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Molecular Cloning, Expression and Characterization of Ribokinase of *Leishmania major*

Patrick O. J. OGBUNUDE[1]*, Nadia LAMOUR[2], and Michael P. BARRETT[3]

[1] *Department of Medical Biochemistry, University of Nigeria, Enugu 1000004, Nigeria;*
[2] *Department of Biochemistry and Massey Cancer Center, Virginia Commonwealth University, Richmond 23298-0614, USA;*
[3] *Institute of Biomedical and Life Sciences, Division of Infection and Immunity, Joseph Black Building, University of Glasgow, Glasgow G12 8QQ, UK*

Abstract Ribokinase (EC 2.1.7.15) from *Leishmania major* was cloned, sequenced and overexpressed in *Escherichia coli.* The gene expressed an active enzyme that had comparable activity to the same enzyme studied in *E. coli*. It specifically phosphorylated *D*-ribose. Under defined conditions, the K_m for the substrates *D*-ribose and ATP were 0.3±0.04 mM and 0.2±0.02 mM, respectively. The turnover numbers of the enzyme for the substrates were 10.8 s^{-1} and 10.2 s^{-1}, respectively. The enzyme product ribose 5-phosphate inhibited the phosphorylation of *D*-ribose with an apparent K_i of 0.4 mM, which is close to the K_m (0.3 mM) of *D*-ribose, suggesting that it might play a role in regulating flux through the enzyme.

Keywords *Leishmania major*; *D*-ribose; ribokinase; molecular cloning

Leishmania are parasitic protozoa belonging to the order kinetoplastida. These parasites cause leishmaniasis, a spectrum of diseases that causes considerable morbidity and mortality in tropical and subtropical regions [1]. Drugs currently available for treatment of leishmaniasis are far from ideal [2]. Development of new antileishmanial drugs is desirable. An understanding of the biochemical physiology of these parasites will underpin development of new drugs and the completion of the *Leishmania major* and *Leishmania infantum* genome sequences is facilitating identification of new drug targets [3]. Ribose plays a number of roles in *Leishmania* metabolism. For example, these protozoa are auxotrophic for purines and they express multiple pathways of purine uptake [4] and salvage [5] that enable them to acquire and use these vital metabolites from their hosts. Ribose 5-phosphate (ribo-5-P) is a key precursor of 5-phospho-*D*-ribosyl-1-α-pyrophosphate, which is used in the synthesis of ribonucleoside 5′-monophosphates. Ribose can also be used directly as an energy source with the pentose phosphate pathway (PPP) [6,7] shunting intermediates into the glycolytic pathway. The PPP can also generate ribo-5-P from glucose. Ribokinase is also capable of generating ribo-5-P using ribose that is either transported directly from its environment [7,8] or liberated from nucleosides using nucleoside hydrolase activities found in these parasites [9,10]. An X-ray crystal structure of ribokinase from *Escherichia coli* [11] shows that it is a homodimer in solution [12] with each 33 kDa subunit composed of two domains [13]. Sequence analyses show that it belongs to the *PfkB* family of carbohydrate kinases [14,15].

Here we report the cloning and functional expression of a ribokinase gene from *L. major*.

Materials and Methods

Design of oligonucleotide primers and polymerase chain reaction amplification

BLAST (http://blast.wustl.edu) searches of the *L. major* genome indicated a gene on chromosome 27 (LmjF27.0420)

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*Corresponding author: Tel, 234-42-301319; Fax, 234-42-303160; E-mail, Ogbunudejim@yahoo.co.uk

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with an open reading frame of 990 bp encoding a 329 amino acid protein orthologous with ribokinase from other organisms. The 3-D structure of the *E. coli* enzyme [12, 13] shows a complex and intricate network of hydrogen bonds involved in the binding of the ribose substrate. Six residues (Asp16, Gly42, Lys43, Asn46, Glu143 and Asp255) involved in this H-bonding network in *E. coli* are all conserved in *L. major*, indicating a conserved catalytic mechanism. Synthetic oligonucleotides to amplify the gene (MWG Biotech, Ebersberg, Germany) comprised a forward primer (5′-AAACATATGCACCGTGTGCAGAACGTT-3′) covering the first methionine of the protein and a reverse primer (5′-AAACTCGAGCTACGTGACACCAGCC-3′) covering the stop codon. The underlined bases represent restriction sites (*Nde*I and *Xho*I) that were inserted to facilitate cloning of the polymerase chain reaction (PCR) amplified products. Amplification was carried out in a DNA thermo cycler (MJ Research, Western Town, USA) using a 100 μl reaction tube containing 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 μM KCl, 200 μM of each dNTP, 40 ng of each primer and 1 U of *Pfu* DNA polymerase. The tube containing the reactants was placed in the thermo cycler programmed for 30 cycles; a single cycle at 94 ºC for 2 min was followed by 30 cycles at 63 ºC for 15 s and 72 ºC for 2 min with a final cycle at 72 ºC for 10 min.

Plasmid construction, insertion and protein overproduction

After agarose gel electrophoresis, the amplified DNA was isolated using the QIAquick Gel Extraction Kit (Qiagen, West Sussex, UK) then cloned into the pGEM-T Easy plasmid (Novagen, Nottingham, UK). After amplification in *E. coli* strain DH5α (Invitrogen, Paisley, UK) the *Nde*I/*Xho*I fragment of pGEM-T was inserted into plasmid pET28a$^+$ (Novagen, Nottingham, UK). The resulting plasmid, pET28a$^+$ribo, was sequenced to confirm the identity, then introduced into *E. coli* strain BL21 (DE3) (Statagene, Amsterdam Zuidoost, Netherlands) for protein overexpression. Overexpression was induced by the addition of 0.5 mM IPTG and incubation with vigorous aeration overnight at room temperature. Cells were harvested by centrifugation, washed once (50 mM HEPES, pH 7.0, 300 mM NaCl, 10 mM EDTA) and lysed by sonication in buffer A (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0). The soluble fraction was recovered by centrifugation at 10,000 *g* for 30 min at 4 ºC. This was applied to a nickel-nitrilotriacetic acid column (BioCAD Vision Workstation; Global Medical Instrumentation, Inc. Ramsey, USA) pre-equilibrated with buffer A. The column was washed with 100 ml of buffer A containing 0.5 mM imidazole, then with buffer A containing 50 mM imidazole. The recombinant protein was eluted with buffer A containing 500 mM imidazole.

Enzyme and inhibitor studies

Enzyme activity was measured spectrophotometrically at room temperature (22 ºC) by a coupled enzyme technique [16]. All the assays were carried out in Tris-HCl buffer at pH 8.5 having established these conditions to be optimal. Ribose with Mg^{2+}-ATP gives ribo-5-P and adenosine diphosphate. The adenosine diphosphate (ADP) is used by phosphoenolpyruvate in a reaction catalyzed by pyruvate kinase to regenerate ATP, and the resulting pyruvate is converted by lactate dehydrogenase to lactate. In the process, NADH is converted to NAD^+. Reaction rates were monitored at 340 nm as a decrease in NADH absorbance when converted to NAD^+. For determination of kinetic constants, the ATP concentrations varied from 0.025 mM to 0.75 mM when the ribose concentration was held constant at 5 mM, and ribose concentrations from 0.0125 mM to 5 mM when ATP concentration was held constant at 0.75 mM. This concentration of ATP (0.75 mM) was determined from preliminary experiments to be the maximal that can be used. The activity of the enzyme began to decline at concentrations above this level. The substrate concentrations, when kept constant, were ATP 0.75 mM, Mg^{2+} 1.0 mM and ribose 5.0 mM. In the experiments where inhibitors were included, the concentrations used are indicated in the figure legend. Reactions were started by the addition of the enzyme (15 μl, which contained 24 μg protein). Reaction volumes were 1 ml. Protein concentration was measured by the method of Bradford [17].

Results and Discussion

The recombinant protein eluted from the nickel-nitrilotriacetic acid column (**Fig. 1**, lane 2) was dialysed overnight in 50 mM Tris-HCl, pH 7.0 at 4 ºC and stored at –70 ºC in buffer/glycerol (1/1). This recombinant enzyme retained more than 50% of its activity after 1 year. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the bacterial extract showed overproduction of ribokinase at the expected molecular weight (34 kDa, **Fig. 1**). The protein content of the extract with enzyme activity was more than 70% of the total bacterial protein extract (**Fig. 1**). The enzyme extract was judged to be over 95% homogenous.

The turnover numbers (k_{cat}) of the enzyme for the sub-

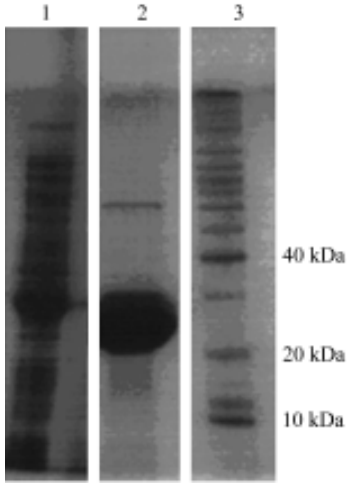

Fig. 1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the protein extract from *Leishmania major*

The SDS-PAGE (12.5% polyacrylamide) analysis of the bacterial extract showed protein overproduction at the expected molecular weight of 34 kDa. Lane 1, total cell lysate; lane 2, fraction from purification; lane 3, molecular weight protein marker.

strates were calculated using the average of three V_{max} values determined from the secondary replots of Y-intercepts and slopes as shown in **Fig. 2**. These values are reported in **Table 1**. The values obtained for both substrates are almost identical. The K_m for ribose and ATP from the replots of the double reciprocal plots were similar to those

Table 1 K_m and kcat value of the ribokinase for various substrates

Varied substrate	K_m (mM)†	k_{cat} (s^{-1})
Ribose	0.3±0.04	10.8
ATP	0.2±0.02	10.2

† Each experiment was repeated three times.

reported for *E. coli* ribokinase [18]; however, the *Leishmania* recombinant enzyme in general appears to be of lower activity than the *E. coli* enzyme. Again, the ribokinase of *E. coli* appears to function maximally at ATP concentrations above 3 mM [18]. This is in contrast to the leishmanial enzyme that is inhibited when the ATP concentration is above 0.75 mM, as determined from our preliminary unpublished studies.

The *L. major* ribokinase specifically phosphorylated *D*-ribose but failed to phosphorylate four, five or six *D*-carbon sugars (erythrose, threose, xylulose, arabinose, fructose and glucose) in the conditions used here. The ribokinase of *E. coli* catalysed phosphorylation of other *D*-sugars like arabinose, xylose and fructose [18] but with efficiencies approximately two orders of magnitude lower than for ribose. Considering that the recombinant leishmanial enzyme was nearly one order of magnitude in activity lower than the *E. coli* enzyme, and also that the *E. coli* enzyme had only marginal activities on the four, five

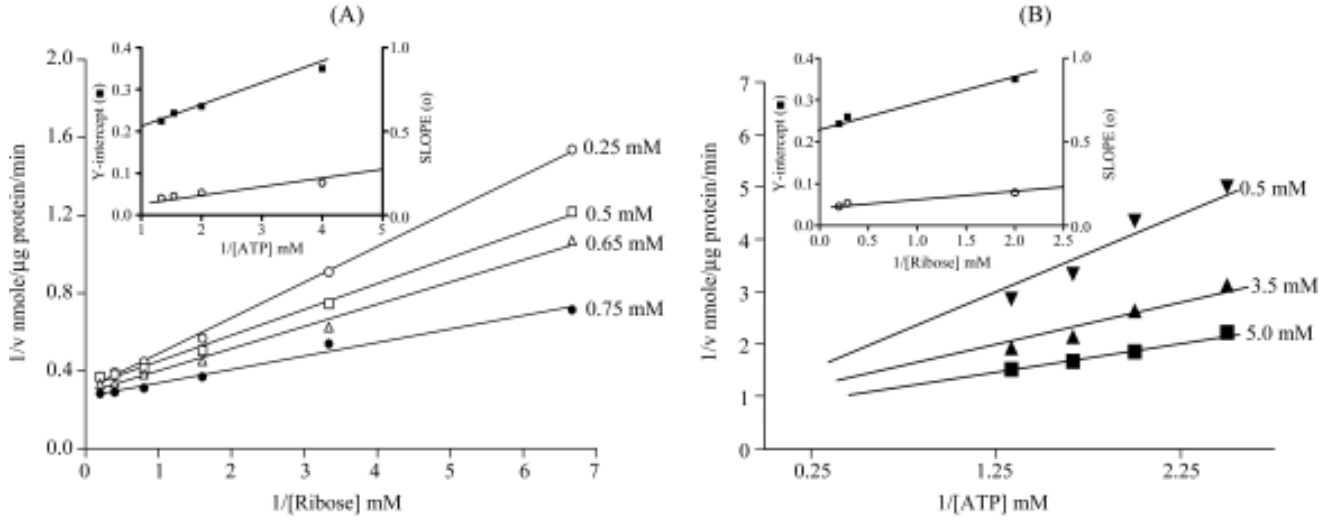

Fig. 2 Double reciprocal plot of initial velocity (V_o) versus [Ribose] at varying fixed [ATP] (A) and versus [ATP] at varying fixed [Ribose] (B)

The graph shows the double reciprocal plot of initial velocity against ribose concentrations at different fixed [ATP] (A) and against ATP concentrations at fixed [Ribose] (B). The inset shows secondary replots of the Y-intercept and the slope from which V_{max} and K_m were obtained. The V_{max} was used to calculate the kcat.

or six carbon sugars, it is therefore unlikely that these sugars would have any physiological relevance to *Leishmania*. The phosphorylation of minor substrates for the *E. coli* enzyme have been considered from a synthetic chemistry perspective, and it was not considered relevant to determine marginal activity in the context of this study. However, it can still be speculated that, as the *E. coli* enzymes use other sugars with a fraction of the efficiency of ribose, the leishmanial enzyme might have similar activity. This could not be detected due to the detention limits of our assay technique, although it is difficult to assign a physiological relevance to those activities.

The phosphorylation of *D*-ribose by *L. major* ribokinase was inhibited when using these sugars at 100-fold excess [**Fig. 3(A)**]. Threose, erythrose and arabinose were better inhibitors than glucose, xylulose, fructose and ribulose. The product, ribo-5-P, also inhibited phosphorylation of ribose with an apparent K_i of 0.4 mM, which is close to the K_m for ribose phosphorylation [**Fig. 3(B)**], and might play a role in regulating flux through this enzyme.

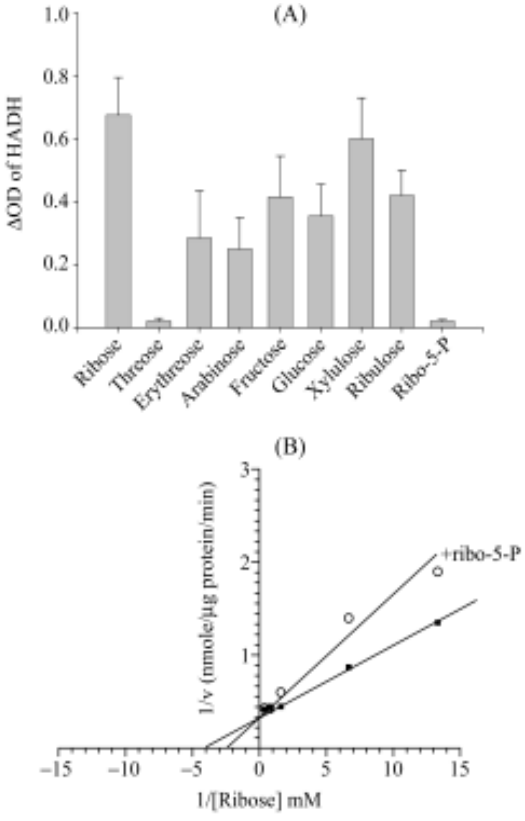

Fig. 3 Inhibition of ribokinase

(A) The incubation mixtures contained the ribose substrate (1.0 mM) without (control) and with different sugars added to 100 mM indicated in the figure. Ribo-5-P, ribose 5-phosphate. (B) Ribose was varied from 0.075 mM to 5 mM in the absence (■) and in the presence (○) of 10 mM inhibitor. The concentration of ATP was fixed at 0.75 mM.

Ribokinase plays important roles in the metabolism of *Leishmania*. Here we show that a gene in these parasites encodes an active ribokinase with comparable activity to the same enzyme studied in *E. coli*. The enzyme's activity is easily measured in a spectrophotometric assay, which could be adapted to screen high throughput inhibition assays should the enzyme prove to be essential to these parasites and thus a target for chemotherapy.

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Minghua XU