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Characterisation of an ATP diphosphohydrolase (Apyrase, EC 3.6.1.5) activity in *Trichomonas vaginalis*

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Abstract

In the present report the enzymatic properties of an ATP diphosphohydrolase (apyrase, EC 3.6.1.5) in *Trichomonas vaginalis* were determined. The enzyme hydrolyses purine and pyrimidine nucleoside 5′-di- and 5′-triphosphates in an optimum pH range of 6.0–8.0. It is Ca^{2+}-dependent and is insensitive to classical ATPase inhibitors, such as ouabain (1 mM), *N*-ethylmaleimide (0.1 mM), orthovanadate (0.1 mM) and sodium azide (5 mM). A significant inhibition of ADP hydrolysis (37%) was observed in the presence of 20 mM sodium azide, an inhibitor of ATP diphosphohydrolase. Levamisole, a specific inhibitor of alkaline phosphatase, and P^1, P^5-di (adenosine 5′-) pentaphosphate, a specific inhibitor of adenylate kinase, did not inhibit the enzyme activity. The enzyme has apparent K_m (Michaelis Constant) values of 49.2 ± 2.8 and 49.9 ± 10.4 μM and V_{max} (maximum velocity) values of 49.4 ± 7.1 and 48.3 ± 6.9 nmol of inorganic phosphate · min^{-1} · mg of $protein^{-1}$ for ATP and ADP, respectively. The parallel behaviour of ATPase and ADPase activities and the competition plot suggest that ATP and ADP hydrolysis occur at the same active site. The presence of an ATP diphosphohydrolase activity in *T. vaginalis* may be important for the modulation of nucleotide concentration in the extracellular space, protecting the parasite from the cytolytic effects of the nucleotides, mainly ATP.

Keywords: *Trichomonas vaginalis*; ATP diphosphohydrolase; Apyrase; Extracellular ATP

1. Introduction

Trichomonas vaginalis is a flagellate protozoan that lives in the human urogenital tract. Trichomoniasis is recognised as a major sexually transmitted disease, whose clinical presentation ranges from a totally asymptomatic infection to a severe vaginitis. The mechanisms of the pathogenicity of *T. vaginalis* are not well defined (Brasseur and Savel, 1982; Alderete and Pearlman, 1984; Rasmussen et al., 1986; Roussel et al., 1991). Biochemical aspects on the surface membrane constituents of these species have been evaluated and may play an important role in the protozoan's mobility and cytoadhesion and in the protection from cytolytic effects (Gillin et al., 1984; Arroyo and Alderete, 1989; Arroyo et al., 1993; Ter Kuile and Müller, 1995; Lopez et al., 2000).

Besides its energetic function in the intracellular environment, extracellular ATP has several effects on many biological processes. These include smooth muscle contraction, neurotransmission, immune response, inflammation, platelet aggregation and pain (Ralevic and Burnstock, 1998; Sitkovsky, 1998; Sneddon et al., 1999; Ding et al., 2000). In addition, extracellular ATP may act as a signalling compound in cytolytic mechanisms (Filippini et al., 1990; Steinberg and Di Virgilio, 1991).

Signalling actions induced by extracellular ATP are directly correlated to the activity of a group of ecto-enzymes, the ectonucleotidases, which includes ecto-ATP diphosphohydrolase (apyrase, EC 3.6.1.5) and ecto-ATPase (EC 3.6.1.3) (Sarkis et al., 1995; Zimmermann et al., 1998). These enzymes hydrolyse a variety of purine and pyrimidine nucleoside di- and triphosphates. Furthermore, they are activated by high concentrations of either Ca^{+2} or Mg^{+2} and are not inhibited by classical inhibitors of intracellular ATPases such as P-type, F-type and V-type or alkaline phosphatase (Plesner, 1995; Zimmermann, 1996). The members of this

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protein family differ in their preference for nucleoside 5′-diphosphates. The recently cloned ecto-apyrase is a non-covalent tetrameric protein (Wang et al., 1998) that hydrolyses both substrates (ATP and ADP) with approximately equal efficiency, whereas an ecto-ATPase has high preference for ATP (Zimmermann et al., 1998). Kegel et al. (1997) reported that an ecto-ATPase is co-expressed with an ecto-ATP diphosphohydrolase in the rat brain.

Several studies have demonstrated the presence of ecto-nucleotidases on the parasite surface. Previous studies investigating the nucleotidases of *Schistosoma mansoni* characterised and purified an ATP diphosphohydrolase activity on the external surface of the parasite tegument (Vasconcelos et al., 1993, 1996). A Mg-dependent ecto-ATPase activity has been described on the external surface of *Leishmania tropica* (Meyer-Fernandes et al., 1997) and, recently, an ectonucleotide diphosphohydrolase was described in intact cells of *Entamoeba histolytica* (Barros et al., 2000). Turner and Lushbaugh (1991) identified three ATPases presenting different optimum pHs, sensitivity to ATPase inhibitors and cation-dependence in the sedimentible fractions of *T. vaginalis*. However, these enzyme activities have not yet been clearly identified and an investigation into the presence of ectonucleotidases in *T. vaginalis* has yet to be published. The present study describes the properties of an ATP diphosphohydrolase activity in *T. vaginalis*.

2. Materials and methods

2.1. Parasite culture

The *T. vaginalis* strain, 30236 (sensitive to metronidazole), from the American Type Culture Collection was used. Trichomonads were cultured axenically in vitro in trypticase–yeast extract–maltose (TYM) medium (Diamond, 1957) without agar (pH 6.0) supplemented with 10% (v/v) heat-inactivated cold serum, penicillin (1000 UI/ml) and streptomycin sulphate (1 mg/ml) in air at 37°C (±0.5). Isolates were subcultured every 48 h in TYM medium. Samples of the original strain were frozen and maintained at −196°C with 5% (v/v) dimethyl sulfoxide (DMSO), as described by Honigberg et al. (1965). Trichomonads in the logarithmic phase of growth and within 48 h of subculture (exhibiting more than 95% mobility and normal morphology) were harvested, washed and centrifuged ($750 \times g$ for 20 min) three times. Parasites were counted with a haemocytometer and adjusted to a concentration of 1×10^6 living organisms/ml in TYM medium. Cell culture was maintained as intact cells or cells were sonicated (40 000 Hz) in order to obtain a homogenate. In experiments using intact cells, cellular viability was assessed, before and after incubations, by mobility. The viability was not affected by incubation conditions.

2.2. Enzyme assays

After preparing the samples, the optimum conditions for nucleotide hydrolysis were determined. Sonicated samples or intact cells of *T. vaginalis* (5–15 μg protein) were added to the reaction mixture containing 50 mM Tris buffer (pH 7.2) and 5 mM $MgCl_2$ or $CaCl_2$. The samples were preincubated for 5 min at 37°C in 200 μl of the reaction mixture. The reaction was initiated by the addition of substrate (ATP or ADP or other as indicated) to a final concentration of 1 mM and stopped by adding 200 μl 10% trichloroacetic acid. The samples were chilled on ice for 10 min before assaying for the release of inorganic phosphate (Pi) (Chan et al., 1986). Incubation times and protein concentration were chosen in order to ensure the linearity of the reactions. Controls with the addition of the enzyme preparation after mixing trichloroacetic acid were used to correct non-enzymatic hydrolysis of substrates. Specific activity is expressed as nmol of Pi released $\cdot$ min^{-1} $\cdot$ mg of $protein^{-1}$. All samples were run in triplicate, with similar results achieved in at least four different cell suspensions.

2.3. Protein determination

Protein was measured by the Coomassie Blue method (Bradford, 1976), using BSA as standard.

2.4. Statistical analysis

Statistical analysis was conducted by Student's *t*-test and one-way analysis of variance (ANOVA), considering a level of significance of 5%.

3. Results and discussion

ATP diphosphohydrolase (apyrase) is an enzyme which has been described in various sources, including the parasite surface (Sarkis et al., 1986; Sarkis and Saltó, 1991; Vasconcelos et al., 1993, 1996; Plesner, 1995). An enzyme with characteristics of an ATP diphosphohydrolase was detected in *T. vaginalis*. The time course for ATP and ADP hydrolysis occurring in sonicated samples of *T. vaginalis* was linear up to 20 min for both activities in the presence of Ca^{2+}. Ca^{2+}-ATP and Ca^{2+}-ADP hydrolysis increased as a function of protein concentration. The product formation was linear in the range of 5–15 μg protein in the incubation medium (data not shown).

Like other ATP diphosphohydrolases, the enzyme was demonstrated to be divalent cation-dependent and its sensitivity to Ca^{2+} and Mg^{2+} is illustrated in Fig. 1. Ca^{2+} was a better activator of ATP and ADP hydrolysis than Mg^{2+} in *T. vaginalis* (Fig. 1A,B). As a consequence, a concentration of 5 mM calcium was selected for subsequent enzyme assays. Cation dependency was confirmed by a dramatic decrease in ATP or ADP hydrolysis in the absence or presence of Ca^{2+} or Mg^{2+} plus 5 mM EDTA (Fig. 1). Furthermore, the

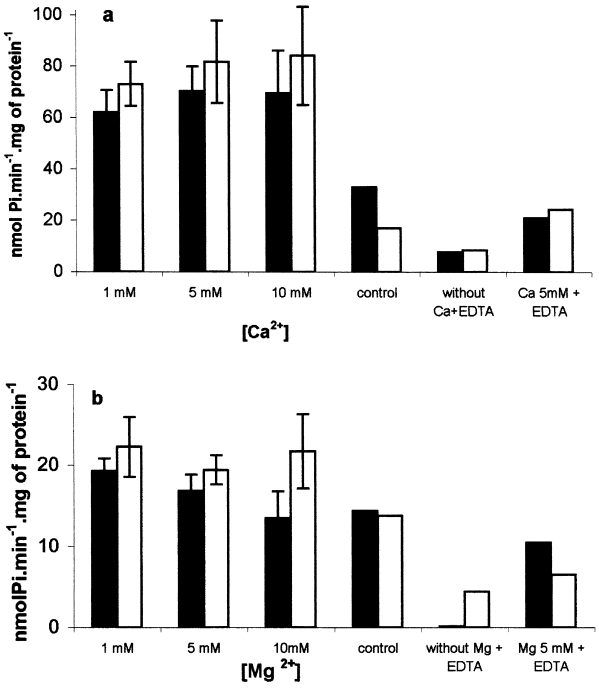

Fig. 1. Effect of $CaCl_2$ (A) or $MgCl_2$ (B) concentration on ATP and ADP hydrolysis promoted by ATP diphosphohydrolase in sonicated samples of *T. vaginalis*. Closed bars represent ATP hydrolysis and open bars represent ADP hydrolysis. Incubation conditions are described in Section 2. The control group was incubated without the addition of Ca^{2+} or Mg^{2+}. Bars represent the mean ± SD of four different determinations using different cell suspensions, each in triplicate.

enzyme showed an optimum pH in the range of 6.0–8.0 for both Ca^{2+}-ATP and Ca^{2+}-ADP hydrolysis (data not shown).

Ca^{2+}-ATP and Ca^{2+}-ADP hydrolysis were determined at substrate concentrations in the range of 50–2500 μM. The enzyme activity increased with increasing concentrations of the nucleotide (Ca^{2+} fixed at 5 mM with variable concentrations of nucleotides). K_m (Michaelis Constant) and V_{max} (maximum velocity) values of sonicated samples of *T. vaginalis* were estimated from the Lineweaver–Burk plot with four different enzyme preparations. The apparent K_m values for Ca^{2+}-ATP and Ca^{2+}-ADP were 49.2 ± 2.8 and 49.9 ± 10.4 μM, respectively. The V_{max} values for Ca^{2+}-ATP and Ca^{2+}-ADP were 49.4 ± 7.1 and 48.3 ± 6.9 nmol Pi · min^{-1} · mg of $protein^{-1}$ for ATP and ADP, respectively.

The parallelism observed for ATP and ADP hydrolysis under several conditions, such as cation and pH dependence, sensitivity to inhibitors and similar apparent K_m values for both substrates suggests the presence of a single enzyme involved in ATP and ADP hydrolysis (Lebel et al., 1980; Sarkis et al., 1986, 1995; Battastini et al., 1991).

ATP diphosphohydrolase has been described as an enzyme with a broad substrate specificity (Sarkis et al., 1986, 1995; Battastini et al., 1991). All nucleotides tested were hydrolysed at a high rate by sonicated samples of *T. vaginalis*. The low rate of AMP hydrolysis observed was probably due to a 5′-nucleotidase activity present in *T. vaginalis* (Table 1).

Table 1
Substrate specificity of ATP diphosphohydrolase of *T. vaginalis*[a]

Substrate	Relative activity
ATP	1.00
ITP	1.09 ± 0.10
CTP	0.58 ± 0.17
ADP	1.19 ± 0.10
UDP	1.04 ± 0.07
CDP	0.61 ± 0.15
IDP	0.67 ± 0.08
GDP	0.60 ± 0.09
AMP	0 23 ± 0.13

[a] Results are expressed as the mean ± SD for at least four experiments. All substrates were used at 1.0 mM (5.0 mM Ca^{2+}). Control Ca^{2+}-ATPase activity was 51.68 ± 3.54 nmol Pi · min^{-1} · mg of $protein^{-1}$.

To evaluate the correlation between the enzyme described in this study with ATPases, ATPase and alkaline phosphatase inhibitors were tested. The following inhibitors had no effect upon ATP and ADP hydrolysis in *T. vaginalis* (Table 2): (a) the Na^+/K^+-ATPase inhibitor, ouabain (Lebel et al., 1980); (b) orthovanadate, an inhibitor of transport ATPases, acid phosphatases and phosphotyrosine phosphatases (Sorensen and Mahler, 1992; Cool and Blum, 1993); (c) the mitochondrial inhibitor, sodium azide (<5.0 mM); (d) the Ca^{2+}, Mg^{2+}-ATPase inhibitor, *N*-ethylmaleimide (NEM); and (e) levamisole, a specific alkaline phosphatase inhibitor. However, 20 mM sodium azide, an inhibitor of ATP diphosphohydrolase from several sources (Plesner, 1995; Knowles and Nagy, 1999), strongly inhibited (37%) ADP hydrolysis (Table 2).

Adenylate kinase activity has been purified and sequenced in *T. vaginalis* (Declerck and Müller, 1987; Lange et al., 1994). The possibility that ADP hydrolysis

Table 2
Effects of inhibitors on ATP and ADP hydrolysis by *T. vaginalis*[a]

Inhibitor	Concentration (mM)	% control enzyme activity	
		ATPase	ADPase
Ap5A	0.01	101 ± 21	99 ± 16
Ouabain	1	98 ± 16	100 ± 12
Azide	5	99 ± 12	87 ± 11
	20	99 ± 11	63 ± 14*
Levamisole	1	107 ± 11	92 ± 11
Orthovanadate	0.1	100 ± 12	96 ± 9
NEM	0.1	88 ± 4	84 ± 3

[a] Control Ca^{2+}-ATPase and Ca^{2+}-ADPase activities were 52.1 ± 4.6 and 56.8 ± 5.9 nmol Pi · min^{-1} · mg of $protein^{-1}$, respectively. Results are expressed as percentages of control activity (100%). Data represent the mean ± SD for at least four experiments and were analysed statistically by one-way ANOVA or by paired sample *t*-test. *Significant difference from control activity (100%) by one-way ANOVA ($P < 0.05$).

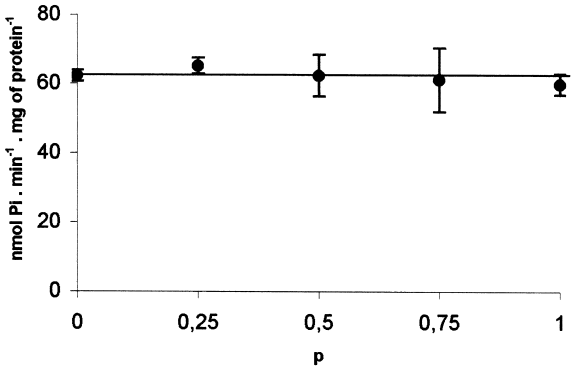

Fig. 2. Competition plot. Assay conditions are described in Section 2. The incubation time was 15 min. Substrate A (ATP) at $P = 0$ was 0.1 mM. Substrate B (ADP) at $P = 1$ was 0.1 mM. Data represent a typical experiment run in triplicate.

occurs by prior conversion to ATP, catalysed by adenylate kinase and later hydrolysis by an ATP-specific enzyme, should be ruled out since this enzyme combination could mimic apyrase activity. The influence of a contaminating adenylate kinase on ADP hydrolysis in our assay conditions was excluded since 10 μM P^1, P^5-di (adenosine 5′-) pentaphosphate (Ap5A), a selective adenylate kinase inhibitor (Sarkis et al., 1995), did not affect either ATP or ADP hydrolysis in *T. vaginalis* (Table 2).

Studies have used the Chevillard plot to characterise the presence of ATP diphosphohydrolase activity, which is a classical method used to determine if two substrates are hydrolysed at the same active site of an enzyme (Chevillard et al., 1993; Kettlun et al., 1994; Pilla et al., 1996). To assay the combination of substrate concentrations in a Chevillard plot, a concentration at which the rate of hydrolysis was the same when either ATP or ADP was used as substrate was chosen (Fig. 2). Series of mixtures containing ATP and ADP were prepared. The P values ranged from 1 to 0. The constant velocity at all substrate combinations tested suggests the participation of a single enzyme involved in ATP and ADP hydrolysis in *T. vaginalis* (Fig. 2).

Since the kinetic behaviour observed here indicated an ATP diphosphohydrolase, investigations as to whether ATP or ADP hydrolysis is mediated by an ecto-enzyme in intact cells of *T. vaginalis* are necessary. Table 3 shows no significant differences in ATP and ADP hydrolysis in disrupted and intact cells of *T. vaginalis*. This result supports the hypothesis that this enzyme activity may correspond to an ecto-enzyme with kinetic characteristics of an ATP diphosphohydrolase.

Table 3
Nucleotide hydrolysis by intact and disrupted cells from *T. vaginalis*[a]

Conditions	Ca^{2+}-ATPase (nmol Pi min^{-1} mg of $protein^{-1}$)	Ca^{2+}-ADPase (nmol Pi min^{-1} mg of $protein^{-1}$)
Intact cells	55.44 ± 8.01	57.88 ± 14.81
Disrupted cells[b]	59.85 ± 2.64	60.00 ± 11.60

[a] Results are expressed as the mean ± SD for at least four experiments.
[b] Disrupted cells were sonicated (40 000 Hz).

Other enzyme activities, such as proteinases and maltase, have been described as ecto- or exoenzymes in *T. vaginalis* (Arroyo and Alderete, 1989; Ter Kuile and Müller, 1995). Furthermore, the sensitivity of the intact organism to non-penetrable thiol reagents has demonstrated that free thiols of surface components are important to the parasite (Gillin et al., 1984). Studies related to the molecular composition of the cell surface of trichomonads and their functional aspects are important for the understanding of their role in cytoadherence and cytotoxicity (Lopez et al., 2000).

Nucleotides are released from dying or destroyed cells under physiological or pathological conditions after massive injury (Ralevic and Burnstock, 1998). Furthermore, it has been shown that extracellular ATP can kill several kinds of cells, except those presenting high level ATP-breakdown activity on their surface (Filippini et al., 1990). An ecto-ATP diphosphohydrolase was localised on the surface of the tegument of *S. mansoni* (Vasconcelos et al., 1993, 1996) and the authors of this study suggested that this enzyme may contribute to the escape mechanisms of the parasite by breaking down ATP or ADP released by activated platelets or cytotoxic T-lymphocytes. Furthermore, *T. vaginalis* was found to contain three distinct ATPases differing in optimum pH, in ion effects, in substrate specificity and in their reactions to inhibitors (Turner and Lushbaugh, 1991). In addition, an ATPase with an optimum pH of 8.0, activated by Ca^{2+}, was demonstrated to react equally well with nucleotide tri- or diphosphates and to be less susceptible to inhibitors (Turner and Lushbaugh, 1991). This enzyme activity shares several of the properties which characterise an ATP diphosphohydrolase (Sarkis et al., 1995). Thus, it may be suggested that the nucleotide hydrolysis observed by these authors in *T. vaginalis* may involve an ATP diphosphohydrolase activity as described herein.

The presence of enzymes performing ATP and ADP hydrolysis in trichomonads is not fully understood and their physiological role in these species is unknown. It has been suggested that these enzymes could be involved in signal transduction, ion transport, protection from the cytolytic effects of extracellular ATP, control of immune response, cellular adhesion, mobility, inflammation and pain mechanisms (Filippini et al., 1990; Dzhandzhugazyan and Bock, 1997; Ralevic and Burnstock, 1998; Sitkovsky, 1998; Sneddon et al., 1999; Ding et al., 2000). The clear identification of these enzymes in the intracellular and extracellular environment of other trichomonad species is important for increasing the understanding of the physiology of these parasites and the mechanisms involved in specific host–parasite interactions. A comparative analysis of these enzyme activities in trichomonads will contribute to the understanding of the physiological significance in regu-

lating the extracellular and intracellular nucleotide levels in the parasites.

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