Resistance of Leishmanial Phosphatases to Inactivation by Oxygen Metabolites

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Leishmania donovani promastigotes produce large quantities of two distinct acid phosphatases; a tartrate-resistant enzyme is localized to the external surface of the plasma membrane, and a tartrate-sensitive enzyme is secreted into the growth medium. It was shown previously that preincubation of human neutrophils and macrophages with the tartrate-resistant phosphatase markedly reduced the ability of these host cells to produce superoxide anions in response to stimulation with the activator formyl-methionyl-leucyl-phenylalanine. The possibility that the cell surface acid phosphatase or the phosphatase that is secreted into the extracellular fluid might compromise other host cell functions, especially intracellular ones, depends on the ability of the enzyme to resist exposure to toxic oxygen metabolites (e.g., superoxide anion, hydrogen peroxide, hypochlorite) generated by phagocytic cells. In the present report, we show that both leishmanial acid phosphatases were relatively resistant to inactivation by oxygen metabolites. At pH 5.5, the activity of the tartrate-resistant phosphatase was reduced 50% by incubation for 1 h with each of the following: 30 mM O_2^- , 500 mM hydrogen peroxide, and 6 mM hypochlorite ion. These concentrations are many fold greater than the concentrations of these substances that are generated by stimulated polymorphonuclear phagocytes. The tartrate-sensitive acid phosphatase differed markedly from the tartrate-resistant phosphatase in that the former was essentially insensitive to even very high concentrations of superoxide anion and hydrogen peroxide. Furthermore, 50% inactivation of the tartrate-sensitive leishmanial phosphatase required exposure to 35 mM hypochlorite for 30 min. These results indicate that the catalytic potential of these two leishmanial acid phosphatases probably survives exposure to toxic oxygen metabolites generated by neutrophils and macrophages.

Leishmania donovani is an intracellular protozoan parasite that infects cells of the reticuloendothelial system. As the etiologic agent of Kala-azar, or visceral leishmaniasis, *L. donovani* is responsible for significant morbidity and mortality in many parts of the world, especially tropical and subtropical areas. The organism possesses a tartrate-resistant acid phosphatase activity which is localized to the outer surface of the extracellular promastigote form (2).

The tartrate-resistant acid phosphatase of the cell surface, which can be solubilized in an active form with detergents such as Triton X-100 (4) or sodium cholate, has been extensively purified (9). A remarkable property of the purified enzyme and one that indicates its potential pathophysiological significance is its ability to block the production of superoxide anions by neutrophils and macrophages (10). Based on this observation, it has been proposed (10) that the acid phosphatase disarms host cells by reducing the level of superoxide anions (O_2^-), thereby allowing the parasite to remain viable as it passes through the gantlet of toxic oxygen metabolites of the normal phagocytic cell, including O_2^- , H_2O_2 , and hypochlorous acid. Since the generation of H_2O_2 and hypochlorous acid is dependent on the availability of O_2^- (6), a reduction in the rate of O_2^- production would likely result in a decrease in the level of the other two oxygen metabolites produced by neutrophils and macrophages. It is noteworthy that when phagocytic cells are infected with *L. donovani*, the production of these oxygen metabolites is reduced but not completely abolished (8). This raises the possibility that the acid phosphatase on the surface of the parasite could be inactivated by exposure to O_2^- , H_2O_2 , or

hypochlorous acid. If so, the catalytic activity of the phosphatase could not be used by the parasite to modify other host cell functions such as phagolysosome formation and endosome acidification. For this reason, we elected to define the sensitivity of the leishmanial acid phosphatase to the specific oxygen metabolites, namely H_2O_2 , superoxide anion, and hypochlorite anion.

In the present report we show that the leishmanial acid phosphatase isolated from the cell surface of promastigotes and the secreted acid phosphatase (3) were both relatively resistant to H_2O_2 and superoxide anion but sensitive to hypochlorite anion.

MATERIALS AND METHODS

Materials. 4-Methylumbelliferyl phosphate (MUP), potassium superoxide, and all of the phosphatases listed in Table 1 were purchased from Sigma Chemical Co., St. Louis, Mo. Hydrogen peroxide and sodium hypochlorite were obtained from Fisher Scientific Co., Pittsburgh, Pa.

Organism and growth conditions. A cloned strain of *L. donovani* (Sudan 1) promastigotes was grown and harvested as described by Gottlieb and Dwyer (2), except for the addition of streptomycin (50 μ g/ml) and penicillin (50 U/ml) to the medium.

Preparation of acid phosphatase. The predominant tartrate-resistant membrane-bound acid phosphatase of *L. donovani* promastigotes was purified to homogeneity, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the final preparation had a specific activity of 4.7×10^6 U/mg of protein in the standard acid phosphatase assay. The purification scheme involved solubilization of the enzyme with sodium cholate followed by chromatography on Seph-

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TABLE 1. Effects of H_2O_2 , KO_2 , and $NaOCl$ on the activity of various phosphatases^a

Acid phosphatase source	% Inhibition ^b by:		
	H_2O_2	KO_2	$NaOCl$
Tartrate-resistant <i>L. donovani</i>	30 (0.81)	20 (1.1)	35 (1.5)
Bovine prostate	90 (1.2)	45 (2.1)	70 (1.6)
Bovine milk	80 (1.5)	35 (2.2)	60 (1.7)
Bovine serum	60 (2.2)	80 (1.2)	90 (1.2)
Potato	60 (1.7)	55 (1.2)	15 (0.81)

^a Sensitivities of different acid phosphatases to various oxygen metabolites were compared by preincubating the phosphatases at pH 7.0 in 0.2 M sodium cacodylate buffer for 30 min at 37°C with 400 mM H_2O_2 , 15 mM KO_2 , (initial concentration), or 3.5 mM OCl^- . At the end of the preincubation period, 10- μ l samples were assayed for phosphatase activity by using the standard acid phosphatase assay. The concentrations of all the enzymes in the assay medium were the same (200 U/ml, measured in the standard acid phosphatase assay at pH 5.5).

^b Each result is the mean ($\pm$ standard deviation) of three determinations.

FIG. 1. Effects of superoxide anion, hydrogen peroxide, and hypochlorite ions on tartrate-resistant acid phosphatase isolated from *L. donovani* membranes. The purified tartrate-resistant enzyme was preincubated with different concentrations of oxygen metabolites in 0.2 M sodium acetate buffer (pH 5.5) at 37°C. Samples (10 μ l) were taken at various times and added to the standard assay medium. A parallel experiment was run each time, which served as the control; in place of oxidants, the buffer was added. Phosphatase activity was determined as described in the text. (A) Treatment with 15 (●) and 30 mM (○) O_2^- for 60 min; (B) treatment with 400 (●), 800 (○), and 1,600 mM (△) H_2O_2 for 60 min; (C) treatment with 3.5 (●), 7.0 (○), and 35 mM (△) hypochlorite for 30 min. Results are expressed as percent inhibition.

adex G-75, concanavalin A-Sepharose, quaternary aminoethyl-Sephadex, phenyl-Sepharose, and Sephadex G-100 (9).

The extracellular tartrate-sensitive acid phosphatase which is secreted into the growth medium was purified as described by Gottlieb and Dwyer (3); the specific activity of the preparation was 4.5×10^6 U/mg of protein.

Standard acid phosphatase assay. Acid phosphatase activity was determined fluorometrically at 37°C with MUP as the substrate in a 0.1-ml reaction mixture that contained 0.2 M sodium acetate buffer (pH 5.5) and 7 mM MUP. One unit of enzyme activity is defined as the amount of enzyme required to convert 1 nmol of substrate to product per h at 37°C. Fluorescence was determined as described elsewhere (11). Superoxide anion, hydrogen peroxide, and sodium hypochlorite, at the concentrations used in this study, had no effect on the fluorescence properties of MUP or the product of the reaction, 4-methylumbelliferone; these oxygen metabolites did not interfere with the phosphatase assay.

Protein determination. The protein concentration was estimated by the method of Bradford (1) with bovine serum albumin as the standard.

RESULTS

Effect of oxygen metabolites on the tartrate-resistant acid phosphatase derived from *L. donovani* membranes. The effects of O_2^- , H_2O_2 , and hypochlorite anion preincubation on the activity of the tartrate-resistant acid phosphatase of *L. donovani* are shown in Fig. 1; the pH of the preincubation medium was 5.5. Preincubation of the enzyme with 30 mM O_2^- for 60 min resulted in about 50% inactivation of phosphatase activity. The enzyme was considerably more resistant to H_2O_2 ; after 50 min, 400 mM H_2O_2 had destroyed less than half of the original activity. Nevertheless, concentra-

FIG. 2. Effect of pH on the inactivation of tartrate-resistant acid phosphatase by superoxide anions, hydrogen peroxide, and hypochlorite ions. The tartrate-resistant acid phosphatase from *L. donovani* was preincubated with 30 mM O_2^- (initial concentration), 1,600 mM H_2O_2 , and 35 mM hypochlorite for 15 min in buffers at various pH. The following buffers were used at 0.2 M final concentration: sodium acetate (pH 4.0, 5.0, and 6.0), sodium cacodylate (pH 7.0), and Tris hydrochloride (pH 8.0). After preincubation, samples (10 μ l) were removed and assayed for phosphatase activity as described in the text; results are expressed in terms of enzyme activity (units/milliliter). At the end of the 15-min preincubation, the concentration of O_2^- at pH 4.0, 6.0, and 8.0 had decreased by 30, 20, and 10%, respectively. The superoxide anion concentration was determined by the method of Newburger et al. (7).

tions of H₂O₂ in excess of 1 M completely inactivated the tartrate-resistant leishmanial phosphatase in 1 h.

We next examined the effect of the pH of the preincubation medium on the ability of O₂⁻, H₂O₂, and hypochlorite anion to inactivate the tartrate-resistant acid phosphatase (Fig. 2). All three substances accelerated inactivation of the phosphatase over the entire pH range of the experiment. Thus, there is no hydrogen ion concentration between pH 4 and 7 where these oxygen metabolites are not capable of inactivating the tartrate-resistant leishmanial acid phosphatase. Although spontaneous dismutation decreased the concentration of O₂⁻ by 30% in 15 min at pH 4.0, compared with only a 10% decrease at pH 8.0, this reduction in O₂⁻ concentration during the preincubation phase of the experiment is probably too little to account for the greater resistance of the leishmanial phosphatase to inactivation by O₂⁻ at acid pH relative to that observed at alkaline pH.

Effect of oxygen metabolites on the tartrate-sensitive acid phosphatase of *L. donovani*. As we were interested in the comparative sensitivity of the two prominent acid phosphatases of *L. donovani*, we investigated the effects of O₂⁻, H₂O₂, and hypochlorite anion on the secreted, tartrate-sensitive acid phosphatase. The tartrate-sensitive acid phosphatase was hardly affected by high concentrations of H₂O₂ (1.6 M) or superoxide anion (30 mM) (Fig. 3). Thus, it seems that in addition to the difference in their sensitivity to tartaric acid, the membrane-associated and secreted phosphatases of

FIG. 3. Effect of superoxide anion, hydrogen peroxide, and hypochlorite ions on the tartrate-sensitive, secreted acid phosphatase of *L. donovani* promastigotes. The purified tartrate-sensitive acid phosphatase secreted by *L. donovani* promastigotes was preincubated for 60 min with 30 mM O₂⁻ (A) and 1.6 M H₂O₂ (B). (C) Results of preincubation of the enzyme with the following three different concentrations (mM) of hypochlorite ion: 35 (Δ), 70 (○), and 700 mM (●) for 30 min. Results are expressed as relative activity.

FIG. 4. Dose-response curve of hydrogen peroxide for tartrate-resistant and tartrate-sensitive acid phosphatase. The purified tartrate-resistant (●) and tartrate-sensitive (×) acid phosphatases were preincubated with 0.2 to 2.0 M hydrogen peroxide in 0.2 M sodium acetate buffer (pH 5.5) at 37°C for 30 min. After preincubation, samples (10 μl) were removed, and phosphatase activity was determined as described in the text. Results are expressed as percent inhibition.

L. donovani differ also in their resistance to inactivation by exposure to O₂⁻ or H₂O₂. The secreted acid phosphatase was also less sensitive than the tartrate-resistant leishmanial phosphatase to inactivation by hypochlorite anion (Fig. 3C); 35 mM sodium hypochlorite caused less than 50% inactivation of the secreted leishmanial acid phosphatase in 30 min.

Comparison of the effect of oxygen metabolites on leishmanial and nonleishmanial acid phosphatases. We were interested in comparing the sensitivity of the leishmanial phosphatases to inactivating oxygen metabolites with the sensitivity of acid phosphatases from plant and animal sources to these inhibitory substances. The tartrate-resistant phosphatase of *L. donovani* is in general significantly more resistant to inactivation by O₂⁻, H₂O₂, and hypochlorite anion than are the bovine and potato acid phosphatases (Table 1). The only exception to this generalization is the greater resistance of potato acid phosphatase activity to hypochlorite ion.

The effects of different concentrations of H₂O₂ on the activity of tartrate-resistant and tartrate-sensitive acid phosphatases of *L. donovani* are shown in Fig. 4; after 30 min 1.6 M H₂O₂ inhibited 80 and 25% of tartrate-resistant and tartrate-sensitive acid phosphatases, respectively.

DISCUSSION

There are at least four good reasons why the tartrate-resistant acid phosphatase of *L. donovani* warrants the attention of investigators interested in increasing our understanding of the pathophysiology and biochemistry of leishmaniasis. First, the enzyme is propitiously located on the outside surface of the promastigote where it is likely to come in direct and early contact with the host cell (2). Second, the phosphatase content of the parasite greatly exceeds that of a dozen other hydrolytic enzymes (4). Third, the cell surface phosphatase is capable of catalyzing the dephosphorylation of phosphoproteins, not only at pH 5.5 but also at neutral pH (S. Das, A. K. Saha, and R. H. Glew, unpublished data). This capacity, together with the fact that mononuclear phagocytic cells possess key regulatory proteins on the outer surface of their plasma membrane, which undergo phosphorylation-dephosphorylation (5, 13), heightens the possibility that the leishmanial phosphatase may utilize host cell

phosphoproteins as substrates, thereby affecting critical host cell functions. Fourth and especially significant, the ability of neutrophils and macrophages to generate germicidal superoxide anions and hydrogen peroxide is markedly reduced by the preincubation of these mononuclear cells for 5 to 15 min at 37°C with small amounts of a pure preparation of the tartrate-resistant leishmanial phosphatase (10).

To date, the little work that has been devoted to the pathophysiologic role of the tartrate-resistant phosphatase has been directed at the effects of the enzyme on the cell surface-dependent processes of host cells. The results of the present study should encourage investigators to consider the possible effects of the enzyme on the intracellular activities of host cells. The major result of our report is the documentation of the resistance of the leishmanial acid phosphatase to exposure to potentially noxious oxygen metabolites, specifically superoxide anions, hydrogen peroxide, and hypochlorous acid. Other acid phosphatases from plant and animal sources are generally more sensitive to these noxious substances (Table 1).

The concentrations of superoxide anion and H_2O_2 required to inactivate 50% of the tartrate-resistant cell surface acid phosphatase of *L. donovani* promastigotes in 1 h are approximately 30 and 500 mM, respectively (Fig. 1A and B). These concentrations are more than 30-fold higher than those generated by mononuclear phagocytes. For example, it has been estimated (12) that neutrophils are capable of generating H_2O_2 at a rate of 16 nmol/min per 10^7 cells. Assuming that 10^7 neutrophils are contained in 1 ml of liquid medium, this capacity for H_2O_2 production would result in an H_2O_2 concentration of 0.96 mM at the end of 1 h. By a similar calculation, the same number of neutrophils could generate a solution that is approximately 0.5 mM in superoxide anions. Based on these estimates, it seems that the amount of H_2O_2 and superoxide anions generated in response to perturbation of the plasma membrane of the neutrophil by promastigotes would have little effect on the activity of the tartrate-resistant acid phosphatase of the parasite.

The concentration of hypochlorite ion needed to reduce the activity of the same phosphatase by 50% is approximately 6 mM (Fig. 1C). Since the rate at which neutrophils carry out halogenation is less than one tenth the rate at which they are capable of generating superoxide anions (12), it is unlikely that significant inactivation of the phosphatase would result from the quantity of hypochlorite produced by neutrophils. This conclusion must be tempered by the fact that the local concentrations of hypochlorite ion, H_2O_2 , and superoxide anions at the parasite-macrophage interface may be considerably higher than those of the bulk solution.

In the pH range 4 to 7, there was no hydrogen ion concentration at which the tartrate-resistant leishmanial phosphatase was unusually sensitive to superoxide anions, H_2O_2 , or hypochlorite ions (Fig. 2). These observations indicate that the catalytic potential of the phosphatase would probably survive exposure to these oxygen metabolites, even at the relatively acidic pH that exists in phagolysosomes.

In summary, these observations and arguments support the idea that the catalytic potential of the cell surface phosphatase of *L. donovani* may survive intracellular transport in the host cell and that it may retain sufficient catalytic activity to act on intracellular phosphorylated constituents

(e.g., phosphoproteins) of the host cell. Thus, although we have demonstrated that this particular phosphatase inactivates one particular function of the host cell surface, namely superoxide anion production (10), the results of the present study leave open the possibility that the phosphatase may compromise other more distal host cell functions, such as phagolysosome formation or endosome acidification.

The present study also showed that the secreted acid phosphatase and the cell surface acid phosphatase of *L. donovani* promastigotes differ markedly in terms of their sensitivity to oxygen metabolites, especially hydrogen peroxide and hypochlorite ions. These results, together with the greatly different sensitivity of the phosphatases to inhibition by tartrate, provide further evidence of the unrelatedness of these two enzymes and reduce the possibility that the cell surface phosphatase represents an intermediate, precursor form of the secreted acid phosphatase.

Future experiments will be aimed at elucidating the effects of the tartrate-resistant leishmanial acid phosphatase on intracellular host cell functions.

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