Characterization of Ectophosphatase Activities in Trypanosomatid Parasites of Plants

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ABSTRACT

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In the present work ectophosphatase activities of three trypanosomatid parasites of plants were characterized using intact cells. *Phytomonas françai*, *Phytomonas mcgheeii*, and *Herpetomonas* sp. hydrolyzed *p*-nitrophenylphosphate at a rate of 5.40, 7.28, and 25.58 nmol Pi/mg of protein per min, respectively. Experiments using classical inhibitors of acid

phosphatases such as sodium orthovanadate (NaVO₃) and sodium fluoride (NaF) showed a decrease in phosphatase activities. Lithium fluoride (LiF) and aluminum chloride (AlCl₃) were also used. Although AlCl₃ had no effect, LiF was able to promote a decrease in the phosphatase activities. Interestingly, the inhibition caused by LiF was enhanced by the addition of AlCl₃ during the reaction, probably due to the formation of fluoroaluminate complexes. This effect was confirmed by cytochemical analysis. In this assay, electron-dense cerium phosphate deposits were visualized on the external surface of the three parasites.

The flagellated trypanosomatids of the genus *Phytomonas* and some *Herpetomonas* spp. are parasites of plants. This parasitism can occur without any apparent pathogenicity but it can also cause diseases of economic significance in plantations of coconut, oil palm, cassava, and coffee (9,10,13). The parasites live mostly in the phloem and laticifers of the infected plants and are thought to be transmitted through the bite of phytophagous insects (9,10). These trypanosomatids have also been detected in edible fruits such as pomegranates, peaches, guavas, and tangerines as well as in their insect vectors (9,10). In the biological cycle of these pathogens, several plant-sucking insects act as intermediate hosts, being the plants main hosts (9,10).

Parasite membranes are involved in many cellular events, including transport of nutrients and ions and protection of the parasites against host immune responses (11,50). The process of phosphorylation/dephosphorylation, involving kinases and phosphatases is of major importance in regulating many cellular processes in eukaryotes (24). Ectoprotein kinases have been identified in *Leishmania major* (41), and ectophosphatase activities have been characterized in members of the family Trypanosomatidae, such as *Trypanosoma* (16,34) and *Leishmania* (19,20,32,38). Recently we demonstrated a novel acid ectophosphatase activity on the external surface of *Herpetomonas muscarum muscarum*, which is highly inhibited by classical phosphatase inhibitors and by fluoroaluminate complexes (14). Although the physiological role of the membrane-bound acid phosphatases has not been well established, they are supposed to be involved with nutrition (20), protection (20,38), and cell differentiation (3,34). Most *Leishma-*

nia spp. are capable of secreting acid phosphatases, and these secreted enzymes are inhibited by sodium tartrate, varying their degree of sensitivity (29). These secreted enzymes have been suggested to be involved with the interaction of the parasites with their host cells (48).

The presence of aluminum in acid soils inhibits plant growth because it may be toxic for many plants (27). Although its toxicity mechanism is not completely understood, aluminum stress promotes an inhibition of the plasma membrane H⁺-ATPase of barley root (26) and of corn root (46). This enzyme is a P-type transport ATPase that plays a fundamental role in nutrition and development of plants (8,42). In the soil, aluminum forms complexes with different anions including fluoride, hydroxide, phosphate, and silicate (2,27). Fluoride anions and fluoroaluminate complexes inhibit different P-type ATPases, including a H⁺-ATPase from maize root (15), a H⁺-ATPase purified from yeast plasma membrane (37), and a vanadate-sensitive ATPase activity found in wheat root microsomes (31). Before reaching the P-type ATPase of plant cells, these complexes could interact with ectoenzymes present on the surface of these plant parasites.

In this work we have characterized ectophosphatase activities on the surface of the trypanosomatids *P. françai*, *P. mcgheeii*, and *Herpetomonas* sp. and studied the effects of fluoride, aluminum, and fluoroaluminate complexes on those activities.

MATERIALS AND METHODS

Microorganisms and growth conditions. We used *P. françai* isolated from *Manihot esculenta* (47), *P. mcgheeii* isolated from *Zea mays* (25), and *Herpetomonas* sp. isolated from the salivary glands of *Phthia picta* (Hemiptera: Coreidae), which was first named as *P. serpens* (7) and further identified as *Herpetomonas* sp. (43). The parasites were grown in Warren medium supple-

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mented with 10% fetal calf serum at 28°C. Three days after inoculation, the cells were harvested by centrifugation, washed twice with 0.9 % saline, washed once with 30 mM Tris-HCl/75 mM sucrose buffer at pH 6.8, and kept in the same buffer before the assays. Cellular viability was assessed before and after incubations by motility and cell dye exclusion (Trypan blue method; 4). The viability of the parasites was not affected by the conditions used in this work.

Fig. 1. Temporal course of the phosphatase activities of *Herpetomonas* sp., *Phytomonas mcgheeii*, and *P. francai* intact cells. The reactions were performed at 30°C in a buffer containing 30 mM Tris-HCl/75 mM sucrose at pH 6.8, 10 mM *p*-nitrophenylphosphate, and 1 mg of protein per ml.

TABLE 1. Maximal velocity (V_{max}) and Michaelis constant (K_m) of phosphatase activities of intact cells of *Herpetomonas* sp., *Phytomonas mcgheeii*, and *P. francai*

Protozoan	V_{max} (nmol Pi mg ⁻¹ min ⁻¹)	K_m (mM <i>p</i> -NPP)
<i>Herpetomonas</i> sp.	30.01 ± 1.05	1.63 ± 0.28
<i>P. mcgheeii</i>	8.54 ± 0.68	1.33 ± 0.27
<i>P. francai</i>	5.34 ± 0.02	0.36 ± 0.06

^a The individual V_{max} and K_m values were obtained by nonlinear regression using Sigma Plot (Version 1.0, Jandel Scientific Software, CA). *p*-NPP = *p*-Nitrophenylphosphate.

TABLE 2. Influence of divalent cations and phosphatase inhibitors on ecto-phosphatase activities of intact cells of *Herpetomonas* sp., *Phytomonas francai*, and *P. mcgheeii*

Addition	% Phosphatase activity ^a		
	<i>Herpetomonas</i>	<i>P. mcgheeii</i>	<i>P. francai</i>
None	100.00	100.00	100.00
Copper sulfate (5 mM)	104.03	247.25	111.11
Magnesium chloride (5 mM)	96.79	112.91	102.41
Calcium chloride (5 mM)	94.14	96.15	104.81
Okadaic acid (2.5 µM)	100.00	100.00	100.00
Aluminum chloride (0.1 mM)	93.11	96.27	102.25
Potassium phosphate (10 mM)	89.17	90.52	87.41
Sodium tartrate (10 mM)	34.28	69.37	77.22
Zinc chloride (1.0 mM)	50.34	31.99	43.00
Sodium fluoride (10 mM)	2.54	17.44	39.81
Lithium fluoride (10 mM)	1.80	12.09	37.22
Sodium orthovanadate (0.1 mM)	10.09	7.84	29.72
Ammonium molybdate (0.1 mM)	2.78	5.36	6.48

^a Phosphatase activity is expressed as a percentage measured under control conditions, i.e., without other additions.

Fig. 2. Influence of pH on the phosphatase activities of **A**, *Herpetomonas* sp., **B**, *Phytomonas mcgheeii*, and **C**, *P. francai* intact cells. The reactions were performed at 30°C in a buffer containing 30 mM Tris-HCl/75 mM sucrose ranging from pH 6.5 to 8.0, 10 mM *p*-nitrophenylphosphate, and 1 mg of protein per ml. The values represent the mean of at least three independent experiments performed in triplicate. The bars represent the standard errors. **Inset**, Influence of pH on the phosphatase activities of **A**, *Herpetomonas* sp., **B**, *P. mcgheeii*, and **C**, *P. francai* membrane-enriched fractions (5) performed as described above.

Phosphatase activity. The phosphatase activity was measured by the rate of inorganic phosphate production. Intact cells were incubated for 1 h at room temperature in 0.5 ml of a reaction mixture containing 30 mM Tris-HCl/75 mM sucrose buffer at pH 6.8, 10 mM *p*-nitrophenylphosphate (*p*-NPP) as substrate, and 1 mg of protein (intact cells or membrane-enriched fractions; 5). Reactions were initiated by the addition of cells and stopped by the addition of 2 ml of 1M NaOH. Controls where cells or membrane-enriched fractions were added after interruption of the reaction were used as a blank. For determining the concentration of released *p*-nitro-

phenol (*p*-NP; product of *p*-NPP hydrolysis), the tubes were centrifuged at $1,500 \times g$ for 20 min and the supernatant was measured spectrophotometrically at 425 nm using a *p*-NP curve as standard. Protein concentration was determined according the method of Lowry et al. (30) with bovine serum albumin (BSA) as standard.

Cytochemical detection of acid phosphatase. The cytochemical assay was performed as previously described (40). Briefly, the parasites were fixed for 20 min at 4°C with glutaraldehyde in 0.1 M cacodylate buffer at pH 7.2, washed in the same buffer, followed by 0.1 mM Tris-maleate buffer at pH 5.0, and incubated for

Fig. 3. Effects of sodium orthovanadate and lithium fluoride (LiF) on the phosphatase activities of **A** and **B**, *Herpetomonas* sp., **C** and **D**, *Phytomonas mcgheeii*, and **E** and **F**, *P. françai* intact cells. The reactions were performed at 30°C in a buffer containing 30 mM Tris HCl / 75 mM sucrose at pH 6.8, 10 mM *p*-nitrophenylphosphate, and 1mg of protein per ml, which corresponds to **A**, **C**, and **E**, sodium orthovanadate, and **B**, **D**, and **F**, LiF concentrations varying as shown on the abscissa. The standard errors were calculated from the absolute activity values of three experiments with different cell suspensions and converted to percentage of the control value.

1 h at 37°C in 2 mM cerium chloride, 5% sucrose, 0.1 mM Tris-acetate buffer at pH 5.0, and 2 mM sodium β -glycerophosphate as substrate. The parasites were washed in Tris-maleate and cacodylate buffers, refixed with 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in a graded acetone series, and embedded in Epon. As a control, the same number of cells were incu-

bated in the absence of substrate. Ultrathin sections were observed unstained in a transmission electron microscope (Zeiss 900 EM, Carl Zeiss Inc., Oberkochen, Germany) operated at 80 kV.

Chemicals. BSA, *p*-NPP, Folin and Ciocalteu's phenol reagent, glutaraldehyde, β -glycerophosphate, and okadaic acid were purchased from Sigma Chemical Co. (St. Louis). Calcium chloride, magnesium chloride, zinc chloride, sodium fluoride (NaF), lithium fluoride (LiF), aluminum chloride, copper sulfate (CuSO_4), potassium phosphate, sodium orthovanadate (Na_3VO_4), cerium chloride, and ammonium molybdate ($[\text{NH}_4]_6\text{Mo}_7\text{O}_{24}$) were purchased from Merck S. A. (São Paulo, Brazil).

Statistical analysis. All experiments were performed in triplicate, with similar results obtained in at least three separate cell suspensions. Maximal velocity (V_{max}) and Michaelis constant (K_m) for *p*-NPP were calculated using a computerized nonlinear regression program (Sigma Plot 1.0; Jandel Scientific Software, San Diego, CA) and data to the Michaelis-Menten equation.

RESULTS

Phosphatase activity. The time courses of each phosphatase activity present on the external surface of *P. françai*, *P. mcgheeii*, and *Herpetomonas* sp. promastigote forms were linear for at least 1 h (Fig. 1). At pH 6.8, those intact parasites were able to hydrolyze *p*-NPP at rates of 5.40 ± 0.15 , 7.28 ± 0.27 , and 25.58 ± 0.11 nmol Pi/mg of protein per min, respectively.

In the pH range from 6.5 to 8.0, in which the cells were viable, the phosphatase activities reached a maximum at pH 6.5, decreasing concomitantly with the increase of pH (Fig. 2). Using membrane-enriched fractions, it was observed that in the pH range from 4.0 to 10.0, the optimum pH was 4.0 for *Herpetomonas* sp. and 5.0 for *P. mcgheeii* and *P. françai* (Fig. 2, inset).

The dependence on *p*-NPP concentration for ectophosphatase activities was observed. The values of V_{max} and apparent K_m for *p*-NPP were 30.01 ± 1.05 nmol Pi/mg of protein per min, 1.63 ± 0.28 mM for *Herpetomonas* sp., 8.54 ± 0.68 nmol Pi/mg of protein per min, and 1.33 ± 0.27 mM for *P. mcgheeii*, and 5.34 ± 0.02 nmol Pi/mg of protein per min and 0.36 ± 0.06 mM for *P. françai*, respectively (Table 1).

Effect of divalent cations and phosphatase inhibitors on the ectophosphatase activities. Different divalent cations and phosphatase inhibitors were tested, and results are shown in Table 2. Magnesium chloride, calcium chloride, aluminum chloride, potassium phosphate, and okadaic acid (a serine-threonine phosphatase inhibitor) had no effect on these ectophosphatase activities. Copper sulfate stimulated the phosphatase activity from *P. mcgheeii* but did not have any effect on phosphatase activities from *P. françai* or *Herpetomonas* sp. The stimulation observed on phosphatase activity from *P. mcgheeii* could be related to the increase of secretion of a phosphatase to lysosomal acid phosphatase in hepatic cells when submitted to high concentrations of copper sulfate (22). Regarding this, sodium tartrate, an inhibitor of secreted acid phosphatases from *L. donovani* (29) and *H. muscarum muscarum* (14), inhibited 65.7, 30.6, and 22.8% in *Herpetomonas* sp., *P. mcgheeii*, and *P. françai* phosphatase activities, respectively (Table 2).

The ectophosphatase activities of the three parasites were sensitive to other phosphatase inhibitors, indicating different patterns of inhibition (Table 2). Ammonium molybdate inhibited almost 100% of the phosphatase activities of the three parasites, while zinc chloride promoted an inhibition of 49.7, 68.0, and 57.0% in *Herpetomonas* sp., *P. mcgheeii*, and *P. françai* enzymatic activities, respectively. The classical phosphatase inhibitors sodium orthovanadate (phosphotyrosin phosphatase inhibitor) and lithium fluoride (Fig. 3) inhibited the enzymatic activities of the three species in a dose dependent manner. The same inhibition pattern was also found for the acid phosphatase inhibitor sodium fluoride (data not shown), which showed a profile of inhibition very simi-

Fig. 4. Effect of aluminum chloride (AlCl_3) and fluoroaluminate complexes (different amounts of AlCl_3 in the presence of 0.1 mM lithium fluoride) on the phosphatase activities of **A**, *Herpetomonas* sp., **B**, *Phytomonas mcgheeii*, and **C**, *P. françai* intact cells. The reactions were performed at 30°C in a buffer containing 30 mM Tris HCl / 75 mM sucrose at pH 6.8, 10 mM *p*-nitrophenylphosphate, and 1 mg of protein per ml, in the absence or in the presence of aluminum chloride (●) and fluoroaluminate complexes (■). Standard error was calculated from the absolute activity values of three experiments with different cell suspensions and converted to percentage of the control value.

lar to lithium fluoride (Fig. 3). *P. françai* was less sensitive to these inhibitors than the two other species, while *Herpetomonas* sp. was the most sensitive to fluoride inhibitors, and inhibited $\approx 97\%$ of that activity (Fig. 3, Table 2). Sodium orthovanadate was more potent than fluoride inhibitors to *P. françai* and *P. mcgheeii* (Fig. 3, Table 2).

Effect of fluoroaluminate complexes on ectophosphatase activity and cytochemical analysis. The fluoroaluminate complexes, which are the result of the addition of increasing concentrations of aluminum chloride to the reaction mixture, in the pre-

sence of lithium fluoride, inhibited 40% of the phosphatase activity of *Herpetomonas* sp. and 25% of the activity of *P. mcgheeii*, while in *P. françai* the fluoroaluminate complexes promoted less than 20% inhibition of the enzymatic activity (Fig. 4). Although aluminum chloride had no effect on those activities, the fluoroaluminate complexes were able to inhibit the ectophosphatase activities (Fig. 4).

Cytochemical detection was performed in the presence and in the absence of fluoroaluminate complexes (0.1 mM lithium fluoride plus 200 μ M aluminum chloride), showing a distinct pat-

Fig. 5. Cytochemical assay. Effect of fluoroaluminate complexes (0.2 mM aluminum chloride [AlCl₃] in the presence of 0.1 mM lithium fluoride [LiF]) on the phosphatase activities of **A and B**, *Herpetomonas* sp., **C and D**, *Phytomonas françai*, and **E and F**, *P. mcgheeii* intact cells. **A, C, and E** = control cells; **B, D, and F** = treated cells (reaction product is absent). Arrows indicate reaction product (in **A, C, and E**); arrowheads indicate inhibition of the reaction (in **B, D, and F**). N = nucleus; K = kinetoplast; FP = flagellar pocket; and bar = 3 μ m.

tern of cerium phosphate deposition (Fig. 5). The parasites that were not treated by fluoroaluminate complexes (control cells; Fig. 5, panels A, C, and E) showed electrondense cerium phosphate deposits homogeneously distributed on the protozoa external surface of the three species, including flagellar pocket and flagellar membrane, visualized by transmission electron microscopy (Fig. 5). The fluoroaluminate complexes partially inhibited the ectophosphatase activities of the three species tested, confirming our biochemical data (Fig. 5, panels B, D, and F).

DISCUSSION

Little is known about the functionality of membrane-bound enzymes in living cells and their possible role in the process of host-parasite interactions. The detection of cell surface-located phosphatase activity is particularly interesting because of its possible role in cell-cell interaction or reception and transduction of external stimuli (33,48). Cellular responses to extracellular stimuli (e.g., parasite adhesion) can evoke signaling pathways including protein phosphorylation/dephosphorylation. It was recently shown that *Leishmania* acid phosphatases can regulate parasite binding to macrophages (33,48). Our paper reports the presence of acid phosphatase activities on the external surface of *P. françai*, *P. mcgheeii*, and *Herpetomonas* sp. The external localization of the *p*-NPP-hydrolyzing site is supported by the use of viable intact cells that were tested after the experiments and by the cytochemical analysis which clearly showed electrondense cerium phosphate deposits on the external surface of these parasites (Fig. 5) and within the flagellar pocket in the case of *Herpetomonas* sp. (Fig. 5A) and *P. françai* (data not shown). A similar pattern has been observed for most *Leishmania* sp. which are capable of secreting acid phosphatases (20,29,48). The trypanosomatid flagellar pocket is a highly specialized area of the parasite membrane that permits internalization of macromolecules, as well as, secretion of macromolecules to the environment (49).

Under the conditions used in this work, we observed that the maximum release of *p*-NP occurred at pH 6.5 for living parasites (Fig. 2) and at $\approx$ pH 5.0 for membrane-enriched fractions (Fig. 2, inset), suggesting an acid phosphatase activity, as previously described for other trypanosomatids (16,19,23,32,34).

Micromolar concentrations of sodium orthovanadate, described as selective phosphotyrosyl phosphatase inhibitor (12,16,18,24,28), was able to promote a decrease of the protozoa ectophosphatase activities (Fig. 3). The addition of fluoride, a putative inhibitor of mammalian phosphoserine phosphatases (1), significantly decreased the ectophosphatase activities of the three species of parasites (Fig. 3, Table 2). Inhibition of phosphotyrosin phosphatase by fluoride (mostly the acid phosphotyrosin phosphatase activity) previously has been reported (6,16,18,44). Similar results have been observed in members of the genera *Leishmania* (19,21,23), *Herpetomonas* (14), and *Trypanosoma* (16,18). The reason for sensitivities toward vanadate and fluoride remains unclear. It is possible that the supposed selective action of the inhibitors depends on the catalytic mechanism of the enzymes, substrate specificity, and association with possible specific regulatory subunits (16). Okadaic acid, a potent inhibitor of serine-threonine phosphatase (24), had no effect on the ectophosphatase activities (Table 2). Aluminum chloride had no effect on these enzymatic activities, but when it was added in the presence of 0.1 mM LiF, the phosphatase activities of *P. mcgheeii* and *Herpetomonas* sp. were significantly inhibited (Fig. 3). This biochemical data was confirmed by cytochemical assay (Fig. 5). Aluminum can form complexes with anions including fluoride (27). Aluminum in acid soils suppressed the germination and chlamidospore production of macroconidia and growth and chlamidospore production of hyphae of the fungus *Fusarium solani* f. sp. *phaseoli* on beans (17). The fluoroaluminate complex acts as a Pi analog that strongly binds to the E₂ conformation (phosphorylated form) of the P-type ATPases in a

quasi-irreversible process that cannot be overcome by high Pi concentrations (39,45). This type of inhibition is similar to that described for sodium orthovanadate (36) and was probably the mechanism of inhibition of the phosphatase activities described here. The possible role of phosphotyrosin phosphatase in the process of metacyclogenesis of *T. cruzi* (3,34,35) and *T. brucei* (3) has been suggested. The same was suggested for *L. donovani* which undergoes phosphotyrosin alteration in the process of cell differentiation (12). A similar role could be expected for the ectophosphatase activities of *P. françai*, *P. mcgheeii*, and *Herpetomonas* sp. described in this work. If this is the case, these enzymes could be good targets for the growth control of the protozoans that live in the vessels of plants, because fluoroaluminate complexes could interact with the ectophosphatases of the parasites before reaching the plant enzymes.

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