Ribokinase from *Leishmania donovani*: purification, characterization and X-ray crystallographic analysis

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Leishmania is an auxotrophic protozoan parasite which acquires D-ribose by transporting it from the host cell and also by the hydrolysis of nucleosides. The enzyme ribokinase (RK) catalyzes the first step of ribose metabolism by phosphorylating D-ribose using ATP to produce D-ribose-5-phosphate. To understand its structure and function, the gene encoding RK from *L. donovani* was cloned, expressed and purified using affinity and size-exclusion chromatography. Circular-dichroism spectroscopy of the purified protein showed comparatively more α -helix in the secondary-structure content, and thermal unfolding revealed the T_m to be 317.2 K. Kinetic parameters were obtained by functional characterization of *L. donovani* RK, and the K_m values for ribose and ATP were found to be 296 ± 36 and $116 \pm 9.0 \mu\text{M}$, respectively. Crystals obtained by the hanging-drop vapour-diffusion method diffracted to 1.95 Å resolution and belonged to the hexagonal space group $P6_1$, with unit-cell parameters $a = b = 100.25$, $c = 126.77$ Å. Analysis of the crystal content indicated the presence of two protomers in the asymmetric unit, with a Matthews coefficient (V_M) of $2.45 \text{ Å}^3 \text{ Da}^{-1}$ and 49.8% solvent content. Further study revealed that human counterpart of this protein could be used as a template to determine the first three-dimensional structure of the RK from trypanosomatid parasites.

1. Introduction

D-Ribose is an abundant pentose sugar in biological systems that can be used as a source of energy as well as as a constituent of RNA, DNA and nucleotide cofactors. Phosphorylation of ribose mediated by ribokinase (RK; EC 2.7.1.15) is the first step in its entrance into metabolic pathways and also into the biosynthesis of nucleotides, tryptophan and histidine (Anderson & Cooper, 1969; Lopilato *et al.*, 1984). Analysis of ribokinase gene sequences from both prokaryotes and eukaryotes identified that they belong to the PfkB family of carbohydrate kinases, also known as the RK family (Bork *et al.*, 1993; Park & Gupta, 2008).

Ribokinase catalyzes the transfer of the γ -phosphate of ATP to O5' of D-ribose to produce ADP and D-ribose-5-phosphate. The catalytic activity of ribokinase is regulated by monovalent and divalent cations. Biochemical studies of ribokinases from *Escherichia coli*, *Arabidopsis thaliana* and human revealed that divalent metal cations are critical for catalytic activity, preferably Mg^{2+} or Mn^{2+} (Chuvikovsky *et al.*, 2006; Quiroga-Roger *et al.*, 2015; Riggs *et al.*, 2016). Metal cations other than Mg^{2+} and Mn^{2+} , such as Co^{2+} , Ca^{2+} , Cu^{2+} , Cd^{2+} , Ni^{2+} and Zn^{2+} , also support the activity of ribokinase (Chuvikovsky *et al.*, 2006; Quiroga-Roger *et al.*, 2015; Schimmel *et al.*, 1974). Divalent metal cations regulate activity by forming a metal–nucleotide complex and also by binding to the conserved NXXE motif present near the active site of

ribokinase (Quiroga-Roger *et al.*, 2015). On the other hand, monovalent cations, preferably K^+ , Cs^+ and NH_4^+ , modulate catalysis by activating ribokinase (Andersson & Mowbray, 2002; Chuvikovsky *et al.*, 2006). The mechanism of activation by monovalent cations has been demonstrated by the binding of a cesium ion between two loops near to the active site in the crystal structures of ribokinase from *E. coli* and *Vibrio cholerae* (Andersson & Mowbray, 2002; Paul *et al.*, 2015). The two structural changes that were associated with the binding of monovalent cation to the enzyme comprised a change in the conformation of the large ATP loop, increasing the affinity for ATP, and the formation of an anion hole to stabilize the transition state (Andersson & Mowbray, 2002; Li *et al.*, 2012).

Visceral leishmaniasis is a clinical syndrome caused by the protozoan parasite *Leishmania donovani*, which belongs to the Trypanosomatidae family. *Leishmania* exists in two morphological forms: a motile promastigote that resides in the midgut of phlebotomine sandflies and an intracellular amastigote that is present in the parasitophorous vacuole of the mammalian macrophage (Sunter & Gull, 2017). For rapid growth and survival, the parasite acquires most of its vital metabolites from the sandfly midgut and the parasitophorous vacuoles of the macrophage during the promastigote and amastigote stages of its lifecycle, respectively (Chang & Dwyer, 1978; Basselin *et al.*, 2000; Burchmore & Hart, 1995; Drew *et al.*, 1995; Ghosh & Mukherjee, 2000; Kündig *et al.*, 1999; Mazareb *et al.*, 1999; Zilberstein & Gepstein, 1993). Among these metabolites, D-ribose accumulates in the promastigotes of *L. donovani* by a carrier-mediated process and also through the degradation of nucleosides by non-specific nucleoside hydrolase (Cui *et al.*, 2001; Pastakia & Dwyer, 1987; Naula *et al.*, 2010). Experiments using radio-labelled D-ribose with *L. mexicana* have identified the importance of ribokinase in the incorporation of free ribose into nucleic acids (Maugeri *et al.*, 2003). Ribokinase plays a key role by phosphorylating the ribose available in the cytosol to enable its entry into *Leishmania* metabolism. To understand the catalytic function using a structure-based approach, the ribokinase gene from *L. donovani* (*LdRK*) was cloned, purified, characterized and crystallographic analysis was carried out.

2. Materials and methods

2.1. Cloning, expression and purification

The full-length ORF for ribokinase was amplified from the genomic DNA of *L. donovani* by polymerase chain reaction using the oligonucleotide primers in Table 1. The amplified PCR product was purified using a gel-extraction kit (Thermo Scientific) and ligated into pJET1.2 cloning vector (Thermo Scientific). The insert was released from the pJET1.2 vector by digestion with the NdeI and XhoI restriction enzymes and was then subcloned into pET-28a(+) (Novagen) expression vector, which adds a hexahistidine tag to the N-terminus of the target protein. The plasmids isolated from the positive clones were verified by restriction digestion followed by sequencing prior

Table 1

Macromolecule-production information.

Source organism	<i>L. donovani</i>
DNA source	Genomic DNA
Cloning vector	pJET1.2
Expression vector	pET-28a(+)
Forward primer†	5'-AATCATATGCACCGTGC GCGGAACGTT-3'
Reverse primer‡	5'-AATCTCGAGCTACATGACACCGCCGCA-3'
Expression host	<i>E. coli</i> BL21 (DE3)
Complete amino-acid sequence of the construct produced§	MGSSHHHHHHSSGLVPRGSHMRARNVRSHTGEY APDILVVGSCFLDYGVYDHPQVGETMHSVS FHKFGGKGANQAVAAGRLGAKVAMVSMVGT GDGSDYIKELERNGVDTAYMFRGKSSSTGLAM ILVDTKSSNNEIVICPNATNHFTPELLRAQTN NYERILHTGLKYLICQNEIPLPTLDTIKEAH SRGVYTVFNSAPAPKPAEVEQIKPFLPVVSLF CPNEVEATLITGVKVTDTESAFSAIKALQQLG VRDVVITLGAAGFVLSAENGAEVHVTKGHVKA VDTTGAGDCFVGSVMYFMSRGRNLLLEACKRAN ECAAISVTRKGTQLSYPHPSLPAGVM

† The NdeI site is underlined. ‡ The XhoI site is underlined. § Non-native residues originating from the vector are underlined.

to transformation into *E. coli* BL21 (DE3) cells. For over-expression, a single colony was inoculated into 30 ml Luria-Bertani (LB) medium containing 50 $\mu\text{g ml}^{-1}$ kanamycin and incubated overnight at 310 K. An overnight-grown culture (20 ml) was diluted to 1% with LB broth, incubated at 310 K to an OD₆₀₀ of 0.6 and then induced with 0.05 mM IPTG for 20 h at 291 K. Induced cells were pelleted for 10 min at 6300g and resuspended in 50 ml lysis buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 40 mM imidazole, 2 mM β -mercaptoethanol, 0.25 mg ml⁻¹ lysozyme, 0.1 mM phenylmethylsulfonyl fluoride) followed by lysis using sonication. The cell lysate was centrifuged at 21 000g for 30 min, and the supernatant was filtered using a 0.2 μm filter (Millipore) and loaded onto a HisTrap HP 5 ml column (GE Healthcare) equilibrated with buffer A consisting of 50 mM Tris-HCl pH 8.0, 300 mM NaCl. Unbound bacterial proteins were eluted with a step gradient of 10, 15, 20 and 30% buffer B (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 500 mM imidazole) and the desired protein was eluted from the column using 80% buffer B. Eluted fractions with high purity were pooled and loaded onto a HiLoad 16/60 Superdex 200 pg column (GE Healthcare) equilibrated with 50 mM Tris-HCl pH 8.0, 125 mM NaCl. The fractions with the highest purity were pooled and then concentrated using Amicon Ultra (10 kDa cutoff) to 8 mg ml⁻¹ for crystallization. All purification steps were performed at 277 K and protein concentrations were determined at 280 nm with a NanoDrop 2000 (Thermo Scientific) using a molar extinction coefficient (ϵ) of 16 390 M⁻¹ cm⁻¹ and the molecular weight.

2.2. Circular dichroism

The circular-dichroism (CD) spectrum was measured using 7.5 mM Tris-HCl pH 8.5 containing 3.2 μM *LdRK* with a J-1500 spectropolarimeter (Jasco) at 298 K from 260 to 190 nm with a scanning speed of 20 nm min⁻¹. The path length of the sample cell was 2 mm, while the data interval and bandwidth were both 1 nm. Two consecutive spectra were recorded and

Table 2
Crystallization.

Method	Hanging-drop vapour diffusion
Plate type	24-well plate
Temperature (K)	296
Protein concentration (mg ml ⁻¹)	8.0
Buffer composition of protein solution	50 mM Tris-HCl pH 8.0, 125 mM NaCl
Composition of reservoir solution	0.1 M citric acid pH 4.3, 3.4 M NaCl, 4.5%(v/v) glycerol
Volume and ratio of drop	4 µl (1:1 protein:reservoir solution)
Volume of reservoir (µl)	500

averaged, and the data were plotted after subtracting the buffer blank in units of mdeg. Secondary-structure content was evaluated using the *CDSSTR* algorithm with reference data set No. 4 from the *DichroWeb* server (Whitmore & Wallace, 2008). Thermal stability was monitored in 10 mM sodium phosphate buffer pH 7.4 by measuring the ellipticity at 222 nm from 293 to 343 K with a temperature gradient of 1 K min⁻¹. The melting temperature (T_m) was calculated by plotting the first derivative of the thermal denaturation curve. All spectra were smoothed before plotting.

2.3. Enzyme kinetics

Enzyme assays were performed in triplicate at 298 K in a 96-well plate using a microplate reader (Molecular Devices) with a path length of 0.596 cm. The activity of ribokinase was measured using the method reported previously (Ogbunode *et al.*, 2007) with some modifications, by coupling the production of ADP to the oxidation of NADH and measuring the change in absorbance at 340 nm. The reaction mixture consisted of 30 mM Tris-HCl pH 8.5, 100 mM KCl, 3 mM MgCl₂, 0.3 mM phosphoenolpyruvate, 0.2 mM NADH, with 0.4 U pyruvate kinase and 1.2 U lactate dehydrogenase as coupling enzymes. For determination of the initial reaction rates, the concentration of ATP was fixed at 0.8 mM and ribose was varied from

Figure 1
12% SDS-PAGE of purified recombinant *LdRK* after gel-filtration chromatography. Lane *M*, molecular-weight markers (labelled in kDa); lane 1, pooled purified fraction from gel-filtration chromatography.

Table 3
Data collection and processing.

Values in parentheses are for the highest resolution shell.

Diffraction source	PX-BL21, Indus-2, RRCAT
Wavelength (Å)	0.9794
Temperature (K)	100
Detector	MAR 225 CCD
Crystal-to-detector distance (mm)	180.00
Rotation range per image (°)	1
Total rotation range (°)	118
Exposure time per image (s)	20
Space group	<i>P</i> 6 ₁
<i>a</i> , <i>b</i> , <i>c</i> (Å)	100.25, 100.25, 126.77
α , β , γ (°)	90.0, 90.0, 120.0
Resolution range (Å)	46.61–1.95 (2.0–1.95)
Total No. of reflections	395500 (27813)
No. of unique reflections	52575 (3698)
Completeness (%)	100 (99.9)
Multiplicity	7.5 (7.5)
Mean <i>I</i> / σ (<i>I</i>)	14.9 (3.4)
<i>R</i> _{merge} (all <i>I</i> + and <i>I</i> −)	0.101 (0.604)
<i>CC</i> _{1/2}	0.998 (0.876)
Overall <i>B</i> factor from Wilson plot (Å ²)	16.7

0.01 to 4 mM; when the concentration of ribose was fixed at 4 mM, that of ATP was varied from 0.005 to 0.8 mM. The reactions were started by the addition of 0.2 µM enzyme and the absorbance was recorded every 10 s for 30 min. The kinetic data were fitted to the Michaelis–Menten equation through nonlinear regression using *GraphPad Prism*.

2.4. Crystallization

Crystallization screening was initially carried out by the sitting-drop vapour-diffusion method at 296 K using a Mosquito protein crystallization robot (TTP Labtech) at IICT, Hyderabad in MRC 96-well plates by mixing 0.15 µl *LdRK* solution with 0.15 µl precipitant solution. Commercially available crystallization kits from Hampton Research (Index, PEG/Ion, SaltRx, Crystal Screen and Crystal Screen 2) and Molecular Dimensions (Structure Screen 1, Structure Screen 2, MORPHEUS, MIDAS, PGALM, PACT *premier* and JCSG *plus*) were used to screen for suitable conditions. Hexagonal rod-shaped crystals appeared after 1 d in a condition consisting of 0.1 M citric acid pH 3.5, 3.0 M NaCl. Further optimization was performed using the hanging-drop vapour-diffusion method by varying the concentration of protein, the pH of the buffer and the ionic strength of NaCl in 24-well plates with a reservoir volume of 500 µl. Crystals suitable for diffraction experiments were obtained after 21 d using 0.1 M citric acid pH 4.3, 3.4 M NaCl, 4.5%(v/v) glycerol (Table 2). Simultaneously, to confirm the presence of RK, crystals were washed with well solution, dissolved in sample-loading buffer and electrophoresed on 12% SDS-PAGE gels followed by silver staining.

2.5. Data collection and processing

A single crystal mounted on a loop was flash-cooled in liquid nitrogen with mother liquor as the cryoprotectant. Diffraction data were collected under cryogenic condition (100 K) on the protein crystallography beamline (PX-BL21)

at the Indus-2 synchrotron, Indore, India using a MAR 225 CCD detector. The collected diffraction data were processed using *XDS* (Kabsch, 2010) and scaled with *AIMLESS* from the *CCP4* program suite (Evans & Murshudov, 2013). Data-collection and processing statistics are given in Table 3.

3. Results and discussion

Recombinant *LdRK* was expressed in *E. coli* BL21 (DE3) and then purified using Ni^{2+} -affinity and gel-filtration chromatography, with a final yield of 15 mg per litre of culture medium. Analysis of the pooled purified fraction by SDS-PAGE indicated that the protein was >95% pure, with an approximate molecular weight of 38 kDa (Fig. 1), which corresponds to the theoretical molecular mass (37.5 kDa) predicted by *ProtParam* (Gasteiger *et al.*, 2005). Before proceeding to the crystallization of *LdRK*, the secondary-structure content of the purified protein was analyzed by far-UV CD spectroscopy. The spectrum specifies α -helix as the dominant secondary structure in *LdRK*, owing to the presence of the characteristic double minimum near 222 and 208 nm and a maximum around 192 nm. Analysis of the data using the *DichroWeb* server predicted the secondary-structure content to be as follows: 27% α -helix, 23% β -sheet, 22% turns and 28% unordered regions (Fig. 2a). The presence of a proportionately greater amount of α -helix in *LdRK* correlates with the crystal structures of ribokinase reported from *V. cholerae* (Paul *et al.*, 2015), *Staphylococcus aureus* (Li *et al.*, 2012) and *E. coli* (Sigrell *et al.*, 1999). To probe the thermal stability of ribokinase, the change in the ellipticity was measured at 222 nm as a function of temperature from 293 to 343 K. The thermal melting (T_m) curve of ribokinase indicates a rapid increase in the ellipticity at 313 K, which begins to level off at 323 K

Table 4

Kinetic parameters of *LdRK*.

The parameters were obtained by performing the assays in triplicate.

Substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu\text{M}^{-1} \text{s}^{-1}$)
Ribose	2.28 ± 0.08	296 ± 36	$(7.70 \pm 0.98) \times 10^{-3}$
ATP	2.15 ± 0.05	116 ± 9	$(18.50 \pm 1.50) \times 10^{-3}$

(Fig. 2b). The T_m of the protein was calculated to be 317.2 K by plotting the first derivative of the thermal melting curve (Fig. 2c). The kinetic parameters (K_m , k_{cat} and k_{cat}/K_m) were obtained by plotting the initial velocities *versus* the respective substrate concentrations (Fig. 3), and the K_m values indicated that *LdRK* has a higher affinity for ATP than ribose (Table 4). *LdRK* binds to ribose with a K_m of 296 μM , which corroborates with the binding affinities (K_m) determined for RK from *L. major* (300 μM) and human (279 μM). The affinity of *LdRK* towards ATP was observed to be similar (116 μM) to those of RK from *L. major* (200 μM ; Ogbunude *et al.*, 2007) and human (70 μM ; Chuvikovskiy *et al.*, 2006).

Although more than 950 conditions were screened to identify the preliminary crystallization conditions, crystals of *LdRK* were only obtained with Index condition A7 consisting of 0.1 M citric acid pH 3.5, 3.0 M NaCl. Since the crystals that were obtained by screening were not suitable for diffraction studies, optimization was performed by varying the protein concentration, the pH and the NaCl concentration of the precipitant solution. After refining the initial crystallization conditions, long hexagonal rod-shaped crystals were obtained using 0.1 M citric acid pH 4.3, 3.4 M NaCl, 4.5% glycerol and reached their maximum size after three weeks (Fig. 4a). The presence of a protein band at the corresponding molecular weight (~ 38 kDa) on a silver-stained gel confirmed the

Figure 2

(a) Far-UV circular dichroism spectrum of *LdRK* measured at 298 K in 7.5 mM Tris-HCl pH 8.5. (b) CD thermal denaturation curve of *LdRK* obtained by measuring the ellipticity at 222 nm in 1.0 K intervals from 293 to 343 K. (c) The first-derivative curve of thermal melting with a peak value (T_m) of 317.2 K.

Figure 3

Kinetic characterization of *LdRK*. (a) Plot of initial velocities against increasing concentrations of ribose. (b) Plot of initial velocities against increasing concentrations of ATP. Each data point represents the average from assays performed in triplicate; the standard error is shown as an error bar.

presence of *LdRK* in the crystals (Fig. 4b). The addition of glycerol tremendously improved the crystal size, and these

Figure 4

Crystallization of *LdRK*. (a) Crystals obtained with 0.1 M citric acid pH 4.3, 3.4 M NaCl, 4.5% (v/v) glycerol using the hanging-drop vapour-diffusion method. (b) Silver-stained SDS-PAGE of the crystals. Lane M, molecular-weight markers (labelled in kDa); lane 1, crystals of *LdRK*.

crystals diffracted to 1.95 Å resolution on the PX-BL21 synchrotron beamline (Fig. 5). The crystal lattice belonged to space group $P6_1$, with unit-cell parameters $a = b = 100.25$, $c = 126.77$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$ (Table 3). Two molecules of *LdRK* were assumed to be present in the asymmetric unit, with a solvent content of 49.8% and a calculated Matthews coefficient (Matthews, 1968) of $2.45 \text{ Å}^3 \text{ Da}^{-1}$. Using a *BLASTP* search of the PDB, ribokinase from *Homo sapiens* (PDB entry 2fv7; Structural Genomics Consortium, unpublished work) was identified as the closest homologous structure, with 42% identity to *LdRK*. Molecular replacement with *MOLREP* (Vagin & Teplyakov, 2010) using chain A of human

Figure 5

Diffraction image of *LdRK* at 1.95 Å resolution obtained using a MAR 225 CCD detector.

RK (PDB entry 2fv7) as the search model suggested the presence of two protomers in the asymmetric unit. The solution obtained in space group $P6_1$ was confirmed by comparing the results of molecular replacement in space groups $P6_1$ (correlation coefficient of 0.431 and R factor of 59.5%) and $P6_5$ (correlation coefficient of 0.364 and R factor of 62.2%). After one round of initial restrained refinement, the R factor decreased to 42.1% in space group $P6_1$. The structure solution and refinement of this protein structure, which are in progress, will help us to understand the detailed catalytic mechanism of this enzyme in the ribose metabolism of leishmanial parasites.

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