

Short sequence-paper

Cloning, characterization and preliminary crystallographic analysis of *Leishmania* hypoxanthine–guanine phosphoribosyltransferase

Paulo S. Monzani^a, Juan D. Alfonzo^b, Larry Simpson^b, Glaucius Oliva^a, Otavio H. Thiemann^{a,*}

^aLaboratory of Protein Crystallography and Structural Biology, Physics Institute of São Carlos, University of São Paulo—USP, Av. Trabalhador São-carlense 400, 13566-590, São Carlos—SP, Brazil

^bHoward Hughes Medical Institute, MacDonal Building, 90095-1662, UCLA, Los Angeles, CA, USA

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Abstract

Hypoxanthine–guanine phosphoribosyltransferase (HGPRT) (EC 2.4.2.8) is an important enzyme involved in the recycling of purine nucleotides in all cells. Parasitic protozoa of the order Kinetoplastida are unable to synthesize purines de novo and use the salvage pathway for the synthesis of nucleotides; therefore, this pathway is an attractive target for antiparasitic drug design. The *hgprt* gene was cloned from a *Leishmania tarentolae* genomic library and the sequence determined. The *L. tarentolae hgprt* gene contains a 633-nucleotide open reading frame that encodes a 23.4-kDa protein. A pairwise alignment of the different HGPRT's sequences revealed a 26%–53% sequence identity with the *Leishmania* sequences and 87% identity to the HGPRT of *Leishmania donovani*. A recombinant protein was expressed in *Escherichia coli*, purified to homogeneity and found to retain enzymatic activity. The steady-state kinetic parameters were determined for the recombinant enzyme and the enzyme is active as a homodimer in solution. Single crystals were obtained for the *L. tarentolae* HGPRT representing the first *Leishmania* HGPRT crystallized and initial crystallographic data were collected. The crystals obtained belong to the orthorhombic space group (P2₁2₁2₁) with unit cell parameters $a = 58.104 \text{ \AA}$, $b = 85.443 \text{ \AA}$ and $c = 87.598 \text{ \AA}$ and diffract to a resolution of 2.3 Å. The availability of the HGPRT enzyme from *Leishmania* and its crystallization suitable for X-ray diffraction data collection should provide the basis for a functional and structural analysis of this enzyme, which has been proposed as a potential target for rational drug design, in a *Leishmania* model system. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Crystallization; *Leishmania tarentolae*; Hypoxanthine–guanine phosphoribosyltransferase; *Trypanosomatida*

Most organisms synthesize purine nucleotides by both the de novo and the salvage pathways. In contrast, protozoan parasites are strict purine nucleotide auxotrophs because of the absence of a purine de novo biosynthetic pathway [1]. Enzymes of the phosphoribosyltransferase (PRTase) family catalyze the biosynthesis of purine nucleotides. Three PRTase enzymes are known to be involved in the recycling of purine nucleotides by the salvage pathway in the kinetoplastid protozoa from the genus *Leishmania*, hypoxanthine–guanine PRTase (HGPRT) (EC 2.4.2.8), adenine PRTase (APRT) (EC 2.4.2.7) and xanthine PRTase (XPRT) (EC 2.4.2.22) [2]. HGPRT is responsible for catalyzing the conversion of guanine and hypoxanthine and α -D-5-phosphoribosyl 1-pyrophosphate (PRPP) into guanine-5-monophosphate (GMP), inosine-5-monophosphate and

pyrophosphate (PPi) [3]. The only *hgprt* gene that has been cloned and characterized from the *Leishmania* genus is that from *Leishmania donovani* [4] and no structural data are known for the enzyme of this parasite genus. The availability of the *Trypanosoma cruzi* HGPRT structure [5] is of great comparative importance and will be used for the molecular substitution approach.

Leishmania tarentolae has been exploited as a model *Leishmania* for a variety of molecular, biochemical and evolutionary studies. The evolutionary data support the inclusion of *L. tarentolae* as a monophyletic clade branching between the *Viannia* and *Leishmania* subgenera [6–8]. As in the case of other trypanosomatids, *L. tarentolae* is a purine nucleotide auxotroph [9]. The ease of cell culture and genetic analysis of *L. tarentolae* should facilitate its use for site-directed mutagenesis of the *hgprt* gene as well as for functional complementation and testing of inhibitory substrates for the rational drug design for future leishmaniasis chemotherapy. We describe in this paper the cloning,

* Corresponding author. Tel.: +55-16-273-9756; fax: +55-16-273-9881.
E-mail address: thiemann@ifsc.usp.br (O.H. Thiemann).

expression, characterization and the first crystallization of a *Leishmania* HGPRT protein. In view of the close phylogenetic relationship, the results will be of general significance as a model for the pathogenic *Leishmania* species.

A 207-bp fragment of *L. tarentolae* *hgp* gene was PCR amplified from genomic DNA with degenerate oligodeoxynucleotides for two conserved regions, one comprising the purine site YLLCVLKGS and the other ILIVEDIV corresponding to the purine/pyrimidine phosphoribosyl transferases signature at positions 121–128 from the *L. donovani* HGPRT sequence. The amplified DNA contained an open reading frame of 69 amino acids with sequence identity to other HGPRT polypeptides. The full-length *hgp* gene was isolated from a *L. tarentolae* UC strain Lambda ZAP Express *Bam*HI–*Sal*3A I genomic library by hybridization screening with the 207-bp PCR-amplified *L. tarentolae*-specific probe. A 1.5-kbp genomic fragment was sequenced and found to contain a 633-nucleotide open reading frame that encodes a protein of 210 amino acids of 23.4 kDa expected molecular mass and a calculated pI of 7.64 (Fig. 1). The *L. tarentolae* HGPRT predicted amino acid sequence shares 87% identity with the HGPRT of *L. donovani* [4], divergence being mostly in the N- and C-terminus regions. Inspection of the sequence alignments in Fig. 1 reveals the conserved purine and PRPP binding domains [4,10] flanked by regions without significant sequence identity among the HGPRTs. The prediction of protein folding motifs from the individual polypeptides and

the aligned sequences indicates a conservation of α -helical (H) and β -sheet (B) structural motifs that has allowed the molecular modeling of the HGPRT from *L. tarentolae* (data not shown). A pairwise alignment of the different sequences revealed a 26–53% sequence identity with the *Leishmania* sequences (data not shown).

The *hgp* open reading frame was amplified by a PCR reaction with oligodeoxynucleotides specific for the *L. tarentolae* *hgp* gene 5' and 3' ends. Oligodeoxynucleotide primers for PCR amplification (GIBCO-BRL) introduce both *Nde*I and *Xho*I restriction sites at its 5' and 3' end, respectively (5'-AGCTCCCATATGAGCAACTCAGTCAAG-3'-*Nde*I; 5'-AGCTAAGCTTGATGTCGAACGAGAGGCG-3'-*Xho*I) for cloning into the pET29a(+) expression vector (Novagen). The PCR reaction containing 2 pmol of each primer and approximately 0.5 ng of the 1.5-kbp genomic fragment was carried out in a GeneAmp 2400 thermocycler (Perkin-Elmer CETUS) with 2.5 U of AmpliTaq DNA polymerase (Promega) according to manufacturer's conditions. The sample was subjected to 2 min denaturation at 94 °C followed by 30 cycles of denaturation at 94 °C for 0.5 min, annealing at 40 °C for 0.5 min, and extension at 72 °C for 1 min. A DNA band of approximately 650 bp in length was gel purified by the NaI-glass powder method [12]. The purified DNA was digested with the restriction enzymes *Nde*I and *Xho*I, cloned into pET29a(+) vector into the *Nde*I/*Xho*I sites and transformed into BL21(DE3) competent cells. The cloned *hgp*



Fig. 1. Multiple alignment of representative HGPRT sequences. The amino acid sequences of several HGPRT proteins are shown aligned with the *L. tarentolae* and *L. donovani* sequences. The amino acid positions are indicated at the top of the alignment. The consensus of the alignment is indicated. “*” Indicates amino acid identities, “:” and “.” indicate conserved substitutions. The boxes indicate the predicted purine binding domain (Site I) and predicted PRPP binding domain (Site II). The sequences are as follows: Ldon (*Leishmania donovani* L25412), Ltar (*Leishmania tarentolae* AF139722), Smans (*Schistosoma mansoni* X13531), Human (*Homo sapiens* NM.000194), Rat (*Rattus norvegicus* M86443), Tbruc (*Trypanosoma brucei* L10721), Tcruz (*Trypanosoma cruzi* L07486), Ttric (*Trichomonas foetus* L08622), Llact (*Lactococcus lactis* X67015), Tgond (*Toxoplasma gondii* U09219) and Clong (*Cricetulus longicaudatus* X17656). The sequences were aligned by the CLUSTALX [11] with further manual refinements.

sequence identity was confirmed by the BigDye-termination method and the T7 and T7-terminator sequencing primers in an ABI377 DNA sequencer (Perkin-Elmer). Cells from a

single colony were grown overnight at 37 °C, 250 rpm in 5 ml LB medium containing 25 µg/ml kanamycin. A larger cell culture was grown in 2XYT with 25 µg/ml kanamycin,

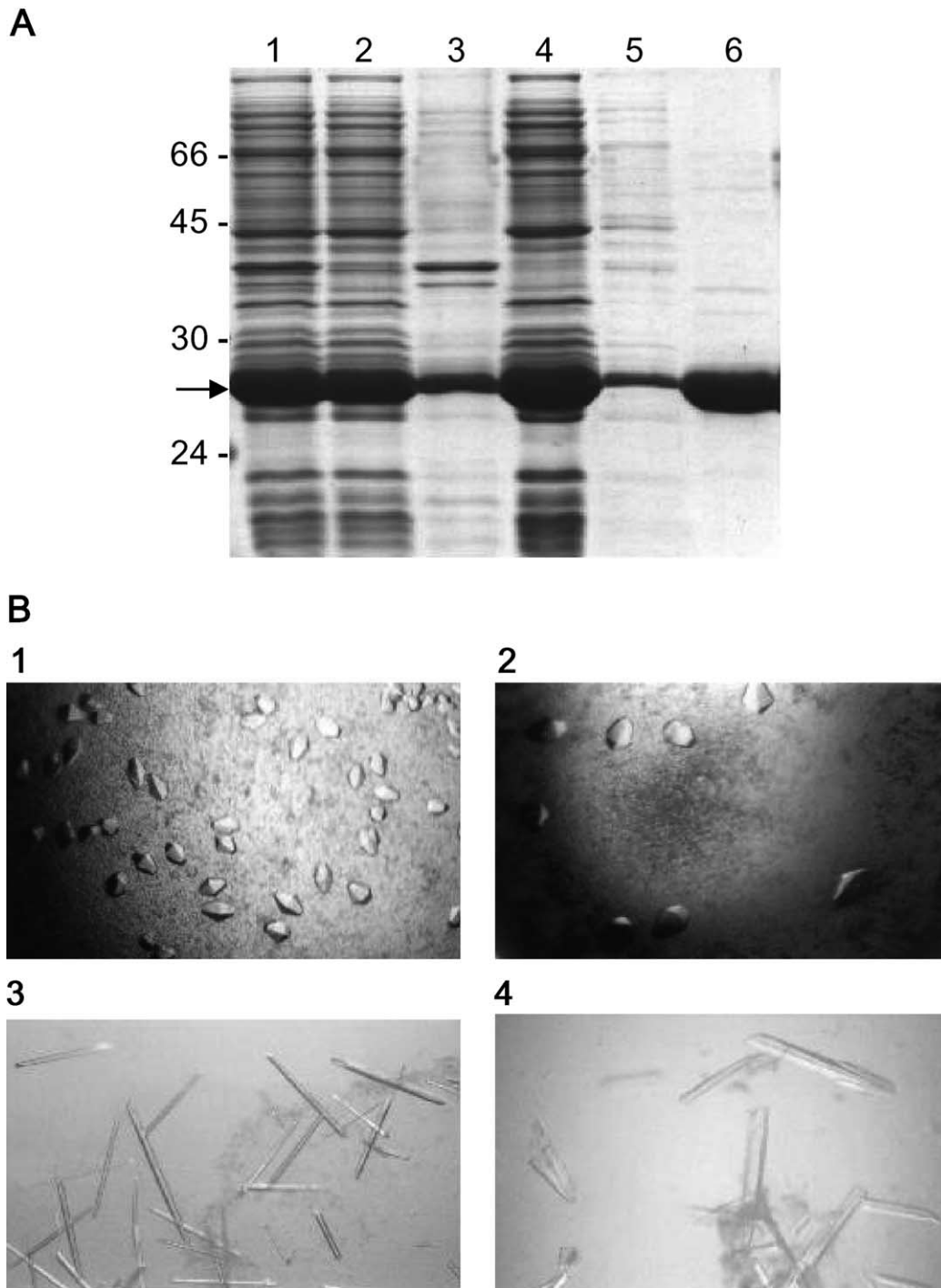


Fig. 2. (A) Expression of *L. tarentolae hgpert* in *E. coli* and purification of the recombinant protein. The *L. tarentolae hgpert* gene was subcloned into the pET29a(+) expression vector and overexpressed in *E. coli* BL21(DE3). The proteins were separated in a 15% SDS-PAGE gel. Lanes: 1—crude lysate, 2—clarified extracts (S20000 supernatant), 3—S20000 protein pellet, 4—ammonium sulfate precipitate fraction, 5—ammonium sulfate supernatant fraction, 6—pooled fractions from POROS 20HQ passage. (B) Crystallization of *L. tarentolae* HGPRT. (1) HGPRT crystals obtained in 20% PEG 6000, 100 mM citric acid and pH 5.0; (2) 15% PEG 6000, 100 mM citric acid and pH of 5.1, at 18 °C; (3) 17% PEG 4000, 5% glycerol, 19% isopropanol, pH 5.6; and (4) in crystals obtained in 19% isopropanol, 19% PEG 4000, 5% glycerol, pH 5.6, at 18 °C. Diffraction data were collected from crystals of condition 4. Interestingly, no diffraction pattern was detectable from the crystals from conditions 1 and 2.

Table 1
Data collection statistics

Space group	P2 ₁ 2 ₁ 2 ₁
Unit cell parameters in angstrom	<i>a</i> = 58.104 <i>b</i> = 85.443 <i>c</i> = 87.598
Resolution	20–2.3 Å
Unique reflections	18,895
<i>R</i> _{merge} * (last shell) (%)	8.1 (25.0)
Completeness (last shell) (%)	94.5 (96.9)
Redundancy	5.67
<i><I/σ></i>	9.9

$$R_{\text{merge}} = \sum |I - \langle I \rangle| / \sum I.$$

at 37 °C, 250 rpm, until OD₆₀₀ = 0.6. The 250-ml culture was then induced for 4 h at 37 °C, 250 rpm, with 0.5 mM IPTG.

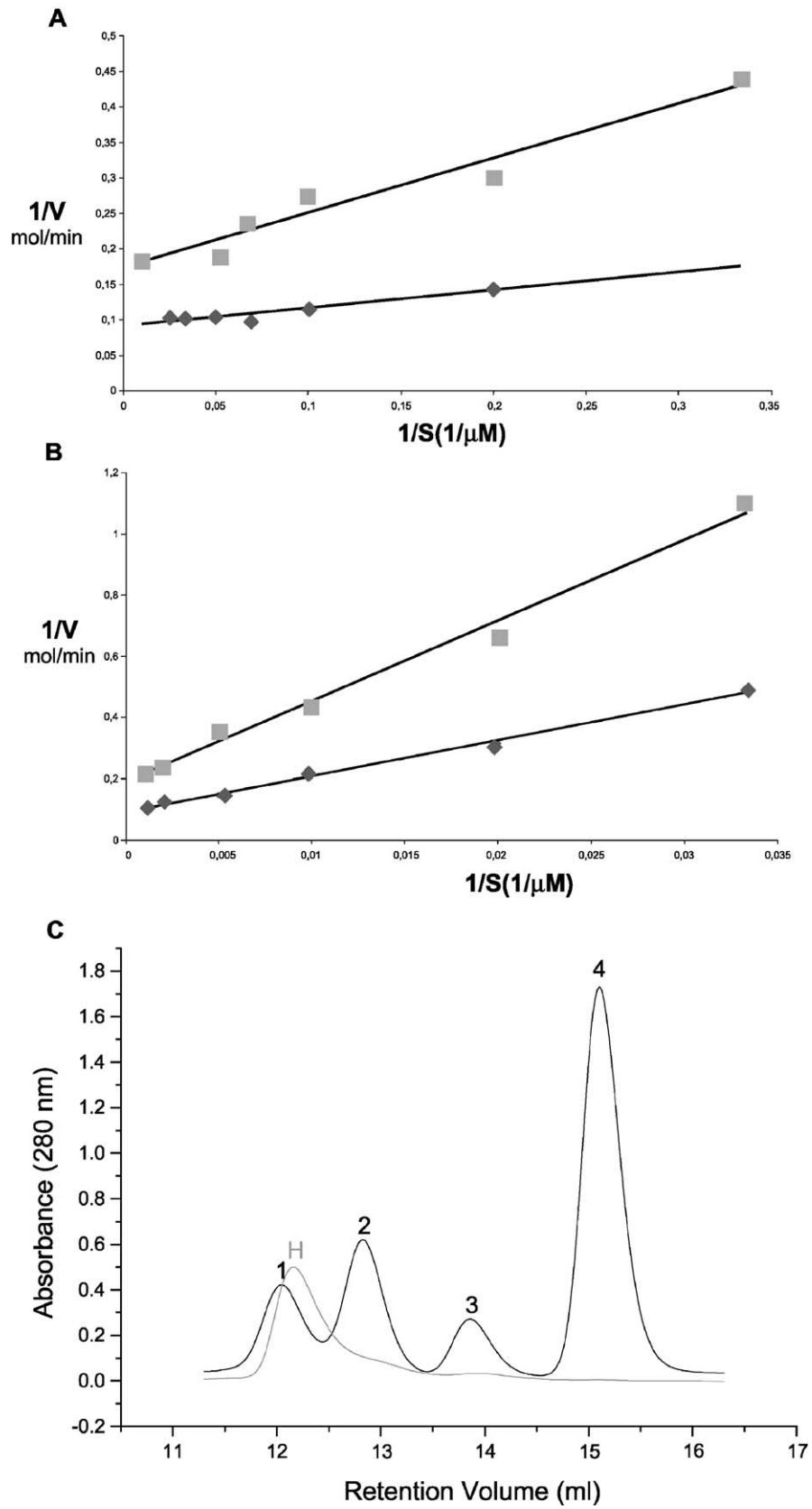
The purification of recombinant HGPRT protein from *L. donovani* has been previously reported [4] using an affinity purification method with a GMP-Agarose column. Interestingly, the *L. tarentolae* HGPRT protein binds with very low affinity to either the GMP or the GTP-Agarose columns. As a result, we developed a new purification protocol. All purification procedures were carried out at 4 °C unless specified. The *Escherichia coli* BL21(DE3)-induced cells were harvested by centrifugation at 4000 × *g* for 15 min. The cells were washed in 100 mM Tris–HCl, 10 mM MgCl₂, pH 7.5 (buffer TMD). Cell lysis was obtained by five cycles of freeze–thaw in TMD buffer plus 1% Triton X-100 followed by six 1-min cycles of sonication in an ice bath. The crude extract was clarified by centrifugation (20,000 × *g*, 30 min) and brought to 60% (w/v) ammonium sulfate for 20 min with slow shaking. The suspension was separated by centrifugation for 20 min at 20,000 × *g*. The protein pellet was dissolved in 8 ml TMD and dialyzed against four changes of 250 ml TMD. The HGPRT from the ammonium sulfate fraction was loaded in a 1.6-ml POROS-20HQ column, equilibrated in TMD buffer adjusted to pH 9.0 with a 1-ml/min flux at room temperature. The HGPRT eluted in the void volume of the column with only trace amounts of contaminants (Fig. 2A). The protein fractions eluted along the NaCl gradient (0–1 M) did not reveal the presence of HGPRT protein by SDS-PAGE or enzymatic activity assay (data not shown). For crystallization purposes, the fractions eluted from the POROS-20HQ column were pooled, concentrated in a Centriprep 10 (10 kDa cutoff) to a

volume of approximately 6 ml and loaded in a second pass through the POROS-20HQ chromatography, as described above. The void volume of the second chromatography contained highly purified HGPRT from *L. tarentolae* (Fig. 2A) yielding approximately 70 mg HGPRT per liter of culture and over 95% purity based on SDS-PAGE analysis (Fig. 2A). The recombinant HGPRT protein migrates as a 23-kDa protein in 15% SDS-PAGE (Fig. 2A) and retained enzymatic activity and substrate specificity (Table 2). The *L. tarentolae* recombinant HGPRT is a homodimer of approximately 50 kDa in the presence of GMP, hypoxanthine or the free enzyme form (Fig. 3C). Samples of the purified *Leishmania* HGPRT were analyzed by isoelectric focusing in a FAST System (Pharmacia Biotech) and a pI of 8.20 was observed (data not shown). This pI is consistent with the result from the POROS-20HQ chromatography where the recombinant HGPRT eluted in the column void volume. The fractions collected in the void volume of the second chromatography were pooled and concentrated to 7 mg/ml in a Centriprep 10 (Millipore).

The crystallization conditions for the *L. tarentolae* HGPRT protein were screened by the hanging-drop vapor-diffusion method. The sparse-matrix kits Crystal Screen I, Crystal Screen II, Crystal Screen PEG6K, Ammonium Sulfate, Cryo-Crystal Screen and Crystal Screen MPD from Hampton Research were used to test initial conditions at 4 and 18 °C. A 3-μl HGPRT solution containing 7 mg/ml enzyme, in TMD buffer pH 7.5, was mixed with an equal volume (3 μl) of the well solutions (500 μl/well) to form the drop. Screening different concentrations of precipitating agent and pH further optimized the initial crystallization conditions. The best crystallization conditions so far obtained for the *L. tarentolae* HGPRT were at 18 °C in 19% *iso*-propanol, 19% PEG 4000, 5% glycerol, pH 5.6 and in 17% PEG 4000, 5% glycerol (Fig. 2B). Minor variations in the crystallization conditions, showed no significant improvements in the crystal quality as shown in Fig. 2B. Crystals of long orthorhombic habit diffracted at 2.3 Å. In 20% PEG 6000, 100 mM citric acid and pH of 5.0 and in 15% PEG 6000, 100 mM citric acid and pH of 5.1 at 18 °C (Fig. 2B) several hexagonal shape HGPRT crystals were obtained. Hexagonal crystals were also obtained at 4 °C with subtle differences in the overall crystal shape. These crystals did not diffract properly and were discarded.

Crystals were mounted in nylon loops (Hampton Research) after quick soaking in a cryo-protectant solution

Fig. 3. Determination of the *K_m* for guanine, hypoxanthine and PRPP of HGPRT and molecular mass. (A) Double reciprocal plots of the HGPRT reaction for guanine (□) and hypoxanthine (◇). The concentration of PRPP was kept constant at 1 mM. Concentrations were held constant at 3, 5, 10, 15, 20, 30 and 40 μM for guanine and 3, 5, 10, 15, 20, 50 and 100 μM for hypoxanthine. (B) Double reciprocal plots of the HGPRT reaction for PRPP at constant concentrations of 40 μM guanine (□) and 100 μM hypoxanthine (◇) while the concentration of PRPP was 10, 30, 50, 100, 200, 500 and 1000 μM. (C) Sizing of the HGPRT. Recombinant *L. tarentolae* HGPRT was chromatographed on a Superose 12 column as described. Different fractions are plotted as a function of OD 280. Proteins of known molecular mass (1–66 kDa bovine serum albumin, 2–45 kDa ovalbumin, 3–29 kDa carbonic anhydrase and 4–13 kDa cytochrome *C*) were used as standards in the calibration of the column. HGPRT free enzyme and with GMP or PRPP bound (H) elute between the BSA (1) and ovalbumin (2) standards, corresponding to approximately a 46-kDa homodimer.



consisting of the same reservoir solution and 15% ethylene glycol. Diffraction data of the long orthorhombic crystals were collected at the Protein Crystallography beamline (PCr) at the National Synchrotron Light Laboratory (LNLS, Campinas, Brazil) using monochromatic X-rays set on 1.537 Å. A total of 75 frames were collected at 100 K, with an exposure time of 5 min in 1° oscillation and a crystal-to-detector distance of 155 mm. The collected diffraction data were processed using DENZO and SCALEPACK [13]. The crystals belong to the primitive orthorhombic space group $P2_12_12_1$ with unit cell parameters of $a = 58.104$ Å, $b = 85.443$ Å, $c = 87.598$ Å. Statistical analysis of the merged reflections indicated that the collected data set is better described as having a resolution of 2.3 Å (Table 1). Such resolution range is sufficient for initial structure determination and modeling. The molecular replacement studies, using *T. cruzi* HGPRT [5] as the search model, indicated a solution for the rotation and translation functions.

The kinetic parameters were measured at 25 °C following the consumption of guanine at 255 nm and hypoxanthine at 243 nm for 1 min in a 1-ml reaction volume containing 100 mM Tris–HCl, 5 mM MgSO₄, pH 7.4, 1 mM PRPP and 0.04 mM guanine or 0.1 mM hypoxanthine [14]. An extinction coefficient of 4.2 for guanine and of 2.2 for hypoxanthine was used. To calculate the first-order rate constants (K_m and V_{max}) for guanine, hypoxanthine or PRPP, the other components were kept constant at 40 μM for guanine, 100 μM for hypoxanthine and 1 mM for PRPP according to the experiment (Table 2). The substrate concentrations for the K_m and V_{max} measurements were of 3–40 μmol guanine, 3–100 μmol hypoxanthine and 10–1000 μmol PRPP. The kinetic parameters of *L. tarentolae* HGPRT were analyzed and K_m and V_{max} values of 2.8 μM/10.9 μM/min for guanine and 4.4 μM/5.7 μM/min for hypoxanthine were obtained. The PRPP K_m and V_{max} were measured with guanine or hypoxanthine as a substrate and the following results were obtained: K_m and V_{max} of 127.1 μM/10.9 μM/min with guanine and 137.6 μM/5.2 μM/min with hypoxanthine (Table 2).

The ability to overexpress and purify the recombinant *L. tarentolae* protein in two chromatographic steps, independent of its binding to GMP or GTP columns, should allow the purification of HGPRT mutants with different affinities for

the substrate that would not bind with the same affinity to those columns. This is an interesting feature for the structural–functional study of the enzyme.

We have characterized the kinetic parameters of the recombinant *L. tarentolae* HGPRT. The values of K_m obtained for the substrate guanine and hypoxanthine, 2.8 and 4.4 μM, respectively, are lower than the values described for the *L. donovani* homologue of 3.8 and 7.6 μM, respectively. Such differences in K_m values can be due to small structure differences between the two enzymes. It is interesting to note that the *L. tarentolae* HGPRT is a homodimer either in the free enzyme form or combined with the product GMP or the substrate PRPP as determined by size exclusion chromatography.

Finally, the successful crystallization of the *L. tarentolae* HGPRT enzyme, suitable for structure determination, should allow us to compare the *Leishmania* enzyme with the other HGPRTases crystallized to date. Such knowledge may be valuable for future structure-based drug design strategies using this enzyme as a model system for the *Leishmania* genus. Interestingly, we have observed differences in K_m and V_{max} between the *L. tarentolae* and *L. donovani* enzymes in spite of the high sequence conservation observed between those enzymes. The structural and functional comparison between the *Leishmania* HGPRT and the available structures of *T. cruzi* [5,15], human [16–18] and other HGPRTases should allow us to investigate this important enzyme from *Leishmania* and possibly explain how subtle structural differences contribute to the enzyme catalytic activity. We are currently refining the structure.

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Table 2

Kinetic constant from *L. tarentolae* and comparison with *L. donovani*

Substrate	K_m <i>L. donovani</i> ^a (μM)	K_m <i>L. tarentolae</i> (μM)	V_{max} <i>L. tarentolae</i> (μmol min ⁻¹)
Guanine ^b –PRPP ^c	3.8	2.8	10.9
Hypoxanthine ^b –PRPP ^c	7.6	4.4	5.7
PRPP ^b –Guanine ^c	ND	127.1	10.9
PRPP ^b –Hypoxanthine ^c	ND	137.6	5.2

ND=Not determined.

^a Published data from *L. donovani* [14].

^b Variable substrate concentration.

^c Constant substrate concentration.

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