Gene 424 (2008) 25–32

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

A biochemical and genetic study of *Leishmania donovani* pyruvate kinase ☆

Will Sandoval [a], Raúl Isea [b], Evelyn Rodriguez [a], Jose Luis Ramirez [a,c,*]

[a] Centro de Biotecnologia, Instituto de Estudios Avanzados, Ministerio de Ciencia y Tecnologia de Venezuela, Carretera Nacional Hoyo de la Puerta, Caracas 1080 A, Venezuela
[b] CeCalCULA Parque Tecnológico de Mérida-Edif Masini, Piso 3, Ofic. B32, Mérida 5101, Venezuela
[c] United Nations University Programme UNU-BIOLAC, Venezuela

ARTICLE INFO

Article history:
Received 24 January 2008
Received in revised form 20 June 2008
Accepted 29 July 2008
Available online 3 August 2008

Keywords:
Visceral leishmaniasis
Glycolysis
In situ mutagenesis
Structural studies

ABSTRACT

Here we present a biochemical and molecular biology study of the enzyme pyruvate kinase (PYK) from the parasitic protozoa *Leishmania donovani*. The PYK gene was cloned, mutagenised and over expressed and its kinetic parameters determined. Like in other kinetoplastids, *L. donovani* PYK is allosterically stimulated by the effector fructose 2,6 biphosphate and not by fructose 1,6 biphosphate. When the putative effector binding site of *L. donovani* PYK was mutagenised, we obtained two mutants with extreme kinetic behavior: Lys453Leu, which retained a sigmoidal kinetics and was little affected by the effector; and His480Gln, which deployed a hyperbolic kinetics that was not changed by the addition of the effector. Molecular Dynamics (MD) studies revealed that the mutations not only altered the effector binding site of *L. donovani* PYK but also changed the folding of its domain C.

1. Introduction

Leishmania donovani is the etiological agent of Old World Visceral leishmaniasis (VL) or Kalazaar, a deadly disease affecting millions of people in Africa and India. Despite the recent introduction of miltefosine, an apparent more effective and little toxic drug against VL, there are early warnings that drug resistant strains can be easily produced (Croft et al., 2006), therefore there is a continuous need to develop newer, cheaper, and more effective drugs against VL.

The trypanosomatid *Leishmania* belongs to a group of protozoa that when living in a mammalian host relies heavily on the glycolytic pathway for the production of ATP (Tielens et al., 1998; Cazzulo, 1992). Trypanosomatids glycolysis is particularly suited as a target for high affinity, non-competitive or irreversible enzyme inhibitors. Moreover, most of the enzymes involved in the conversion of glucose into pyruvate are located in an organelle called the glycosome (Opperdoes and Borst, 1977; Michels et al., 2000) One consequence of this organization is that the glycolytic flux through this organelle seems unregulated (Nwagwu and Opperdoes, 1982). In trypanosomatids hexokinase (EC 2.7.1.1) and phosphofructokinase (EC 2.7.1.11), key regulatory enzymes of glycolysis (van Schaftingen et al., 1985), are not affected by the typical effectors described for other cells. On the other hand, trypanosomatid PYK (EC 2.7.1.40) is a cytosolic enzyme separated from the rest of the glycolytic enzymes. Contrary to mammalian PYKs, which are allosterically regulated by F1,6BP, trypanosomatids PYKs are activated by submicromolar concentrations of fructose 2,6–biphosphate (F2,6BP) a typical effector of mammalian PFK and are relatively unaffected by fructose 1,6-biphosphate (F1,6BP) (van Schaftingen et al.,1985; Ponte-Sucre et al., 1993; Ernest et al., 1994a,b, 1998). PYK plays an important role in regulating glycolytic flux from F1,6BP to pyruvate and catalyzes the irreversible final step of this pathway by which the phosphor group of phosphoenolpyruvate (PEP) is transferred to ADP to form pyruvate and ATP. Like in any other organism, trypanosomatids (*Trypanosoma brucei*, *L. mexicana* and *Trypanoplasma borelli*) (van Schaftingen et al., 1985; Ponte-Sucre et al., 1993; Ernest et al., 1994a,b, 1998) PYKs are homotetrameric with a subunit molecular mass of approximately 54,500 and share 47–51% amino acids sequence identity with other eukaryotic PYKs (Ernest et al., 1994b, 1998; Rigden et al., 1999). The crystal structure of *L. mexicana* PYK has been reported (Rigden et al., 1999). *L. mexicana* PYK shares the same overall secondary structure with *Escherichia coli*, rabbit muscle and yeast. Each subunit contains four domains: N-terminal, A, B, and C. The active site lies in a pocket found between domains A and B; and in *L. mexicana* domain C contains binding site for the allosteric effector.

In *Leishmania*, PYK represents an important metabolic crossroad linking intermediaries of the incipient trypanosomatid's Krebs cycle, lipid synthesis and the glycolytic pathway. Although in a recent report RNAi gene inactivation seems to be possible in *L. braziliensis* (Peacock et al., 2007), in general, studies to assess the metabolic importance of *Leishmania* glycolytic enzymes have been hampered by the difficulty in implementing in vivo gene inactivating techniques. In *T. brucei* where

Abbreviations: CHEF, Contour Clamped Hexagonal Electric Field; FIGE, Field Inversion Gel Electrophoresis; F1,6BP, fructose 1,6 biphosphate; F2,6BP, fructose 2,6 biphosphate; MD, Molecular Dynamics; MEM, Minimum Essential Medium; PYK, Pyruvate Kinase; RMSD, relative displacements; $S_{0.5}$, substrate concentration at half-maximum velocity; VL, Visceral Leishmaniasis; WT, Wild type.

☆ The DNA and protein sequence of *Leishmania donovani* has been deposited at GenBank with accession number EU024521.

* Corresponding author. Centro de Biotecnologia, Instituto de Estudios Avanzados, Ministerio de Ciencia y Tecnologia de Venezuela, Carretera Nacional Hoyo de la Puerta, Caracas 1080 A, Venezuela. Tel.: +58 212 903 5084; fax: +58 212 903 5086.
E-mail address: ramjoseluis@gmail.com (J.L. Ramirez).

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doi:10.1016/j.gene.2008.07.034

RNAi inactivating experiments are feasible, the essentiality of PYK on parasite survival has been firmly established (Coustou et al., 2003).

In the present work we have studied biochemically and genetically *L. donovani* PYK, the gene has been cloned, over expressed and mutated. When *L. donovani* PYK was mutated in critical amino acids of the putative binding site for the allosteric effector F2,6BP, mutated enzymes lacked the normal allosteric transition of the enzyme exhibiting two extreme kinetic behaviors.

2. Materials and methods

2.1. Parasite cells

L. donovani MHOM/SD/00/Khartoum (LSB-52-1) and *L. donovani* MHOM/SD/00/Khartoum (LSB-52-1) clone 4.1.3 were kindly donated by Dr. Kenneth Stuart from Seattle Biomedical Research Institute (SBRI), Seattle, Washington, U.S.A. *Leishmania* promastigotes were cultured at 24 °C in RPMI 1640 (Gibco-BRL) medium supplemented with inactivated fetal bovine serum at 10% (vv^{-1}) (Gibco-BRL), essential amino acids at 5% in minimum essential medium (vv^{-1}) (MEM Gibco-BRL) without L-glutamine, sodium pyruvate in MEM, non-essential amino acids in MEM and 0.2% glucose (wv^{-1}).

2.2. Genomic DNA isolation

Leishmania epimatigotes cells in log phase were collected by centrifugation and washed twice with saline solution. Cell pellet was resuspended in 0.5 mL of saline phosphate buffer ($NaPO_4$ pH 8; 65 mM NaCl, 1% Glucose (wv^{-1})). Parasite disruption was promoted by the addition of an equal volume of lysis buffer (10 mM Tris–HCl pH 7.5, 100 mM NaCl, 0.5% SDS (wv^{-1}), 1 mM EDTA) and incubated overnight at 37 °C with proteinase K at 10 μg ml^{-1}. The lysate was extracted twice with an equal volume of a mixture of chloroform: phenol (1:1 vv^{-1}), and once with a mixture of chloroform: isoamylic alcohol (24:1 vv^{-1}). The aqueous phase was transferred to a new tube and the DNA precipitated by the addition of 2.5 volumes of ethanol at 99% (vv^{-1}) and storage at −20 °C overnight. The DNA precipitated was collected by centrifugation at 10,000 ×*g* for 15 min, vacuum dried and resuspended in sterile bi-distillated water.

2.3. Preparation of high molecular weight DNA

Leishmania chromosome DNA was isolated from early log epimastigotes cells following the protocol described by (Galindo and Ramirez, 1989). Cells were collected by centrifugation at 4000 ×*g* for 12 min at 4 °C, and the pellet was resuspended (4×10^8 cells mL^{-1}) in saline phosphate buffer. Cell suspension was mixed with an equal volume of low melting point agarose 2%, prepared in saline phosphate buffer. Agarose blocks containing *Leishmania* chromosomal DNA were lysated in buffer (0.5 M EDTA pH 9.0, 1% sodium N-lauryl sarcosinate, and proteinase K 2 mg mL^{-1}) for 12 h at 37 °C. Blocks were stored in 0.5 M EDTA pH 9.0 solution at 4 °C.

2.4. Plasmid DNA isolation

Recombinant plasmidic DNA was isolated from bacteria according to the protocol of Birnboim and Doly, 1979. For DNA sequencing, plasmids were isolated with the kit Wizard® Plus SV Minipreps DNA Purification System, following (Promega) indications.

2.5. DNA gel electrophoresis

DNA gel electrophoresis was done on agarose gels of various concentrations in a horizontal chamber using either l× TBE buffer (80 mM Tris–HCl pH 7.6, 90 mM boric acid, 2 mM EDTA) or 1× TAE buffer (40 mM Tris–acetate pH 8, 0.5 M EDTA). Samples were loaded on gels after adjusting them to l× loading buffer (0.042% bromophenol blue (wv^{-1}), 0.042% xylene-cyanol (wv^{-1}), 5% (vv^{-1}) glycerol). Electrophoretical runs were done with voltage gradient of 5 V. cm^{-1}. After runs, gels were stained with ethidium bromide at 0.5 μg mL^{-1} and DNA bands were observed at 302 nm in a UV mini-transilluminator Gel Doc 1000® (BioRad).

2.6. Pulse field electrophoresis

2.6.1. Field Inversion Gel Electrophoresis (FIGE)

This system was used to purify cosmid 11A7. Samples were loaded on 8% agarose gels in 0.5× TBE in an electrophoresis chamber BRL model H3. Electrophoresis was done with a voltage gradient of 4 V cm^{-1} for 10 h at 16 °C. Field inversion pulse times were controlled with the system PPI-200 (MJ Research, Inc.) using the following program: initial pulse ramp 0:0.01 s with 0.05 s increments on each step. Steps were repeated 20 times.

2.6.2. Contour clamped hexagonal electric field (CHEF) electrophoresis

Leishmania chromosomal size DNA was separated in 1% agarose gels in 0.5× TBE buffer using a CHEF-DR (BioRad) apparatus (Chu et al., 1986). The electrophoresis run was done at 14 °C with a voltage gradient of 4 V cm^{-1}, and the following pulse program: initial time 60 s, final time 120 s, run length 20 h, electric field angle 120°.

2.7. Isolation of DNA fragments from agarose gels

DNA bands resolved in low melting point agarose (Gibco-BRL) were sliced out with a razor blade and the agarose blocks were covered with solution of 3 M sodium acetate and 1× TE pH 5.2 and melted by incubation at 65 °C for 10 min. DNA was extracted twice with 500 μL of saturated phenol in Tris–HCl pH 7.5 at 65 °C. To precipitate the DNA from the aqueous phase, two volumes of 99% (vv^{-1}) ethanol were added and incubated overnight at −20 °C. DNA was collected by centrifugation at 10,000 ×*g* for 15 min, and the pellet was washed with 500 μL of 70% (vv^{-1}) ethanol and air dried.

2.8. Southern transfers

DNA was vacuum transferred into nylon Hybond-N^+filters (Amersham) using a LKB 2016 VacuGene instrument with a pressure of 50 cm Hg. Gel was equilibrated with 10X SSC (3 M NaCl, 0.3 M sodium citrate) for 10 min. Then the gel was washed in 0.25 N HCl during 7 min. After this incubation, this solution was removed and replaced by alkaline solution (0.5 N NaOH, 1.5 M NaCl). The gel was equilibrated in a buffer solution (0.5 N Tris–HCl pH 7.5, 1.5 M NaCl) for 10 min. Finally, the transference was done running 20× SSC solution through the gel during 45 min. The filter was removed, and the DNA was fixed by heating for 2 h at 80 °C.

2.9. Probe labeling

Radioactive labeling: Probes were labelled with [α-^{32}P] dCTP (3000 Ci $mmol^{-1}$) using the Random Primer kit Megaprime® (Amersham) and following Manufacturer's protocol. Non-radioactive labeling was done by adding dUTP-digoxigenine tails to the oligos using terminal transferase (Boehringer Mannheim).

2.10. Cloning L. donovani PYK

A genomic library of *L. donovani* (kindly donated for Dr. Theo De Vos of the Seattle Biomedical Research Institute) constructed in Supercos 7 vector was screened with a probe of *L. mexicana* whole gene kindly donated by Dr. Paul Michels, Institute of Cellular Pathology, Université Catholique de Louvaine, Brussels, Belgium. One positive colony was grown for cosmid DNA purification.