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Leishmania amazonensis: Characterization of an ecto-phosphatase activity

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Abstract

We have characterized a phosphatase activity present on the external surface of *Leishmania amazonensis*, using intact living parasites. This enzyme hydrolyzes the substrate *p*-nitrophenylphosphate (*p*-NPP) at the rate of 25.70 ± 1.17 nmol Pi $\times$ h^{-1} $\times$ 10^{-7} cells. The dependence on *p*-NPP concentration shows a normal Michaelis–Menten kinetics for this ecto-phosphatase activity present a V_{max} of 31.93 ± 3.04 nmol Pi $\times$ h^{-1} $\times$ 10^{-7} cells and apparent K_m of 1.78 ± 0.32 mM. Inorganic phosphate inhibited the ecto-phoshatase activity in a dose-dependent manner with the K_i value of 2.60 mM. Experiments using classical inhibitor of acid phosphatase, such as ammonium molybdate, as well as inhibitors of phosphotyrosine phosphatase, such as sodium orthovanadate and [potassiumbisperoxo(1,10-phenanthroline)oxovanadate(V)] (bpV-PHEN), inhibited the ecto-phosphatase activity, with the K_i values of 0.33 μM, 0.36 μM and 0.25 μM, respectively. Zinc chloride, another classical phosphotyrosine phosphatase inhibitor, also inhibited the ecto-phosphatase activity in a dose-dependent manner with K_i 2.62 mM. Zinc inhibition was reversed by incubation with reduced glutathione (GSH) and cysteine, but not serine, showing that cysteine residues are important for enzymatic activity. Promastigote growth in a medium supplemented with 1 mM sodium orthovanadate was completely inhibited as compared to the control medium. Taken together, these results suggest that *L. amazonensis* express a phosphohydrolase ectoenzyme with phosphotyrosine phosphatase activity.
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Index Descriptors and Abbreviations: *Leishmania amazonensis*; Ecto-phosphatase activity; Phosphotyrosine phosphatase

1. Introduction

Leishmaniasis is a parasitic disease caused by protozoa of the genus *Leishmania* affecting more than 12 million people worldwide (Desjeux, 2004). These parasites exist as motile flagellate promastigotes that live in the alimentary tract of blood-sucking sand flies and as nonflagellate intracellular amastigote mostly within the macrophages of mammalian hosts (Vannier-Santos et al., 2002). The severity of the disease produced by several *Leishmania* species varies widely, ranging from cutaneous and/or mucosal to visceral infection. *Leishmania amazonensis*, formerly described in the Amazon region, also occurs in many parts of Brazil and is the ethiological agent of cutaneous lesions or diffuse cutaneous (Grimaldi and Tesh, 1993), but it is noteworthy that *L. amazonensis* may cause the complete clinical spectrum of leishmaniasis, including the diffuse cutaneous and the visceral infection (Barral et al., 1991).

The plasma membrane of cells may display enzymes whose active sites face the external medium rather than the cytoplasm. The activities of these enzymes, referred to as ectoenzymes, can be measured by assaying intact cells (Fernandes et al., 2003; Lemos et al., 2002; Jesus et al., 2002; Meyer-Fernandes et al., 1997). Membrane-bound acid phosphatase activities have been characterized in some species of the family Trypanosomatidae, such as *Trypanosoma*

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spp. (Bakarala et al., 2000; Fernandes et al., 1997; Furuya et al., 1998; Meyer-Fernandes et al., 1999), *Herpetomonas muscarum muscarum* (Dutra et al., 1998), *Leishmania donovani* (Glew et al., 1982; Remaley et al., 1985) and in *Leishmania mexicana*, where this enzyme was purified (Menz et al., 1991; Wiese et al., 1996).

Protein phosphorylation comprises a major mechanism in the control of biological phenomena in most organisms. Phosphorylation–dephosphorylation of serine, threonine, and tyrosine residues triggers conformational changes that modulate protein biological properties (Hunter, 1995). The signaling regulation of the stimulus–response coupling during differentiation and proliferation is largely mediated by protein phosphorylation in eukaryotes (Hunter, 1995), including Trypanosomes (Parsons et al., 1993). In various tissues and cells, the presence of phosphotyrosyl protein phosphatases, which are also active toward low molecular weight, nonprotein phosphoesters such as alkyl and aryl phosphates, including *p*-nitrophenylphosphate (*p*-NPP), has been described (Lau et al., 1989). The presence of protein tyrosine phosphatase activities in *L. donovani* (Cool and Blum, 1993), *Trypanosoma brucei* (Bakarala et al., 2000; Fernandes et al., 2003) and *Trypanosoma cruzi* (Furuya et al., 1998; Meyer-Fernandes et al., 1999) has been demonstrated. Although the physiological role of the membrane-bound acid phosphatases has not been well established, they are supposed to be involved with nutrition (Gottlieb and Dwyer, 1981) escape (Gottlieb and Dwyer, 1981; Martiny et al., 1999; Remaley et al., 1985; Remaley et al., 1985; Saha et al., 1985), virulence (Furuya et al., 1998; Katakura and Kobayashi, 1988; Singla et al., 1992;), and cell differentiation (Meyer-Fernandes et al., 1999).

Recently, it has been shown the presence of a membrane-bound protein tyrosine phosphatase on the surface of *Leishmania major* (Aguirre-García et al., 2006). Here, we have characterized an ecto-phosphatase activity in *L. amazonensis* and demonstrated its modulation by compounds that can modify SH groups and specific inhibitors of protein tyrosine phosphatase ($ZnCl_2$, sodium orthovanadate, and bpV-PHEN). We also demonstrated the inhibitory effect of sodium orthovanadate in the cell proliferation.

2. Materials and methods

2.1. Microorganisms

The MHOM/BR/75/Josefa strain of *L. amazonensis* was used throughout this study. It was isolated from a human case of diffuse cutaneous leishmaniasis in Brazil and provided by Dr. Cuba-Cuba (Universidade de Brasília, Brazil) and has been maintained in our laboratory since then in axenic culture. Promastigotes were cultured in Warren's medium (Warren, 1960) supplemented with 10% heat-inactivated fetal bovine serum at 22 °C. Parasites were harvested at the stationary phase of growth by centrifugation, washed twice and kept in 116 mM NaCl, 5.4 mM KCl, 5.5 mM D-glucose, and 50.0 mM Hepes–Tris buffer, pH 7.2. Cellular viability was assessed before and after incubations, by motility and by trypan blue dye exclusion (Dutra et al., 1998). The viability was not affected under the conditions employed here.

2.2. Phosphatase measurements

The phosphatase activity was measured by the rate of *p*-nitrophenol (*p*-NP, *p*-NPP hydrolysis product), production. Intact cells were incubated for 1 h at 30 °C in 0.5 ml of reaction mixture containing 116.0 mM NaCl, 5.4 mM KCl, 5.5 mM glucose, 50.0 mM Hepes–Tris buffer, pH 7.0, 5.0 mM *p*-nitrophenylphosphate (*p*-NPP) as substrate and 2×10^7 cells ml^{-1}. The reaction was initiated by addition of cells and stopped by the addition of 1.0 ml 1 N NaOH. Controls, where cells were added after interruption of the reaction, were used as blank. For determining the concentration of released *p*-NP, the tubes were centrifuged at 1500*g* for 15 min and the supernatants were measured spectrophotometrically at 425 nm, using an extinction coefficient of $14.3 \times 10^3\ M^{-1}\ cm^{-1}$. We also tested phosphoamino acids as substrates. In this case, the hydrolytic activities were spectrophotometrically analyzed by measuring the released inorganic phosphate from these substrates, under the same condition employed above (Fiske and Subbarow, 1925). The values obtained for *p*-nitrophenylphosphatase activity measured using these methods were exactly the same. The values shown represent average ± SE of three independent experiments.

2.3. Inhibition assays

Phosphatase activity of intact cells of *L. amazonensis* was analyzed with specific inhibitors of protein tyrosine phosphatase ($ZnCl_2$, sodium orthovanadate and bpV-PHEN). We also analyzed the effect of ammonium molybdate (an inhibitor of acid phosphatase) and inorganic phosphate (the natural product of the phophatase activities). The phosphatase activity in the absence of inhibitors (25.70 ± 1.17 nmol Pi $\times\ h^{-1} \times 10^{-7}$ cells) was taken as 100%.

2.4. Reagents

All reagents were purchased from E. Merck (São Paulo, Brazil) or Sigma–Aldrich (Sigma Co. St. Louis, MO). Deionized distilled water from a MilliQ system of resins (Millipore Corp., Bedford, MA) was used in the preparation of all solutions including substrates and inhibitors.

2.5. Statistical analysis

The data were analyzed statistically by means of Stundent's *t*-test. *P* values of 0.05 or less were considered significant. Apparent K_m and V_{max} values were calculated using a computerized non-linear regression fit of the data to the Michaelis–Menten equation. K_i values for the inhibitors were calculated using a computerized non-linear regression

program (Sigma Plot 2000–Jandel Scientific Software, 1986–2000, San Diego, CA, USA) of the data.

3. Results

The time-course of phosphatase activity present on the external surface of *L. amazonensis* was linear for at least 1 h (Fig. 1A). Similarly, in assay to determine the influence of cell density on the ecto-phosphatase activity, it was observed that this activity was directly proportional to the number of cells (Fig. 1B). At pH 7.2, intact cells were able to hydrolyze *p*-NPP at a rate of 25.70 ± 1.17 nmol Pi × h^{-1} × 10^{-7} cells. To exclude the possibility that the observed *p*-NPP hydrolyzed was due to secreted soluble enzymes, cells were incubated in the absence of *p*-NPP. Subsequently, the suspension was centrifuged to remove cells and the supernatant was assayed for phosphatase activity. This supernatant failed to show *p*-NPP hydrolysis (data not shown). These data also rules out the possibility that the phosphatase activity here described could be derived from lysed *L. amazonensis* cells.

The dependence on *p*-NPP concentration showed a normal Michaelis–Menten kinetics for this phosphatase activity and values of V_{max} and apparent K_m for *p*-NPP were 31.93 ± 3.04 nmol Pi × h^{-1} × 10^{-7} cells and 1.78 ± 0.32 mM, respectively (Fig. 2). Different phosphatase inhibitors were tested and the results are shown in Fig. 3. The apparent K_i values for ammonium molybdate (Fig. 3A), sodium orthovanadate (Fig. 3B), bpV-PHEN (Fig. 3C), inorganic phosphate (Fig. 3D) and zinc chloride (Fig. 3E) were, respectively, 0.33 μM, 0.36 μM, 0.25 μM, 2.60 mM and 2.62 mM. Lineweaver–Burk plot analysis (Table 1) revealed that ammonium molybdate, sodium orthovanadate, bpV-PHEN and inorganic phosphate were competitive inhibitors while zinc chloride was a noncompetitive inhibitor. Living *L. amazonensis* were also able to hydrolyze *P*-tyrosine, *P*-serine and *P*-threonine at a rate of 10.01 ± 2.31, 6.58 ± 1,48 and 2.30 ± 0.26 nmol Pi × h^{-1} × 10^{-7} cells, respectively (Fig. 4). However, as *p*-NPP, only *P*-tyrosine hydrolysis was inhibited by sodium orthovanadate (Fig. 4).

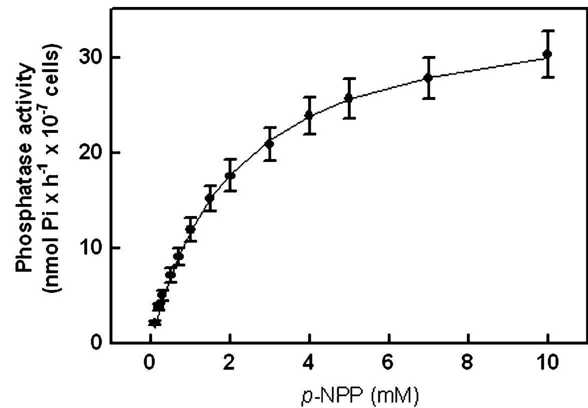

Fig. 2. Dependence of *p*-NPP concentration on the phosphatase activity of *L. amazonensis* intact cells. Cells were incubated for 1 h at 30 °C in the same reaction medium (final volume: 0.5 ml) described in Fig. 1, with different *p*-NPP concentrations. Curves represent the fit of experimental data by non-linear regression using the Michaelis–Menten equation as described in Section 2. The values represent means ± standard errors of at least ten independent experiments performed in triplicate.

These data could be indicating that the enzyme responsible for the hydrolysis of *p*-NPP and *P*-tyrosine would be a phoshotyrosine phosphatase, different from that responsible of the *P*-serine and *P*-threonine hydrolysis.

Reduced glutathione (GSH) and cysteine were also tested. Interestingly, both GSH (Fig. 5A) and cysteine (Fig. 5C) were able to revert the inhibition promoted by zinc, in a dose-dependent manner (Fig. 5B and D). However, serine, an amino acid that presents a hydroxyl group instead of a thiol group, did not exert such effect (Fig. 5E). We also examined the effect of 1 mM of sodium orthovanadate in the cell growth of *L. amazonensis*. As shown in Fig. 6, promastigote growth in a medium supplemented with orthovanadate was completely inhibited as compared to control.

4. Discussion

The protein tyrosine phosphatase (PTP) contains a signature sequence (I/V) HCXAGXXR(S/T)G, which includes

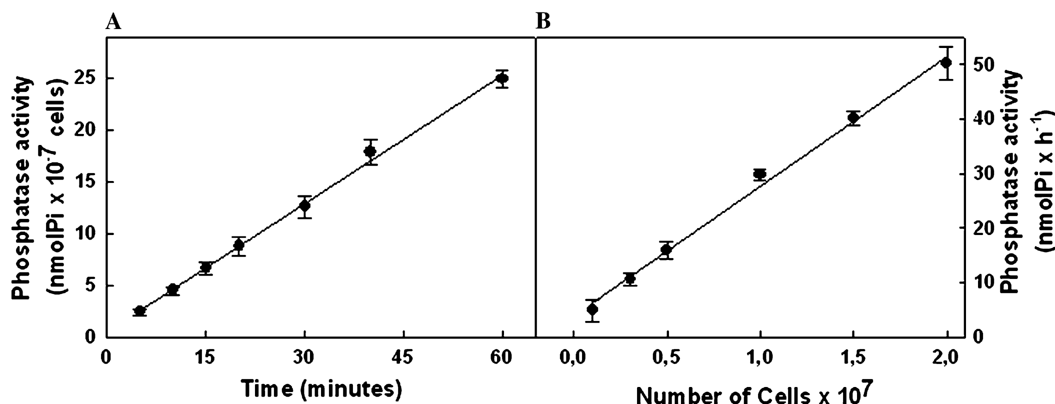

Fig. 1. Time-course (A) cell density-dependence (B) of ecto-phosphatase activity of *Leishmania amazonensis*. Intact parasite were assayed at 30 °C in the reaction medium (final volume: 0.5 ml) containing 116.0 mM NaCl, 5.4 mM KCl, 5.5 mM glucose, 50.0 mM Hepes–Tris buffer, pH 7.0, 5.0 mM *p*-NPP. The values represent means ± standard errors of at least five independent experiments performed in triplicate.

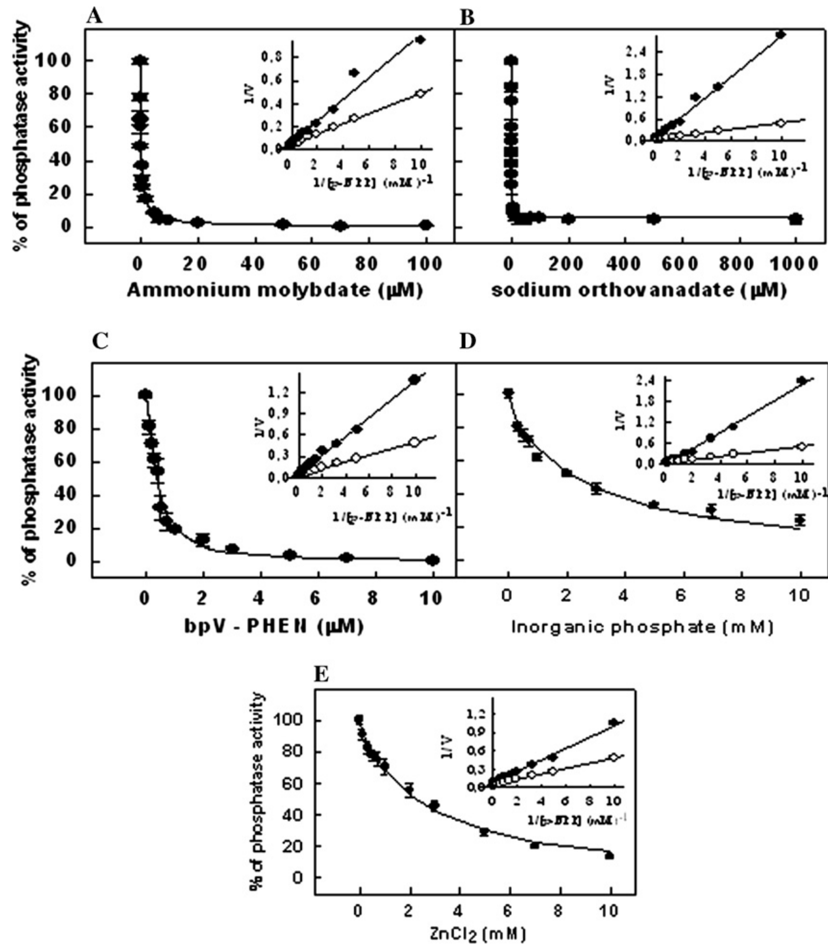

Fig. 3. Effects of different inhibitors on the phosphatase activity of *L. amazonensis* intact cells. Cells were incubated for 1 h at 30 °C in the same reaction medium (final volume: 0.5 ml) described in Fig. 1 with different concentrations of ammonium molybdate (A), sodium orthovanadate (B), bpV-PHEN (C), inorganic phosphate (D) and $ZnCl_2$ (E) as shown on the abscissa. The values expressed as relative activity represent the mean of at least three independent experiments, which were performed in triplicate. Curves represent the fit of experimental non-linear regression data as described in Section 2. Inset shows Lineweaver–Burk plot of the inhibition of *p*-nitrophenylphosphate hydrolysis by the different inhibitors. Cells were incubated as described above in the absence (open circles) or presence (closed circles) of 0.3 μM ammonium molybdate (A), 0.3 μM sodium orthovanadate (B), 0.3 μM bpV-PHEN (C), 2 mM inorganic phosphate (D) and 3 mM $ZnCl_2$ (E).

Table 1
Kinetic parameters of phosphatase inhibitors on the ecto-phosphatase activity of *L. amazonensis* intact cells

Inhibitors	Kinetic parameters	
	K_m (mM)	V_{max} (nmol Pi × h × 10^{-7} cells)
Control	1.78 ± 0.32	31.93 ± 3.04
Inorganic phosphate	3.92 ± 0.41*	27.96 ± 1.36
Ammonium molybdate	5.53 ± 0.75*	34.27 ± 2.16
Sodium orthovanadate	12.37 ± 2.06*	33.76 ± 3.76
bpV-PHEN	4.57 ± 0.04*	36.50 ± 1.19
$ZnCl_2$	1.53 ± 0.31	15.97 ± 0.14*

Cells were incubated as described in Section 2. The values represent means ± standard errors of at least three independent experiments, performed in triplicate. The Kinetic parameters of apparent K_m and V_{max} values were calculated using a computerized non-linear regression fit of the data to the Michaelis–Menten equation. *$p < 0.05$.

an essential cysteine residue that comprises the active nucleophilic site (Fischer et al., 1991). The cysteine active site is the key for the PTPs catalytic function. The cysteine residue may be regulated by reversible reduction/oxidation involv-

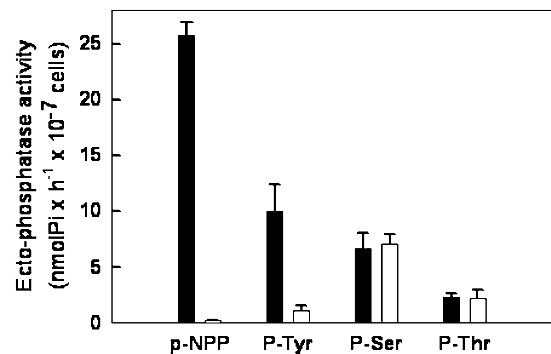

Fig. 4. Substrate specificity in the absence (closed bar) or in the presence of 1 mM sodium orthovanadate (hatched bar). Cells were incubated for 1 h at 30 °C in the same reaction medium (final volume: 0.5 ml) described in Fig. 1 with 5 mM of each substrate *p*-nitrophenylphosphate (*p*-NPP) or *P*-tyrosine (*P*-Tyr) or *P*-serine (*P*-Ser) or *P*-threonine (*P*-Thr). The data are means ± standard errors of three determinations with different cells suspensions.

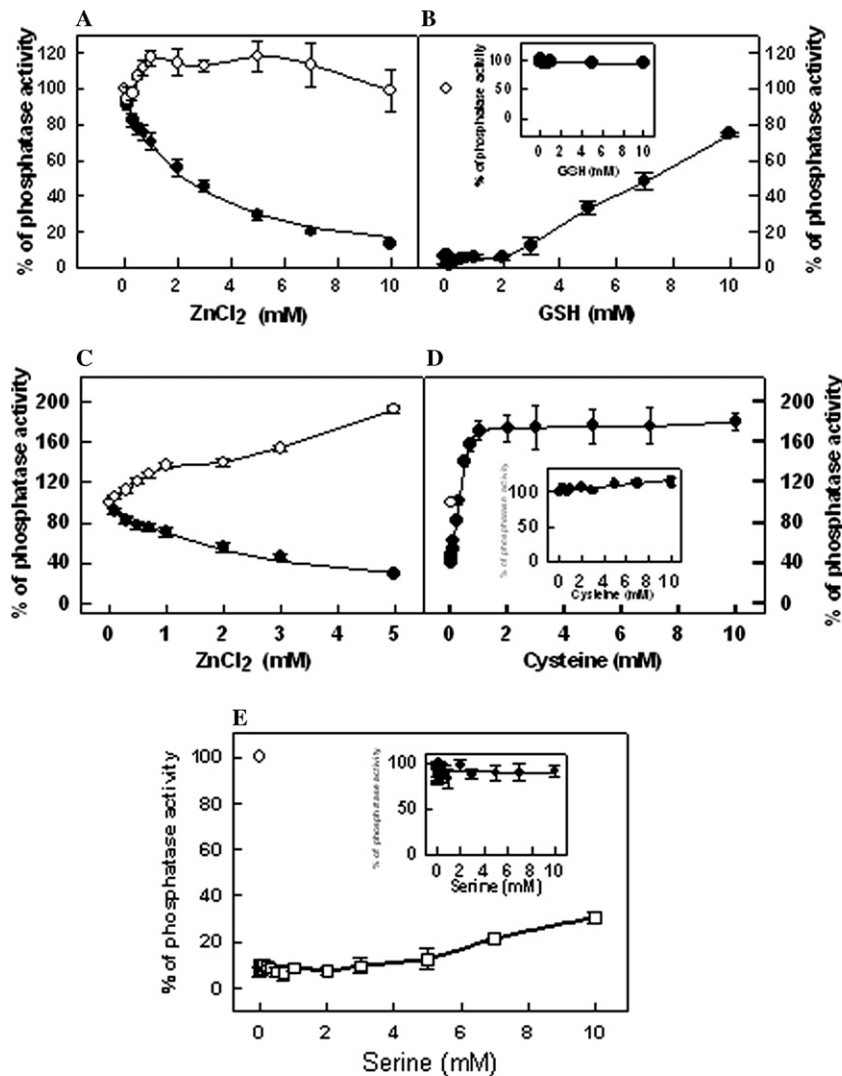

Fig. 5. Effects of reduced glutathione, cysteine and serine in the $ZnCl_2$ inhibition of the phosphatase activity of *L. amazonensis* intact cells. Cells were incubated as described above with different concentrations of $ZnCl_2$ (A and C), reduced glutathione (B), cysteine (D) and serine (E). (A) $ZnCl_2$ with (open circles) and without (closed circles) 10 mM of reduced glutathione; (B) $ZnCl_2$ with different concentrations of reduced glutathione (closed circles). Control was performed in the absence of 10 mM $ZnCl_2$ and reduced glutathione (open circle); (C) $ZnCl_2$ with (open circles) and without (closed circles) 10 mM cysteine; (D) $ZnCl_2$ with different concentrations of cysteine (closed circles). Control was performed in the absence of 10 mM $ZnCl_2$ and cysteine (open circle); (E) $ZnCl_2$ with different concentrations of serine (open squares). Control was performed in the absence of 10 mM $ZnCl_2$ and serine (open circle). Insets: Effects of different concentrations of reduced glutathione (B), cysteine (D) and serine (E) on the phosphatase activity of *L. amazonensis* intact cells. The values were expressed as mean percentual activities ± standard errors of at least three independent experiments, performed in triplicate.

ing cellular oxidants (Denu and Tanner, 1998). The study of the effect of metal ions on phosphatase activity such as zinc is interesting, because it has redox properties or tight binding to sulfhydryl groups. Zinc inhibition was protected dose-dependently by coincubation with reduced glutathione and cysteine, two thiol compounds capable to bind zinc (DeChatelet et al., 1971; Giroux and Henkin, 1972; Prasad and Oberleas, 1970), whereas serine, an amino acid that does not present the thiol group, did not exert such effect (Fig. 4). This result suggests a possible role for the thiol group in the mechanism of zinc inhibition. The inhibitory effect exerted by zinc may be through the coordination of some residues in a Zn^{2+} binding site (Kim et al., 2000).

Specific inhibitors of protein phosphatases were used for the characterization of the ecto-phosphatase present on the surface of *L. amazonensis*. This enzyme was strongly inhibited by ammonium molybdate (Fig. 3A), classical inhibitor of acid phosphatase (Dutra et al., 1998; Ferraro et al., 2004; Jesus et al., 2002; Lemos et al., 2002), and was also sensitive to sodium orthovanadate (Fig. 3B) and bpV-PHEN (Fig. 3C) two well-known specific phosphotyrosine protein phosphatase inhibitors (Cool and Blum, 1993; Furuya et al., 1998; Posner et al., 1994), suggesting that this enzyme would be a phosphotyrosine phosphatase, as *L. major* (Aguirre-García et al., 2006) and other parasitic protozoa (Bakarala et al., 2000; Fernandes et al., 2003; Furuya et al., 1998; Jesus et al., 2002; Meyer-Fernandes et al., 1999). Although intact promastigotes were able to hydrolyze *P*-tyrosine, *P*-serine and *P*-threonine, only the *P*-tyrosine hydrolysis was inhibited by vanadate. Vanadate is a

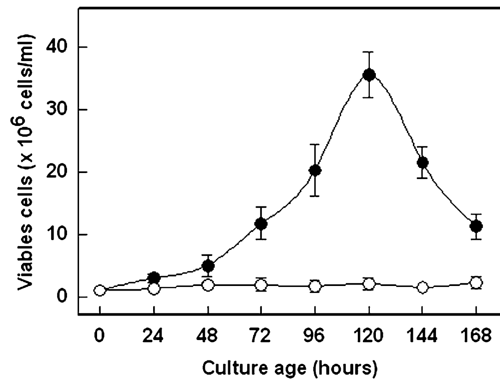

Fig. 6. Growth of *L. amazonensis* cultivated in Warren's medium at 22 °C as described in Section 2 for 7 days, in the absence (closed circles) or in the presence of 1 mM sodium orthovanadate (open circles). Values shown are means ± standard errors of three different experiments. In the control curve (absence of vanadate) the same volume of water (solvent of vanadate solution) was added to the growth medium.

phosphate analog and is generally thought to bind as a transition state analog to the phosphoryl transfer enzymes that it inhibits, since it can easily adopt a trigonal bipyramidal structure (Huyer et al., 1997), an analysis of sodium orthovanadate inhibition showed that it behaves as a competitive inhibitor (Fig. 3B, inset; Table 1), with a K_i of 0.36 μM similar to the one demonstrated in protein–tyrosine phosphatase 1B (PTP1B) (Huyer et al., 1997).

We have recently demonstrated that *Fonsecaea pedrosoi*, the principal causative agent of human chromoblastomycosis, uses a cell wall ecto-phosphatase, sensitive to vanadate, to interact with host epithelia and fibroblasts (Kneipp et al., 2004). Accordingly, we showed that the incubation of *L. amazonensis* promastigotes with sodium orthovanadate, increased the parasite adhesion to macrophage (Martiny et al., 1996). We have also demonstrated that *L. amazonensis* phosphatase activity can upregulate parasite attachment (Vannier-Santos et al., 1995) and promoted intracellular survival within macrophages (Martiny et al., 1996, 1999). The physiological role of the ecto-phosphatase in protozoa parasites is still largely unknown, but a possible involvement in the cell proliferation has also been proposed (Bernardes et al., 2000). The mechanism of inhibition promoted by ammonium molybdate and bpV-PHEN, was similar to demonstrated by sodium orthovanadate. This is not unexpected, considering the fact that like vanadate the ammonium molybdate and bpV-PHEN are phosphate structural analogs. The inorganic phosphate, product of the reaction, showed to be a competitive inhibitor (Fig. 3D, inset; Table 1), as observed in human liver acid phosphatase (Taga and Van Etten, 1982).

The ecto-phosphatase through phosphomonoester hydrolysis could supply inorganic phosphate to the parasite proliferation (Gottlieb and Dwyer, 1981; Hassan and Coombs, 1987). Accordingly, the addition of 1 mM orthovanadate to the culture medium inhibited completely the proliferation of *L. amazonensis.* It is important to note that vanadate can affect other orthophosphate-mediated processes (Klarlund, 1985), being a potent inhibitor of cation transport P-ATPases (Cunha et al., 1992; O'Neal et al., 1979). However, vanadate is not a potent inhibitor of these processes on intact cells as the oxidation–reduction reactions which take place in the cytoplasm diminish its inhibitory effect (Cantley and Aisen, 1979).

The precise role of ecto-phosphatases is not well established, but it has been related to cell growth, providing the cell with a source of nutrients, as well as protecting the parasite by preventing the protozoan digestion in the alimentary tract of the invertebrate host (Wiese et al., 1996). Its accessibility on cell surfaces and possible involvement in essential cellular functions suggest that the ecto-phosphatase might provide a promising target for chemotherapy.

Acknowledgments

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