CrATP as a new inhibitor of ecto-ATPases of trypanosomatids

O. C. MOREIRA, P. F. RIOS, F. F. ESTEVES, J. R. MEYER-FERNANDES *and* H. BARRABIN*

Instituto de Bioquímica Médica, Programa de Biologia Estrutural, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro Rio de Janeiro, Brasil

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SUMMARY

Trypanosomatid protozoa include heteroxenic species some of them pathogenic for men, animals and plants. Parasite membrane contains ecto-enzymes whose active sites face the external medium rather than the cytoplasm. *Herpetomonas* sp. displayed a Mg^{2+}-dependent ecto-ATPase activity, a Mg-independent ecto-ADPase and an ecto-phosphatase activity. Both, the ecto-ADPase and phosphatase activities were insensitive to CrATP (chromium(III) adenosine 5′-triphosphate complex). Ecto-ATPase activity was reversibly inhibited. At 2 mM ATP the apparent Ki was $4{\cdot}7 \pm 1{\cdot}0\ \mu$M but a fraction of about 40–50% was insensitive to CrATP. Remarkably, at low substrate concentration (0·2 mM) more than 90% of the ecto-ATPase was inhibited with $Ki = 0{\cdot}33 \pm 0{\cdot}10\ \mu$M. These parameter dependences are interpreted as the presence of 2 ecto-ATPases activities, one of them with high ATP apparent affinity and sensitivity to CrATP. DIDS (4,4 diisothiocyanatostilbene 2,2′ disulfonic acid), suramin and ADP were also effective as inhibitors. Only ADP presented no additive inhibition with CrATP. The pattern of partial inhibition by CrATP was also observed for the ecto-ATPase activities of *Leishmania amazonensis*, *Trypanosoma cruzi* and *Trypanosoma rangeli*. CrATP emerges as a new inhibitor of ecto-ATPases and as a tool for a better understanding of properties and role of ecto-ATPases in the biology of parasites.

Key words: ecto-ATPase, *Herpetomonas*, CrATP, trypanosomatids, ecto-phosphatase.

INTRODUCTION

Trypanosomatid protozoa include heteroxenic species that are pathogenic for men (e.g. disease-causing parasites such as *Leishmania* and *Trypanosoma*), pathogenic for plants (genus *Phytomonas* and some *Herpetomonas* spp.) and non-pathogenic species that develop strictly in the insect midgut (e.g. *Crithidia*).

Over 20 different species of the genus *Leishmania* are known to be pathogenic for humans. It is estimated that more than 2 million new cases of leishmaniases arise each year (Desjeux, 2004). Chagas disease is widespread throughout Latin America, where nearly 20 million people are infected by *Trypanosoma cruzi* and 90 million are at risk in endemic areas (World Health Organization, 2002).

Infection of plants with trypanosomatids has been known since 1909, when Lafont (1909) described these flagellates in laticiferous plants. The genus *Phytomonas* and some *Herpetomonas* parasitize plants without apparent pathogenicity but they can also cause diseases of economic significance in plantations of coconut, oil palm, cassava and coffee (Dollet, 1984; Camargo *et al*. 1990; Redman *et al*. 1995; Camargo, 1999; Catarino *et al*. 2001). These trypanosomatids have been detected in various edible fruits, such as guavas, peaches, pomegranates and tangerines and in their insect vectors (Redman *et al*. 1995). The parasites live mostly in the xylem and phloem of the infected plants and are transmitted through the bite of phytophagous insects (Dollet, 1984; Camargo *et al*. 1990; Redman *et al*. 1995; Camargo, 1999; Catarino *et al*. 2001). In the biological cycle of these pathogens, several plant-sucking insects act as intermediate hosts and the plant acts as the main host (Jankevicius *et al*. 1989). The promastigotes form predominates in both axenic cultures and in isolates from plants and insects and shows ultrastructural features typical of the family Trypanosomatidae, containing kinetoplast, glycosomes, endoplasmic reticulum, acidocalcisomes and a single tubular mitochondrion (Attias *et al*. 1988; Freymuller *et al*. 1990; Redman *et al*. 1995; Soares de Medeiros *et al*. 2005).

Surface-mediated interactions between any of these parasites and the media environment are of critical importance from the physiological point of view. Parasite membrane contains enzymes whose active sites face the external medium rather than the cytoplasm. The activities of these enzymes, referred to as ecto-enzymes, can be measured using intact cells (De Pierre and Karnowsky, 1973, 1974; Furuya *et al*. 1998; Meyer-Fernandes, 2002).

* Corresponding author: Instituto de Bioquímica Médica, CCS, Universidade Federal do Rio de Janeiro, Cidade Universitária, CEP 21941-590, Rio de Janeiro, RJ, Brazil. Fax: +55 21 2270 8647. E-mail: barrabin@bioqmed.ufrj.br

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The ecto-NTPases (EC 3.6.1) were referred to usually as ecto-nucleoside triphosphate diphosphohydrolases 2 (ecto-NTDPase 2) type or ecto-ATPases (E-type ATPases) since they hydrolyse ATP better than other trinucleotides while ADP is hardly hydrolysed. Cell membrane ecto-ATPases are integral membrane glycoproteins, millimolar divalent cation-dependent, that hydrolyse all nucleoside triphosphates (Plesner, 1995; Kirley, 1997; Meyer-Fernandes *et al*. 1997). The ecto-apyrases (EC 3.6.1.5) or NTDPases 1, on the other hand, can hydrolyse ADP to AMP + Pi as efficiently as ATP to ADP + Pi. Although both enzymes exhibit a slightly different sensitivity to inhibitors (Plesner, 1995; Heine *et al*. 1999), they share close sequence homology (Handa and Guidotti, 1996). For a review about the identity and the function of mammalian ecto-NTDPases see Robson *et al*. (2006). Both enzymes are unaffected by the ecto-phosphatase inhibitors (Meyer-Fernandes, 2002). It has been reported that the ecto-ATPases are sensitive to dicyclohexylcarbodiimide (DCCD) and suramin, which are compounds that do not cross the cellular membrane.

Ecto-ATPases have been described in several protozoa such as *Toxoplasma gondii* (Asai and Suzuki, 1990; Bermudes *et al*. 1994; Asai *et al*. 1995; Nakaar *et al*. 1998), *Entamoeba histolytica* (Bakker-Grunwald and Parduhn, 1993; Barros *et al*. 2000), *Tetrahymena thermophila* (Smith *et al*. 1997), *Leishmania tropica* (Meyer-Fernandes *et al*. 1997), *L. amazonensis* (Berredo-Pinho *et al*. 2001), *Trypanosoma cruzi* (Bernardes *et al*. 2001; Bisaggio *et al*. 2003; Meyer-Fernandes *et al*. 2004), *Herpetomonas muscarum* (Alves-Ferreira *et al*. 2003), *Tritrichomonas foetus* (de Jesus *et al*. 2002), *Crithidia deanei* (dos Passo Lemos *et al*. 2002).

In mammals, the ecto-ATPases seem to have multiple roles in extracellular nucleotide metabolism and in the regulation of nucleotide-based intercellular signalling (Plesner, 1995; Robson *et al*. 2006). However, their physiological roles in protozoa parasites are poorly understood. Experimental evidence suggests their involvement in the adenosine acquisition mechanisms and in the mechanism of parasite infection (for a review see Meyer-Fernandes, 2002).

The bidentate complex of ATP with Cr^{3+}, CrATP, is an MgATP analogue that is known to form a stable complex with P-ATPases provoking their inhibition. This mechanism has been described for Na^+,K^+-ATPase (Pauls *et al*. 1980; Linnertz *et al*. 1995) sarcoplasmic reticulum Ca^{2+}-ATPase (Vilsen and Andersen, 1992; Vilsen, 1995; Einholm *et al*. 2004) and the PMCA (Moreira *et al*. 2005). CrATP has also been shown to inhibit other enzymes that have ATP as a substrate, like phosphoglycerate kinase (Serpersu *et al*. 1992), fructokinase (Raushel and Cleland, 1977), ADP-glucose pyrophosphorylase (Binderup *et al*. 2000) and hexokinase (Danenberg and Cleland, 1975).

The molecular diversity and cellular functions of ecto-NTDPases in protozoa parasites are still an open question (Zimmermann, 1999). In this regard, the development of specific enzyme inhibitors promises to offer important tools for basic research and therapy. Here we show that CrATP is an efficient inhibitor of the ecto-ATPase from *Herpetomonas* sp. and other pathogenic trypanosomatids, but does not inhibit either the Mg-independent ADPase or the *p*-nitrophenylphosphatase activities. Data suggest that the ecto-ATPase in *Herpetomonas* is not a single enzyme but the mixture of 2 different isozymes, only one of them being sensitive to CrATP.

MATERIALS AND METHODS

Reagents

All reagents were purchased from E. Merck (Darmstadt, Germany) or Sigma Chemical Co. (St Louis, MO). [γ-^{32}P]ATP was synthesized as described by Walseth and Johnson (1979).

CrATP synthesis

CrATP was synthesized from the sodium salt of ATP and $CrCl_3$, as described by De Phamphilis and Cleland (1973). The bidentate isomer was isolated through a cation-exchange column (Dowex 50-H^+ resin) and the product confirmed by the absorption spectrum between 400 and 800 nm (De Phamphilis and Cleland, 1973) and quantified by its absorption at 610 nm ($\varepsilon_{610\ nm} = 20\ M^{-1}.\ cm^{-1}$). The final product is free of contaminating Cr^{3+} since addition of 1 mM citrate (an efficient Cr^{3+} chelator) to the reaction medium does not modify the inhibition by CrATP (not shown).

Culture methods

Herpetomonas sp. promastigotes (CT.IOC 13; kindly provided by Dr Reginaldo Brazil, Instituto Rene Rachour, FIOCRUZ, MG, Brazil) were grown at 25 °C in a medium containing 20 g/l sucrose, 20 g/l KCl, 3 g/l yeast extract, 3 g/l peptone, 1 mg/l folic acid, supplemented with 10 mg/l haemin and 10% (v/v) fetal bovine serum. At 2 days after inoculation, cells were harvested by centrifugation, washed twice with 5 mM phosphate buffer, pH 7·0, containing 150 mM NaCl (PBS) and resuspended in the same solution. *Trypanosoma rangeli* (Macias strain, kindly supplied by Dr Wanderley De Souza, UFRJ, Brasil) was used throughout this study. The parasites were grown in liver infusion tryptose medium supplemented with 20% fetal calf serum at 28 °C. Five days after inoculation, long epimastigotes, predominantly (>98%), were collected by centrifugation, washed twice and kept in 50 mM Tris-HCl buffer, pH 7·2, 100 mM sucrose and 20 mM KCl and resuspended in

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the same solution. *Trypanosoma cruzi* (Y strain) were grown in liver infusion tryptose medium supplemented with 10% fetal calf serum at 28 °C. Seven days after inoculation, the parasites were harvested by centrifugation, washed twice with 0·9% saline and once with 30 mM Tris-HCl/75 mM sucrose buffer, pH 6·8, and kept in the same buffer before the assays. The MHOM/BR/75/Josefa strain of *Leishmania amazonensis* was used throughout this study. It was isolated from a human case of diffuse cutaneous leishmaniasis in Brasil and provided by Dr Cuba-Cuba (Universidade de Brasilia, Distrito Federal, Brasil) and has been maintained in our laboratory in axênica culture. Promastigotes were cultured in Warren's medium supplemented with 10% heat-inactivated fetal calf serum at 22 °C. Five days after inoculation, promastigotes were collected by centrifugation, washed twice and kept in 116 mM NaCl, 5·4 mM KCl, 5·5 mM D-glucose, and 50 mM Hepes-Tris (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid adjusted with hydroxymethyl aminomethane) buffer, pH 7·2, and kept in the same buffer before the assays. For all parasites the cell number was measured in a Neubauer chamber. Cellular viability was assessed before and after incubation using motility and trypan blue dye exclusion assessments.

Ecto-ATPase activity

All intact parasites were incubated for 1 h at 30 °C in 500 μl of a mixture (standard medium) containing, unless otherwise specified, 116 mM NaCl, 5·4 mM KCl, 5·5 mM D-glucose, 50 mM Hepes-Tris buffer (pH 7·2), 2 mM ATP and $1{\cdot}0 \times 10^8$ cells/ml in the absence or presence of 2 mM $MgCl_2$. Mg^{2+}-dependent ecto-ATPase activity was calculated from the total activity, measured in the presence of 5 mM $MgCl_2$, minus the basal activity, measured in the absence of $MgCl_2$. ATPase activity was determined by measuring the hydrolysis of [γ-^{32}P]ATP (10^4 Bq/nmol ATP).

The experiments were started by the addition of living cells and stopped by the addition of 1 ml of a cold mixture containing 200 mg charcoal in 1 M HCl. The tubes were centrifuged at 1500 ***g*** for 10 min at 4 °C. Aliquots (500 μl) of the supernatants containing the released inorganic phosphate (^{32}Pi) were deposited on filtering paper and, after drying, transferred to scintillation vials containing 9 ml of scintillation fluid (2 g 2,5-diphenyloxazole (PPO) in 1 l of toluene). ATPase activity was calculated by subtracting the non-specific ATP hydrolysis measured in the absence of cells. ATP hydrolysis was linear with time under the assay conditions used and was proportional to cell numbers for all parasites.

Alternatively, using ADP or ATP as substrates, the hydrolytic activities, under the same conditions as described above, were assayed spectrophotometrically by measuring the release of Pi from the nucleotides (Lowry and Lopes, 1946). The values obtained for ATPase activities measured using both methods (spectrophotometric and radioactive) were exactly the same.

E-Phosphatase activity. In addition to the measurements of ecto-ATPase activity, ecto-para-nitrophenylphosphatase activity was determined in the same medium as that for ATP hydrolysis, except that ATP was replaced by 5 mM p-nitrophenylphosphate. The reaction was determined spectrophotometrically at 425 nm, using a molar extinction coeficient of $\varepsilon = 14{\cdot}3 \times 10^3\ M^{-1}.cm^{-1}$.

RESULTS

As described for other trypanosomatids, *Herpetomonas* sp. presented a Mg^{2+}-dependent ecto-ATPase activity on its external surface (Fig. 1). In the intact parasites, whose viability was assessed before and after the reactions by motility and by trypan blue dye exclusion, ATP hydrolysis was low ($4{\cdot}5 \pm 0{\cdot}9$ nmol Pi/h/10^8 cells) in the absence of any divalent metal (addition of 1 mM ethylenediaminetetraacetic acid (EDTA)). At pH 7·2, the addition of 2 mM $MgCl_2$ stimulated the ATP hydrolysis up to $80{\cdot}2 \pm 3{\cdot}0$ nmol Pi/h/10^8 cells, resulting in a Mg^{2+}-dependent ecto-ATPase activity of $75{\cdot}7 \pm 3{\cdot}1$ nmol Pi/h/10^8 cells. The time-course of ATP hydrolysis by the *Herpetomonas* sp. ecto-ATPase was linear for at least 60 min (Fig. 1A). Similarly, in assays to determine the influence of cell density, Pi formation was linear over a nearly 10-fold range of cell density (Fig. 1B). To check the possibility that the observed ATP hydrolysis was the result of secreted soluble enzymes, as seen in other parasites, we prepared a reaction mixture with cells that were incubated in the absence of ATP. Subsequently, the suspension was centrifuged to remove cells and the supernatant was checked for ATPase activity. This supernatant failed to show ATP hydrolysis either in the absence or in the presence of $MgCl_2$ (data not shown). These data also rule out the possibility that the ATPase activity here described could be from lysed *Herpetomonas* sp. cells.

Addition of CrATP to the hydrolytic media partially inhibited the Mg^{2+}-dependent ecto-ATPase activity with a Ki of $4{\cdot}8 \pm 1{\cdot}0$ μM (Fig. 2A). The inhibition was not affected by pre-incubation of the cells for 1 h with CrATP (not shown). Furthermore, washing the cells after 1 h exposure with 200 μM CrATP fully restored the ecto-ATPase activity, indicating that the inhibition was reversible. The Mg^{2+}-independent ecto-ATPase activity was insensitive to CrATP (Fig. 2A). Tests performed with 1 mM CrATP, in the absence of other nucleotides, showed that this analogue was not hydrolysed by the ecto-enzymes of *Herpetomonas* sp. (not shown).

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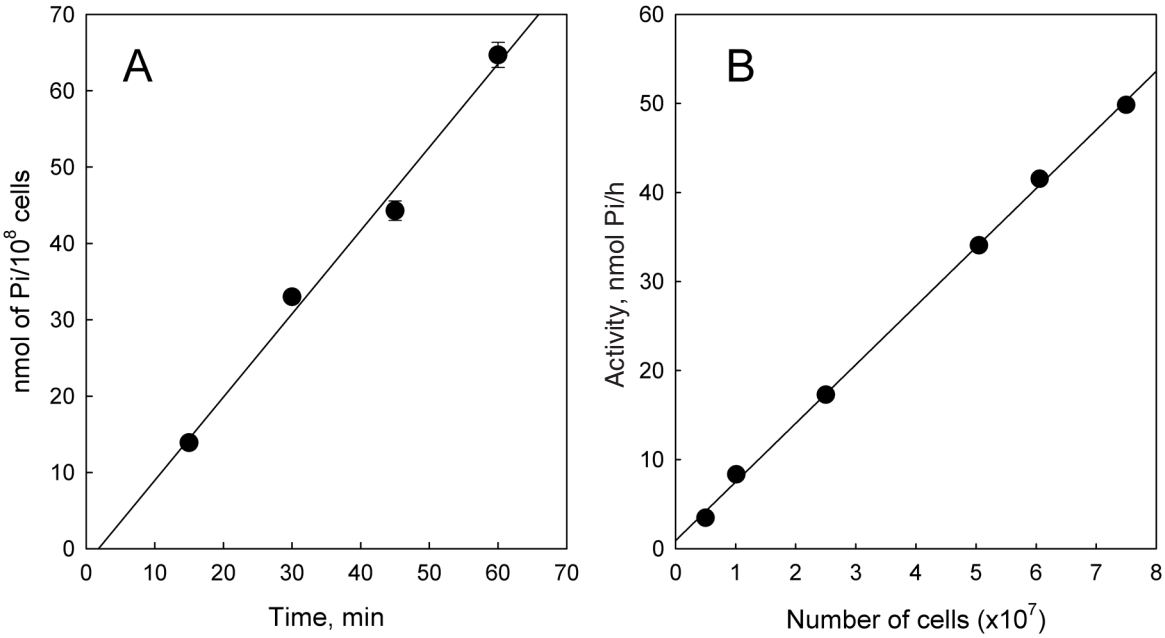

Fig. 1. Time-course (A) and effect of cell density (B) on the ecto-ATPase activity by intact cells of *Herpetomonas* sp. Cells were incubated for different periods of time (A) or for a fixed period of 1 h (B) at 37 °C. The reaction medium contained 50 mM Tris–HCl buffer, pH 7·2, 2 mM Tris–ATP [γ-32P]ATP (sp. act 10^4 Bq/nmol ATP), 2 mM $MgCl_2$ and 1 mg/ml of protein which corresponds to $2{\cdot}2 \times 10^8$ cells/ml. Data are means ± S.D. of 3 determinations with different cell suspensions.

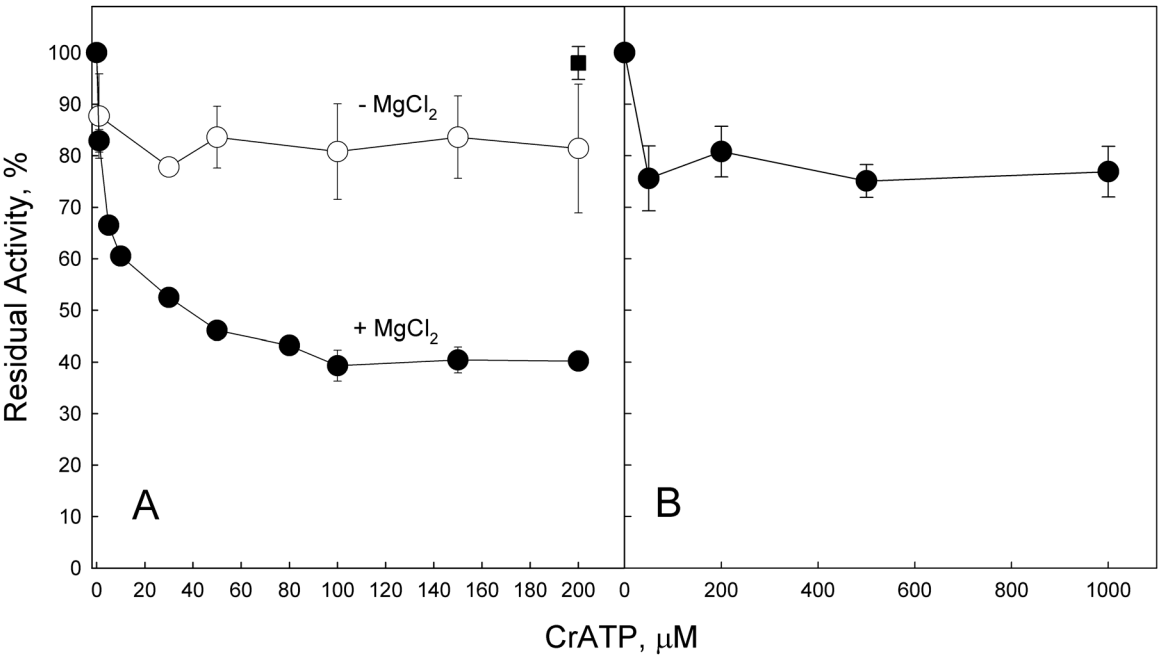

Fig. 2. Effect of increasing concentrations of CrATP on the ecto-ATPase (A) and phosphatase (B) activities of *Herpetomonas* sp. (A) Intact cells were incubated for 1 h at room temperature, in a medium containing 50 mM Hepes-KOH buffer, pH 7·2, 65 mM KCl, 100 mM sucrose, 2 mM [γ-32P]ATP (4000 cpm/nmol), in the absence (○) or presence (●) of 2 mM $MgCl_2$. In the case of absence of $MgCl_2$, 300 μM EDTA was added. Data are means ± S.E. of 3 determinations with different cell suspensions. The black square shows the reversibility of ecto-ATPase inhibition. Cells were incubated at room temperature with 200 μM CrATP as above and washed by centrifugation. Ecto-ATPase activity was assayed with 2 mM [γ-32P]ATP. Values representing 100% activities were $4{\cdot}90 \pm 0{\cdot}04$ (○) and $79{\cdot}5 \pm 15$ (●) nmol Pi/10^8 cel × h, respectively. (B) Intact cells were incubated for 1 h, at room temperature, in medium containing 50 mM Hepes-KOH buffer, pH 7·2, 65 mM KCl, 100 mM sucrose, 2 mM $MgCl_2$ and increasing concentrations of CrATP, in the presence of 5 mM *p*-nitrophenylphosphate (*p*NPP). Data are means ± S.E. of 3 determinations with different cell suspensions. 100% activity was 94 ± 10 nmol pNP/10^8 cel × h.

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Several ecto-enzymes were described in trypanosomatids that can hydrolyse phosphorylated compounds: ecto-ATPase (NTPDase1), ecto-nucleoside triphosphate diphosphohydrolases (NTPDase2) and ecto-phosphatase. To check if these enzymes were affected by CrATP different substrates were used. The ecto-phosphatase activity, followed by pNPP hydrolysis, was only slightly inhibited (10%) by the ATP analogue (Fig. 2B). The NTPDases2 are enzymes that can hydrolyse ATP to ADP as well as ADP to AMP. The *Herpetomonas* sp. cells were able to hydrolyse ADP at low levels; however, this activity was not stimulated by Mg^{2+} so it must not be a truly NTPDase2. Futhermore, the CrATP did not inhibit the Mg^{2+} independent ADP hydrolysis at concentrations able to cause maximal inhibition of ecto-ATPase (results not shown).

The curve of inhibition of ecto-ATPase at a 10-fold lower ATP concentration (0·2 mM) gives a Ki approximately 10 times lower ($0{\cdot}33 \pm 0{\cdot}04\ \mu$M) suggesting a competitive mechanism (Fig. 3A). Curiously, the degree of maximal inhibition increased with the decrease of substrate concentration. This effect is better described in Fig. 3B, where the full ecto-ATPase activities, the maximally inhibited (residual) and the CrATP sensitive activities measured at different ATP concentrations were plotted. With the increase in the ATP concentration appeared a CrATP-resistant ecto-ATPase activity that seemed to have Km = $3{\cdot}49 \pm 0{\cdot}19$ mM and Vmax = $115{\cdot}6 \pm 3{\cdot}2$ nmol Pi/10^8 cel × h. The CrATP sensitive activity seemed to have a much higher apparent affinity for ATP (Km = $85 \pm 39\ \mu$M) and a lower maximal velocity than the insensitive one (Vmax = $39{\cdot}2 \pm 3{\cdot}9$ nmol Pi/10^8 cel × h).

DIDS and suramin are impermeable compounds that had been used as inhibitors of ecto-ATPases of trypanosomatids (Bisaggio *et al*. 2003). The ecto-ATPase activity of *Herpetomonas* sp. was effectively inhibited by these two polyanions (Fig. 4A and B). The apparent Ki's were $19{\cdot}0 \pm 5{\cdot}1$ and $15{\cdot}2 \pm 7{\cdot}6\ \mu$M respectively. It should be noted that inhibition by DIDS was also partial and higher at 0·2 mM (82%) than at 2 mM ATP (42%).

Bearing in mind that the compounds used here have partial inhibitory effects and taking into consideration the possibility of having more than one enzyme with different inhibitor sensitivities, the maximal inhibitory effects of associations of CrATP + DIDS or CrATP + suramin were measured (Fig. 5). Additive effects of inhibitors were observed, suggesting that they act on different isoenzymes or that they act on different regions of one enzyme, each one having a partial and independent inhibitory effect. The dependence of inhibition on ATP concentration seems to argue against this last idea.

ADP also inhibited the ecto-ATPase of *Herpetomonas* sp. with apparent Ki of 1 mM and 40% maximal inhibition (not shown), comparable to the pattern described for *Herpetomonas muscarum muscarum* (Meyer-Fernandes *et al*. 2004). The ADP and CrATP maximal inhibitions were not additive (Fig. 6).

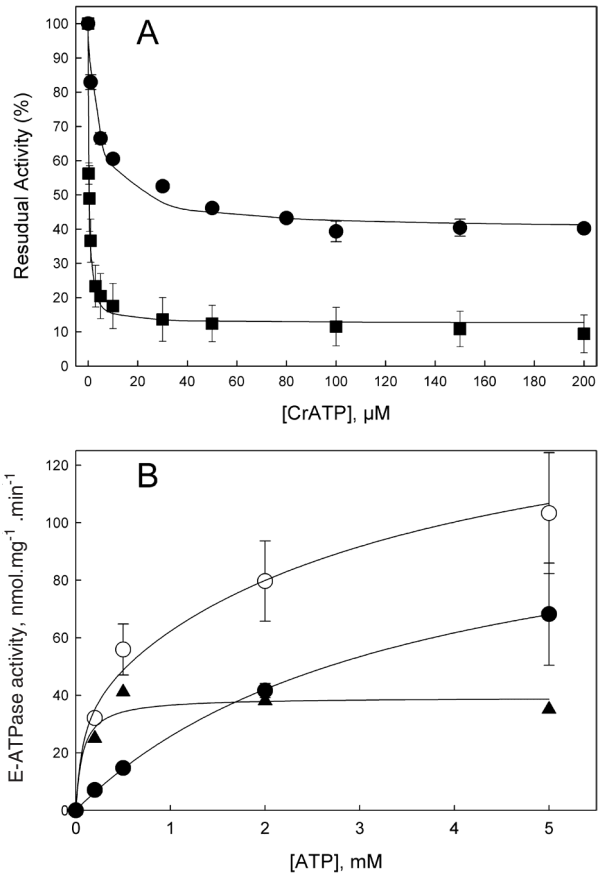

Fig. 3. ATP dependence of the CrATP inhibition of the ecto-ATPase activity. (A) Intact cells were incubated for 1 h at room temperature, in a medium containing 50 mM Hepes-KOH buffer, pH 7·2, 65 mM KCl, 100 mM sucrose, 2 mM $MgCl_2$ and increasing concentrations of CrATP, in the presence of (■) 200 μM [γ-32P]ATP (4000 cpm/nmol) or (●) 2 mM [γ-32P]ATP (400 cpm/nmol). Data are means ± S.E. of 3 determinations with different cell suspensions. Data were fitted by non-linear regression using the equation $v = 100 - As/(1 + Ki/[\text{CrATP}])$, where v is enzyme activity, As represents the sensitive maximal activity (in %) and Ki the concentration of CrATP to inhibit half of the As. The activity at 'infinite' CrATP concentration, $Ai = 100 - As$, represents the percentage of insensitive activity. In (■) 100% activity was $32{\cdot}1 \pm 1{\cdot}4$ nmol Pi/10^8 cel × h cells and Ki = $0{\cdot}33 \pm 0{\cdot}10\ \mu$M; in (●) 100% activity was $79{\cdot}5 \pm 15$ nmol Pi/10^8 cel × h and Ki = $4{\cdot}7 \pm 1{\cdot}0\ \mu$M. (B) Activities measured in the absence (○) and in the presence (●) of 200 μM CrATP at different ATP concentrations. The difference between the curves (CrATP sensitive ecto-ATPase) was calculated (▲). The lines represent fitting of the data with the Michaelis-Menten equation (▲,○). The continuous line, representing the fit of the total activity, was calculated as the sum of the fitted sensitive and insensitive activities.

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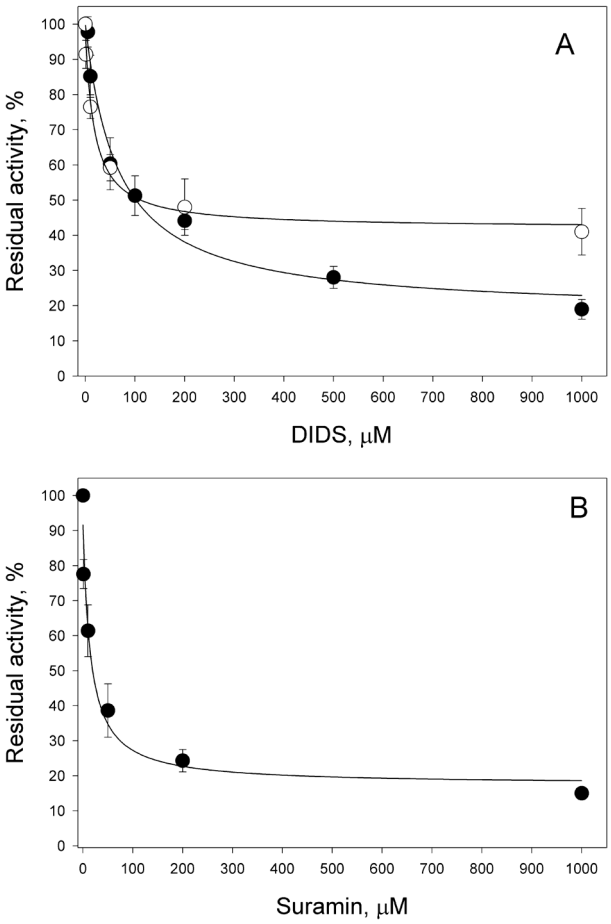

Fig. 4. Effect of DIDS (A) and suramin (B) on ecto-ATPase activity of *Herpetomonas* sp. Intact cells were incubated for 1 h at room temperature, in a medium containing 50 mM Hepes-KOH buffer, pH 7·2, 65 mM KCl, 100 mM sucrose, 2 mM $MgCl_2$ and increasing concentrations of (A) DIDS in the presence of (○) 200 μM or (●) 2 mM [γ-32P]ATP or (B) suramin in the presence of 2 mM [γ-32P]ATP. Data are means ± S.E. of 3 determinations with different cell suspensions.

The inhibition observed with CrATP on the ecto-ATPase of *Herpetomonas* sp. was also observed in *Trypanosoma cruzi*, *Trypanosoma rangeli* and *Leishmania amazonensis* (Fig. 7). In all cases the inhibitions were partial (especially obvious in the case of *L. amazonensis*) and with apparent Ki in the few micromolar range.

DISCUSSION

CrATP has been used as a kinetic and structural tool because of its capacity to inhibit a series of enzymes that use the MgATP complex as substrate. The catalysis of most of the enzymes inhibited by CrATP involves transference of a phosphate group from ATP either to a second substrate (like glucose in hexokinase) or to the enzyme itself, forming a phosphoenzyme that is part of the catalytic cycle of the enzyme. P-ATPases are within this last case. With these enzymes CrATP sits at the ATP site and forms

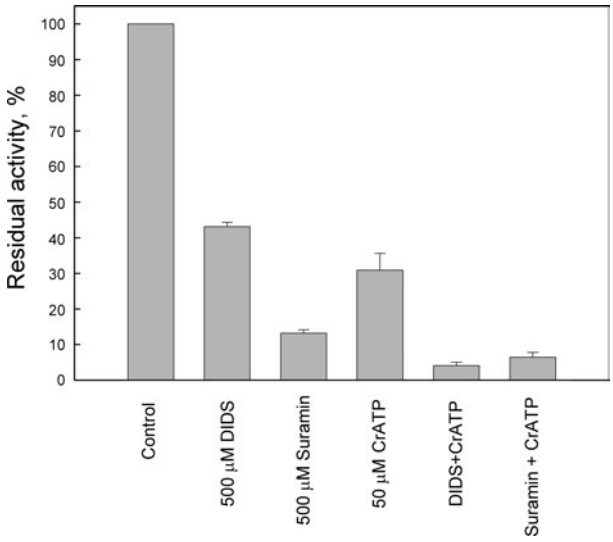

Fig. 5. Additive effects of DIDS or suramin and CrATP on ecto-ATPase activity of *Herpetomonas* sp. Intact cells were incubated for 1 h at room temperature with 500 μM DIDS or suramin in the presence or absence of 50 μM CrATP in a medium containing 50 mM Hepes-KOH buffer, pH 7·2, 65 mM KCl, 100 mM sucrose, 2 mM $MgCl_2$ and 2 mM ATP. Data are means ± S.E. of 3 determinations with different cell suspensions.

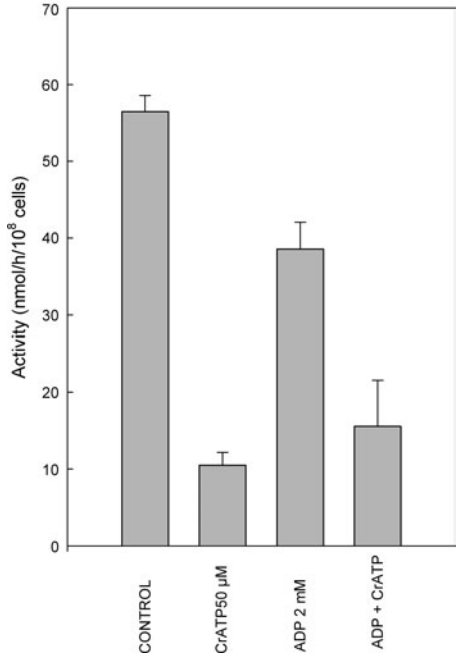

Fig. 6. Effects of CrATP and ADP on the ecto-ATPase activity of *Herpetomonas* sp. Intact cells were incubated for 1 h at room temperature with 2 mM [γ-32P]ATP (400 cpm/nmol), in the presence of 200 μM CrATP, 2 mM ADP or both, in a medium containing 50 mM Hepes-KOH buffer, pH 7·2, 65 mM KCl, 100 mM sucrose and 2 mM $MgCl_2$. Data are means ± S.E. of 3 determinations with different cell suspensions.

stable complexes with the proteins leading to an irreversible enzyme inhibition. Here we show that ecto-ATPases from different trypanosomatids are

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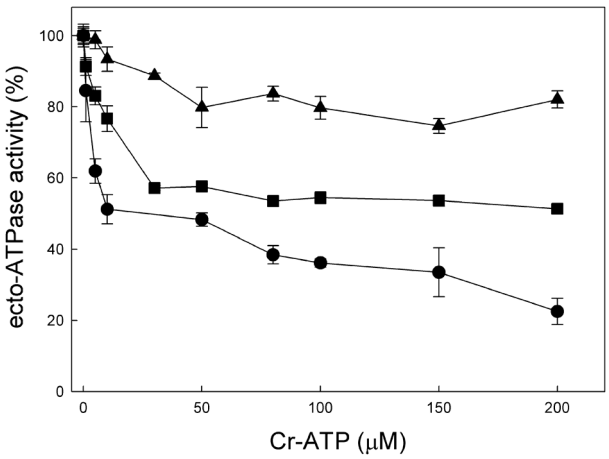

Fig. 7. Effect of increasing concentrations of CrATP on the ecto-ATPase activity of pathogenic trypanosomatids. Culture of intact cells of the trypanosomatids *Leishmania amazonensis* (▲), *Trypanosoma cruzi* (■) and *Trypanosoma rangeli* (●) were washed with PBS and incubated in medium containing 50 mM Hepes-KOH buffer, pH 7·2, 65 mM KCl, 100 mM sucrose, 3 mM $MgCl_2$, 3 mM [γ-32P]ATP and increasing CrATP concentrations to estimate inhibition of ecto-ATPase activity. Activities in the absence of CrATP were (nmol $Pi/10^8$ cel × h cells) (▲) 62·3, (■) 12·3, and (●) 16·3. Data are means ± S.E. of 3 determinations with different cell suspensions.

also sensitive to CrATP, though in a reversible manner. The reversibility suggests that ecto-ATPases do not form phosphorylated intermediates during catalysis. This is also supported by their insensitivity to vanadate (Attias *et al*. 1988; Meyer-Fernandes, 2002; Alves-Ferreira *et al*. 2003).

The *Herpetomonas* sp. does not exhibit any Mg-dependent hydrolysis of ADP, indicating the absence of a true ecto-NTPDase2 activity. By chemical assays some Mg-independent ATP or ADP hydrolysis was detected, nevertheless they were insensitive to CrATP. The ecto-phosphatase activity was also almost unaffected by CrATP.

The apparent affinities for CrATP (Ki) of the ecto-ATPases from trypanosomatids are in the order of a few μM, lower than those usually obtained for the other known inhibitors like DIDS and suramin (Meyer-Fernandes *et al*. 1997; Berredo-Pinho *et al*. 2001; Bernardes *et al*. 2001; de Jesus *et al*. 2002). The value of Ki decreased near 10-fold when a 10-fold lower substrate concentration was used, suggesting a true competitive mechanism. However, the inhibition of the ecto-ATPase activities in *Herpetomonas*, *Leishmania* and *Trypanosoma cruzi* were not total. The maximal inhibitions attained were 30–60% at 2 mM ATP and 40–50 μM CrATP, suggesting that CrATP is a universal inhibitor of trypanosomatid ecto-ATPase activities. Regarding *Herpetomonas* sp., inhibition was maintained constant at least up to 200 μM, which is 50 times the apparent Ki. Intriguingly, the values of maximal inhibition were different depending on substrate concentration. Incomplete inhibition was also observed with DIDS in *Herpetomonas* sp., *T. cruzi* (Bernardes *et al*. 2001) and with ADP in *Herpetomonas muscarum muscarum* (Alves-Ferreira *et al*. 2003).

The most plausible explanation for the pattern of partial inhibition is to consider the presence of 2 different enzymes, one sensitive to CrATP, which also has high ATP apparent affinity (Km = 85 μM), and another less sensitive (or insensitive) to CrATP with low substrate apparent affinity (Km = 3·5 mM). It is noteworthy that CrATP is considered to bind at 'high affinity ATP binding-sites' in enzymes as distinct as P-type ATPases (Vilsen and Andersen, 1992; Vilsen, 1995; Einholm *et al*. 2004; Moreira *et al*. 2005) and glycokinase (Danenberg and Cleland, 1975). These data, the absence of additive effects with ADP as inhibitor, and the reversible competitive inhibition observed here suggest that the CrATP binding site may be the catalytic site of (at least some) ecto-ATPases. A complex MgATP dependence for ecto-ATPase activity has also been described in *Tritrichomonas foetus* (Alves-Ferreira *et al*. 2003). In this parasite the activity increases with 2 kinetic components with respect to MgATP concentration, one with Km = 30 μM and a second with Km = 2 mM. Ecto-ATPases have been classified into several groups according to their phylogenetic relationships (Robson *et al*. 2006; Zimmermann, 1999). All of them share a number of highly conserved domains. Small differences in the primary structures are enough for a considerable impact on catalytic properties and to account for the differences observed between related ecto-ATPases enzymes (Asai *et al*. 1995; Nakaar *et al*. 1998; Heine *et al*. 1999). The alternative to 2 enzymes is the possibility of one enzyme in two different states of aggregation. It is known that ecto-NTPDase enzymes exist as homoligomers (Stout and Kirley, 1996; Lewis-Carl *et al*. 1998; Wang *et al*. 1998) and agents that disturb the degree of aggregation affect their enzymatic properties.

In protozoa, several hypothesis have been proposed for the function of ecto-ATPases. They include acquisition of adenosine from the media, necessary for normal growth, modulation of parasite infection and virulence, and involvement in cellular adhesion (Meyer-Fernandes, 2002). However, their function is still an open question and CrATP has potential as a valuable selective tool for a better understanding of properties and role of ecto-ATPases in the biology of parasites. Furthermore, the use of CrATP as a new inhibitor can help to select among distinct activities of ATP hydrolysis on the trypanonomatids surface. In this paper, CrATP unravelled the existence of an ecto-ATPase with a high apparent affinity for ATP on a trypanosomatid surface. Considering the low amount of extracellular

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ATP such an enzyme may be of physiological importance.

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