Cloning and expression of the hypoxanthine-guanine phosphoribosyltransferase gene from *Trypanosoma brucei*

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ABSTRACT

The hypoxanthine-guanine phosphoribosyltransferase (HGPRT) enzyme of *Trypanosoma brucei* and related parasites provides a rational target for the treatment of African sleeping sickness and several other parasitic diseases. To characterize the *T. brucei* HGPRT enzyme in detail, the *T. brucei* *hgprt* was isolated within a 4.2 kb *SalI-KpnI* genomic insert and sequenced. Nucleotide sequence analysis revealed an open reading frame of 630 bp that encoded a protein of 210 amino acids with a $M_r = 23.4$ kd. After gap alignment, the *T. brucei* HGPRT exhibited 21–23 % amino acid sequence identity, mostly in three clustered regions, with the HGPRTs from human, *S. mansoni*, and *P. falciparum*, indicating that the trypanosome enzyme was the most divergent of the group. Surprisingly, the *T. brucei* HGPRT was more homologous to the hypoxanthine phosphoribosyltransferase (HPRT) from the prokaryote *V. harveyi* than to the eukaryotic HGPRTs. Northern blot analysis revealed two trypanosome transcripts of 1.4 and 1.9 kb, each expressed to equivalent degrees in insect vector and mammalian forms of the parasite. The *T. brucei* *hgprt* was inserted into an expression plasmid and transformed into $S\phi 606$ *E. coli* that are deficient in both HPRT and xanthine-guanine phosphoribosyltransferase activities. Soluble, enzymatically active recombinant *T. brucei* HGPRT was expressed to high levels and purified to homogeneity by GTP-agarose affinity chromatography. The purified recombinant enzyme recognized hypoxanthine, guanine, and allopurinol, but not xanthine or adenine, as substrates and was inhibited by a variety of nucleotide effectors. The availability of a molecular clone encoding the *T. brucei* *hgprt* and large quantities of homogeneous recombinant HGPRT enzyme provides an experimentally manipulable molecular and biochemical system for the rational design of novel therapeutic agents for the treatment of African sleeping sickness and other diseases of parasitic origin.

INTRODUCTION

Trypanosoma brucei is a protozoan parasite that is the etiologic agent of African sleeping sickness, a devastating and often fatal disease in humans, and nagana, a deadly disease of domestic animals. Transmitted to humans by the bite of an infected tsetse fly, *T. brucei* inhabit and replicate exclusively within extracellular milieus of the host. The conventional therapeutic agents for the treatment of African sleeping sickness are far from ideal since these drugs are ineffective after central nervous system involvement and have numerous undesirable side effects due to their lack of selectivity for the metabolic machinery of the parasite. Furthermore, treatment of African sleeping sickness is often complicated by chemotherapeutic failure, a predicament that is often attributed to drug resistance (1–3).

Selective and rational therapies for parasitic diseases have been generally difficult to devise since many of the major metabolic pathways in these lower eukaryotes are similar, if not identical, to those in their vertebrate host. One metabolic pathway that is conspicuously different between parasites and humans is that for the production of purine nucleotides. Whereas mammalian cells synthesize purine nucleotides *de novo*, all genera of parasitic protozoa that have been examined are auxotrophic for purines (4–12). In order to satisfy their purine requirements, each genus of parasite has evolved a unique complement of purine salvage enzymes for which no identical mammalian counterpart exists. Biochemical and metabolic studies have substantiated the existence of a plethora of purine salvage enzymes in *T. brucei*, including phosphoribosyltransferases (PRTs), nucleoside kinases, and nucleoside cleavage activities (10,13,14). The unique features of the purine salvage system of *T. brucei* and the related parasites *Trypanosoma cruzi* and *Leishmania* cause these pathogenic hemoflagellates to be selectively susceptible to the cytotoxic effects of several pyrazolopyrimidine analogs of hypoxanthine and inosine (15,16). For instance, *T. b. brucei*, *T. b. gambiense*, and *T. b. rhodiense* bloodstream forms are all sensitive to allopurinol (HPP) (17), a pyrazolopyrimidine isomer of hypoxanthine that is nontoxic to humans and used extensively in the treatment of hyperuricemia and gout (18).

Metabolic activation of HPP in *T. brucei*, as well as in *Leishmania* and *T. cruzi*, is initiated by the enzyme hypoxanthine-

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guanine phosphoribosyltransferase (HGPRT: IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8). The unique substrate specificity of the HGPRT enzyme of these parasites suggests that the enzyme may serve as a rational target for antiparasitic drugs. To characterize this therapeutically germane enzyme further and to develop molecular reagents for future studies directed toward structure-based drug design, we have cloned and sequenced the *hgprt* from *T. brucei*. Furthermore, the parasite *hgprt* has been inserted into an expression vector, expressed to very high levels in *E. coli*, and large quantities of recombinant HGPRT have been purified to homogeneity.

MATERIALS AND METHODS

Chemicals and reagents

[α -³²P]dCTP (3000 Ci/mmol) and [α -³⁵S]dATP (1320 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA). [¹⁴C]Hypoxanthine (56 mCi/mmol), [¹⁴C]guanine (56 mCi/mmol), and [¹⁴C]xanthine (56 mCi/mmol) were bought from Moravsek Biochemicals (Brea, CA). [¹⁴C]HPP (46 mCi/mmol) was obtained from Research Products International Corp. (Mount Prospect, IL). Synthetic oligonucleotides were prepared by OLIGO's ETC. (Wilsonville, OR). Thermostable AmpliTaq DNA polymerase was obtained from the Perkin-Elmer Corp. (Norwalk, CT). All restriction and DNA modifying enzymes were acquired from either Bethesda Research Laboratories Life Technologies Inc. (Gaithersburg, MD) or Boehringer Mannheim Biochemicals (Indianapolis, IN). Guanosine 5'-triphosphate covalently attached through its ribose hydroxyl groups to an agarose resin by an adipic acid dihydrazide spacer (catalog number, G 9768), phosphoribosylpyrophosphate (PRPP), and all purine bases were purchased from the Sigma Chemical Co. (St. Louis, MO). All other materials, chemicals, and reagents were of the highest quality commercially available.

Cell culture

Procyclic forms of the pleomorphic *T. b. brucei* TREU667 strain were propagated in SDM-79 medium (19) supplemented with 10 % heat-inactivated fetal calf serum acquired from HyClone Inc. (Logan, UT).

Nucleic acid isolation

Genomic DNA was isolated from 1.0×10^{10} *T. brucei* procyclic forms as described (20). Total RNA was prepared by the phenol-chloroform extraction protocol reported by Landfear and Wirth (21).

Isolation of a *T. brucei hgprt* fragment by the polymerase chain reaction

To generate a homologous DNA probe for the isolation of the *T. brucei hgprt*, a fragment of the gene was amplified from genomic DNA in the polymerase chain reaction (PCR) using degenerate oligonucleotide primers created from completely conserved amino acid sequences on the mammalian (22,23), *Schistosoma mansoni* (24), and *Plasmodium falciparum* (25) HGPRT proteins. A sense primer, 5'-TCTGGATCC-GAG-GA[CT]-AT[CT]-AT[CT]-GA[CT]-AC-3', was constructed with an 9 nucleotide leader encompassing a *Bam*HI site 5' to a degenerate oligonucleotide sequence corresponding to amino acids 134–139, EDIIDT, of the human HGPRT. The antisense primer, TCTGAATTC-[AG]TA-[ACG]CC-[ACG]AC-[ACG]AC-[A-G]AA-3', was synthesized with an *Eco*RI site preceded by a

3 nucleotide leader 5' to a mixed oligonucleotide generated from residues 187–191, FVVG Y, of the human HGPRT. Amplification of the specific *hgprt* fragment was accomplished on a Coy Instruments (Ann Arbor, MI) thermocycler using the amplification assay mixture described by Hanson *et al.* (26). The sample was subjected to 30 cycles of denaturation at 94°C for 0.5 min, annealing at 40°C for 0.5 min, and extension at 72°C 1.0 min. The PCR products were ligated into pBluescript KS⁺ vector obtained from Stratagene (San Diego, CA), and transformed into XL-1 Blue *Escherichia coli*. Large scale plasmid preparations were prepared according to the large scale alkaline lysis protocol (27). The 174 bp *hgprt* fragment was sequenced according to the dideoxy chain termination method (28) with [³⁵S]dATP as the radiolabel and the Sequenase 2.0 sequencing kit from United States Biochemical Corp (Cleveland, OH).

Libraries of *T. brucei* DNA

The cDNA library in λ -ZAP (Stratagene, La Jolla, CA) and the genomic library in EMBL3 were constructed from cDNA and genomic DNA, respectively, prepared from the IsTAR 1 clone of the EATRO 164 strain of *T. b. brucei*. Both the cDNA and genomic libraries were generously provided by Drs. Parsons and Peter Myler of the Seattle Biomedical Research Institute (Seattle, WA).

Isolation of the *hgprt* from *T. brucei* libraries

The *T. brucei hgprt* was isolated from the *T. brucei* genomic library in two steps. First, the amplified *hgprt* PCR product was employed as a probe to screen the *T. brucei* cDNA library, and second, an *hgprt* cDNA that lacked the 5' protein coding portion of the *hgprt* was used to screen the genomic library. The prehybridization, hybridization, and washing conditions employed to isolate the *hgprt* cDNAs and genomic locus were identical to those described previously by this laboratory for the isolation of the *L. donovani* ornithine decarboxylase (ODC) gene (26). Positive bacteriophage from each library were carried through a tertiary plaque purification, and their DNA was isolated by CsCl density gradient centrifugation (29).

Subcloning and sequencing of *T. brucei hgprt*

The lengths of the *hgprt* inserts from the cDNA library were determined on 0.8% agarose gels, and the longest cDNA, a 1.4 kb fragment, was ligated into pBluescript KS⁺ and transformed into XL-1 Blue *E. coli*. DNA from the genomic clones was probed with the 1.4 kb *hgprt* cDNA under the same high stringency conditions employed for screening the libraries and a 4.2 kb *Sal*I-*Kpn*I genomic fragment was isolated as described for the cDNAs. Large scale plasmid preparations and nucleotide sequencing of DNA were performed as described above for the PCR fragment.

Computer assisted DNA and amino acid sequence analysis

Analyses of nucleotide and predicted amino acid sequences were performed on an IBM compatible AT computer using the Sequence Analysis Program from International Biotechnologies, Inc. (New Haven, CT). Protein sequences were aligned using the CLUSTAL V multiple sequence alignment program, a modification of the CLUSTAL package described by Higgins and Sharp (30). Amino acid similarity scores between nonidentical amino acid pairs were obtained from the log-odds amino acid similarity matrix of Dayhoff (31).

Southern and Northern analyses

Southern blots of restriction enzyme-cleaved TREU667 genomic DNA were performed under standard conditions (29). A Northern blot of RNA isolated from bloodstream and procyclic forms of *IsTAR 1 T. brucei* was hybridized to the *hgprt* under the same high stringency conditions described for probing the Southern blots. The Northern blot was contributed by Dr Parsons.

Mapping the 5' terminus of the mature *hgprt* mRNA

The 5' terminus of the mature *T. brucei hgprt* transcript was defined by a modification of the PCR amplification protocol reported by Hanson *et al.* (26). cDNA synthesis from total RNA was achieved with random hexamer primers using the reverse transcriptase (RT) protocol described by Kawasaki (32). The RT mixture was then adjusted to 10 mM Tris-HCl, pH 8.3, 50 mM KCl, and 1.5 mM MgCl₂, and 10 pmol of each oligonucleotide primer and 5 units of *Taq* DNA polymerase were added. The sense primer, 5'-CTCGGGATCCCAACGCTATATAAGTATCAGTTTCTGTACTTTATTG-3', contained an 11 nucleotide leader containing a *Bam*HI restriction site followed by 31 nucleotides matching positions 5–35 of the *L. donovani* mini-exon (33). The mini-exon is an RNA that is *trans*-spliced onto the 5' end of all mRNAs from *Trypanosoma* and *Leishmania* species (34,35). Of the 31 nucleotides of the sense primer, the *T. brucei* and *L. donovani* mini-exon genes differ in only 4 positions. The antisense primer, 5'-CTCGAATTCGCTGCCC-TTCAATACAGA-3', was constructed to nucleotides 151–168 of the protein coding portion of the *T. brucei hgprt* and contained a 9 nucleotide leader enclosing an *Eco*RI recognition site. The PCR sample was incubated at 94, 50, and 72°C, respectively, for 35 cycles in the thermocycler. A 250 bp PCR product that hybridized to the *T. brucei hgprt* was subcloned and sequenced as described above.

Overexpression of *T. brucei hgprt* in *E. coli*

The *T. brucei hgprt* was ligated into the pBACE expression vector constructed by Craig *et al.* (36). An *Nde*I restriction site was introduced at the initiation Met codon and a *Sal*I cleavage site was inserted 3' to the termination signal by PCR mutagenesis in two separate amplification reactions, necessitated by the presence of an internal *Nde*I site within the *T. brucei hgprt*. The 5' end of the *hgprt* was amplified using 5'-TCTCATATGGAA-CCAGCTTGCAAATA-3' as the mutagenic primer and 5'-GT-AGGAATTCCACACGTGTGG-3' as the antisense primer containing an internal *Eco*RI cleavage site. The 3' end of the gene was amplified using 5'-TGTGGAATTCCTGCGGGCCT-C-3' containing the same internal *Eco*RI site as the sense primer and the mutagenic 5'-TCTGTGACACAAGGCACAAGAGC-TACCTG-3' as the antisense primer. The two PCR fragments were digested with the appropriate restriction enzymes and ligated simultaneously into pBACE that had been cleaved with *Nde*I and *Sal*I. The ligation mixture was transformed into the *E. coli* K-12 strain Sφ606 (Δ *gpt-pro-lac*, *thi*, *hpt*) that was initially described (37) and generously provided to this laboratory by Dr Per Nygaard of the University Institute of Biological Chemistry B (Copenhagen, Denmark) through Dr Sidney Craig of the University of California (San Francisco, CA). This strain contains a deletion in the gene encoding the bacterial xanthine-guanine phosphoribosyltransferase (XGPR) activity and is also deficient in hypoxanthine phosphoribosyltransferase (HPRT) activity. DNA was prepared from the transformants, and the 630 bp *Nde*I-

*Sal*I fragment containing the entire *T. brucei hgprt* was sequenced in both directions.

500 ml cultures of Sφ606 cells transformed with the recombinant expression plasmid were incubated in a low phosphate medium modified from that previously described (36) by reducing the concentration of 'vitamin free' Casamino acids (Difco Laboratories, Detroit, MI) to 0.1 % and deleting the equimolar mixture of NaH₂PO₄ and Na₂HPO₄. The adjustments were crucial for maximizing expression of recombinant protein, as the 'vitamin free' Casamino acids contained 100 μmol/g of inorganic phosphate. The final concentration of phosphate in the modified induction medium was, therefore, ~100 μM.

Inorganic phosphate assay

Inorganic phosphate levels in the *hgprt* expression medium were determined as described (38).

HGPRT assay

HGPRT activity was assayed as previously reported (39). The rates of [¹⁴C]HPP phosphoribosylation were measured by replacing hypoxanthine or guanine in the HGPRT cocktail with HPP.

HGPRT purification

Recombinant *T. brucei* HGPRT was purified by a modification of the method described by Allen *et al.* (39) for the purification of native HGPRT from *L. donovani*. 500 ml cultures of Sφ606 *E. coli* were grown for 12 hr at 37°C to an OD₆₀₀ = 0.9–1.2. The cells were washed several times with phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, and 1.0 mM KH₂PO₄) and lysed by French press in 20 mM Tris, pH 7.4, 5 mM MgCl₂, and 1mM dithiothreitol (TMD) buffer. After centrifugation at 40,000×g for 30 min at 4°C, the cell supernatant was chromatographed over a 10 ml GTP-agarose affinity column. The column was then washed with 10 column volumes of TMD and the protein eluted in TMD containing 1.0 mM PRPP.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis on 12 % acrylamide slab gels was performed as described (29).

RESULTS

Isolation and sequencing of the *hgprt* from *T. brucei*

Preliminary attempts to isolate the *hgprt* from *T. brucei*, as well as from other parasites of the Trypanosomatidae family, by cross hybridization under low stringency conditions to either the mouse (23), *S. mansoni* (24), or *P. falciparum* (25) *hgprt* cDNAs were unsuccessful. Consequently, the PCR was exploited to amplify fragments of the *T. brucei hgprt* from genomic DNA using primers to conserved amino acid sequences among other eukaryotic HGPRT proteins (22–25). A 174 bp fragment that encoded a fragment of the *T. brucei hgprt* was amplified and used as a probe for the isolation of *hgprt* cDNAs from a cDNA library. Nucleotide sequence analysis of the longest cDNA, a 1.4 kb fragment, confirmed that it lacked the 5' end of the protein coding segment of the *T. brucei hgprt*. Therefore, the 1.4 kb cDNA was employed in turn to isolate the *hgprt* from a genomic library. A 4.2 kb *Sal*I-*Kpn*I fragment was subcloned into pBluescript, and the protein coding segment of *hgprt* was sequenced in both directions. The nucleotide and predicted amino

Figure 3. Northern blot analysis of *hgprt* transcripts. Nytran filters to which RNA isolated from bloodstream, stumpy, and procyclic forms of IsTAR 1 *T. brucei* was blotted and probed with the 774 bp *T. brucei* *NdeI-SalI* *hgprt* fragment from the pBAce expression vector under conditions of high stringency. Stumpy forms were induced with DFMO as described (61). This digestion released two fragments, a 248 bp *NdeI-NdeI* and a 526 bp *NdeI-SalI* fragment, that together encompassed the entire *T. brucei* *hgprt* coding region. The RNA standards are from the 1.4–9.5 kb RNA ladder supplied by GIBCO BRL (Gaithersburg, MD). The sizes of the *hgprt* transcripts are indicated.

domain lies a central Arg residue and a Thr-Ala dipeptide in the *P. falciparum* enzyme (25) that distinguishes the malarial purine binding site from those of the human (22), *S. mansoni* (24), *T. brucei*, and *V. harveyi* (40). A conserved aromatic amino acid and an Ala-Asp are found at these positions in the other HPRTs. These striking differences may account for the unique ability of the malarial HGPRT to recognize xanthine as a substrate (44). The other eukaryotic HGPRTs, as well as the prokaryotic HPRT, do not phosphoribosylate xanthine. A third region located proximal to the COOH termini, a stretch of amino acids for which no function has been ascribed, is also highly conserved among the aligned PRT sequences. Between residues 162 and 181 of the *T. brucei* HGPRT, there are 7 amino acid identities and 9 similarities within this region. Although no known function has been imputed to this region of the protein, several naturally occurring missense mutations in this regions confer HGPRTase deficiency in humans and elevate apparent K_m values for both substrates dramatically (45), indicating the importance of these sequences in catalytic competence and substrate binding. It is worth noting that the amino acid regions outside the 3 conserved areas are highly divergent among the PRT proteins with only an occasional single amino acid identical among the 5 aligned PRT sequences. Among the aligned sequences (Figure 2), the longest stretch of amino acid identity outside the 3 conserved regions is 2 amino acids, a Ser-Tyr dipeptide corresponding to residues 83 and 84 of the *T. brucei* HGPRT.

Size and quantitation of *T. brucei* *hgprt* transcripts

A Northern blot of total RNA isolated from trypomastigote, stumpy, and procyclic forms of IsTAR 1 *T. brucei* was probed with the 774 bp *NdeI-SalI* fragment that contains the protein coding portion of the *hgprt*. Two transcripts of 1.4 and 1.9 kb, each large enough to encode the entire trypanosome HGPRT, were recognized by this probe in all 3 developmental stages of the parasite (Figure 3). The hybridization signals to both bands

Figure 4. Southern blot analysis of the *hgprt* locus. 10 μ g of genomic DNA from *T. brucei* strain TREU667 were incubated overnight with the indicated restriction enzymes, fractionated on a 0.8 % agarose gel, transferred to a Nytran filter, and probed with the 248 bp *NdeI-NdeI* and the 526 bp *NdeI-SalI* *hgprt* fragments as described in the legend to Figure 3. The protein coding region of the *T. brucei* *hgprt* contains a single restriction site each for *NdeI*, *EcoRI*, and *SacII*, while *BamHI*, *AvaI*, *HindIII*, and *KpnI* do not cleave within the *hgprt* coding region. DNA markers are products of λ phage DNA digested with *AvaI* and were obtained from United States Biochemical (Cleveland, OH).

were equivalent in the 3 life forms, and the level of *hgprt* expression did not appear to be developmentally regulated. The same two transcripts are observed in RNA from TREU667 procyclic forms (data not shown).

Southern analysis of the *hgprt* locus in *T. brucei*

As a number of genes in *T. brucei* are arranged in tandemly repeated arrays (44,45), the *hgprt* locus in TREU667 *T. brucei* was analyzed for repeated sequences by restriction enzyme analysis and Southern blotting (Figure 4). *T. brucei* DNA was digested with a variety of restriction enzymes, blotted onto Nytran filters, and probed with the 774 bp *NdeI-SalI* fragment. Restriction enzymes *NdeI*, *EcoRI*, and *SacII*, each of which cleaves the *hgprt* once, excised 3 or more fragments of dissimilar sizes that hybridized to the *hgprt* probe (Figure 4). If *hgprt* was organized as a tandem repeat within the *T. brucei* genome, this strategy would yield a prominent band of identical size in all lanes of a Southern blot in which the genomic DNA had been digested with a single cutter. Moreover, digestion of *T. brucei* DNA with either *AvaI*, *HindIII*, or *KpnI*, which do not cleave within *hgprt*, generates two DNA fragments that hybridize to the *hgprt*. These data suggest that *hgprt* is present in at least two copies within the *T. brucei* genome. Although the restriction pattern depicted in Figure 4 does not rule out the possibility that the *hgprt* locus consists of two very different alleles, this conjecture requires restriction fragment length polymorphisms for every restriction enzyme tested.

Overexpression of *hgprt* in *E. coli*

After appropriate PCR mutagenesis, the *T. brucei* *hgprt* was ligated into the *E. coli* expression plasmid pBAce and transformed into *S*ph606 *E. coli* that are genetically deficient in both the HPRT and XGPRT activities. Nucleotide sequencing demonstrated that the primary structures of the recombinant and native HGPRT enzymes were identical. After inoculation into low phosphate medium, the transformed *E. coli* were grown for ~12 hr to an

Figure 5. SDS polyacrylamide gel electrophoresis of expressed and purified trypanosomal HGPRT. The *T. brucei* *hgprt* ligated into the prokaryotic expression vector pBAce was transformed into *E. coli* K-12 strain Sϕ606 (Δ *gpt-pro-lac*, *thi*, *hprt*) and expressed in the low phosphate medium described in Materials and Methods. A pBAce plasmid lacking *hgprt* was transformed into Sϕ606 cells as a negative control. After a 12 hr of growth, the transformed *E. coli* were harvested and the proteins fractionated by SDS polyacrylamide gel electrophoresis. Recombinant *T. brucei* HGPRT was purified by GTP-agarose affinity chromatography as described in Materials and Methods. Lanes: A, proteins from Sϕ606 *E. coli* transformed with the control pBAce plasmid; B, proteins from Sϕ606 cells transformed with the *hgprt*-containing pBAce vector; C, PRPP-eluate of *T. brucei* HGPRT chromatographed over GTP-agarose column. ~10 μ g of protein was loaded onto each lane of the SDS gel.

OD₆₀₀ = 0.9–1.2. Further growth of cells in this induction medium to stationary phase densities severely diminished *hgprt* expression. Sϕ606 *E. coli* transformed with the *hgprt* construct expressed very high levels of HGPRT activity, whereas the same cells transformed with a control pBAce plasmid lacking *hgprt* expressed no HGPRT activity. Proteins from the Sϕ606 *E. coli* were then fractionated by SDS polyacrylamide gel electrophoresis and stained with Coomassie Blue. As demonstrated in Figure 5, the recombinant *T. brucei* HGPRT is the predominant protein in *E. coli* lysates transformed with the *hgprt*-containing plasmid and is not detected in cells transformed with the control plasmid. The recombinant HGPRT migrates slightly more slowly than that expected from its predicted M_r .

Purification and substrate specificity of *T. brucei* HGPRT

Recombinant *T. brucei* HGPRT protein was purified by GTP-agarose affinity chromatography followed by elution with 1.0 mM PRPP (data not shown). No HGPRT activity was present either in the void volume or in washes prior to the addition of the PRPP. As demonstrated in Figure 5, passage over the affinity column resulted in a one step purification of the protein to homogeneity. ~100 mg of pure *T. brucei* HGPRT could be obtained by this strategy per liter of bacterial culture. The substrate specificity of the pure recombinant trypanosome HGPRT was also evaluated. Clearly, *T. brucei* HGPRT prefers the naturally occurring nucleobases, hypoxanthine and guanine, as substrates but is also capable of phosphoribosylating HPP. K_m values of 2.3 ± 0.9 , 4.8 ± 0.6 , and $243 \pm 35 \mu$ M and k_{cat} values of 32 ± 4.7 , 53 ± 2.9 , and $4.4 \pm 0.6 \mu$ mol/min/mg protein were calculated for the recombinant enzyme by Lineweaver–Burk analysis for hypoxanthine, guanine, and HPP, respectively (Figure 6). Neither xanthine nor adenine were

Figure 6. Lineweaver–Burk analysis of recombinant *T. brucei* HGPRT. The ability of the homogeneous *T. brucei* HGPRT to phosphoribosylate hypoxanthine and guanine (Panel A), and HPP (Panel B) was determined at a number of substrate concentrations and the data analyzed by the method of Lineweaver–Burk.

phosphoribosylated by the purified *T. brucei* HGPRT. Inhibition studies also revealed that a variety of nucleotide effectors were inhibitors of the *T. brucei* HGPRT. GTP, IMP, GMP, ATP, and AMP at a concentration of 1.0 mM, all reduced, in order of diminishing effectiveness, the capacity of the enzyme to phosphoribosylate 20 μ M [¹⁴C]hypoxanthine. Moreover, the conversion of [¹⁴C]hypoxanthine to the nucleotide level was obliterated by excess hypoxanthine or guanine but unaffected by adenine or xanthine (data not shown).

DISCUSSION

A full length *hgprt* gene has been isolated from a genomic library of *T. brucei* DNA. Unlike previously isolated parasite *hgprt* cDNAs that were isolated by cross hybridization to the murine *hgprt* cDNA, the nucleotide sequence disparity of the *T. brucei* *hgprt* gene precluded its recognition by the heterologous *hgprt* probes. Thus, the PCR was exploited to amplify a specific *hgprt*

fragment from *T. brucei* using oligonucleotide primers generated to the PRPP binding and COOH terminal sequences that are highly conserved among the mammalian (22,23), *S. mansoni* (24), and *P. falciparum* (25) HGPRTs. Nucleotide sequence analysis revealed that the primary structure of the *T. brucei* HGPRT protein contained more amino acid identities with the *V. harveyi* HPRT than to its counterpart from higher eukaryotes. This homology cannot be explained by substrate specificity variations, since the prokaryotic enzyme phosphoribosylates hypoxanthine exclusively, while xanthine and guanine are recognized by a biochemically and genetically distinct protein (37,40,48). The lack of homology between the *T. brucei* and eukaryotic HGPRTs is somewhat surprising in view of the extensive homology observed between the primary structures of the ODC (49) and IMP dehydrogenase (50) proteins from *T. brucei* and mouse. The *T. brucei* and murine ODC enzymes exhibit a 69 % amino acid identity over a core region of 376 amino acids with no gaps in the alignment, whereas the *T. brucei* and human IMP dehydrogenase proteins are identical in 53 % of their residues with only a few small gaps in the computed alignment. The amino acid sequence divergence among the HGPRT proteins from phylogenetically diverse organisms suggests that conservation of primary structure is not critical for enzyme function. Despite the extensive divergence of primary structures, analyses of the secondary structures of mammalian and parasite HGPRTs using the algorithm of Chou and Fasman (51), predict several common features, including a β - α - β structure characteristic of a mononucleotide fold involved in PRPP binding to PRTs (24,42, and data not shown).

The *hgprt* gene is not differentially expressed in *T. brucei*. The lack of differential expression of HGPRT is unsurprising in view of the facts that both the procyclic and bloodstream forms of *T. brucei* are auxotrophic for purines (10,13) and that hypoxanthine is a predominant environmental source of purines for the parasite (52). Whether the two transcripts are derived from the same or different genetic loci is not known in view of the fact that these parasites appear to contain more than one *hgprt* copy. As Southern blot and sequence analysis of cDNA generated and amplified from the 5' portion of *hgprt* mRNA revealed only a single fragment, the data suggest that the *hgprt* transcripts are heterogeneous in their 3' termini. This conjecture is supported by the results of experiments in which PCR of reverse transcribed RNA, using a sense primer 3' to the *hgprt* protein coding region and oligo (dT), amplified two DNA fragments that differed in size by ~500 bp.

The ability to overexpress and purify the *T. brucei* HGPRT in an *E. coli* background genetically deficient in both HPRT and XGPRT has established that the enzyme is specific for hypoxanthine and guanine and does not recognize either xanthine or adenine. The existence of separate PRTs for xanthine and adenine is supported by experimental evidence demonstrating the differential incorporation of radiolabeled purine bases into nucleotide pools in both procyclic and bloodstream forms of *T. brucei* species and by the fact that *T. brucei* lack adenine deaminase activity (10,13), an enzyme that is expressed to high levels in promastigotes of *Leishmania* (53). Moreover, expression of the recombinant *T. brucei* HGPRT in the S ϕ 606 *E. coli* establishes that the enzyme can recognize HPP as a substrate. The procyclic forms of *T. b. brucei*, *T. b. gambiense*, and *T. b. rhodesiense* can further convert HPP to aminopyrazolopyrimidine nucleotides and incorporates the triphosphate of the latter into RNA (17). This metabolic sequence

does not exist in humans or mammals, and the procyclic forms of the parasite are selectively sensitive to HPP. HPP exhibits no toxicity toward humans and is widely used as an hypouricemic agent in the treatment of hyperuricemia and gout (18). Although, the curative effects of HPP against African trypanosomiasis in humans have not been established, the pyrazolopyrimidine exhibits demonstrable therapeutic efficacy in preliminary clinical trials against both cutaneous leishmaniasis (54) and Chagas disease (55). Moreover, as hypoxanthine is the salvageable purine present in the highest concentrations in human blood and cerebrospinal fluid (52), the milieus inhabited by the parasite, HGPRT is likely to provide a critical nutritional function in *T. brucei*. The selective toxicity of HPP toward *T. brucei* and the potentially central role of HGPRT in purine salvage imply that the parasite HGPRT may provide a biochemically rational target for the therapeutic manipulation of African sleeping sickness.

Although virtually all antiparasitic regimens are empirical in design, a precedent for rationally based treatments of African trypanosomiasis exists. α -Difluoromethylornithine (DFMO), an irreversible inhibitor of ODC, the rate limiting step in polyamine biosynthesis (56), has shown considerable promise in the treatment of some forms of African trypanosomiasis (57). The therapeutic efficacy of DFMO is based on the rapid rate of turnover of the mammalian enzyme in contrast to the *T. brucei* ODC (49,58). The instability of the mammalian ODC is conferred by a COOH terminus rich in PEST sequences, a sequence missing in the *T. brucei* ODC. Although, DFMO treatment does not manifest the toxic side effects of the conventional antitrypanosomal reagents and is effective after central nervous system involvement, its administration is prolonged and expensive, and the drug is not particularly effective toward *T. b. rhodesiense* (57). Moreover, resistance to DFMO has been demonstrated *in vitro* (59,60). Thus, the search for newer and improved therapies for the treatment of African trypanosomiasis are imperative. The isolation of the molecular clone encoding the *T. brucei* HGPRT and the functional overexpression and purification of large quantities of pure recombinant HGPRT enzyme from *E. coli* provide an avenue for a structure-based strategy for the design and discovery of novel HGPRT substrate analogs or HGPRTase inhibitors that can serve as effective agents in the treatment of African trypanosomiasis or of any other parasitic disease.

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REFERENCES

1. Williamson, J. (1970) In Mulligan, H.W., (ed.), The African Trypanosomiasis. Wiley-Interscience, New York, pp. 125-221.
2. Leach, T.M. and Roberts, C.J. (1981) *Pharmacol. Ther.* **13**, 91-147.
3. Geary, T.G., Edgar, S.A. and Jensen, J.B. (1986) In Campbell, W.C. and Rew, R.S., (eds.), Chemotherapy of Parasitic Diseases. Plenum Press, New York, pp. 209-236.
4. Marr, J.J., Berens, R.L. and Nelson, D.J. (1978) *Biochim. Biophys. Acta* **544**, 360-371.

5. Heyworth, P.G., Gutteridge, W.E. and Ginger, C.D. (1978) *J. Protozool.* **25**, 9B.
6. Sherman, I.W. (1979) *Microbiol. Rev.* **43**, 453–495.
7. Boonlayangoor, P., Albach, R.A. and Booden, T. (1980) *Arch. Invest. Med. (Mex)* **11**, 83–88.
8. Berens, R.L., Marr, J.J., LaFon, S.W. and Nelson, D.J. (1981) *Mol. Biochem. Parasitol.* **3**, 187–196.
9. Wang, C.C. and Simashkevich, P.M. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 6618–6622.
10. Fish, W.R., Marr, J.J. and Berens, R.L. (1982) *Biochim. Biophys. Acta* **714**, 422–428.
11. Schwartzman, J.D. and Pfefferkorn, E.R. (1982) *Exp. Parasitol.* **53**, 77–86.
12. Wang, C.C. and Aldritt, S. (1983) *J. Exp. Med.* **158**, 1703–1712.
13. Fish, W.R., Looker, D.L., Marr, J.J. and Berens, R.L. (1982) *Biochim. Biophys. Acta* **719**, 223–231.
14. Schmidt, G., Walter, R.D. and Konig, E. (1975) *Tropenmed. Parasitol.* **26**, 19–26.
15. Marr, J.J. and Berens, R.L. (1983) *Mol. Biochem. Parasitol.* **7**, 339–356.
16. Ullman, B. (1984) *Pharmaceut. Res.* **1**, 194–203.
17. Fish, W.R., Marr, J.J., Berens, R.L., Looker, D.L., Nelson, D.J., LaFon, S.W. and Balber, A.E. (1985) *Antimicrob. Agents Chemother.* **27**, 33–36.
18. Palella, T.D. and Fox, I.H. (1989) In Scriver, C.S., Beaudet, A.L., Sly, W.S. and Valle, D. (eds.), *The Metabolic Basis of Inherited Diseases*. McGraw Hill Book Co., New York, 6th edition, pp. 965–1006.
19. Brun, R. and Schonenberger, M. (1979) *Acta Trop.* **36**, 289–292.
20. Wilson, K., Collart, F.R., Huberman, E., Stringer, J.L. and Ullman, B. (1991) *J. Biol. Chem.* **266**, 1665–1671.
21. Landfear, S.M. and Wirth, D.F. (1985) *Mol. Biochem. Parasitol.* **15**, 61–82.
22. Jolly, D.J., Okayama, H., Berg, P., Esty, A.C., Filpula, D., Bohlen, P., Johnson, G.G., Shively, J.E., Hunkapillar, T. and Friedman, T. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 477–481.
23. Konecki, D.S., Brennard, J., Fuscoe, J.C., Caskey, C.T. and Chinault, A.C. (1982) *Nucl. Acids Res.* **10**, 6763–6775.
24. Craig, S.P.III, McKerrow, J.H., Newport, G.R. and Wang, C.C. (1988) *Nucl. Acids Res.* **16**, 7087–7101.
25. King, A. and Melton, D.W. *Nucl. Acids Res.* **15**, 10469–10481.
26. Hanson, S., Adelman, J. and Ullman, B. (1992) *J. Biol. Chem.* **267**, 2350–2359.
27. Davis, L.G., Dibner, M.D. and Battey, J.F. (1986) *Basic Methods in Molecular Biology*, Elsevier, New York.
28. Sanger, F., Nicklen, S. and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
29. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor University Press, Cold Spring Harbor.
30. Higgins, D.G. and Sharp, P.M. (1988) *Gene* **73**, 237–244.
31. Dayhoff, M.O. (1978) In Dayhoff, M.O. (ed.), *Atlas of Protein Sequence and Structure*. National Biomedical Research Foundation, Washington DC, Vol. 5, Suppl. 3., pp. 345–358.
32. Kawasaki, E.S. (1990) In Inns, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J. (eds.), *PCR Protocols*. Academic Press, Inc., San Diego, pp. 21–27.
33. Wilson, K., Hanson, S., Landfear, S.M. and Ullman, B. (1991) *Nucl. Acids Res.* **19**, 5787.
34. Murphy, W.J., Watkins, K.P. and Agabian, N. (1986) *Cell* **47**, 517–525.
35. Sutton, R.E. and Boothroyd, J.C. (1986) *Cell* **47**, 527–535.
36. Craig, S.P.III, Yuan, L., Kuntz, D.A., McKerrow, J.H. and Wang, C.C. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 2500–2504.
37. Jochimsen, B., Nygaard, P. and Vestergaard, T. (1975) *Mol. Gen. Genet.* **143**, 85–91.
38. Ames, B.N. and Dubin, D.T. (1960) *J. Biol. Chem.* **235**, 769–775.
39. Allen, T., Henschel, E.V., Coons, T., Cross, L., Conley, J. and Ullman, B. (1989) *Mol. Biochem. Parasitol.* **33**, 273–281.
40. Showalter, R.E. and Silverman, M.R. (1990) *Nucl. Acids Res.* **18**, 4621.
41. Sogin, M.L., Elwood, H.J. and Gunderson, J.H. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 1383–1387.
42. Hershey, H.V. and Taylor, M.W. (1986) *Gene* **43**, 287–293.
43. Dush, M.K., Sikela, J.M., Khan, S.A., Tischfield, J.A. and Sambrook, P.J. (1987) *Proc. Natl. Acad. Sci. USA* **82**, 2731–2735.
44. Queen, S.A., Jagt, D.V. and Reyes, P. (1988) *Mol. Biochem. Parasitol.* **30**, 123–134.
45. McKusick, V.A. (1992) *Mendelian Inheritance in Man*. The Johns Hopkins University Press, Baltimore, pp. 1873–1880.
46. Tomashow, L.S., Milhausen, M., Rutter, W.J. and Agabian, N. (1983) *Cell* **32**, 35–43.
47. Tschudi, C. and Ullu, E. (1988) *EMBO J.* **7**, 455–463.
48. Richardson, K.K., Fostel, J. and Skopek, T.R. (1983) *Proc. Natl. Acad. Sci. USA* **11**, 8809–8816.
49. Phillips, M.A., Coffino, P. and Wang, C.C. (1987) *J. Biol. Chem.* **262**, 8721–8727.
50. Wilson, K., Berens, R.L., Sifri, C.D. and Ullman, B., manuscript submitted.
51. Chou, P.Y. and Fasman, G.D. (1974) *Biochemistry* **13**, 222–245.
52. Eells, J. and Spector, R. (1983) *Neurochem. Res.* **8**, 1451–1457.
53. Looker, D.L., Berens, R.L. and Marr, J.J. (1983) *Mol. Biochem. Parasitol.* **9**, 15–28.
54. Martinez, S. and Marr, J.J. (1992) *New Engl. J. Med.* **326**, 741–744.
55. Gallerano, R.H., Sosa, R.R. and Marr, J.J. (1990) *Am. J. Trop. Med. Hyg.* **43**, 159–166.
56. Metcalf, B.W., Bey, P., Danzin, C., Casera, P. and Vevert, J.P. (1978) *J. Am. Chem. Soc.* **100**, 2661–2553.
57. Schechter, P.J., Barlow, J.L.R. and Sjoerdsma, A. (1987) In McCann, P.P., Pegg, A.E., and Sjoerdsma, A. (eds.), *Inhibition of Polyamine Metabolism, Biological Significance and Basis for New Therapies*. Academic Press, New York, pp. 345–364.
58. Ghoda, L., Phillips, M.A., Bass, K.F., Wang, C.C. and Coffino, P. (1990) *J. Biol. Chem.* **265**, 11823–11826.
59. Bellofatto, V., Fairlamb, A.H., Henderson, G.B. and Cross, G.A.M. (1987) *Mol. Biochem. Parasitol.* **25**, 227–238.
60. Phillips, M.A. and Wang, C.C. (1987) *Mol. Biochem. Parasitol.* **22**, 9–17.
61. Aboagye-Kwarteng, T., Ole-Moiyoi, O.K., and Lonsdale-Eccles, J.D. (1991) *Biochem. J.* **275**, 7–14.