The regulation of phosphofructokinase in epimastigote *Trypanosoma cruzi*

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Received 17 April 1986

Glycosomal (microbody)-enriched fractions prepared from epimastigote *Trypanosoma cruzi* were used as a partially purified source of phosphofructokinase. D-Fructose 6-phosphate showed sigmoidal kinetics at pH 7.0, but hyperbolic kinetics at pH 8.0. Various adenosine nucleotides were positive effectors; 5′-AMP was the most powerful. ATP showed hyperbolic kinetics under all conditions tested. Several described inhibitors and activators of mammalian phosphofructokinase were without significant effect on the trypanosomal enzyme; the absence of effect of D-fructose 2,6-bisphosphate is of particular note.

Phosphofructokinase *D-Fructose 2,6-bisphosphate* (Trypanosoma cruzi, *Parasitic protozoon*)

1. INTRODUCTION

Chagas' disease is a major cause of suffering and death in Latin America. The aetiological agent is the parasitic protozoon, *Trypanosoma cruzi*. There is no effective chemotherapy for established cases [1]. Recognition of differences in host and parasite biochemistry may help change this fact.

The early glycolytic enzymes in the related *T. brucei* are contained in a microbody-like organelle, the glycosome [2]. Phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11; PFK) is particulate and shows detergent latency in all three developmental stages of *T. cruzi* [3]. A glycosomal location has now been established [4].

PFK is a key regulatory enzyme of glycolysis in mammalian cells [5], and recently D-fructose 2,6-bisphosphate ($F26P_2$) has been described as a powerful positive effector of PFK from many sources [6]. PFK shows a broadly similar pattern of regulation in several other members of the order Kinetoplastida related to *T. cruzi*; D-fructose 6-phosphate (F6P) kinetics are sigmoidal, 5′-AMP is a positive effector, but no other important effectors have been identified. $F26P_2$ is without effect on the enzyme from *T. brucei* [7]. PFK studied in *T. cruzi*, however, failed to show sigmoidal kinetics with F6P; the effect of $F26P_2$ was not examined [8].

In this paper, glycosomal fractions prepared from epimastigote *T. cruzi* cultures were used as a source of partially purified PFK. Sigmoidal kinetics were demonstrated with F6P as substrate under some assay conditions and no stimulation by $F26P_2$ was detected.

2. MATERIALS AND METHODS

Media components were obtained from Difco. Biochemicals and enzymes were obtained from Boehringer or Sigma. Chemicals were supplied by BDH or Fisons.

The Sonya strain of *T. cruzi* [9] was maintained in liquid medium [10] by serial passage. Mid-log

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

cultures were harvested by centrifugation and washed once in buffered saline then once in breakage medium (0.25 M sucrose, 1 mM disodium edetate, 25 mM Tris-HCl, pH 7.6). After resuspension in breakage medium, cells were broken by grinding with silicon carbide [11]. After a low-speed centrifugation to remove abrasive, a 5000 × *g* (10 min) supernatant was prepared and applied to the top of a linear 0.8–2.0 M sucrose gradient; volume 20 ml. After centrifugation at 130000 × *g* for 180 min the gradient was fractionated and glycosomal fractions with an average density of 1.23 g/ml were pooled and stored in liquid nitrogen until assayed. Preparative centrifugations were performed using an MSE Prepspin 50 ultracentrifuge.

Two PFK assays were employed: (a) contained 66 mM Hepes (pH in text), 5 mM $MgSO_4$, 1 mM disodium edetate, 0.2 mM NADH, 3 U/ml aldolase, 4.5 U/ml triosephosphate isomerase, 4.5 U/ml glycerol-3-phosphate dehydrogenase,

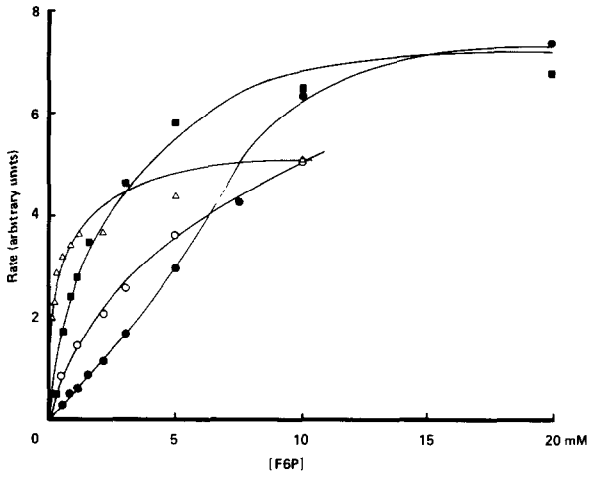

Fig.1. The effects of varying pH and of the addition of 5′-AMP on F6P kinetics. All assays contained fixed [ATP] = 0.5 mM; all other assay conditions were those given in section 2. ■, pH 8.0; ●, pH 7.0; △, pH 7.0, 3 mM AMP; ○, pH 7.0, 30 μM AMP.

Table 1

Effector screen

No effect	Stimulatory effect	Inhibitory effect
2-Phosphoglycerate	5′-AMP	calcium chloride (5 mM)
2,3-Diphosphoglycerate	3′-AMP	D-glucose 1,6-bisphosphate
Phospho*enol*pyruvate	cyclic AMP	D-fructose 2,6-bisphosphate
6-Phosphogluconate	ADP	oleate (0.25 mM)
Calcium chloride	3-phosphoglycerate	
Potassium chloride		
Ammonium acetate		
Inosine 5′-diphosphate		
Inorganic phosphate		
Aspartic acid		
Glutathione (oxidised and reduced forms)		
Citrate		
Isocitrate		
Succinate		
D-Fructose 1,6-bisphosphate		
Oleate (50 μM)		

Working at fixed assay conditions, at pH 7.5, PFK activity in the presence or absence of various possible effectors was measured. Each rate was performed in triplicate then the mean values compared. Stimulatory and inhibitory effectors were defined as those causing greater than 25% change in reaction rate. Each putative effector was added to a final concentration of 1 mM unless otherwise specified

0.1% (w/v) Triton X-100, and ATP and F6P to final concentrations as specified; (b) contained 66 mM Hepes (pH in text), 5 mM $MgSO_4$, 10 mM KCl, 1 mM disodium edetate, 0.2 mM NADH, 0.71 mM phospho*enol*pyruvate, 7 U/ml pyruvate kinase, 10 U/ml lactate dehydrogenase, 0.1% (w/v) Triton X-100, and ATP and F6P to final concentrations as specified.

In both assays, water and glycosomal extract were added to a final volume of 1.0 ml. Assay (b) was only used when D-fructose 1,6-bisphosphate was added. All coupling enzymes had been extensively dialysed to remove ammonium sulphate. As the glycosomal extracts contained contaminating phosphoglucose isomerase activity, all assays contained both F6P and D-glucose 6-phosphate in molar ratio 1:2.5 [12]. Reaction rates were measured by recording the decrease in A_{340} caused by the coupled oxidation of NADH. Assays were performed on a Unicam SP8000 double beam recording spectrophotometer with the cuvette holder maintained at 28°C.

3. RESULTS

The effect of pH on F6P kinetics is shown in fig.1. Sigmoidal kinetics were obtained at pH 7.0, whilst they were hyperbolic at pH 8.0. Hill plots (not shown) gave Hill coefficients of 1.9 at the lower pH, and 0.84 at the higher value.

The results of a screen for possible effectors are shown in table 1. Of the glycolytic and tricarboxylic acid cycle intermediates examined, only 3-phosphoglycerate appreciably changed the reaction rate (it was weakly stimulatory). All the adenosine nucleotides examined were strongly stimulatory. Various other described positive effectors of PFK from other sources were either without effect or were inhibitory.

Fig.1 also shows that the addition of 5′-AMP caused F6P kinetics at pH 7.0 to become hyperbolic, with an increase in affinity of PFK for F6P. The K_m for F6P at pH 8.0 is 1.9 mM; at pH 7.0 in the presence of 3 mM 5′-AMP it is 50 μM; the K_{mapp} at pH 7.0 in the absence of 5′-AMP is 5.8 mM.

ATP kinetics at fixed [F6P] were hyperbolic at both pH 7.0 and pH 8.0; Hill coefficients were less than unity. No marked inhibition was seen at concentrations of ATP up to 5 mM. The addition of 5′-AMP (0.1 mM) caused the K_m of ATP to fall from 1.75 mM to 0.80 mM at pH 7.0; at pH 8.0, K_m = 0.40 mM (not shown).

The relative K_{mapp} values of various adenosine phosphate stimulators of PFK are shown in table 2. 5′-AMP is the most potent stimulator of activity, cyclic AMP (adenosine 3′,5′-cyclic monophosphate) the least. The effect of adding D-fructose 2,6-bisphosphate on F6P kinetics is shown in fig.2. Some inhibition is visible at $F26P_2$ =

Table 2

Effectiveness of different adenosine nucleotides as stimulators of PFK

Adenosine nucleotide	K_{mapp}
Cyclic AMP	2.6 mM[a]
ADP	0.28 mM
3′-AMP	0.77 mM
5′-AMP	33 μM

[a] Determined at pH 7.0; other nucleotide K_{mapp} values were determined at pH 8.0

Low [F6P] and [ATP] were chosen such that PFK activity was negligible. The addition of different concentrations of each effector caused a hyperbolic stimulation of activity in each case, from which K_{mapp} values were calculated using double reciprocal plots (not shown)

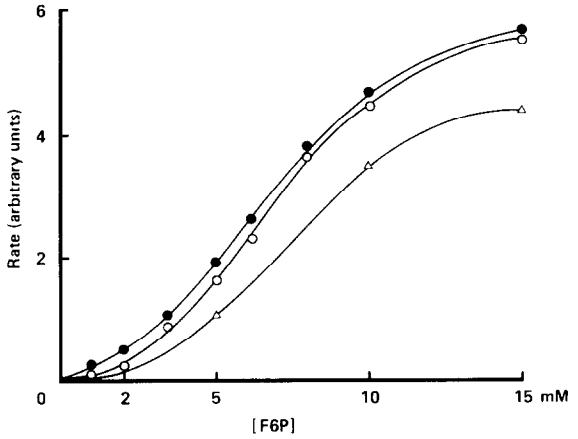

Fig.2. The effect of adding $F26P_2$ on F6P kinetics. Each assay contained fixed [ATP] = 0.5 mM, and was performed at pH 7.0. Other assay conditions were as given in section 2. ●, no $F26P_2$ added; ○, 0.1 mM $F26P_2$; △, 0.5 mM $F26P_2$.

0.1 mM; it is more marked at 0.5 mM. These data support the result for $F26P_2$ shown in table 1. The $F26P_2$ used stimulated a commercially obtained rabbit muscle PFK; mixing experiments failed to demonstrate any inhibitor of this action in the glycosomal preparation used (not shown).

4. DISCUSSION

PFK examined in a partially purified glycosomal fraction from epimastigote *T. cruzi* showed cooperativity of binding of F6P at low pH, but no evidence of ATP cooperative binding, nor was there any significant inhibition by high [ATP] (up to 5 mM). Adenosine nucleotides were stimulators of PFK activity, 5′-AMP being the most potent. Various other effectors of mammalian PFK did not act similarly on the *T. cruzi* enzyme. In particular, the sugar bisphosphates including $F26P_2$ had no stimulatory effect; the inhibition seen with $F26P_2$ was probably non-allosteric in nature. Raising the pH increased the affinity of PFK for both substrates.

This pattern of regulation resembles that seen for PFK in other members of order the Kinetoplastida including *Leishmania donovani* and *L. braziliensis* [13], *Crithidia fasciculata* [14], and *T. brucei* [7,15]. Lowering the pH inhibits PFK from *T. cruzi*, which is also seen in mammalian PFK [5] and that in *C. fasciculata* [14], although the opposite is observed in *L. donovani* [13]. PFK purified from epimastigote *T. cruzi* by sonication followed by ammonium sulphate precipitation showed no cooperativity of F6P binding [8] at pH 7.4; possible explanations include the method of purification, or contamination of ATP by 5′-AMP. It is also of note that PFK purified from *T. brucei* by a method similar to that employed in this paper showed a K_{mapp} for 5′-AMP of 7 μM [15], whilst PFK purified to homogeneity from the same organism [7] had a K_{mapp} for 5′-AMP at 0.64 mM. The glycolytic glycosomal enzymes of *T. brucei* are very tightly associated [16], and over-purification may alter the kinetic properties of PFK, a phenomenon noted when the enzyme is obtained from various other sources [17]. Glycosomal fractions in *T. cruzi* contain low hydrolase activities [4] and the interference caused by copurified phosphoglucose isomerase was eliminated (v.s.).

Mammalian PFK is modulated in a highly complex fashion, and possesses at least nine different regulatory sites [18]. The isofunctional enzyme in *T. cruzi* has a far simpler pattern of regulation resembling related members of the order Kinetoplastida previously examined. The lack of stimulatory effects of $F26P_2$ shown here agrees with a similar lack of effect on the purified enzyme from *T. brucei* [7]. $F26P_2$ is the most potent positive effector known for PFK from most eukaryotic sources [6]; perhaps the glycosomal localisation of the enzyme in members of the order Kinetoplastida reflects an early evolutionary divergence of modes of control of PFK in this order. A proton gradient across the glycosomal membrane could regulate PFK; such a gradient is maintained in vacuoles in *Saccharomyces carlsbergensis* by an ATP-dependent membrane proton pump [23].

The high value for K_{mapp} for cAMP suggests that this compound is not a physiological effector of PFK in *T. cruzi*; it is of note that cAMP-receptor proteins in *T. cruzi* [20] and *T. brucei* [21] are predominantly cytosolic, and that adenyl cyclase is associated with the plasma membrane of *T. cruzi* [22].

Inhibition of PFK by oleate occurs at 0.25 mM but not at 50 μM. The former concentration of free fatty acid is probably grossly unphysiological, and PFK is unlikely to be modulated in *T. cruzi* by this compound; it is of relevance that β-oxidation in this organism appears to have no glycosomal component [4].

Glucose is the most important exogenous energy substrate for the trypomastigote (bloodstream) form of *T. cruzi*, and the same is probably true of the amastigote (intracellular, tissue) stage [19]. All three morphological forms possess particulate PFK, presumably glycosomal [3]. The pattern of regulation of PFK in the easily obtained culture epimastigote is probably mirrored in the other two stages described above, which parasitise man. Antileishmanial antimonial drugs probably act by inhibiting PFK [19], and it is to be hoped that the trypanosomal enzyme here described may also be selectively inhibited by a new drug some day.

ACKNOWLEDGEMENT

We would like to thank Dr C.G. Proud for helpful discussions at the beginning of this project.

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