Trypanosoma brucei: Ecto-Phosphatase Activity Present on the Surface of Intact Procyclic Forms

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The results presented in this paper indicate that procyclic forms of Trypanosoma brucei possess a phosphatase activity detected in the external cell surface able to hydrolyze about 0.7 nmol · mg^{-1} · min^{-1} *p*-nitrophenylphosphate. A faster rate of hydrolysis was observed when membrane-enriched fractions were used. This activity is weakly sensitive to 1 mM NaF, 10 mM tartrate and 10 mM levamizole but strongly inhibited by 0.1 mM vanadate. Inhibition by both NaF and vanadate have a competitive character. This phosphatase activity decreases by increasing the pH from 6.8 to 8.4, a pH range in which cell viability was maintained during at least 1 hour. In the membrane-enriched fractions this phosphatase activity showed to be an acid phosphatase. In addition, intact cells could catalyze the dephosphorylation of [^{32}P]phosphocasein phosphorylated at serine and threonine residues.

Introduction

Reversible phosphorylation of proteins is recognized to be a major mechanism for the control of intracellular events in eukaryotic cells. Phosphorylation-dephosphorylation of serine, threonine, and tyrosine residues triggers conformation changes in regulated proteins that alter their biological properties (Cohen, 1989; Walton and Dixon, 1993). In higher eukaryotes, regulation of the complex interactions required for differentiation and proliferation is mediated in part by protein phosphorylation (Parsons *et al.,* 1993). The African *Trypanosoma brucei* is a protozoan parasite that passes through several extracellular cycles through mammalian hosts and in the tsetse fly vector (Vickerman, 1986), with morphologically and biochemically distinct stages. Recently it has been demonstrated that *Trypanosoma brucei* possesses several protein kinases such as protein kinase C-like (Keith *et al.,* 1990), casein kinase-like (Aboagye-Kwartaeng *et al.,* 1991), a class of 90-kDa autophosphorylating serine/threonine protein kinases (Hide *et al.,* 1994) and tyrosine kinase (Wheeler-Alm and Shapiro, 1992). Recent data show that extensive phosphorylation of the largest subunit of RNA polymerase II is a conserved feature between trypanosomes and higher eukaryotes (Chapman and Agabian, 1994).

In *L. donovani,* acid phosphatase activity was suggested as a marker of virulence (Singla *et al.,* 1992). Cloning and characterization of a protein phosphatase in *L. chagasi* showed it to be conserved among *Leishmania* and a member of the four classes of eukaryotic serine/threonine protein phosphatases (Burns *et al.,* 1993). The presence of cytosolic and plasma-membrane-bound tyrosine phosphatase activities in *T. brucei* (Bakalara *et al.,* 1995a, 1995b) has been reported.

Since important regulatory mechanisms via protein phosphorylation were recently reported to occur in *T. brucei* (Parsons *et al.,* 1994), the objective of this work was to investigate the possible exis-

Abbreviations: CDTA, trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid; MEF, membrane-enriched fractions; PCMB, *p*-chloromercuribenzoate; pNP, *p*-nitrophenol; pNPP, p-nitrophenylphosphate; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazoly)]-benzene); PMSF, phenylmethanesulfonyl fluoride; Tris, tris (hydroxymethyl)aminomethane.

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tence of corresponding phosphatase activities in these cells. The data presented indicate that *T. brucei* procyclic forms contain ecto-phosphatase activity which is capable to hydrolyze phosphoamino acids and casein phosphorylated at serine and threonine residues.

Materials and Methods

Parasites

T. brucei brucei procyclic forms (ILTar 1 procyclics) were grown at 28 °C in SDM-79 medium (Cunningham, 1977) supplemented with hemin (7.5 mg/ml) and 10% heat-inactivated fetal bovine serum. Two to three days after inoculation, cells were collected by centrifugation, washed twice and kept in 50 mM Tris (tris (hydroxymethyl)aminomethane)-HCl pH 7.2, 20 mM KCl and 100 mM sucrose. Parasite membrane-enriched fractions were prepared by 3 cycles of freezing and thawing (-130/37 °C) in the same buffer. After centrifugation (12,000 x *g* for 20 min), the supernatant was discarded, the pellet was washed twice and ressuspended in the same buffer above with 0.2% Triton X-114. The enzyme activity of the membrane-enriched fractions was enriched by 5-fold when compared to the cells suspension. The protein concentration was determined by biuret assay.

Preparation of [^{32}P]-labeled casein

Phosphorylated [^{32}P]casein was prepared by mixing 5 mg/ml of previously dephosphorylated casein with casein kinase II obtained from *Rhodnius prolixus* oocytes (Silva-Neto and Oliveira, 1993). The following reaction medium was used: 50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 150 mM NaCl, 1 mM NaF, 1.2 mM EDTA, 1.2 mM EGTA, 100 μM [^{32}P]ATP (10^5 Bq / nmol). When phosphorylation was completed, aliquots were analyzed for incorporation of phosphate using the method of Sahal and Yamaguchi-Fujita (1987). The reaction mixture was then passed through spin columns, equilibrated in 20 mM Tris-HCl, pH 8.0 in order to remove remaining [^{32}P]ATP. ^{32}P-labeled casein was then adjusted to 4.6 mg/ml using the following buffer: 20 mM Tris-HCl pH 8.0, 15 mM NaCl, casein 10 mg/ml, 2 mM benzamidine and 100 μM PMSF.

p-Nitrophenylphosphate hydrolysis

Unless otherwise specified, pNPP hydrolysis was measured as follows: the reaction mixtures (0.5 ml) contained 10 mM pNPP, 50 mM Tris-HCl pH 7.2, 100 mM sucrose, 20 mM KCl and 1 mM CDTA to chelate divalent cations. Reactions were initiated by the addition of intact cells (1 mg of protein / ml which corresponds to 1.2 x 10^8 cells/ml) or membrane-enriched fraction (0.2 mg/ml) to the reaction mixtures, incubated at 28 °C with gentle shaking (40 oscillations/min) and terminated after 30/60 min by the addition of 1 ml of 1 N NaOH. After centrifugation at 1000 x *g* for 20 min the supernatant was used for determination of the *p*-nitrophenol released at 425 nm. Specific activity is expressed as nmol of *p*-nitrophenol released $\cdot min^{-1} \cdot mg^{-1}$ of protein. The liberation of *p*-nitrophenol was linear with time in the assay conditions used and was directly proportional to the enzyme concentration in all cases. A control without cells was used as a blank (the extinction coefficient for *p*-nitrophenolate ion is 1.75 x 10^4 M^{-1} cm^{-1}).

Phosphoamino acids hydrolysis

Hydrolysis of the phosphorylated amino acids was measured in the same conditions used for pNPP. The free phosphate released was determined in the end of reaction.

Phosphoprotein hydrolysis. Hydrolysis of ^{32}P-labeled casein was measured in the same condition used for pNPP hydrolysis except that pNPP was replaced by ^{32}P-labeled casein. The reactions were initiated by the addition of cells and terminated by the addition of 0.5 ml 20% trichloroacetic acid. The ^{32}Pi released was measured as a phosphomolybdate complex using a mixture of benzene and isobutyl alcohol (Vieyra *et al.*, 1985). The organic supernatant (0.5 ml) was added to 9 ml of scintillation liquid (2 g PPO, 1 g POPOP in 1 l toluene) and counted in a liquid scintillation counter.

Chemicals

All chemicals were obtained from Sigma Chemical Co., St. Louis, MO. SDM-79 was from J. R. H. Biosciences, Kansas City, KS.

Results

The time course of a phosphatase activity independent of divalent cations present on the external surface of intact *Trypanosoma brucei* procyclic forms (Fig. 1. panel A, line a) and on the membrane-enriched fractions (Fig. 1. A, line b) is linear until at least one hour. At pH 7.2 these intact cells were able to hydrolyze about 0.67 nmol pNPP · $mg^{-1} \cdot min^{-1}$. Membrane-enriched fractions were able to hydrolyze 3.2 and 9.3 $nmol \cdot mg^{-1} \cdot min^{-1}$ pNPP at pH 7.2 and 4.5, respectively, with a K_m of 0.36 ± 0.05 mM at pH 7.2 and 0.84 + 0.06 mM at pH 4.5. Cells were tested at stationary phase and at first, second and third inoculation day; no differences were observed in the activity intensity. In addition, this ecto-phosphatase activity, found in intact *T. brucei* procyclic forms, showed the ability to dephosphorylate the phosphocasein phosphorylated on serine and threonine residues (Fig. 1. panel B). These intact cells, in a medium free from divalent cations, were able to hydrolyze *o*-phospho-L-serine (1.86 nmol phosphate free · $mg^{-1} \cdot min^{-1}$), but did not hydrolyze *o*-phospho-L-tyrosine or *o*-phospho-L-threonine in an appreciable manner.

The detection of this phosphatase activity in intact cells suggested that it might be an ecto-phosphatase. A criterion used to confirm the integrity of the cells and the sideness of the phosphatase activity was the demonstration that the plasma membrane maintained its impermeability to ethidium bromide during the course of the experiments. As shown in Fig. 1.b (inset), the addition of the cells to a medium containing ethidium bromide, after they were preincubated during one hour under the experimental conditions of the phosphatase assays, was not followed by a fast fluorescence increase unless digitonin, a known plasma-membrane permeabilizing agent, was included in the medium (Vercesi *et al.*, 1991). The observation of parasite morphology and mobility by counting the viable cells in a Neubauer chamber, confirmed the integrity and viability of the cells. The possibility that a soluble phosphatase secreted by the cells could be responsible for the observed pNPP hydrolysis was tested by incubation of pNPP in a reaction medium in which the cells had been preincubated during 60 min. No pNPP hydrolysis could be detected in this medium after the cells were removed by centrifugation (data not shown).

As shown in Fig. 2, the membrane-enriched fractions treated with Triton X-114 showed that this is an acid phosphatase activity. In the pH range from 6.8 to 8.4, in which the cells were viable, the ecto-phosphatase activity decreased linearly (inset).

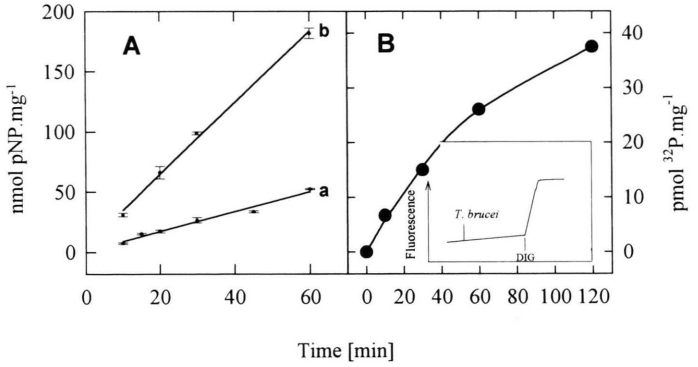

Fig. 1. Time course of phosphatase activity in *T. brucei* procyclic forms. Intact cells (panel A, line a) and membrane-enriched fractions (panel A, line b) were assayed in a reaction medium (0.5 ml) containing 50 mM Tris-HCl buffer pH 7.2 , 100 mM sucrose, 20 mM KCl, 1 mM CDTA, 10 mM pNPP and 1 mg/ml protein at 30 °C. For the time course of phosphoprotein hydrolysis by *T. brucei*, intact cells (panel B.), experimental conditions were identical to those used for pNPP hydrolysis with [^{32}P]casein used as substrate. The ^{32}Pi released was measured as indicated under Materials and Methods. **Inset:** *T. brucei* procyclic cells impermeability to ethidium bromide after incubation during one hour under the experimental conditions used for ecto-phosphatase assays. The cells (2 mg protein/ml) were incubated during 1 hour under the conditions of the phosphatase assays and the changes in ethidium bromide fluorescence upon its binding to DNA were followed at 365–580 nm. Ethidium bromide (EB, 100 mM) and 1.6 mM digitonin (DIG) were added where indicated by the arrows.

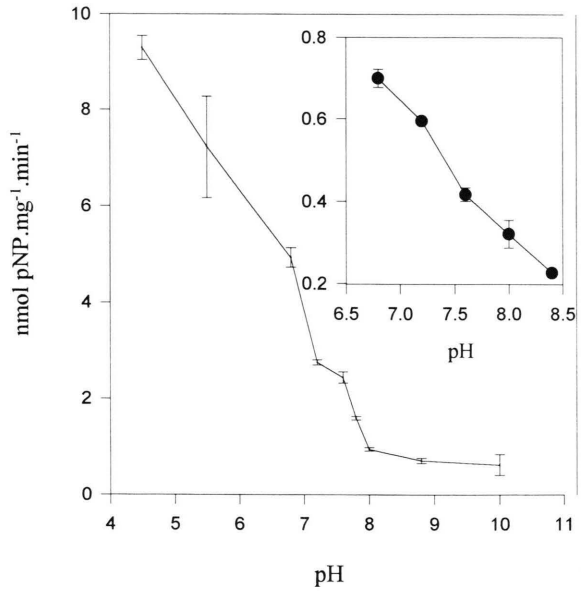

Fig. 2. Effect of pH on *T. brucei* ecto-phosphatase activity. Phosphatase activity in the membrane-enriched fractions was measured under the conditions of Fig. 1, panel A, line b, except that 50 mM sodium citrate and 50 mM sodium acetate were used for pH values of 4.5 and 5.5, respectively. Tris-HCl buffer was used for other pH values. Inset shows the effect of pH on phosphatase activity in the intact cells (pH 6.8 to 8.4).

Different phosphatase inhibitors were tested and Table I shows that NaF 0.1 and 1 mM has practically no effect on the activity, both in intact cells or in membrane-enriched fractions, but at 10 mM, NaF inhibits it by about 50% under all conditions. Only vanadate, a potent inhibitor of acid phosphatase and phosphotyrosine phosphatases (Chernoff and Li, 1985; Gordom, 1991; Cool and Blum, 1993; Hunter, 1995) strongly inhibited this ecto-phosphatase activity more effectively at pH 7.2 than at pH 4.5. Other phosphatase inhibitors such as levamizole and tetramizole (alkaline phosphatase), tartrate (secreted phosphatase (Lovelace and Gottlieb, 1986)) failed to inhibit this activity; levamizole and tetramizole at 10 mM showed stimulatory effects. PCMB, that is known to be inhibitory for SH-dependent enzymes, at concentrations of 0.1 and 1 mM, decreases the control activity to 50%. The inhibitions by vanadate and by NaF were dose-dependent; 50% inhibition of control activity being attained at a vanadate concentration of 0.5 μM (Fig. 3A.) and at a NaF concentration of 15 mM (Fig. 4A.). Both vanadate and NaF showed to be competitive inhibitors, with Ki values of

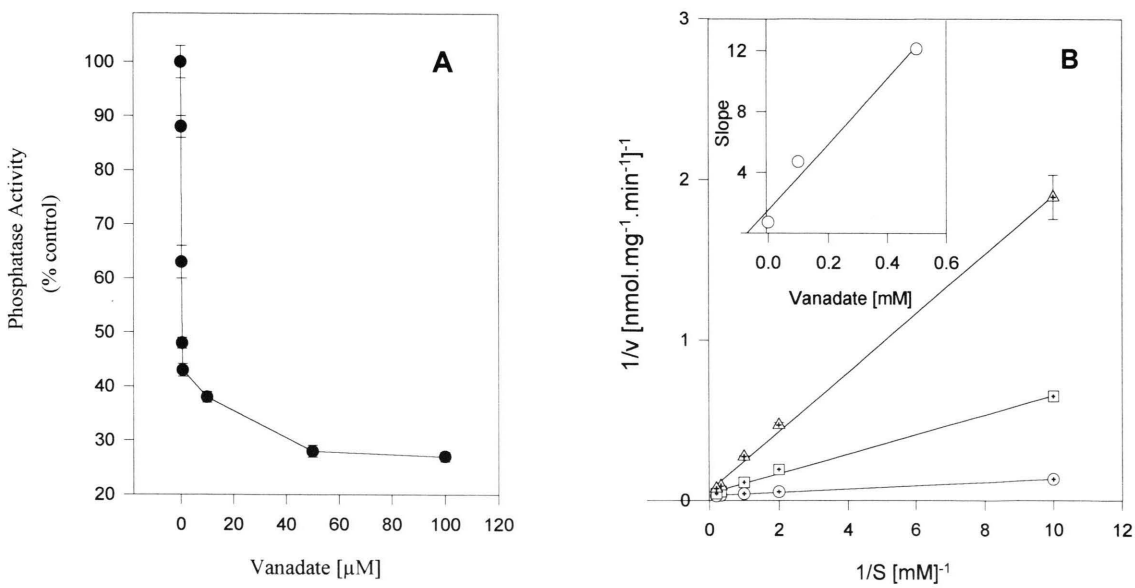

Fig. 3A. Inhibition of ecto-phosphatase activity in intact cells by sodium vanadate. The reaction was measured under the conditions of Fig. 1 (panel A, line a), intact cells. 100% activity corresponds to 0.7 nmol .mg^{-1}. min^{-1}.
Fig. 3B. Double reciprocal plot of the inhibition of p-nitrophenyl phosphate hydrolysis by sodium vanadate. Conditions of the assay are identical to those described for membrane-enriched fractions, pH 4.5 (Fig. 1, panel A), in the absence (○) or presence of 0.1 mM (□) or 0.5 mM (△) vanadate. Inset shows slope vs. [vanadate].

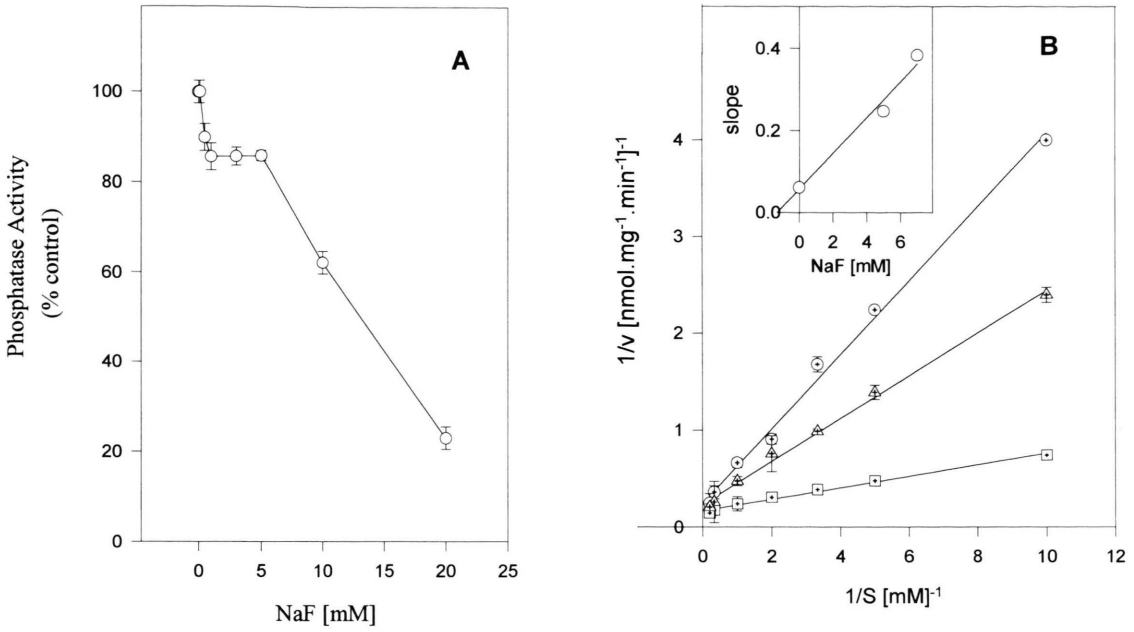

Fig. 4A. Inhibition of ecto-phosphatase activity in intact cells by sodium fluoride. The reaction was measured under the conditions of Fig. 1 (panel A, line a), intact cells. 100% activity corresponds to 0.7 nmol .mg^{-1}. min^{-1}.
Fig. 4B. Double reciprocal plot of the inhibition of p-nitrophenyl phosphate hydrolysis by sodium fluoride. Conditions of the assay are identical to those described for membrane-enriched fractions, pH 4.5 (Fig. 1, panel A),in the absence (□) or presence of 5 mM (△) or 7 mM (○) fluoride. Inset shows slope vs. [fluoride].

0.07 mM and 1.40 mM for vanadate and NaF, respectively. Both inhibition constants were determined by a replot of the slopes of Fig. 3B and 4B. against inhibitor concentrations (insets).

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 protein phosphatase activity is particularly interesting due to its possible role in cell-cell interaction or reception and transduction of external stimuli. Cellular response to extracellular stimuli (e.g., parasite adhesion) can evoke signaling pathways including protein phosphorylation/dephosphorylation. The external surface of *Leishmania* and *Trypanosoma cruzi* contains membrane-bound acid phosphatase activities (Remaley *et al.*, 1985; Nakagura *et al.*, 1985; Das *et al.*, 1986). It was recently shown that *Leishmania* acid phosphatases can regulate parasite binding to macrophages (Vannier-Santos *et al.*, 1995; Martiny *et al.*, 1996). Other protein phosphatases such as the so-called receptor protein tyrosine phosphatase (RPTP) were shown to have an important role in the process of homofilic cell-cell adhesion (Fischer *et al.*, 1991; Gebbink et al, 1993).

In the procyclic forms of *T. brucei* this is, to our knowledge, the first demonstration of an ecto-phosphatase with P-casein phosphatase activity. Although acid phosphatase activities have been described in *T. brucei* bloodstream forms (Steiger *et al.*, 1980; Schell *et al.*, 1990), in contrast to the phosphatase activity described here, one of them (the acid phosphatase activity present in the flagellar pocket) was tartrate-sensitive and their capacity to hydrolyze P-proteins were not investigated. Protein-serine/threonine-phosphatase activity in the cytosol of mammalian cells can be accounted for by four distinct enzymes which have been classified into two types (Ingerbritsen and Cohen, 1983). The cation independent enzyme type 1 is blocked by inhibitors 1 and 2 whereas types 2B and 2C have absolute requirement for divalent cations (Cohen, 1989). This indicates that

Table I. Effect of inhibitors on the phosphatase activity present on the surface of *T. brucei* procyclic forms.

Addition to the reaction medium:	% Activity		
	Intact cells	MEF[1]	
		pH 4.5	pH 7.2
None	100	100	100
NaF 0.1 mM	100	94	100
NaF 1 mM	60	90	100
NaF 10 mM	20	40	70
Levamizole[2] 1 mM	90	95	125
Levamizole[2] 10 mM	–	100	325
Tetramizole[3] 1 mM	60	95	155
Tetramizole[3] 10 mM	–	100	300
Tartrate 0.1 mM	–	90	100
Tartrate 1 mM	100	75	100
Tartrate 10 mM	–	65	85
PCMB 0.01 mM	–	90	120
PCMB 0.1 mM	–	45	40
PCMB 1 mM	50	45	40
Vanadate 0.001 mM	65	100	80
Vanadate 0.01 mM	40	90	10
Vanadate 0.1 mM	25	70	10
Vanadate 1 mM	10	20	10

The experimental conditions were identical to those of Fig. 1.
1- MEF is the membrane-enriched fraction.
Levamizole, (L[-]-2,3,5,6-tetrahydro-6-phenylimidazol[2,1-b]thiazole).
Tetramizole, ([±]-2,3,5,6-tetrahydro-6-phenylimidazol[2,1-b]thiazole).
The results represent the average of two experiments, performed in duplicate, with two different cell preparations. 100% activity corresponds to 0.7 nmol pNP. $mg^{-1}.min^{-1}$ in intact cells, 3.2 nmol $pNP.mg^{-1}.min^{-1}$ in MEF at pH 7.2 and 9.3 nmol $pNP.mg^{-1}.min^{-1}$ in MEF at pH 4.5.

the described phosphatase activity has similarity only with type 1 and type 2A. Various characteristics of this enzyme such as the similar time course for pNPP and ^{32}P-casein hydrolysis (Fig. 1) and high sensitivity to vanadate (Fig. 3), a potent inhibitor of phosphotyrosine phosphatase activities (Chernoff and Li, 1985; Gordom, 1991; Cool and Blum, 1993; Hunter, 1995) suggest that this enzyme present on the membrane surface of *T. brucei* procyclic forms has similarities with the tyr/ser protein phosphatase present in vaccinia virus (Guan *et al.*, 1991).

The results presented here are similar to those obtained with a tartrate-resistant cell surface acid phosphatase (ACP1) of *L. donovani* promastigotes, where this enzyme dephosphorylates several phosphoproteins phosphorylated on serine-residues but has a low activity on phosphohistone phosphorylated at tyrosine residues (Remaley *et al.*, 1985). A 42 kDa *Leishmania* phosphatase was recently shown to have remarkable structural and functional similarities with the P-casein phosphatase of 2C class protein phosphatases (Burns *et al.*, 1993). The ecto-phosphatase present on the surface of *T. brucei* procyclic forms, active within a range of physiological pH (Fig. 2, inset) and able to hydrolyze phosphoproteins (Fig. 1, panel B), may have physiological role in the regulation of the cyclical development of *T. brucei*, as protein kinases present in these cells do (Parsons *et al.*, 1993). On the other hand, a nonmetal-requiring form of phosphatase offers an opportunity to study the relation between a well studied chemical system, as the hydrolysis of phosphates in the absence of metal, and the enzymatic catalysis on the surface of parasites.

Regarding to the kinetic characteristics of the phosphatase activity described here, a tartrate sensitive phosphatase activity purified from culture supernatants of *L. donovani* promastigotes showed a very similar value of K_m (0.79 mM for pNPP) but Ki of 0.030 mM and 3.5 mM for NaF and vanadate, respectively (Lovelace *et al.*, 1986). Intact *T. cruzi* epimastigotes, at pH 7.2, showed a K_m of 22.2 mM towards pNPP; this activity being strongly inhibited by fluoride in the sub-cellular

fractions, both acid and alkaline activities (Letelier *et al.,* 1985). Interesting, the K_m value of *T. brucei* procyclic forms membrane-enriched fractions, toward pNPP, 0.36 at pH 7.2, is very similar to that described by Schell et al (1990) in the flagellar pocket from *T. brucei* bloodstream forms (0.4 mM pNPP, at pH 5.0), although part of this activity showed to be tartrate-sensitive. Tartrate sensitive and resistant phosphatase activities were also described for *T. rhodesiense* (McLaughlin, 1986), however the km values toward pNPP (approximately 2.0) were much higher than we obtained for the phosphatase described here.

With respect to the effects of vanadate and fluoride, it must be stressed that micromolar concentrations of vanadate, described as selective phosphotyrosyl phosphatase inhibitor (Chernoff and Li, 1985; Gordom, 1991; Cool and Blum, 1993; Hunter, 1995), decreases the presented *T. brucei* procyclic forms phosphatase activity although we did not find hydrolytic activity toward phosphoaminoacid phosphotyrosine. Fluoride is a putative inhibitor of mammalian phosphoseryl phosphatases (Antoniw and Cohen, 1976), however there have been exceptions since several phosphotyrosine protein phosphatases, associated with acid activity, were unusually sensitive to fluoride inhibition (Boivin and Galand, 1986; Tamura *et al.,* 1986). The reason for the different sensitivities toward both of these inhibitors remains unclear. It is possible that the supposed selective action of vanadate and fluoride depends on the catalytic mechanism of the enzymes, substrate specificity and association with possible specific regulatory subunits. In this work, both inhibitors showed to compete with pNPP for the active site of the enzyme(s). Experiments are in progress to characterize a similar ectophosphatase in bloodstream forms of *T. brucei.*

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