinetics at higher enzyme concentrations under conditions where substrate concentrations were not limiting. Optimal concentrations of H_2O_2 and iodide were found to be 40 and 25 μM, respectively, for both enzymes. Higher concentrations of H_2O_2 inhibited the cytolytic activity. The pH dependence of the cytolytic reaction is also very similar for both enzymes, showing maximal activity at about pH 6.3. Moreover, the cytolytic activities of both enzymes were inhibited by tyrosine, tryptophan, cysteine, and to a lesser extent by histidine. We conclude from these data that the mechanisms of horseradish peroxidase and lactoperoxidase in promoting the lysis of erythrocytes are closely related if not identical.

Various peroxidases, including myeloperoxidase, lactoperoxidase, and horseradish peroxidase, have been shown to exert cytotoxic activities in the presence of hydrogen peroxide and certain halide ions (1–6). For some time it was believed that certain active forms of oxygen, including hydroxyl radicals and singlet oxygen, were formed by the peroxidases and were responsible for the cytotoxic activity (7–9). More recently, however, many investigators believe that the toxic products are hypohalous acids (10–12), although some still consider the involvement of singlet oxygen a distinct possibility (13).

Various reports indicate, however, that considerable structural differences exist among these peroxidases. Whereas lactoperoxidase and horseradish peroxidase contain a single heme moiety per molecule, myeloperoxidase contains 2 heme moieties per molecule which are not equivalent. This is indicated by the observations that the formation of Compound I and Compound III of myeloperoxidase involves changes at one of the heme moieties with the other remaining in the ferric form (14, 15). However, at high H_2O_2 concentrations both heme moieties react with the peroxide (16), indicating that the second heme is not inert and may participate in some of the reactions catalyzed by myeloperoxidase.

Recent studies by Courtin *et al.* (17) indicate that significant differences also exist in the heme moieties of lactoperoxidase and horseradish peroxidase. These authors found that the structure of Compound I of lactoperoxidase is similar to the structure of Compound II of horseradish peroxidase; i.e., the 1-electron deficiency in the porphyrin ring of horseradish peroxidase Compound I is not present in lactoperoxidase Compound I but is presumably present in the protein portion of the enzyme.

These observations indicate that the intermediates formed by these three peroxidases in the presence of H_2O_2 are structurally quite different and it seems therefore reasonable to assume that their reactivity may be equally different. Therefore, although each of these peroxidases exerts cytotoxic activity in the presence of H_2O_2 and a halide, it appears inappropriate to assume without further evidence that in each case this occurs by the same chemical and/or kinetic mechanism.

Based on these considerations we compared the cytotoxic properties of lactoperoxidase and horseradish peroxidase under identical conditions. From the data presented here we conclude that the two enzymes exert their cytotoxic activity by a very similar, if not identical, chemical mechanism.

Materials and Methods. *Materials.* Lactoperoxidase and horseradish peroxidase, type VI, as well as 3-aminotyrosine, mimosine, and cetyltrimethylammonium bromide, were purchased from Sigma Chemical Company

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(St. Louis, Mo.). Amino acids were obtained from General Biochemicals (Chagrin Falls, Ohio). Hydrogen peroxide (30%) was a product of Matheson, Coleman and Bell (Norwood, Ohio). Dilutions of H_2O_2 were made daily and kept at 0°C.

Methods. Cytolytic activities were determined with a Beckman Model 24 spectrophotometer as previously described (18). Briefly, a typical assay mixture contained 1 μg lactoperoxidase (or 100 μg horseradish peroxidase), 40 μ*M* H_2O_2, 25 μ*M* KI, and 2×10^6 erythrocytes in 9.5 m*M* phosphate buffer, pH 7.2, containing 140 m*M* NaCl. Total volume was 2 ml. Reference cuvettes contained all of the above, except the peroxidase. Assays were done by placing the sample cuvette in the reference light beam and the reference cuvette in the sample light beam. All assays were done at 37°C. Erythrocytes were obtained from freshly drawn rabbit blood. The rate of cell lysis was calculated from the linear portion of the spectrophotometer recordings.

Results. *Cytolytic assay.* Erythrocyte lysis, as promoted by horseradish peroxidase in the presence of H_2O_2 and iodide, proceeds via several distinct phases as illustrated in Fig. 1. Following the start of the reaction a certain amount of time passes before lysis of the erythrocytes becomes apparent. Following this lag period the erythrocytes lyse at a virtually constant rate until lysis is complete. No lysis is observed when either hydrogen peroxide or iodide is omitted from the reaction mixture. A similar profile as that shown in Fig. 1 was obtained when the horseradish peroxidase was replaced by lactoperoxidase or myeloperoxidase (18).

No lag periods were observed when erythrocytes were lysed by the addition of cetyltrimethylammonium bromide (CTAB). Under otherwise identical conditions the rate of cell lysis was found to be proportional to the CTAB concentration between 0.2 and 5 μ*M*, but no observable lag time was present. These results suggest that the lytic profiles obtained with the peroxidases could be characteristic for the damaging action of peroxidases.

Cytolytic rate as a function of peroxidase concentration. The dependence of the cytolytic rate on the concentration of lactoperoxidase is shown in Fig. 2a. The rate of erythrocyte lysis increases with increasing lactoperoxidase concentration from 0.02 to 0.10 μg LPO/ml. At higher lactoperoxidase concentrations, however, the rate is no longer dependent on the enzyme concentration and no further increase in rate is observed even at a peroxidase concentration as high as 100 μg/ml. In order to assure that the hydrogen peroxide concentration did not become rate limiting at the higher lactoperoxidase concentrations, the experiment was done at two different H_2O_2 concentrations.

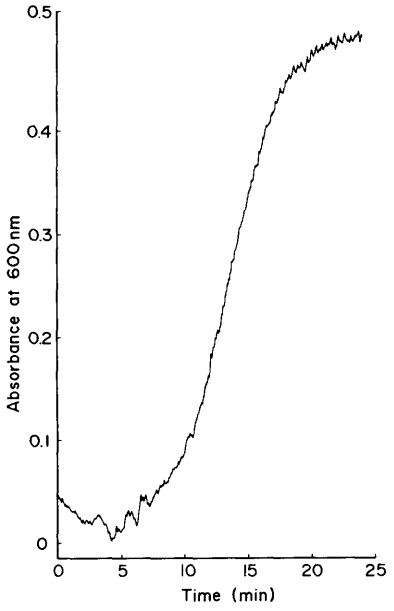

FIG. 1. Profile of horseradish peroxidase-mediated lysis of erythrocytes. Assay cuvettes contained 100 μg horseradish peroxidase, 40 μ*M* H_2O_2, 25 μ*M* KI, 2×10^6 rabbit erythrocytes, 9.5 m*M* phosphate, and 0.14 *M* NaCl. Total volume, 1 ml; pH, 7.2.

Increasing the peroxidase concentration also resulted in a decrease in the initial lag time, approaching a minimum value of about 5 min under our assay conditions (Fig. 2b). The lag time did not change with peroxidase concentrations varying between 1 and 100 μg/ml. These results indicate that the cytolytic activity of lactoperoxidase toward erythrocytes displays saturation kinetics; at higher enzyme concentrations the rate of cytolysis becomes independent of the peroxidase concentration as well as of the substrate concentrations.

Comparable results were obtained when horseradish peroxidase was used instead of

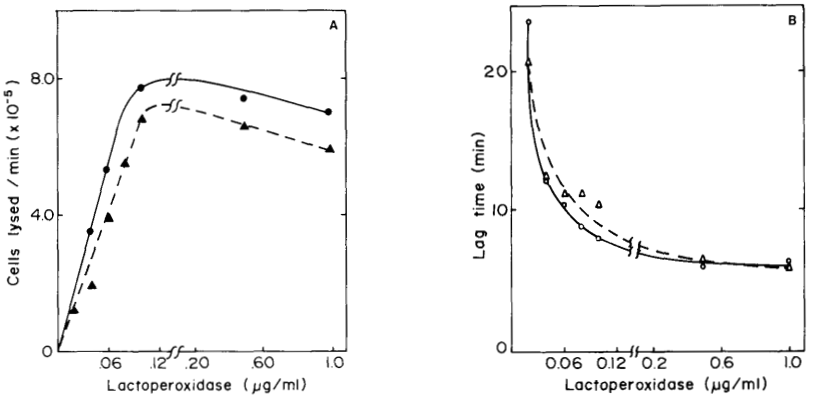

FIG. 2. Rate of erythrocyte lysis (A) and length of the lag time (B) as a function of lactoperoxidase concentration. Lactoperoxidase concentrations as indicated: H_2O_2, 40 μM (—) or 60 μM (---); KI, 25 μM. Other conditions as indicated in Fig. 1. Each point represents the average of three independent determinations.

lactoperoxidase in a similar series of experiments. However, the concentrations of horseradish peroxidase required to promote a comparable rate of cell lysis were about 100 times higher than those of lactoperoxidase.

Dependence of the cytolytic rate on H_2O_2 concentration. First order kinetics were observed at H_2O_2 concentrations between 1 and 20 μM and mixed order kinetics were obtained at H_2O_2 concentrations between 20 and 40 μM (Fig. 3). At concentrations above 50 μM, H_2O_2 exhibited substrate inhibition with respect to the rate of cell lysis. These data are consistent with the lower rates of cell lysis that were observed in the presence of 60 μM H_2O_2 as compared with those observed in the presence of 40 μM H_2O_2 (Fig. 2a). Using Lineweaver–Burk analysis, an apparent K_m value of 5.6 μM with respect to H_2O_2 was calculated for lactoperoxidase at pH 7.2. Virtually identical results were obtained with horseradish peroxidase.

Curiously, the inhibition of the lysis by high concentrations of H_2O_2 was not reflected in a corresponding lengthening of the lag time. Instead, the lag time was inversely proportional to the H_2O_2 concentrations between 1 and 200 μM (Fig. 4). The figure indicates that at infinite H_2O_2 concentrations a minimum but definite lag time is obtained (9 min in this set of experiments).

Dependence of cytolytic activity on iodide. The cytolytic activity of lactoperoxidase to-

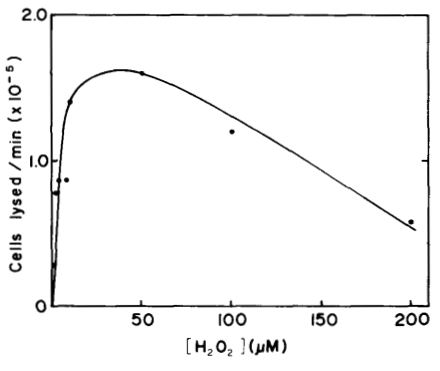

FIG. 3. Rate of erythrocyte lysis as a function of hydrogen peroxide concentration. Other conditions as indicated in Figs. 1 and 2.

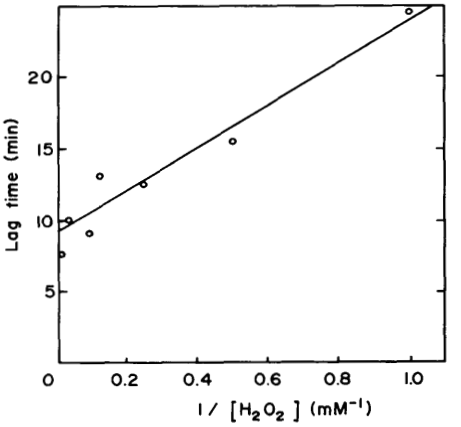

FIG. 4. Relationship between lag time and the reciprocal H_2O_2 concentration.

ward erythrocytes is fully dependent on the presence of iodide ions. This is illustrated in Fig. 5, which shows the rate of cell lysis as a function of iodide concentration. No toxic activity is found in the absence of iodide. The rate of the cytolytic activity increases with increasing iodide concentration, reaching a maximum at about 25 μM. At higher iodide concentrations the rate becomes independent of the iodide concentration. Virtually identical results were obtained with horseradish peroxidase.

The cytolytic activity of both enzymes also appears to be specific for iodide. No lysis was observed with either enzyme when iodide was replaced by bromide, thiocyanate, or fluoride, at either 25 or 250 μM.

pH dependence of the cytolytic reaction. The cytolytic activity of the peroxidases is strongly dependent on pH as illustrated in Fig. 6. The activity of both horseradish peroxidase and lactoperoxidase decreased rapidly and virtually linearly with increasing pH above pH 6.5. Horseradish peroxidase appears to have maximal activity at about pH 6.3, whereas no maximum was observed for lactoperoxidase. Measurements outside the pH range indicated in Fig. 6 were not reliable due to an increase in the spontaneous lysis of the erythrocytes.

Inhibition of cytolysis by peroxidase inhibitors. Several compounds inhibit the cytolytic activity of the peroxidases, as illustrated in Table I. The amino acids tryptophan, tyrosine, cysteine, and histidine inhibit the reaction of both peroxidases, whereas all other naturally occurring amino acids are without effect at concentrations up to 10 mM. It is noteworthy that tryptophan is as good an inhibitor as tyrosine, whereas histidine is a relatively weak inhibitor. In each case the potency of the amino acids as inhibitors is somewhat higher for horseradish peroxidase than for lactoperoxidase, but the relative potency is the same for both peroxidases (try = tyr > cys > his). Furthermore, in each case the decrease in the rate of cytolysis was accompanied by a proportionate increase in the lag time.

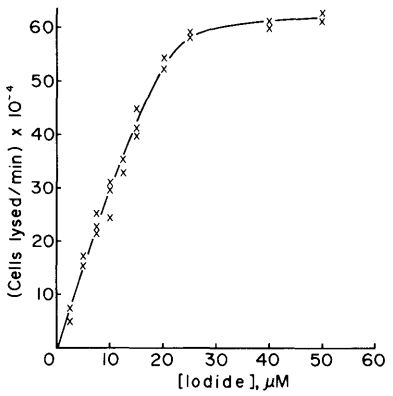

FIG. 5. Rate of erythrocyte lysis as a function of iodide concentration. Other conditions as indicated in Figs. 1 and 2.

Discussion. The cytotoxic activity displayed by various peroxidases in the presence of hydrogen peroxide and certain halide ions has been recognized for a long time; the exact mechanisms of these cytotoxic reactions, however, are still largely unknown. Although a large amount of information has been accumulated through the work of many investigators, the use of different target cells or molecules and different reaction conditions prevents one from utilizing much of this information in attempting to compare the mechanisms by which various peroxidases exert their toxic activity.

The cytolytic assay utilized here has the advantage that kinetic information concerning the lytic reaction can readily be obtained. Thus, information concerning the presence of and length of a lag time, the rate of cell lysis, any relationship between the length of the lag time and the rate of cell lysis, and whether cell lysis reaches completion are parameters that are readily measured and can be considered when the lytic activity (or inhibition thereof) by various substrates is being compared.

In the present communication we report that no significant differences are observed in the mechanism of the cytotoxic activities of lactoperoxidase and horseradish peroxidase, except that under otherwise identical conditions the lactoperoxidase is about 100 times more potent than horseradish peroxidase in the lytic reaction. No significant differences were found with respect to the lytic profiles obtained, the optimal H_2O_2 and iodide concentrations, and the inhibition by high concentrations of H_2O_2. Both enzymes displayed saturation kinetics and both enzymes were inhibited by the same amino acids. Finally, there is considerable similarity in the pH depen-

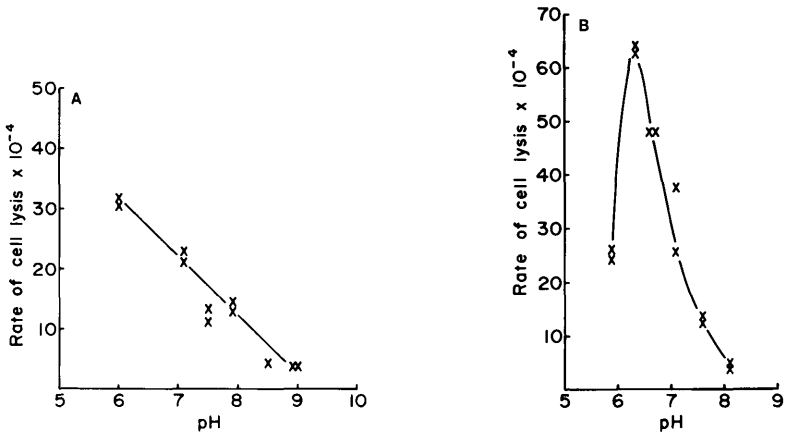

FIG. 6. pH dependence of the cytolytic rate of lactoperoxidase (A) and horseradish peroxidase (B). One milliliter of the reaction mixture contained 1 μg lactoperoxidase or 100 μg horseradish peroxidase, 40 μM H_2O_2, 25 μM KI, 9.5 mM Na-phosphate, 140 mM NaCl, and 2×10^6 rabbit erythrocytes.

dence of the cytolytic reactions. We conclude from these results that the reported differences in the structures of Compounds I of the two enzymes (17) are not reflected in the mechanisms by which these two enzymes exert their cytotoxic activity under our conditions.

TABLE I. INHIBITION OF THE CYTOLYTIC ACTIVITY OF HORSERADISH PEROXIDASE (HRP) AND LACTOPEROXIDASE (LPO) BY VARIOUS AMINO ACIDS[a]

Amino acid added (μM)	Percentage inhibition[b]	
	LPO	HRP
Histidine		
100	51	80
10	15	40
Tyrosine		
20	78	N.L.[c]
10	45	96
1	N.S.[d]	60
Tryptophan		
20	74	N.L.
10	40	84
Cysteine		
30	78	N.L.
20	49	N.L.
10	30	85

[a] Reaction conditions as described under Methods, except for the addition of the amino acid.

[b] Calculated from the decrease in lytic rate. Values are averages of three determinations.

[c] N.L., no lysis occurred within 1 hr after start of the reaction.

[d] N.S., no significant inhibition.

Several other interesting, and perhaps unexpected, observations should be noted. First, with both peroxidases saturation kinetics were obtained with respect to enzyme concentrations. This indicates that the enzymes may not just function as a simple catalyst, but that a binding step (e.g., to the erythrocyte membrane) may be involved. Alternatively, other reactions taking place in the cell membrane may become rate limiting at high peroxidase concentrations.

Second, the cytolytic activity of both enzymes toward erythrocytes is fully dependent on the presence of iodide ions; no lysis occurs when the iodide is omitted. Such a complete dependence on the presence of a halide ion has previously been observed with myeloperoxidase and lactoperoxidase (1, 19–21). Instead of iodide, myeloperoxidase can also use chloride, bromide, and thiocyanate ions as cofactors for its antimicrobial activity (1) and lactoperoxidase has been shown to use thiocyanate as well as iodide for its antibacterial action (20, 21). In contrast, we found that none of these cofactors except iodide supported the cytolytic activity of lactoperoxidase against erythrocytes, even at high concentrations. This observation may suggest that the ability of a given halide to function as a cofactor in the cytotoxic reaction may at least in part depend on the nature of the target cell.

We found that the cytolytic activity of lactoperoxidase and horseradish peroxidase are

inhibited by concentrations of H_2O_2 higher than 50 μM. Inhibition by high concentrations of hydrogen peroxide was also found by Bakkenist *et al.* (12), who investigated the chlorination of monochlorodimedone by myeloperoxidase and H_2O_2. This could be due to formation of the enzymatically inactive Compound III form, which is promoted at high H_2O_2 concentrations (14, 22–24). The fact that high concentrations of H_2O_2 cause a decrease in the rate of cytolysis without causing a corresponding increase in the lag time is puzzling, however, especially since this was not observed with other inhibitors of the cytolytic reaction. This finding requires further study.

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