Ecto-phosphatase Activity on the Cell Surface of *Crithidia deanei*

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Z. Naturforsch. **57 c,** 500–505 (2002); received January 14/February 12, 2002

Crithidia deanei, Phosphotyrosyl Phosphatase, Vanadate Inhibition

In the present work we have partially characterized an ecto-phosphatase activity in Crithidia deanei, using viable parasites. This enzyme hydrolyzed *p*-nitrophenylphosphate at a rate of 3.55 ± 0.47 nmol Pi/h × 10^8 cells. The dependence on *p*-NPP concentration shows a normal Michaelis-Menten kinetics for this phosphatase activity and the value of the apparent K_m for *p*-NPP was 5.35 ± 0.89 mM. This phosphatase activity was inhibited by the product of the reaction, the inorganic phosphate. Experiments using classical inhibitors of acid phosphatases, such as $ZnCl_2$ and sodium fluoride, as well as inhibitors of phosphotyrosine phosphatase, such as sodium orthovanadate and ammonium molybdate, showed a decrease in this phosphatase activity, with different patterns of inhibition.

Introduction

Trypanosomatid protozoa include heteroxenic species whose life cycle alternates between invertebrate and vertebrate hosts (*e.g.* disease-causing parasites such as *Leishmania* and *Trypanosoma*) and non-pathogenic species that develops strictly in the insect midgut (*e.g. Crithidia*). Parasites of the genus *Crithidia* have been widely used as a model for biochemical studies on eukaryotic cells, as they are non-pathogenic, can be easily grown axenically *in vitro* in chemically defined media, and share several metabolic pathways with higher eukaryotes (Gero, 1998; De Souza and Motta, 1999). A few members of this genus, such as *C. deanei,* harbor an endosymbiontic bacterium in its cytoplasm. The endosymbiont supplies the host trypanosomatid with essential growth factors, such as purine bases, hemin and aminoacids (De Souza and Motta, 1999). The close metabolic relationship of endosymbiont-bearing trypanosomatids accounts for less stringent growth requirements in axenic medium, while naturally occurring symbiont-free species, such as pathogenic parasites, scavenge many of the essential nutrients they require from their hosts. A knowledge of which nutrients are scavenging and an understanding of how scavenging is accomplished can lead to the identification of unique biochemical pathways used by the parasite and, therefore, potential targets for rational drug design (Cohn and Gottlieb, 1997).

The plasma membranes of cells contain 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 living cells (Meyer-Fernandes *et al*., 1997; Furuya *et al*., 1998; Peres-Sampaio *et al*., 2001). Reversible phosphorylation of proteins is recognized to be a major mechanism for the control of intracelular events in eukaryotic cells. Phosphorylation-dephosphorylation of serine, threonine, and tyrosine residues triggers conformational changes in proteins that alter their biological properties (Hunter, 1995). The regulation of the complex interactions required for differentiation and proliferation is mediated in part by protein phosphorylation in higher eukaryotes (Hunter, 1995), as well as in Trypanosomatidae family (Parsons *et al*., 1993). Such phosphorylations are reversible, and several phosphatases active towards phosphotyrosyl [Tyr(P)]-proteins have been described as acid and alkaline phosphatases (Lau *et al*., 1989).

In various protozoa parasites ecto-phosphatases have been described, which are also active toward low molecular weight, non-protein phosphoesters

such as alkyl and aryl phosphates, including *p*-nitrophenylphosphate, *O*-phospho-L-tyrosine and phosphoproteins (Fernandes *et al.,* 1997; Dutra *et al*., 1998, 2000; Meyer-Fernandes *et al.,* 1999; Bakalara *et al.,* 2000). Although the physiological role for ecto-phosphatases has not been well established, they are supposed to be involved with nutrition (Rodrigues *et al*., 1999; Dutra *et al.,* 2000) and with cell differentiation (Bakalara *et al*., 1995; 2000; Meyer-Fernandes *et al.,* 1999). In the present work we have characterized a phosphatase activity present on the external surface of living monoxenic trypanosomatid protozoa *C. deanei* and demonstrated that *C. deanei* grown in a medium supplemented with adenosine present lower level of phosphatase activity.

Materials and Methods

Microorganisms

The normal strain of *Crithidia deanei* contained its endosymbiont (De Souza and Motta; 1999) was grown in Warren liquid culture medium (Warren, 1960) supplemented with 10% fetal bovine serum at 28 °C. After 24 h of inoculation, cells were collected by centrifugation, washed twice, and kept in 50 mM Hepes (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]), pH 7.2, 116 mM NaCl, 5.4 mM KCl, and 5.5 mM glucose. Cellular viability was assessed, before and after incubations by mobility and Trypan Blue methods (Dutra *et al*., 1998). The viability was not affected under the conditions employed here.

Phosphatase measurements

Intact cells were incubated for 1 hour at 30 °C in 0.5 ml of a mixture containing, unless otherwise specified, 116.0 mM NaCl, 5.4 mM KCl, 5.5 mM D-glucose, 50.0 mM Hepes-Tris (tris[hydroxymethyl]aminomethane) buffer, pH 7.2, 5.0 mM *p*-NPP, and 3.0×10^8 cells/ ml. The experiments were started by the addition of living cells and terminated by the addition of 1.0 ml of 1.0 N NaOH. The tubes were then centrifuged at $1{,}500 \times g$ for 10 min at 4 °C. The phosphatase activity was also calculated by subtracting the nonspecific *p*-NPP hydrolysis measured in the absence of parasites. The reaction was determined spectrophotometrically at 425 nm using an extinction coefficient of 14.3×10^3 M^{-1} cm^{-1} (Rodrigues *et al*., 1999).

Statistical analysis

All experiments were performed in triplicate, with similar results obtained in at least three separate cell suspensions. Apparent K_m for *p*-NPP and V_{max}. values were calculated using a computerized nonlinear regression analysis of the data to the Michaelis-Menten equation (Saad-Nehme *et al*., 1997). Statistical significance was determined by Student's *t* test. Significance was considered as $P < 0.05$.

Reagents

All reagents were purchased from E. Merck (D-6100 Darmstadt, Germany) or Sigma Chemical Co. (St. Louis, MO). Distilled water was deionized using a MilliQ system of resins (Millipore Corp., Bedford, MA) and was used in the preparation of all solutions.

Results and Discussion

Crithidia deanei presented an ecto-phosphatase activity on its external surface. At pH 7.2, intact cells of *C. deanei* were able to hydrolyze *p*-NPP (3.55 ± 0.47 nmol Pi/h $\times 10^8$ cells). The time course of *p*-NPP hydrolysis by the ecto-phosphatase was linear for at least 60 minutes (Fig. 1A). Similarly, in assays to determine the influence of cell density, phosphatase activity measured over 60 minutes was linear over a nearly 3-fold range of cell density (Fig. 1B). To check the possibility that the observed *p*-NPP hydrolysis was the result of secreted soluble enzymes, as seen in other parasites (Rodrigues *et al*., 1999; Dutra *et al*., 2001), cells were incubated in the absence of *p*-NPP. Subsequently, the suspension was centrifuged to remove cells and the supernatant was checked 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 from lysed *C. deanei* cells.

The dependence on *p*-NPP concentration shows a normal Michaelis-Menten kinetics for this phosphatase activity and the values of V_{max} and apparent K_m for *p*-NPP were 6.93 ± 0.42 nmol Pi/h $\times$

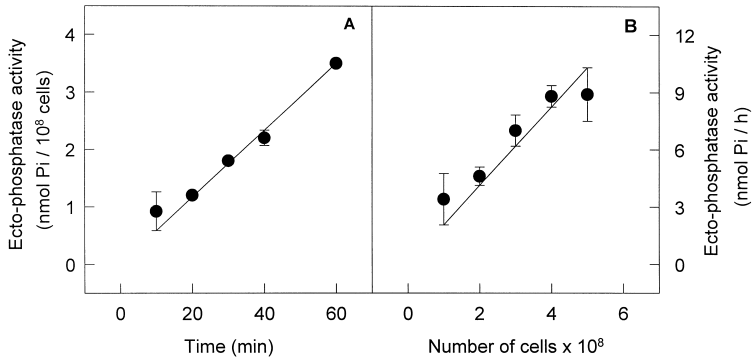

Fig. 1. Time course (A) and cell density dependence (B) of the ecto-phosphatase activity of intact cells of *C. deanei*. Cells were incubated for different periods of time (A) or for 1 h (B) at 30 °C, in a reaction medium containing 50 mM Hepes-Tris buffer, pH 7.2, 116 mM NaCl, 5.4 mM KCl, 5.5 mM D-glucose and 5 mM *p*-NPP. Data are means ± SE of three determinations with different cell suspensions.

10^8 cells and 5.35 ± 0.84 mM, respectively (Fig. 2, open circles). This enzyme was inhibited partially by the product of the reaction, the inorganic phosphate (Fig. 2, closed circles). Different phosphatase inhibitors were tested and the results are showed in Figure 3. The acid phosphatase inhibitors zinc chloride (Fig. 3A) and sodium fluoride (Fig. 3B) (Dutra *et al.*, 1998, 2000) inhibited respectively 70% and 40% of this enzyme activity in a dose dependent manner. Although NaF is a serine-threonine phosphatase inhibitor (Hunter, 1995), inhibition of phosphotyrosin phosphatase by fluoride has been previously described (Bakalara *et al.,* 2000). The classical phosphotyrosine phosphatase inhibitors, sodium orthovanadate (Fig. 3C) and ammonium molybdate (Fig. 3D) (Mikalsen and Kaalhus, 1998) inhibited about 80% and 95% of this enzyme activity in a dose dependent manner. These data suggest that this ecto-phosphatase of *C. deanei* could be a phosphotyrosin phosphatase. The high sensitivity to vanadate (Fig. 3C) and molybdate (Fig. 3D), two known potent and specific phosphotyrosyl protein phosphatases inhibitors (Lau *et al.,* 1989; Mikalsen and Kaalhus, 1998), suggest that this phosphatase has similarities with the tyr/ser protein phosphatase present in *vaccinia* virus (Guan and Dixon, 1991) and might dephosphorylate phosphoproteins phosphorylated in tyrosine and serine residues on host cell. The reason for the no complete

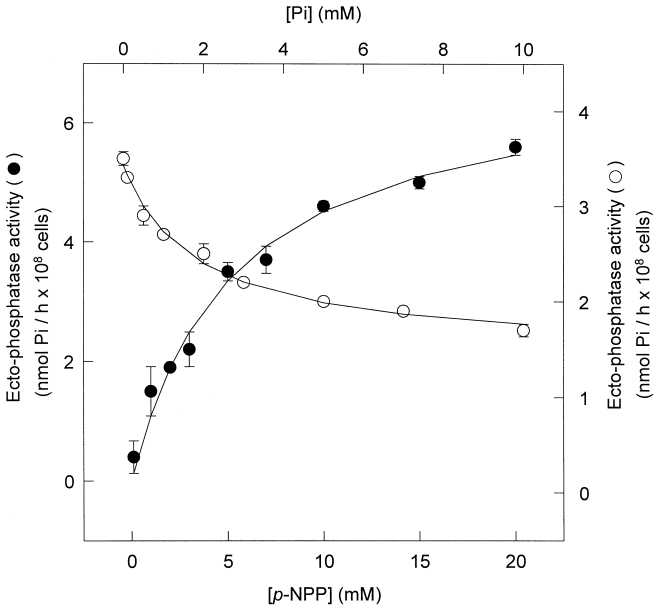

Fig. 2. Dependence on *p*-NPP concentrations (●) and inhibition of ecto-phosphatase activity by inorganic phosphate (○) in intact cells of *C. deanei*. Cells were incubated for 1 h at 30 °C, in the same reaction medium (final volume: 0.5 ml) as that described in the legend to Fig. 1. Data are means ± SE of three determinations with different cell suspensions.

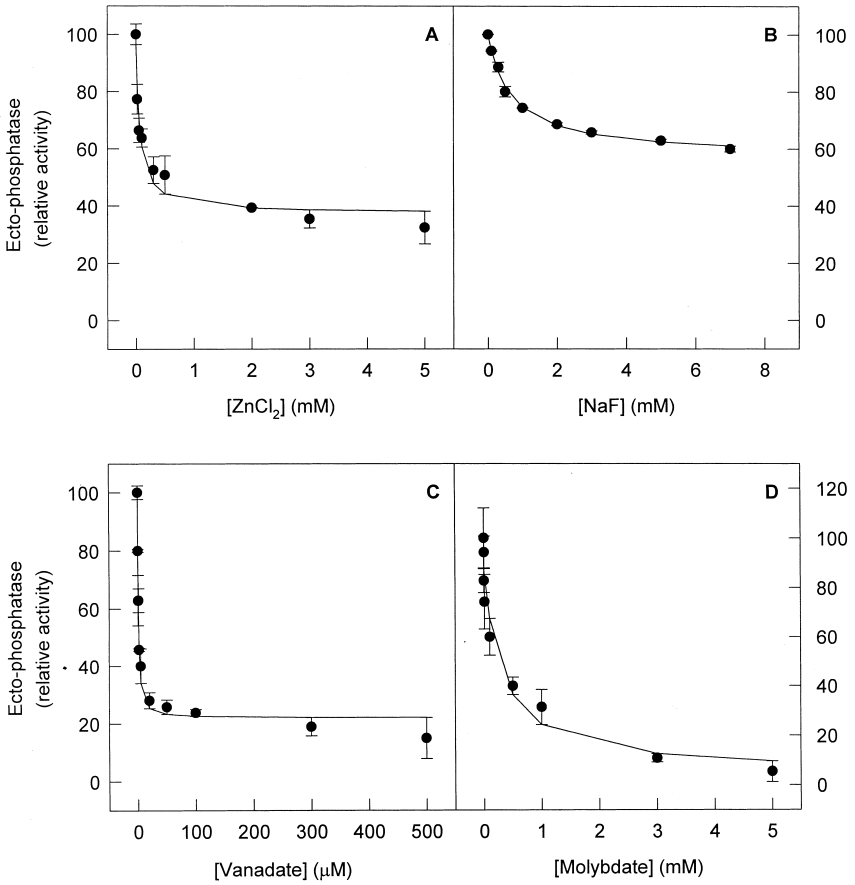

Fig. 3. Inhibition of *C. deanei* ecto-phosphatase activity by acid-phosphatase and phospho-tyrosine phosphatase inhibitors. Cells were incubated for 1 h at 30 °C, in the same reaction medium (final volume: 0.5 ml) as that described in the legend to Fig. 1, which corresponds to inhibitors concentrations varying as shown on the abscissa. $ZnCl_2$ (A), NaF (B), vanadate (C) and molybdate (D). The ecto-phosphatase (3.55 ± 0.47 nmol Pi/h × 10^8 cells) activity was taken as 100%. 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.

inhibition of the phosphatase activity by inorganic phosphate (Fig. 2, closed circles), $ZnCl_2$ (Fig. 3A), NaF (Fig. 3B) and vanadate (Fig. 3C) remains unclear. It is possible that the supposed selective action of these inhibitors depends on the catalytic mechanism of the enzyme, substrate specificity and association with possible specific regulatory subunits. Other protein phosphatases such as the receptor protein tyrosine phosphatase (RPTP) were shown to have an important role in the process of homophilic cell-cell adhesion (Fischer *et al.*, 1991). We suggest that this phosphatase present on the surface membrane which externally dispose the active site, is active within the range of physiological pH and able to hydrolyze *p*-NPP may have physiological role in the interactions between the parasite and the insect host cells.

Extracellular adenosine is a normal component of extracellular milieu. Parasite ectoenzymes such as 3′nucleotidase/nuclease (3′NTase) and 5′nucleotidase (5′NTase) associated with purine nucleotide degradation in the external environment are increased under purine stress (Gottlieb, 1985; Gero, 1998). The ecto-phosphatase activity present on the surface of *C. deanei* might dephosphorylate nucleotides, making adenosine available to *C. deanei* from nucleotides, which, because of their charge, are not permeable to the plasma mem-

brane. *C. deanei* grown in a medium supplemented with 5 mM adenosine were compared to the *C. deanei* grown in a control medium concerning the presence of the ecto-phosphatase activity. It was observed that *C. deanei* grown in control medium presented twofold more ecto-phosphatase activity (3.55 ± 0.47 nmol Pi/h $\times 10^8$ cells) than the *C. deanei* grown in a medium supplemented with 5 mM adenosine (1.60 ± 0.049 nmol Pi/h $\times 10^8$ cells). Reversible phosphorylation of proteins and hydrolysis of extracellular phosphorylated molecules play a role on the regulation of several cellular processes, including cell proliferation and differentiation in higher eukaryotic organisms (Hunter, 1995), as well as in trypanosomatids (Parsons *et al*., 1994; Berrêdo-Pinho *et al*., 2001). Our data suggest a relationship between *C. deanei* nutrition and ecto-phosphatase activity, as it has been observed in other parasites (Bernardes *et al*., 2000; Dutra *et al*., 1998). 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).

Acknowledgement

We would like to acknowledge the excellent technical assistance of Fabiano Ferreira Esteves. This work was partially supported by grants from the Brazilian agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Financiadora de Estudos e Projetos (FINEP), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) and Programa de Núcleos de Excelência (PRONEX, grant 0885).

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