12

Cloning the Ribokinase of Kinetoplastidae: *Leishmania Major*

Patrick Ogbunude[1], Joy Ikekpeazu[1], Joseph Ugonabo[2],
Michael Barrett[4] and Patrick Udeogaranya[3]
[1]*University of Nigeria, Enugu-Campus, Enugu*
[2]*Department of Microbiology, University of Nigeria, Nsukka*
[3]*Department of Pharmacy, University of Nigeria, Nsukka,*
[4]*University of Glasgow, Glasgow,*
[1,2,3]*Nigeria*
[4]*Scotland*

1. Introduction

The kinetoplastidae are flagellated protozoans that are widely distributed in nature and cause diseases in both plants and vertebrates. They are distinguished by presence of kinetoplast, that is, the DNA-containing region in their single large mitochondrion. The diseases in crops and lifestocks cause considerable economic loss while serious human suffering and death occur in infections in man. In humans, the diseases include trypanosomiasis, leishmaniasis and chagasis. Trypanosomiasis is caused by infection with two of the three subspecies of *Trypanosoma brucei*; Chagas disease is caused by infection with *Trypanosoma cruzi* and various forms of leishmaniasis are caused by different species of *Leishmania*. These forms of diseases have been classified by the World Health Organization (WHO) as major tropical diseases. According to WHO estimates, about 12 million people suffer from the disease but close to 350 million people in 80 countries world-wide are at risk (Anon, 1990). They are endemic in tropics, subtropics and southern Europe in settings ranging from rain forests in the Americas to deserts in Asia and Middle East. The cellular biology of these kinetoplastids is essentially similar, for example, they are all motile protozoans with a single flagellum that originates close to their large single mitochondrion. They all have glycosomes, that is, micro-bodies that perform glycolysis. All typically grow asexually although sexual recombination has been shown or inferred but is not obligate in any one of them. They divide by binary fission during which their nucleus does not undergo membrane dissolution or chromosome condensation. They are well adapted to their hosts and evade immune elimination by antigenic variation, and alteration of immune responsiveness. There is no effective immune response against human trypanosomiasis which invariably results in fatality. In the case of *T. cruzi* and Leishmania spp., the immune response tends to control rather than eliminate them.

Leishmaniases constitute a broad spectrum of diseases, including the localized cutaneous and the disseminated visceral and mucocutaneous forms. Cutaneous and mucosal leishmaniases cause chronic skin sores and facial disfigurement respectively while untreated

visceral leishmaniasis, otherwise known as kala-azar, causes life-threatening systemic infection. There are a total of about 21 leishmanial species that cause leishmaniases and these are transmitted by about 30 species of phlebotomine sandflies (Desjeux 1996, Ashford 1997). Globally, leishmaniasis caused approximately 59000 deaths and 2.4 million disability adjusted life years in 2001 (World Health Organization Report, 2001). The life cycle of Leishmania involves two stages, a stage in the sand fly vectors where they exist mostly as promastigotes and another stage in the mammalian host. The promastigotes are injected into human host where they invade the macrophages by receptor-mediated endocytosis, transforming into amastigotes that multiply by binary fission.

Leishmaniasis is a treatable disease, however, the antileishmanial therapy is bewildering largely because of the complexities of the disease. The few effective agents available generally are potentially toxic and mostly are difficult to administer. Furthermore the treatment of cutaneous leishmaniasis, for example, is often complicated by rapid self healing making it difficult to assess efficacy of trials. The pentavalent antimony compounds, sodium stibogluconate (Pentostam, Glaxo Wellcome, UK) and meglumine antimonite (Glucantime, Rhône-Poulenc Rorer, France) have been the mainstays of antileishmanial therapy since 1940s (Berman, 1997, Herwaldt & Berman 1999). These drugs, although, effective suffer from disadvantages of long duration of therapy, parenteral mode of administration, almost always reversible toxic effects among other disadvantages. Other new approaches to management of leishmaniasis have some merits but unfortunately most of the non-parenteral agents that have been assessed at best have modest activity against a limited range of species and strains (Herwaldt & Berman, 1992). New effective, safe and affordable drugs are needed for all kinetoplastids. It will be better to have more than one new drug so that combination therapy can be employed whenever drug resistance arises and also provide back-up when resistance emerges.

Kinetoplastids have been useful for study of fundamental molecular and cellular phenomena like antigenic variation, RNA editing and mRNA trans-splicing (Borst & Rudenko, 1994, Stuart, 1991, Perry & Agabian, 1991). The comparison of their sequences with other eukaryotes may be useful in identification of ancient conserved motifs considering their early evolutionary divergence. Also their protein sequence may be a useful source of diversity for protein engineering.

Rational development of anti-leishmanial drugs that will exploit biochemical differences between host and parasite is most desirable.

There are challenges to achieving this goal but with current available technologies, it is possible. With post-genomic bioinformatics and experimental research, it is possible to identify drug targets, vaccine candidates and pathogenic processes. It is also possible to identify candidate diagnostics which realistically should be non-invasive, inexpensive and deployable at the poor resource sites. The new diagnostics in addition should discriminate between types of diseases.

Ribose metabolism in *Leishmania* is of interest because like in other protozoa parasites, it is auxotrophic for purines and expresses multiple pathways for purine uptake (Landfear *et al.*, 2004) and salvage (Hwang & Ulman, 1997) that enable it to acquire and use these vital metabolites from the hosts. A metabolic pathway of interest is that involved in production of ribose 5-phosphate (R5P) required for the synthesis of 5-phosphoribosyl-1-pyrophosphate used with nucleobases for the synthesis of nucleic acids. Classically ribose 5-phosphate is generated in cells by one or combination of the following pathways:

- Ribokinase conversion of ribose (reaction 1)
- Condensation of fructose 6-phosphate and glyceraldehyde 3-phosphate mediated by transaldolase and transketolase (reaction 2).
- Conversion of glucose via oxidative pathway of pentose phosphate pathway (reaction 3).
- Hydrolysis of nucleoside to nucleobase and ribose followed by reaction 1

The stoichiometric expressions of these reactions are:

1. Ribose + ATP $\longrightarrow$ R5P + ADP
2. 2F6P + G3P $\longrightarrow$ 3R5P
3. G6P + 2NADP$^+$ + H_2O $\longrightarrow$ R5P + 2NADPH 2H$^+$ + H_2O
4. Nucleoside $\longrightarrow$ Base + Ribose $\longrightarrow$ R5P

Reaction 4 is unique to trypanosomatids including *Leishmania* .

The consequence of reaction 4, that is the nucleoside hydrolase activity, is the abundance of intracellular ribose available to *Leishmania* parasites. The extracellular ribose has been shown previously to be efficiently incorporated into the nucleic acids of leishmania (Maugeri *et al*., 2003). Ribose may also serve as a source of energy for organisms (Berens *et al*., 1980) hence there is every need to study the enzyme (ribokinase) that is involved in the mobilization of ribose. Ribokinase is an ATP-dependent phosphoribosyl kinase (EC 2.1.7.15), which catalyses the conversion of ribose to ribose 5-phosphate, a substrate of 5-phosphoribosyl 1-pyrophosphate synthatase, that uses nucleobases and ribose 5-phosphate to synthesize nucleic acids. X-ray crystallography of *Escherichia coli* ribokinase shows the protein to be a homodimer in solution with each subunit having two domains. Each subunit has a molecular weight of about 33 KDalton (Sigrell *et al*., 1997, Sigrell *et al*., 1998, Sigrell *et al*., 1999). The turnover numbers for *Leishmania* ribokinase for the substrates D-ribose and ATP are respectively10.8 s^{-1} and 10.2 s^{-1} and the catalytic activity is strongly dependent on the presence of monovalent cations(Ogbunude *et al*., 2003, Chuvikovsky *et al*., 2006).

Because of these important roles played by ribokinase in *Leishmania* metabolism, we cloned the genes for the enzyme and expressed it in *E coli* as previously published (Ogbunude *et al*., 2007). Here we summarize the method used in cloning and expression of the enzyme.

2. Materials and methods

2.1 Chemicals

Synthetic oligonucleotides were obtained from MWG-Biotech AG, UK. All restriction and DNA modifying enzymes were obtained from Promega (UK) or Roche, Roche Diagnostics GmbH, Germany. All other chemicals and reagents were of the highest quality commercially available.

2.2 Methods

2.2.1 Culture of *L.major* promastigotes

The *L. major* promastigotes (MHOM/JL/80/Friedlin) were grown in tissue culture flasks in HOMEM medium supplemented with heat-inactivated 10% fetal bovine serum at 27^{o}C. Promastigotes were harvested at approximately 10^7 cells/ml at the end of logarithmic growth phase. Following isolation and washing in wash buffer (50 mM Tris-HCl, pH 8.0), the genomic DNA of the promastigotes was isolated by published procedures with some

modifications (Wilson *et al*., 1991). Briefly, promastigotes were resuspended to a density of 5 x 10^9 per 450 µl in 50 mM Tris-HCl, pH8, 50 mM EDTA, 100 mM NaCl. 50 µl of 10% SDS was added to this mixture with 50 µl of proteinase type IV (40 mg ml^{-1} stock) and 2000 U ml^{-1} RNAse inhibitor. The mixture was incubated at 37°C for 2 hours with intermittent shaking. To the mixture an equal quantity of phenol was added and then the supernatant collected and an equal volume of 3:1 v/v phenol/chloroform added to this supernatant. After mixing and separation of the phases by centrifugation the aqueous phase was again taken and this time mixed with 4 volumes of chloroform. The supernatant was collected, and 0.1 volumes of 3 M sodium acetate (pH 7.0) added followed by 2.5 volumes of ethanol. Nucleic acid was left to precipitate at –20°C overnight and then collected by centrifugation. The precipitate was washed once in 70% ethanol, air-dried and then dissolved in 50 µl of double distilled H_2O.

2.2.2 Design of oligonucleotide primers

The gene coding a putative ribokinase was identified in the *L. major* genome using BLAST searches with ribokinase of diverse organisms (Fig 1). Oligonucleotide primers were designed against conserved regions of proteins, which represented an open reading frame. The forward primer (5'-AAACATATGCACCGTGTGCAGAACGTT-3') was designed from the first peptide of the protein while the reverse (5'-AAACTCGAGCTACGTGACACCAGCC-3') was designed from the second peptide. The underlined bases represent restriction sites (*Nde1* and *xho1*) that were inserted to facilitate cloning of the PCR amplified products. The oligonucleotides were synthesized by MWG-Biotech AG, UK.

2.2.3 PCR amplification

Amplification was carried out in a DNA thermal cycler (MJ Research Inc., Western Town, MA, USA). PCR was routinely performed in 100 µl reaction containing 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 µM KCl, 200 µM each of dNTPs, 40 ng of each of the primer and 1 unit of Pfu DNA polymerase. The tube containing the reactants were placed in the thermo cycler programmed for 30 cycles, a single cycle at 94°C for 120 s; was followed by 30 cycles at 63°C for 15 s and 72°C for 120 s; with a final cycle at 72°C for 600 s.

2.2.4 Detection of PCR products

Five micro liters of the PCR product was electrophoresed in a 1% agarose gel containing 0.1 µg of ethidium bromide per ml, and bands were visualized by UV transillumination.

2.2.5 Preparation of cloning vector and insertion

The amplified DNA, after gel electrophoresis was isolated using the commercially available QIAquick Gel Extraction Kit (Qiagen, West Sussex). The gel-purified PCR product (1.2-kb amplicon) was cut with *Nde1* and *xho1* at the primer sequence sites underlined. This was ligated with the pGEM-T Easy plasmid (Novagen) cut with the same enzymes. The recombinant plasmid was introduced into *E. coli* strain DH5α (Invitrogen) by heat transformation in Luria broth (LB) medium at 42°C for 50 sec. Agar plates with LB medium supplemented with ampicillin, isopropyl-1-thio-β-D-galactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-galactoside (X-gal) were used to test the recombinant strain. Positive strain was used for recombinant protein over-expression experiments.

2.2.6 Protein overproduction

For recombinant protein over expression, the Nde 1 and xho 1 digest of pGEM-T was inserted between the same sites of plasmid pET28a$^+$ (Novagen). The resulting plasmid pET28a$^+$ribo construct harbors the rbk gene under the control of a hybrid promoter-operator region, consisting of sequence of T7 promoter and a *lac* operator. The pET28a$^+$ribo was introduced into the BL21 (strain DE3)(Statagene) for protein over expression. Expression of the His-tagged ribokinase was induced by 0.5 mM IPTG overnight at room temperature. Cells were harvested by centrifugation, washed once and stored at –20^{o}C in 5 ml of 50 mM Hepes buffer, pH 7.0 containing 300 mM NaCl and 10 mM EDTA. The cells were lysed by sonication in buffer A (50 mM NaH_2PO_4 containing 300 mM NaCl, pH 8.0) and the soluble fraction recovered by centrifugation at 10,000 g for 30 min at 4^{o}C. This was applied to nickel-nitrilotriacetic acid column (bioCAD) pre-equilibrated with the buffer A. The column was washed with 100 ml of the buffer A containing 0.5 mM imidazole and then with the same buffer containing 50 mM imidazole and finally the his-tagged recombinant protein was eluted with 500 mM imidazole in the buffer A. The eluant was dialysed overnight in 50 mM Tris-HCl, pH 7.0 at 4^{o}C and stored at –70^{o}C in buffer/glycerol (1:1).

3. Results

Data base searches of *L. major* ribokinase sequence revealed a gene in chromosome 27 (LmjF27.0420) with an open reading frame of 990 base pairs encoding a 329 amino acid protein orthologous with ribokinase from other organisms . Figure 1 shows the alignment of this protein with ribokinase from phylogenetically diverse organisms. The alignment revealed a high degree of conservation of primary structure among the four aligned proteins.

The SDS-Page analysis of the bacterial extract shown in figure 2 demonstrated protein overproduction at the expected molecular weight of 34 Kdalton. The Coomassie staining indicated that the recombinant protein represented more than 90% of the total protein. After purification, the enzyme was judged to be over 95%homogenous.

The figure 3 graph shows time dependence of conversion of ribose to R5P. In this coupled assay system, ADP is utilized by phosphoenolpyruvate in reaction catalyzed by pyruvate kinase to regenerate ATP and the resulting pyruvate is converted by lactate dehydrogenase to lactate and in the process NADH is oxidized to NAD^+. The reaction was followed at 340 nm as a decrease in NADH absorbance when converted to NAD^+. The reaction demonstrates that the recombinant protein is indeed a functional enzyme. The enzyme when stored at -70^{o}C in buffer/glycerol (1:1) retained more than 50% of activity after a year. It retained full activity for two months when stored at -70^{o}C with or without glycerol. The enzyme is highly specific for D-ribose in species so far studied although it insignificantly phosphorylates other five and six carbon sugars like D-arabinose, D-xylose, D-galactose and D-fructose. The catalytic efficiency for catalysis of D-ribose ranges from 10.8 s^{-1} mM^{-1} to 40.6 s^{-1} mM^{-1} while it is in the order of 0.05 s^{-1} mM^{-1} to 1.8 s^{-1} mM^{-1} for other D-sugars. The ribokinae activity depends absolutely on the presence of inorganic phosphate. Omission of inorganic phosphate from reaction mixture reduced the enzyme activity to insignificant level.

S. cerevisiae (1) ----------MGITVIGSLNYDLDTFTDRLPNAGETFRANHFETHAGGKGLNQAAAIGKLKNPSS
E. coli (1) ---------MQNAGSLVVLGSINADHILNLQSFPTPGETVTGNHYQVAFGGKGANQAVAAGRSG----
H. sapiens (1)
MAASGEPQRQWQEEVAAVVVVGSCMTDLVSLTSRLPKTGETIHGHKFFIGFGGKGANQCVQAAARLG----
L. major (1)
MHRVQNVQSHVGEYAPDILVVGSCFLDYVGYVDHMPQVGETMHSESFHKGFGGKGANQAVAAGRLG----

S. cerevisiae (56)
RYSVRMIGNVGNDTFGKQLKDTLSDCGVDITHVGTYEGINTGTATILIEEKAGGQNRILIVEGANSKTIY
E. coli (56) -ANIAFIACTGDDSIGESVRQQLATDNIDITPVSVIKGESTGVALIFVNGEGEN--
VIGIHAGANAALSP
H. sapiens (67) -AMTSMVCKVGKDSFGNDYIENLKQNDISTEFTYQTKDAATGTASIIVNNEGQN–
IIVIVAGANLLLNT
L. major (67) -AKVAMVSMVGTDGDGSDYIKELERNGVHTAYMLRTGKSSTGLAMILVDTKSSNN-
EIVICPNATNYFTP

S. cerevisiae(126) DPKQLCEIFPEGKEE--EEYVVFQHEIPDPLSIIKWIHANRPNFQIVYN---
PSPFKAMPKKDWELVDL
E. coli (123) ALVEAQRE--RIAN--ASALLMQLESPLESVMAAAKIAHQNKTIVALN---PAPARELPD-------
H. sapiens (134) EDLRAAAN--VISR--AKVMVCQLEITPATSLEALTMARRSGVKTLFN---PAPAIADLDP------
L. major (135) ELLRAQTSNYEKILHTGLKYLICQNEIPLPTTLDTIKEAHSRGVYTVFNSAPAPKPAEVEQIK-----
-

S.cerevisiae (190)
LVVNEIEGLQIVESVFDNELVEEIREKIKDDFLGEYRKICELLYEKLMNRKKRGIVVMTLGSRGVLFCSH
E. coli (176) -----ELLALVDIITPNETEAEKLTGIRVENDEDAAKAAQVLHEKGIR-----TVLITLGSRGVWASVN
H. sapiens (188) ------QFYTLSDVFCCNESEAEILTGLTVGSAADAGEAALVLLKRGCQ-----
VVIITLGAEGCVVLSQ
L. major (198) -----PFLPYVSLFCPNEVEAALITGMKVTDTESAFRAIKALQQLGVR-----DVVITLGAAGFALSEN

S.cerevisiae (260) ESPEVQFLPAIQNVSVVDTTGAGDTFLGGLVTQLYQGET--
LSTAIKFSTLASSLTIQRKGAAESMPLYK
E. coli (235) G–EGQRVPGFR-VQAVDTIAAGDTFNGALITALLEEK–
PLPEAIRFAHAAAAIAVTRKGAQPSVPWRE
H. sapiens (247) TEPEPKHIPTEK-
VKAVDTTGAGDSFVGALAFYLAYYPNLSLEDMLNRSNFIAAVSVQAAGTQSSYPYKK
L. major (257) G-AEPVHVTGKH-VKAVDTTGAGDCFVGSMVYFMSRGR--
NLLEACKRANECAAISVTRKGTQLSYPHPS

S. cerevisiae (328) DVQKDA----
E. coli (300) EIDAFLDRQR
H. sapiens (316) DLPLTLF---
L. major (323) ELPAGVT---

Fig. 1. Alignment of a putative ribokinase gene product from *L. major* with ribokinase sequences from *S. cerevisiae* *E. coli* and *H. sapiens*.

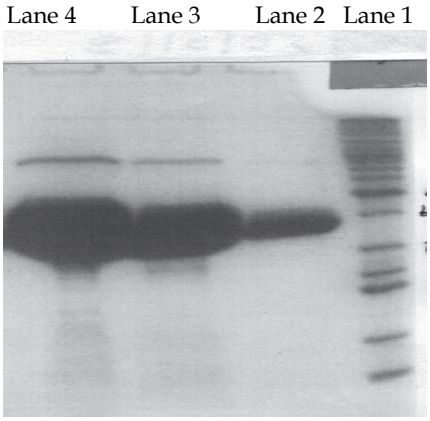


Fig. 2. SDS-Page analysis of bacterial extract

The SDS-Page (12.5% polyacrylamide) analysis of the bacterial extract shows protein overproduction at the expected molecular weight of 34,000 daltons. The 1kb molecular weight marker which served as a control is in Lane 1. Lanes 2, 3 and 4 are fractions collected at intervals from the nickel-nitrilotriacetic acid column.

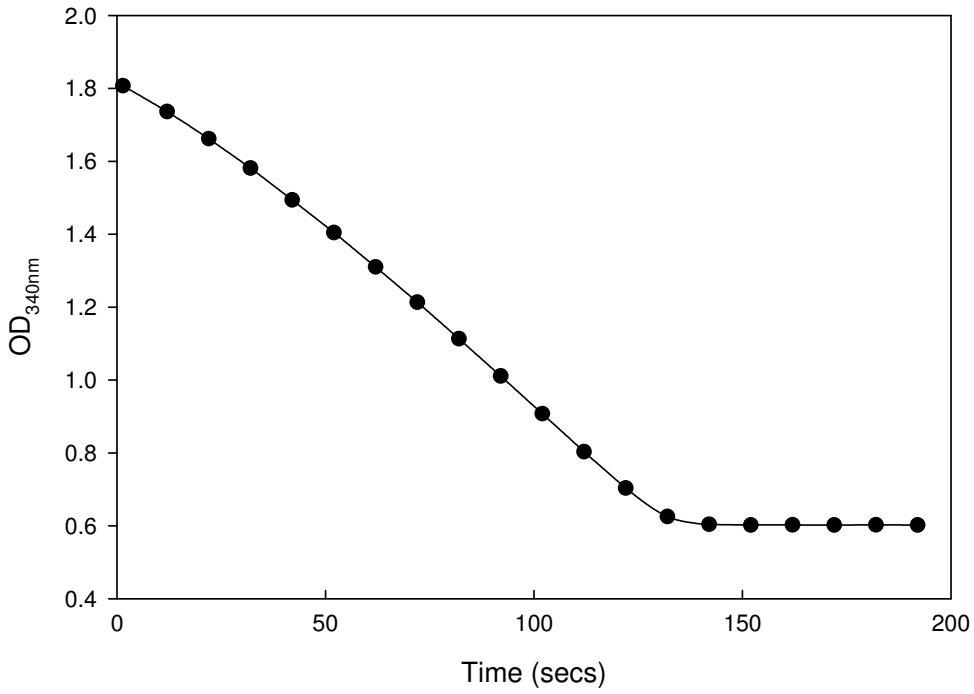


Fig. 3. Time dependence of conversion of ribose to ribose 5-phosphate by ribokinase

4. Conclusion

The interest in the uptake and metabolism of ribose as a potential metabolic supplement has justified the cloning and characterization of the enzyme. Administration of exogenous ribose for instance have been shown to increase both the repletion and maintenance of high level of ATP in various types of cells (Smolenski *et al*., 1998, St Cyr et *al*., 1989) and maintenance of higher levels of ATP in rat heart and dog kidney during transplantation experiments (Muller *et al*, 1998). Thus ribose has potential therapeutic applications involving cardiovascular cells. Studies into the major carbon sources used by intracellular *Leishmania* amastigotes show that *L. mexicana* mutant lacking the three high affinity hexose transporters was unable to establish infection in macrophages or susceptible mice (Burchmore *et al*., 2003), indicating that hexose uptake is essential for intracellular growth. The levels of hexose in the phagolysosome may, however, be limiting as mutant *L. major* amastigotes that have defect in gluconeogenesis was also found to be poorly virulent in macrophages and susceptible mice (Nadereer *et al*., 2006). These studies indicate that intracellular amastigotes depend on both salvage and *de novo* synthesis of hexoses. The phagolysosome of macrophages could however contain a range of other sugars including ribose which could be used directly as an energy source with the pentose phosphate pathway (Barrett 1997, Maugeri *et al*., 2003) shunting intermediates into the glycolytic pathway.

In parasitic organism such as *Leishmania* which, lacks purine nucleoside phosphorylase enzyme and instead has high activity of purine nucleoside hydrolase, the organism will generate high concentration of intracellular ribose. As well, it accumulates ribose from the host via a specific carrier-mediated protein (Pastakia & Dwyer, 1987). That ribose is encountered throughout the life cycle of the parasite is well known (Burchmore & Barrett, 2001). In absence of glucose in culture medium, ribose served as a substitute and became essential for the growth of the *Leishmania* promastigotes (Steiger & Black, 1980). Thus ribose plays an important role in the metabolism of *Leishmania*. For ribose to be utilized, it must be converted to ribose 5-phosphate by ribokinase, hence the interest in identifying and cloning of this enzyme responsible for this critical first step in ribose metabolism.

The ribokinase gene has been identified from variety of sources including human, *E. Coli* and *Leishmania*. There is a high degree of similarity between the ribokinase gene from animal (human) and that from *E. Coli* (Park et al., 2007). The structure of ribokinase as revealed by X-ray crystal structure of *E.coli* shows that it is a homodimer in solution with each subunit composed of two domains (Sigrell *et al*., 1997, Sigrell *et al*., 1998, Bork *et al*., 1993). Also the crystallographic studies show that oxygen atoms of the α-D-pentofuranose ring are involved in hydrogen bonding interactions between the enzyme and the substrates, ribose and ADP (Andersson & Mowbray 2002, Maj & Gupta 2001). The amino acids residues, asparagines and glutamic acid, at sequence positions 187 and 190, appear to be conserved in all the species studied and have been shown to be the site for phosphate binding (Park *et al*. 2007). Site directed mutation of these amino acid residues have led to formation of mutant enzymes with altered $MgATP^{2+}$ and phosphate requirements (Parducci *et al*., 2006).

In summary, *L. major* has a gene that expressed functional ribokinase. The enzyme was specific for D-ribose and did not phosphorylate related sugars unlike what was seen with *E.coli* ribokinase that phosphorylated related sugars to a minor degree. Presently, it is not known whether the enzyme is essential. A lot more information is still required before specific compounds are designed to target the enzyme for chemotherapy.

5. Acknowledgement

The authors appreciate the financial support of The University of Nigeria in defraying part of the cost of publishing this article. The research was supported by a grant from the European Commission INCO-DC Program (ERBIC18CT980357).

6. References

Andersson, CE. & Mowbray, SL. (2002) Activation of ribokinase by monovalent cations. *Journal of Molecular Biology*, 315, 409-419

Anon (1990) World Report on Tropical Diseases, World Health Organization

Ashford, RW. (1997) The leishmaniases as model zoonoses. *Annals of Tropical Medicine Parasitology*. 91: 693-701.

Barrett, MP. (1997) The pentose phosphate pathway and parasitic protozoa . *Parasitology Today* 13, 11-16.

Berens, RL. Deutsch-King, LC. & Marr, JJ. (1980) *Leishmania donovani* and *Leishmania braziliensis*: hexokinase, glucose 6-phosphate dehydrogenase, and pentose phosphate shunt activity. *Experimental Parasitology* 49, 1-8.

Berman, JD. (1997) Human leishmaniasis: clinical, diagnostic and chemotherapeutic developments in the last 10 years. *Clinical Infectious Diease* 24:684-703.

Bork, P. Sander, C. & Valencia, A. (1993) Convergent evolution of similar enzymatic function on different protein folds: The hexokinase, ribokinase, and galactokinase families of sugar kinases. *Protein Science* 2, 31-40

Borst, P. & Rudenko, G. (1994) Antigenic variation in African trypanosomes. *Science* 264, 1872-1873

Burchmore, RJ. & Barrett, MP. (2001) Life in vacuoles-nutrient acquisition by *Leishmania* amastigotes. *International Journal Parasitology* 31, 1311-1320.

Chuvikovsky, DV. Esipov, RS. Skoblov, YS. Chupova, LA. Muravyova, TI. Miroshnikov, AI. Lapinjoki, S. & Mikhailopulo, IA. (2006) Ribokinase from *E. Coli*: Expression, purification, and substrate specificity. *Bioorganic Medicinal Chemistry* 14, 6327-6332.

Desjeux, P. (1996) Leishmaniasis: public health aspects and control. *Clinical Dermatololgy*, 14:417-423.

Herwaldt, BL. Berman, JD. (1992) Recommendations for treating leishmaniasis with sodium stibogluconate (Pentosam) and review of pertinent clinical studies. *American Journal of Tropical Medicine and Hygiene* 46: 296-306.

Hwang, HY. & Ullman, B. (1997) Genetic analysis of purine metabolism in *Leishmania donovani*. *Journal of Biological Chemistry* 272, 19488-19496.

Landfear, SM. Ullman, B. Carter, NS. Sanchez,MA. (2004) Nucleoside and nucleobase transporters in parasitic protozoa. *Eukaryotic Cell* 3, 245-254

Maj, MC. & Gupta, RS. (2001) The effect of inorganic phosphate on the activity of bacterial ribokinase. *Journal of Protein Chemistry* 20, 139-144.

Maugeri, DA. Cazzulo, JJ. Burchmore,JS, Barrett, MP. Ogbunude, POJ. (2003) Pentose phosphate metabolism in *Leishmania Mexicana*. *Molecular and Biochemical Parasitology* 130, 117-125.

Muller, C. Zimmer, H. Gross, M. Gresser, U. Brotsack, I. Wehling, M. & Plim, lW. (1998) Effect of ribose on cardiac adenine nucleotides in a donor model for heart transplantation. *European Journal of Medical Research* 3, 554-558.

Naderer, T. Ellis, MA. Sernee, MF. De Souza DP. Curtis, J. Handman, E. & McConville, MJ. (2006) Virulence of Leishmania major in macrophages and mice requires the gluconeogenic enzyme fructose-1,6-bisphosphatase. Proceedings of National Academy of Science USA 103, 5502-5507.

Ogbunude, POJ. Lamour, N. Barrett, P. (2007) Molecular cloning, expression and characterization of ribokinase of *Leishmania major*. *Acta Biochimica Biophysica (Sinica)* 39, 462-466.

Parducci, RE. Cabrera, R. Baez, M. & Guixe, V. (2006) Evidence for catalytic Mg2+ ion and effect of phosphate on the activity of *Escherichia coli* phosphofructokinase-2: regulatory properties of a ribokinase family member. *Biochemistry* 45, 9291-9299.

Park, J. Koeverden, P. Singh, B. & Gupta, RS. (2007) Identification and characterization of human ribokinase and comparison of its properties with *E. Coli* ribokinase and human adenosine kinase. *FEBS Letters* 581, 3211-3216.

Pastakia, KB. Dwyer, DM. (1987) Identification and characterisation of a ribose transport system in *Leishmania donovani* promastigotes. *Molecular Biochemical Parasitology* 26, 175-182.

Perry, K.& Agabian, N. (1991) mRNA processing in the trypanosomatidae. Experientia 47, 118-128

Shi, W. Schramm,VL., Almo, SC. (1999) Nucleoside hydrolase from *Leishmania major*. Cloning, expression, catalytic properties, transition state inhibitors and 2.5-Å crystal structure. *Journal of Biological Chemistry* 274, 21114-211120.

Sigrell, JA. Cameron, AD. Jones, TA. & Mowbray, SL. (1997) Purificatioin, characterization and crystallization of *Escherichia coli* ribokinase. *Protein Science* 6, 2474-2476

Sigrell, JA. Cameron,AD. Jones, TA.& Mowbray, SL. (1998) Structure of *Eschericia coli* ribokinase in complex with ribose and dinucleotide determined to 1.8 Å resolution: Insights into a new family of kinase structures. *Structure* 6, 183-193.

Sigrell, JA. Cameron,AD. & Mowbray, SL. (1999) Induced fit on sugar binding activities ribokinase. *Journal of Molecular Biology* 290, 1009-1018.

Smolenski, RT. Kalsi, KK. Zych, M. Kochan, Z. & Yacoub, MH. (1989) Adenine/ribose supply increases adenosine production and protects ATP pool in adenosine kinase-inhibited cardiac cells. Journal of Molecular Cell Cardiology, 30,673-683.

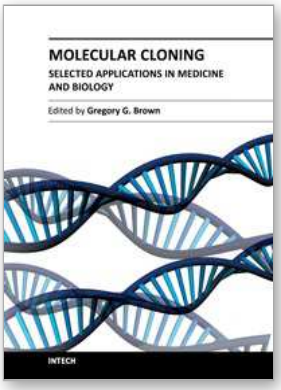
St Cyr, JA. Bianco, RW. Schneider, JR. Mahony Jr, JR. Tveter, K. Einzig, S. & Foker, JE. (1989) Enhanced high energy phosphate recovery with ribose infusion after global myocardial ischemia in a canine model. *Journal of Surgery Research* 46, 157-162.

Steiger, RF. & Black, CD. (1980) Simplified defined media for cultivating *Leishmania donovani* promastigotes. *Acta Tropica*. 37, 195-198

Stuart K (1991) RNA editing in trypanosomatid mitochondria *Annual Review of Microbiology* 45, 327-344.

Wilson, K. Collart, FR. Huberman, E. Stringer, JL. & Ullman, B. (1991) Amplification and molecular cloning of the IMP dehydrogenase gene of *Leishmania donovani*. *Journal of Biological Chemistry* 266, 1665-1671.

World Health Organization, Annex 3: Burden of disease in DALYs by cause, sex and mortality stratum in WHO regions, estimates for 2001. In: *The world health report, Geneva: WHO*, 2002: 192-7 (www.who.int/whr/2002/whr 20002_annex3.pdf).



Molecular Cloning - Selected Applications in Medicine and Biology

Edited by Prof. Gregory Brown

ISBN 978-953-307-398-9
Hard cover, 324 pages
Publisher InTech
Published online 12, October, 2011
Published in print edition October, 2011

The development of molecular cloning technology in the early 1970s created a revolution in the biological and biomedical sciences that extends to this day. The contributions in this book provide the reader with a perspective on how pervasive the applications of molecular cloning have become. The contributions are organized in sections based on application, and range from cancer biology and immunology to plant and evolutionary biology. The chapters also cover a wide range of technical approaches, such as positional cloning and cutting edge tools for recombinant protein expression. This book should appeal to many researchers, who should find its information useful for advancing their fields.

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821