ENZYMES OF PURINE SALVAGE IN *TRYPANOSOMA CRUZI*

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1. Introduction

Previous experiments in which we examined the incorporation of radiolabelled bicarbonate, formate and glycine by washed cell suspensions of blood trypomastigote, intracellular amastigote and culture epimastigote forms of *Trypanosoma cruzi* suggested that no form of the organism could biosynthesize de novo significant amounts of purine [1]. Absence of such biosynthesis implies dependence on salvage and in agreement with this, we detected substantial incorporation of radiolabelled purine bases and nucleosides, though not nucleotides [1]. Here, we report the presence in cell-free homogenates of the three main forms of *T. cruzi* of phosphoribosyltransferase, aminohydrolase, kinase, hydrolase and phosphorylase activities which might be involved in this salvage. A preliminary report of some of the data has been presented [2].

2. Materials and methods

2.1. *Radiotracers*

[8-^{3}H]Adenine, [8-^{3}H]guanine sulphate, [G-^{3}H]-hypoxanthine, [G-^{3}H]adenosine, [8-^{3}H]guanosine and [U-^{14}C]inosine were all obtained from the Radiochemical Centre, Amersham, Bucks.

2.2. *Parasites*

The Sonya strain of *Trypanosoma cruzi* was maintained in male and female ASH/XP mice (~20 g body wt). Blood from these animals was used to infect subcutaneously male and female chinchillas (300–500 g body wt; 10^7 trypomastigotes injected/100 g body wt). Parasites were harvested 14 ± 1 days after infection. Blood stages (mainly broad forms) were isolated using a technique involving differential centrifugation to remove most of the erythrocytes and DEAE-cellulose chromatography (with phosphate/saline/glucose buffer (pH 7.5) ionic strength = 0.206) to remove the remaining blood cells [3].

Intracellular stages (mainly amastigote forms) were isolated from hind limb muscle tissue. This was disrupted in an MSE tissue homogenizer and the homogenate incubated with DNase, collagenase and trypsin. Parasites, contaminated only by a few blood cells, were obtained by differential centrifugation. They were further purified in a linear sucrose gradient (0.25–0.70 M sucrose, 225 × *g* for 5 min) [3]. Culture epimastigote forms of the organism were grown at 28°C in a modified LIT-medium [4]. Cultures were harvested by centrifugation while in the mid-logarithmic phase of growth.

2.3. *Preparation of enzyme extracts*

Organisms (~5 × 10^9 cells/ml) were suspended in 50 mM Tris buffer (pH 7.0) containing 0.1 mM dithiothreitol and (except phosphoribosyltransferase and kinase activities) 1.0 mM EDTA and disrupted by 3 cycles of freezing (liquid nitrogen) and thawing (in a 37°C water bath). Homogenates were centrifuged at 100 000 × *g* (27 000 × *g* for hydrolase and phosphorylase assays) for 60 min and the supernatant fraction used as the source of enzyme activity. For some experiments, this was first dialysed overnight against 1000 vol. homogenisation buffer or desalted with a column of Sephadex G-25.

2.4. *Enzyme assays*

Phosphoribosyltransferase activity: the reaction mixture, based on [5] and in a final vol. 50 μl contained: Tris–HCl buffer (pH 7.0) 30 mM; $MgCl_2$, 2 mM; PRPP, 2 mM; [^{3}H]adenine, 0.4 mM, 1 μCi; or [^{3}H]hypoxanthine, 0.8 mM, 1 μCi; or [^{3}H]guanine, 0.05 mM, 1 μCi; ~30 μg protein.

2.4.1. Nucleoside kinase activity

The reaction mixture, based on [6] and in a final vol. 250 μl contained: Tris–HCl buffer (pH 7.0) 50 mM; dithiothreitol, 0.1 mM; KCl, 50 mM; $MgCl_2$, 0.5 mM; ATP, 0.7 mM; PEP, 0.25 mM; pyruvate kinase from rabbit muscle (Boehringer), 0.005 mg; radiolabelled nucleoside, 0.25 mM, 5 μCi ^3H or 0.25 μCi ^{14}C; ~150 μg protein.

2.4.2. Nucleoside hydrolase/phosphorylase activity

The reaction mixture, based on [7] and in a final vol. 50 μl contained: Tris–HCl buffer (pH 7.4) 20 mM; dithiothreitol, 0.1 mM; EDTA, 1 mM; radiolabelled nucleoside, 1 mM, 1–3 μCi ^3H or 0.05–0.15 μCi ^{14}C; ~100 μg protein; phosphate, 40 mM (phosphorylase assay only).

2.4.3. Adenine/adenosine aminohydrolase activity

The reaction mixture, based on [8] and in a final vol. 50 μl contained: Tris–HCl buffer (pH 7.4), 20 mM; dithiothreitol, 0.1 mM; EDTA, 1 mM; radiolabelled adenine/adenosine, 0.20/0.50 mM, 3 μCi ^3H; ~100 μg protein.

Incubations were carried out at 28°C (epimastigote extracts) or 37°C (trypomastigote and amastigote extracts). Reactions were stopped by heating the mixtures in a water bath at 95°C for 4 min. They were then centrifuged at 1200 × *g* for 10 min and the products in the centrifugal supernatants separated in the presence of internal markers by thin-layer chromatography on PEI cellulose plates with fluorescent indicator using water (0.1 M LiCl for hydrolases, phosphorylases and aminohydrolases) as solvent [5,9]. Spots were located with an UV lamp, cut out and estimated for radioactivity in a liquid scintillation system with a counting efficiency of 8–20% for ^3H and 40–80% for ^{14}C.

3. Results

Key enzymes involved in purine salvage in mammalian cells and the reactions which they mediate are shown in fig.1 [10].

We have shown that *T. cruzi* has an apparent preference for the salvage of purine bases, rather than nucleosides, and so initially we looked for enzyme activities which might be involved. We found in homogenates of all 3 main forms of *T. cruzi* adenine, hypoxanthine and guanine phosphoribosyltransferase activities which

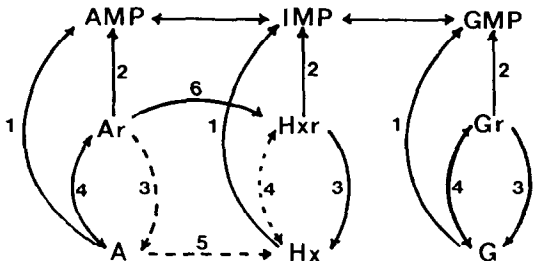

Fig.1. Enzyme activities involved in purine salvage [10]. Solid lines indicate reactions known to occur in *T. cruzi* (this paper or [1,12]). Dashed lines indicate reactions apparently absent for the organism. Enzyme activities: (1) phosphoribosyltransferase; (2) nucleoside kinase; (3) nucleoside hydrolase; (4) nucleoside phosphorylase; (5) adenine aminohydrolase; (6) adenosine aminohydrolase.

were markedly greater than those required to explain the rates of utilisation of the radiotracers by whole cells (~0.1 nmol . min^{-1} . mg $protein^{-1}$) (table 1). Note that as guanine is relatively insoluble, saturating concentrations may not have been reached in this assay (0.05 mM); the activities therefore may be below maximal.

It has been shown in *Crithidia fasciculata* [8] and *Leishmania* [11,12] that adenine and guanine are not only salvaged directly by phosphoribosyltransferases but also indirectly by prior deamination to hypoxanthine and xanthine, respectively. We have been unable to detect adenine aminohydrolase activity in our homogenates, but we were able to detect adenosine aminohydrolase (table 1). Levels in amastigotes were particularly high; lymphocyte contamination of these preparations (<0.1% by numbers) were far too low to explain these high values.

Nucleoside kinase activities were found in all 3 forms of the organism (table 1). They were at a lower level than those found for the phosphoribosyltransferase activities but were higher than those required to explain the rates of purine nucleoside utilisation by whole cells.

Finally, we looked for enzyme activities which might convert nucleosides to bases. These were found at relatively high levels in all 3 forms of the organism (table 1). We used 3 parameters to assign these activities to hydrolase or phosphorylase categories:

(i) We measured them in extracts prepared in Tris buffer and subsequently dialysed or desalted using columns of Sephadex G-25.

Table 1
Activities of purine salvage enzymes in homogenates of *T. cruzi*

Enzyme activity	Purine substrate	Specific activity[a]		
		Trypomastigote	Amastigote	Epimastigote
Phosphoribosyl transferase	Adenine	9.40 ± 5.20 (7)	8.04 ± 3.39 (2)	8.93 ± 2.40 (4)
	Hypoxanthine	14.20 ± 8.60 (7)	7.63 ± 6.70 (2)	12.00 ± 3.39 (4)
	Guanine	2.16 ± 1.89 (7)	3.53 ± 2.60 (3)	1.54 ± 0.56 (4)
Nucleoside kinase	Adenosine	0.29 ± 0.19 (3)	0.85 ± 0.37 (3)	0.71 ± 0.38 (12)
	Inosine	0.88 ± 0.17 (3)	1.23 ± 0.52 (3)	1.43 ± 0.20 (5)
	Guanosine	0.08 ± 0.06 (3)	0.14 ± 0.03 (2)	0.28 ± 0.15 (5)
Nucleoside hydrolase	Adenosine	n.d.[b]	n.d.[b]	n.d.[b]
	Inosine	5.18 ± 1.90 (3)	5.68 ± 0.42 (2)	7.84 ± 4.90 (10)
	Guanosine	2.17 ± 0.37 (3)	1.56 ± 1.20 (3)	1.50 ± 0.90 (3)
Nucleoside phosphorylase (catabolic direction)	Adenosine	1.106 ± 0.61 (6)	2.02 ± 1.12 (5)	2.39 ± 1.31 (4)
	Inosine	n.d.	n.d.	n.d.
	Guanosine	n.d.[b]	n.d.[b]	n.d.[b]
Nucleoside phosphorylase (anabolic direction)	Adenine	12.89 ± 10.63 (3)	42.3 ± 30.0 (2)	113 ± 49 (6)
	Hypoxanthine	n.d.	n.d.	n.d.
	Guanine	0.26 ± 0.02 (3)	2.09 ± 0.31 (2)	0.21 ± 0.09 (6)
Aminohydrolase	Adenine	n.d.	n.d.	n.d.
	Adenosine	0.05 ± 0.04 (3)	4.70 ± 2.20 (6)	0.12 ± 0.03 (6)

[a] nmol . min^{-1} . mg $protein^{-1}$ ± SD (no. determinations); [b] see text; n.d. not detectable

(ii) We looked for a stimulation of these activities in the presence of phosphate (40 mM).
(iii) We looked for nucleoside production when radiolabelled bases (1 mM) were incubated with ribose 1-phosphate (20 mM) under the conditions used for the hydrolase assay.

On the basis of these parameters, the bulk of the adenosine activity was identified as a phosphorylase, although we could not exclude the possibility that a small amount of it was due to a hydrolase. We noted that the activity measured in the anabolic direction was much higher than that measured in the catabolic direction. In contrast to the situation with adenosine, the activity with inosine was identified as that of a hydrolase. The rate of conversion of guanosine to guanine was reduced in the presence of phosphate, suggesting that it too was occurring by a hydrolase mechanism. However, we were able also to detect phosphorylase activity in the anabolic direction indicating that some phosphorylase activity is also present.

4. Discussion

The enzyme activities concerned with purine salvage which we have found to be present in homogenates of *T. cruzi* are indicated in fig.1. Together they form pathways which explain the utilisation of purine bases and nucleosides by washed cell suspensions of the organism [1]. The specific activities in all cases are about equal to or in excess of those required to explain the rates of utilisation by whole cells. Since the phosphorylases detected can function anabolically, there are at least 2 potential routes of assimilation of all the purine bases and nucleosides tested, with the exception of hypoxanthine.

The simplest explanation for the apparent presence of both guanosine hydrolase and phosphorylase activities is that guanosine is an alternative substrate both for the adenosine phosphorylase and the inosine hydrolase. We have yet to test this hypothesis experimentally.

Results were essentially the same for blood trypomastigote, intracellular amastigote and culture epimastigote forms of the organism. Our results thus provide further evidence for the view that there is little variation in purine and pyrimidine metabolism during the life cycle of *T. cruzi* [13].

Some of our results are in disagreement with published data. In particular, the adenosine phosphorylase activity found here has been described as being due to a hydrolase and the apparent absence of adenosine aminohydrolase in epimastigotes has been reported [12]. The presence of the former is best shown, as here, by demonstration of the reversibility of the reaction. The existence of the latter is seen most clearly in the amastigote forms of the organisms. This was not attempted by the previous investigators.

The presence of various purine phosphoribosyltransferase activities have also been described in other parasitic protozoa, including *Leishmania* [14,15], *Crithidia fasciculata* [16], *Trypanosoma gambiense* [9] and *Plasmodium chabaudi* [5,17]. The same authors have inferred the presence of nucleoside kinases. Inosine and guanosine but not adenosine hydrolase activities have been described in *Leishmania* together with a hint of the presence of an adenosine phosphorylase activity [18]. In contrast, purine nucleoside hydrolases of much broader specificity have been described in *Crithidia fasciculata* [19] and *T. gambiense* [9]. Adenine aminohydrolase appears to be present in *C. fasciculata* and *Leishmania* but adenosine aminohydrolases are apparently absent [8,11,12].

The main purine in the circulatory system of the mammal is hypoxanthine [14]. The apparent absence of inosine phosphorylase activity in *T. cruzi*, which might function in the anabolic direction together with an inosine kinase to form an alternative pathway for the salvage of this purine base, means that the only pathway by which this can be salvaged involves a phosphoribosyltransferase activity. Inhibitors of this activity should therefore block the growth of all forms of *T. cruzi*. In addition, it has not escaped our notice that the purine phosphoribosyltransferase activities provide a mechanism for the generation of the metabolites of allopurinol which are believed to be responsible for growth inhibitory effects of that compound on *T. cruzi* [20,21]. We are now engaged therefore in purifying, characterising and looking at the inhibitor sensitivity profiles of the purine phosphoribosyltransferase activities.

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