Molecular Cloning and Expression of a Purine-specific N-Ribohydrolase from *Trypanosoma brucei brucei*

**SEQUENCE, EXPRESSION, AND MOLECULAR ANALYSIS**

(Received for publication, July 22, 1997, and in revised form, November 5, 1997)

Roger Pellec, Vern L. Schramm§, and David W. Parkin¶¶

*From the United International Livestock Research Institute, Nairobi, Kenya and the §Department of Biochemistry, Albert Einstein College of Medicine, Bronx, New York 10461*

N-Ribohydrolases, including the inosine-adenosine-guanosine-prefering (IAG) nucleoside hydrolase, have been proposed to be involved in the nucleoside salvage pathway of protozoan parasites and may constitute rational therapeutic targets for the treatment of these diseases. Reported is the complete sequence of the *Trypanosoma brucei brucei* iagnh gene, which encodes IAG-nucleoside hydrolase. The 1.4-kilobase iagnh cDNA contains an open reading frame of 981 base pairs, corresponding to 327 amino acids. The iagnh gene is present as one copy/haploid genome and is located on the size-polymorphic pair of chromosome III or IV in the genome of *T. b. brucei*. In Southern blot analysis, the iagnh probe hybridized strongly with *Trypanosoma brucei gambiens*, *Trypanosoma brucei rhodesiens*, *Trypanosoma evansi*, *Trypanosoma congolense*, and *Trypanosoma vivax* and, to a lesser extent, with *Trypanosoma cruzi* genomic DNA. The iagnh gene is expressed in bloodstream forms and procyclic (insect) life-cycle stages of *T. b. brucei*. There are no close amino acid homologues of IAG-nucleoside hydrolase outside bacterial, yeast, or parasitic organisms. Low amino acid sequence similarity is seen with the inosine-uridine-prefering nucleoside hydrolase isozyme from *Crithidia fasciculata*. The *T. b. brucei* iagnh open reading frame was cloned into *Escherichia coli* BL21(DE3), and a soluble recombinant IAG-nucleoside hydrolase was expressed and purified to >97% homogeneity. The molecular weights of the recombinant IAG-nucleoside hydrolase, based on the amino acid sequence and observed mass, were 35,735 and 35,737, respectively. The kinetic parameters of the recombinant IAG-nucleoside hydrolase are experimentally identical to the native IAG-nucleoside hydrolase.

Protozoan parasites rely on preformed purine nucleosides or bases for the biosynthesis of purine ribonucleotides, since they do not contain *de novo* purine biosynthetic pathways (1–3). Parasites and host cells have several common steps in purine salvage pathways, but unique intermediate steps are determined by the response of the parasite to the purine composition of the host’s cells or bloodstream. Protozoan parasites have evolved pathways to utilize any purine nucleoside or base in the environment (1). Intracellular hemoparasites, exemplified by the American trypanosomes, have different purine nucleosides or bases available than the extracellular hemoprotein protozoan parasites, such as the African trypanosomes. In addition, the various life-cycle stages of the African trypanosomes are exposed to diverse environments, which vary from the mouth and midgut of tsetse flies to the extracellular bloodstream of livestock, wildlife, and humans (4, 5).

The purine salvage pathway can be divided into stages: 1) the biosynthesis of IMP from free nucleosides or bases, and 2) the conversion of IMP to adenyate and guanulate nucleotides (1). The biosynthetic enzymes of the purine salvage pathway are purine nucleoside kinases, hypoxanthine-guanine-xanthine phosphoribosyl-1-pyrophosphate transferases, and the purine N-ribohydrolases and/or phosphorylases. N-Ribohydrolases found in both African and American trypanosomes are potential chemotherapeutic targets, since no N-ribohydrolase activity or encoding genes have been identified in mammals (6). N-Ribohydrolases catalyze the hydrolysis of the N-ribosidic bond between N-9 of the purine base and C-1’ of the (deoxy-) ribose, as shown by Reaction 1.

\[
\text{(Deoxy-)nucleoside} + H_2O \rightarrow \text{base} + \text{(deoxy-)ribose}
\]

**REACTION 1**

These enzymes have been identified and characterized, to a greater or lesser extent, from *Trypanosoma brucei brucei* (7), *Trypanosoma brucei gambiens* (8), *Trypanosoma cruzi* (9), *Leishmania donovani* (10), *Leishmania mexicana* (11), and *Crithidia fasciculata* (12–14). In *C. fasciculata*, over 90% of nucleoside salvage occurs through the inosine-uridine-prefering (IU)-¹ nucleoside hydrolase and guanosine-inosine-prefering nucleoside hydrolases, establishing a role for these isozymes in the purine nucleoside salvage pathway (14).

The IU- (13) and guanosine-inosine (14) nucleoside hydrolase from *C. fasciculata* and the inosine-adenosine-guanosine-prefering (IAG) nucleoside hydrolase from *T. b. brucei* (7) have been extensively characterized. The IU-nucleoside hydrolase has been purified, the chemical and kinetic mechanisms determined (13), the transition state determined from kinetic isotope effects (15), the cDNA cloned and overexpressed in *Escherichia coli* (6), and the recombinant enzyme has been characterized by x-ray crystallography (16).

Recent research has focused on the IAG-nucleoside hydrolase sequences of trypanosomes and leishmanias. The IAG-nucleoside hydrolase from *T. b. brucei* has been identified and characterized, and its sequence is similar to that of the enzyme from *C. fasciculata*. The IAG-nucleoside hydrolase from *T. b. brucei* has been expressed and purified, and its kinetic parameters have been determined. The enzyme is specific for inosine, adenosine, and guanosine, with minimal activity towards the corresponding deoxyribonucleosides. The enzyme has been shown to be involved in the biosynthesis of purine nucleotides in the parasitic forms of the trypanosomes.

---

*This work was supported by an African Regional Research Fulbright Award (to D. W. P. for 1994–1995), by National Institutes of Health Research Grant GM41916 (to V. L. S.), and by the International Livestock Research Institute (ILRI), Nairobi, Kenya. This is ILRI Publication 97065. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AP017231.

¶ To whom correspondence should be addressed: International Livestock Research Institute, P. O. Box 30709, Nairobi, Kenya. Tel.: 254-2-630-743; Fax: 254-2-631-499; E-mail: d.parkin@cgnet.com.

---

¹ The abbreviations used are: IU-, inosine-uridine-prefering; IAG-, inosine-adenosine-guanosine-prefering; bp, base pair(s); PCR, polymerase chain reaction; ORF, open reading frame; PFGE, pulsed field gel electrophoresis; MES, 4-morpholineethanesulfonic acid.
lase from \textit{T. b. brucei}, a hemoproteozan parasite of livestock (7). Studies of the \textit{T. b. brucei} enzyme have established that \textit{IAG}-nucleoside hydrolase has different substrate and inhibitor specificity, kinetic mechanism, and transition state structure than \textit{IU}-nucleoside hydrolase from \textit{C. fasiculata} (7). Reported in this paper is the nucleotide sequence of the \textit{iagnh} open reading frame, the predicted amino acid sequence, genetic distribution among several parasitic organisms, the relative level of expression of the \textit{iagnh} mRNA in different life-cycle stages of \textit{T. b. brucei}, and a comparison of the kinetic parameters of the purified nucleoside substrates for the native and recombinant \textit{IAG}-nucleoside hydrolase. The overexpression of \textit{IAG}-nucleoside hydrolase in \textit{E. coli} and the purification of the recombinant enzyme provide sufficient protein for structural and transition state studies.

**MATERIALS AND METHODS**

**Parasites**—The different bloodstream forms of \textit{T. b. brucei}, ILTat1.1, a pleomorphic strain (17); \textit{T. b. gambiensi}, IL3250; \textit{Trypanosoma brucei} rhodesiense, IL3953; \textit{Trypanosoma congolense}, IL1180; \textit{Trypanosoma vivax}, IL2160; were grown and isolated as described (18). Procytic \textit{T. b. brucei} ILTat1.1 (bleed fly midgut stage) were cultured in vitro and isolated as described (19). The trypanosomes were used immediately, or stored frozen as cell pellets at \(-70^\circ\text{C}\). \textit{Theileria parva} (piroplasm form) DNA was a generous gift from Dr. ole-Moloi, International Livestock Research Institute, Nairobi, Kenya (20).

**spectrophotometric Assay**—A continuous spectrophotometric assay for \textit{IAG}-nucleoside hydrolase was performed, at the appropriate wavelength, with inosine, guanosine, adenosine, or \textit{p}-nitrophenylriboside as substrate, in 50 mM phosphate, pH 7.2 (7).

**Amino Acid Sequence of the N-terminal and CNBr Fragments**—Native \textit{IAG}-nucleoside hydrolase was isolated from the longer bloodstream form of \textit{T. b. brucei} and purified to near homogeneity (7). The \textit{IAG}-nucleoside hydrolase was dialyzed extensively against 1 M phosphate, pH 7.2 (7) for 48 h. Themino acid sequence were obtained for the N-terminal region and CNBr fragments isolated using reverse-phase high performance liquid chromatography, eluting with a gradient of acetonitrile in 0.1% trifluoroacetic acid. The amino acid sequences were obtained for the N-terminal region and CNBr fragments using automated Edman gas-phase sequencing. The protein fragmentation, sequencing, and mass analysis were provided by the Laboratory of Macromolecular Analysis, Albert Einstein College of Medicine, Bronx, NY.

**RNA and DNA Preparation**—Total parasitic RNA was isolated by the modified guanidinium thiocyanate-phenol-chloroform extraction procedure (21, 22). Poly(A\(^{+}\)) RNA was purified from total RNA by oligo(dT) chromatography and genomic DNA was isolated by the SDS-proteinase K-phenol method (23). Phage DNA was isolated by the LambdaorbTM phage adsorbent (Promega) protocol. Plasmid DNA was purified from overnight bacterial culture, by the Wizard™ miniprep (Promega) DNA purification system.

**DNA Library Construction**—A genomic DNA library from \textit{T. b. brucei} clone ILTat1.1 (long slender actively dividing bloodstream form) was constructed in the cloning vector \textit{agt}11. Genomic DNA was sheared by sonication to 2-8-kilobase pair DNA fragments, which were purified on an agarose gel and converted to DNA polymerase. The fragments were ligated with EcoRI adaptors and to \textit{agt}11 vector dephosphorylated arms, followed by packaging into phage heads and plating on \textit{E. coli} Y1090 cells (24). Clones and subclones were sequenced using the fmol™ DNA sequencing system (Promega).

**Cloning of the IAG-Nucleoside Hydrolase Open Reading Frame**—Two cDNA fragments encoding the 5' and 3' halves of the full-length cDNA of the \textit{iagnh} gene were generated by PCR amplification of total single-stranded cDNA from actively dividing long slender bloodstream form of ILTat1.1 (25). The 5'-region was cloned by PCR amplification using the synthetic sense primer (5'-TGGACACAGCTCTGCTATTATGT-3') for the mini-exon-derived sequence (common to all African trypanosome mRNAs) and the codon-based antisense primer (5'-TCACGTAACAGTTATAGTATGTTTGC-3') for the internal CNBr amino acid fragment, PNFVIGE, of \textit{IAG}-nucleoside hydrolase. The 3'-region was cloned using the degenerate sense primer (5'-CCACGCTTCTGCTAATACT-3') designed from the CNBr fragment and the antisense degenerate primer based on the oligo(dT) primer 5'-CCCTGCTG-3'.

The above PCR products (550 and 850 bp, respectively) were used as \textit{[^32P]}-labeled probes to isolate a 3.5-kilobase pair \textit{agt}11 clone (24) containing the \textit{iagnh} open reading frame (ORF) with the ATG initiation codon located 300 bp downstream of the 5' end. This clone was used as the template in PCR amplification to generate the full-length \textit{iagnh} sequence. Primers 5'-GGCCCATGCGAAACAGCTGCCTATT-3' and 5'-CCCCCTGACCTGCTAAACCGCGTGGAGAC-3', based on the nucleotide sequence of the 5' and 3' regions, were designed to incorporate the underlined unique \textit{NcoI} and \textit{PstI} sites and to create the ATG initiation and the TCA termination codons (in bold), respectively. The double-stranded DNA was purified from agarose gel using the GeneClean™ system (BI0101, Inc.) and cloned into the pMOS-T vector and used to transform \textit{E. coli} JM109. A modified \textit{PET}-28a (+) expression vector was constructed by excising the \textit{BanHI}-HistDIII cloning site sequence and replacing it with a linker containing 5'-\textit{BanHI}-PstI-HindIII-3' sites, thus introducing a unique \textit{PstI} site in the expression site. The \textit{iagnh} ORF was excised from the pMOS-T expression vector with \textit{NcoI} and \textit{PstI} and subcloned into the modified \textit{PET}-28a expression vector. Both DNA strands were sequenced using overlapping primers. The modified \textit{PET}-28a expression vector, containing the \textit{iagnh} ORF, was used to transform \textit{E. coli} BL21(D3) cells made competent using the CaCl\(_2\), method (26).

**Chromosomal Localization of the iagnh Gene**—A filter blot containing pulsed field gel electrophoresis (PFGE)-separated chromosomes from \textit{T. b. brucei} isolate TREU927/4 was a generous gift of Dr. Sarah Melville and Vanessa Leech (Cambridge University, Cambridge, United Kingdom). Briefly, chromosomes were separated on an agarose gel by PFGE as described (27). Following staining of the gel with ethidium bromide to visualize the chromosomes bands under UV light, chromosomal DNA was transferred onto a nylon filter using standard methods (23), after nicking the DNA by soaking the gel in 0.25 M HCl for 15 min. The filter was prehybridized, hybridized with \textit[^32P]-labeled \textit{iagnh} probe, and washed under the conditions described for Southern blots.

**Expression of T. b. brucei IAG-Nucleoside Hydrolase**—An overnight culture was generated by incubating 50 ml of 2 \times YT (tryptophic soy broth 16 g/liter; yeast extract, 10 g/liter; NaCl, 5.0 g/liter) containing 30 mg/ml kanamycin monosulfate (kanamycin A), with a loop of liquid culture \textit{E. coli} BL21 (DE3) cells, carrying the \textit{iagnh} ORF on the \textit{PET}-28a vector, and incubated overnight at 37 °C in a shaking incubator. The next morning, 5 ml of this overnight culture was added to 300 ml of Terrific Broth (tryptic soy broth 12 g/liter; yeast extract, 24 g/liter; glycerol, 4.0 ml/liter, containing 50 mg/ml Heps, pH 7.2, containing 30 mg/ml kanamycin A) and incubated at 37 °C with shaking until the cells reached a \textit{A}_{600} between 1 and 2. The cells were then transferred to 15 liters of Terrific Broth, containing 30 mg/ml of kanamycin A, grown in a 16-liter New Brunswick Fermentor, with agitation at 37 °C. Upon reaching \textit{A}_{600} = 4-5, the culture was cooled to 20 °C and isopropyl-\textit{l}-thio-\textit{b}-galactopyranoside, dissolved in 50% ethanol, was added to the medium to give a final concentration of 0.2 mg/ml isopropyl-\textit{l}-thio-\textit{b}-galactopyranoside. The cells were incubated with stirring and aeration overnight at 20 °C. The next morning, the cells were harvested by centrifugation, washed once with 10 ml phosphate buffer, pH 7.5, containing 140 mM NaCl, 3 mM KCl, 1 mM \textit{CaCl}\(_2\), 0.4 mM MgCl\(_2\) (Dubesco's phosphate-buffered saline), and stored at -20 °C.

**Recombination of Recombinant IAG-Nucleoside Hydrolase**—Purification of the recombinant \textit{IAG}-nucleoside hydrolase (rIAG-nucleoside hydrolase) was based on the previous published scheme (7) with two additional chromatography steps to obtain highly purified protein. Supernatant of the bacteria in the \textit{PET}-28a vector was used for chromatography. The enzyme was flash-frozen in dry ice/ethanol and stored at -70 °C.

**Northern and Southern Blot Hybridizations**—Northern blot analysis of poly(A\(^{+}\)) RNA (\(-1 \mu\text{g}\)) was performed as described (28). Southern blot analysis of purified genomic DNA (2 \mu g) digested with a variety of restriction endonucleases was performed as described (23). Two \textit[^32P]-labeled probes from either the \textit{iagnh} ORF (\textit{iagnh} probe) or the 663-bp 5'-fragment of the ORF for the \textit{iuhn} gene (\textit{iuhn} probe) were generated by random priming (29). The \textit[^32P]-labeled probe based on the \textit{\beta}-tubulin gene (\textit{\beta}-tubulin probe) was used as an internal control to determine the amount of mRNA transferred to the nylon filters (30).
The legends and Roman numerals on the left to the chromosomal bands indicate the corresponding chromosome equivalents of each band. The chromosomal nomenclature has been defined by the *T. b. brucei* Genome Project Initiative, with the chromosomes labeled according to increasing size in PFGE using *Roman numerals* (27). A filter containing PFGE-separated chromosomes was hybridized with the following *T. b. brucei* ILTat 1.1 DNA probes: cysteine protease (lane 2), cyclophilin A (lane 3), glyceraldehyde-3-phosphate dehydrogenase (lane 4), and *iagnh* probe (lane 5). CZ indicates the compression zone. Panel B, genomic DNA (2 μg) from *T. b. brucei* ILTat 1.1 was digested with the indicated restriction enzymes, fractionated on a 0.8% agarose gel, transferred to a Nytran filter, and probed with the *iagnh* probe. Post-hybridization washes in 1 × SSC, 0.1% SDS at 65 °C were followed by autoradiography. The ORF of the *T. b. brucei iagnh* contains a single restriction site each for *Hae* III (lane 5), *Kpn* I (lane 6), *Pvu* II (lane 7), *Rsa* I (lane 8), and *Sal* I (lane 9), while BamHI (lane 1), EcoRI (lane 3), HindIII (lane 2), and *Nco* I (lane 4) do not cleave within the *iagnh* ORF. Panel C, Southern blot analysis of various parasitic genomic DNA. Total genomic DNA (~2 μg) from *T. b. brucei* (lane 1), *T. b. gambiense* (lane 2), *T. b. rhodesiense* (lane 3), *T. evansi* (lane 4), *T. vivax* (lane 5), *T. congolense* (lane 6), *T. cruzi* (lane 7), *T. parva* (lane 8), and *C. fasciculata* (lane 9) were digested to completion with *Pst*I and then analyzed by Southern blot hybridization with the *iagnh* probe at 55 °C. Post-hybridization washes was in 2 × SSC, 0.1% SDS at 55 °C, followed by autoradiography at −70 °C for 3 days (lanes 1–5) and 14 days (lanes 6–9). Numbers on left are DNA size markers in kilobases (kbp).

**RESULTS**

**Genomic Analysis of the *iagnh* Locus in *T. b. brucei***—Genomic DNA from *T. b. brucei* (ILTat 1.1) was digested with restriction endonucleases, which cleave internally and/or externally to the *iagnh* ORF, and hybridized with the *iagnh* probe (Fig. 1, panel B). Endonucleases with one cleavage site within the *iagnh* ORF yielded two unequal size hybridization bands. A time-dependent cleavage using *Rsa*I did not produce a pattern expected for tandemly repeated genes (data not shown). All endonucleases (except *Nco*I) which have no restriction sites in the *iagnh* locus produced a single hybridizing fragment. *Nco*I, which does not contain a restriction site in the *iagnh* locus, cleaved the genomic DNA to generate two distinct hybridization bands on a highly resolving long range separation gel (data not shown).

The chromosomal localization of the *iagnh* gene was determined by Southern blot hybridization of PFGE-separated chromosomes of *T. b. brucei* isolate TREU927/4 (27) using the *iagnh* probe. The ethidium bromide-stained gel showed the chromosomal band profile (Fig. 1, panel A, lane 1). The autoradiograph showed that the *iagnh* probe hybridized to two bands with the larger-sized band containing chromosomes III, IV, V, and VI and the smaller-sized band containing chromosomes III and IV. The *iagnh* probe hybridization signal to the III, IV chromosome band was of the same intensity as that to the III, IV, V, VI chromosome band. Three control probes were used: a cDNA probe containing the *T. b. brucei* cyclophilin A homologue (cyclophilin probe), which hybridized to the compression zone (lane 3), and a cysteine protease probe (lane 2) (31) and glyceraldehyde-3-phosphate dehydrogenase probe (lane 4) (32), which hybridized to the band containing chromosome VI and the band containing chromosome III, IV, V, and VI, as expected (27).

The *Pst*I-digested genomic DNA from several protozoan parasites was probed with the *iagnh* probe at 55 °C and washed under medium stringency conditions (2 × SSC, 0.1% SDS at 55 °C) and showed strong hybridization signals with *T. b. brucei* gambiense and rhodesiense, *T. evansi*, *T. vivax*, *T. congolense*, and *T. cruzi* DNA and a faint signal with *Th. parva* and *C. fasciculata* DNA (Fig. 1 panel C). There were only weak hybridization bands observed against genomic DNA from *T. vivax*, *C. congolense*, or *T. b. brucei* using the IU-nucleoside hydrolase probe (data not shown).

**Primary Gene Sequence of IAG-Nucleoside Hydrolase**—The full-length cDNA for IAG-nucleoside hydrolase contains an ORF of 981 bases, a 5′ mini-exon 186 bases upstream to the ATG start codon, and a poly(A) tail extending 90 bases downstream from the TGA stop codon (Fig. 2). The coding region is 53% GC, which is consistent with the average coding region GC content of 51.6% from 31 previously reported *T. b. brucei* genes (33).

**Sequence Alignment**—A BlastX search of the amino acid sequence, deduced from the *iagnh* ORF (Fig. 3), revealed homology to only two ORF, one that encodes the IU-nucleoside hydrolase from *C. fasciculata* (32% amino acid identity) and one that encodes the YEIK *E. coli* hypothetical 33.7-kDa protein in the *nfi-fruA* intergenic region (33% amino acid identity) (34, 35). Regions of significant amino acid sequence similarity include the N-terminal 20 amino acids and the region from amino acids 158–196. The N-terminal motif KXXXXLDXXDXXD of the IAG- and IU nucleoside hydrolases are conserved, as is a P residue at amino acid 26. Another conserved motif (amino acid 182–196) is AEXNXDPXAXXXV. The N-terminally processed IAG-nucleoside hydrolase contains 326 amino acids compared with 314 amino acids from the N-terminally processed IU-nucleoside hydrolase of *C. fasciculata* (6). The additional 12 amino acids in the IAG-nucleoside hydrolase

---

2 R. Pelle and N. B. Murphy, manuscript in preparation.
are located around positions 5, 170, and 260 relative to the similar proteins.

Expression during Development of mRNA of One iagnh Gene—The mRNAs from the actively dividing long slender and intermediate bloodstream forms, non-dividing short stumpy bloodstream form, and the procyclic tsetse midgut form life-cycle stages of *T. b. brucei* were hybridized with the iagnh probe (Fig. 4, panel A). The relative amount of mRNA loaded in each lane was determined by hybridization to the same blot of a β-tubulin (Fig. 4, panel B). The iagnh mRNA was expressed equally in the actively dividing long slender and intermediate bloodstream forms and was slightly less expressed in the procyclic culture form. The non-dividing short stumpy bloodstream form showed a decrease in the levels of expression of the iagnh mRNA. However, the levels of stable mRNA expressed between the life-cycle stages is similar to that observed for β-tubulin.

Overexpression, Purification, and Kinetic Analysis of IAG-Nucleoside Hydrolase—E. coli (BL21(DE3)) transformed with the modified pET-28a-IAG-nucleoside hydrolase construct (Fig. 5) expressed over 10% of the soluble protein as IAG-nucleoside hydrolase (Fig. 6, panels A and B). About 200 mg of >97% homogenous protein was obtained from 150 g (wet weight) of E. coli (Table I and Fig. 6, panel C). The protein eluted at a *Ve/Vo* corresponding to a *Mr* of 72,000 by Superdex 200 Gel chromatography, while SDS-PAGE showed a single band of a *Mr* of 35,000, consistent with the native protein being a dimer of *Mr* 35,000 subunits. The calculated molecular weight, based on the predicted amino acid sequence from the cDNA, was 35,735 for the rIAG-nucleoside hydrolase. This is within experimental error of the observed molecular weight for the rIAG-nucleoside hydrolase determined by mass spectrometry, *Mr* = 35,737. The observed molecular weight of the native IAG-nucleoside hydrolase, as determined by mass spectrometry, is 35,759, which is 22 atomic mass units different from the rIAG-nucleoside hydrolase. A single codon change introduced by the PCR cloning could account for the difference, so kinetic analysis was conducted to ensure that the recombinant and native IAG-nucleoside hydrolase have identical kinetic properties.

The kinetic parameters for the recombinant IAG-nucleoside hydrolase are compared with the published data of the native IAG-nucleoside hydrolase in Table II. The *Km* and *kcat* for all the naturally occurring purine nucleoside substrates and the synthetic substrate, *p*-nitrophenylriboside, were experimentally identical.

DISCUSSION

Genomic DNA Sequence and Analysis of the iagnh Gene—The cDNA for IAG-nucleoside hydrolase, a purine salvage enzyme from the African trypanosome *T. b. brucei*, contains both the mini-exon found in the mRNA of all trypanosomes and the poly(A) tail. Both native and recombinant IAG-nucleoside hydrolases have a processed N-terminal Met as established by N-terminal sequencing data. The calculated molecular weight, based on the translated amino acid sequence of the cDNA and the observed molecular weight, determined by mass spectroscopy, was 35,735 and 35,737, respectively. Thus, the ORF and the DNA sequence assignment are established.

The iagnh locus in *T. b. brucei* (ILTat 1.1) was analyzed for both copy number and tandem repeats using restriction enzyme analysis and Southern blotting. A number of genes in *T.*...
b. brucei, including tubulins (36), calmodulin (37), and glycer-aldehyde-3-phosphate dehydrogenase (32), are arranged as tandem repeat sequences, while others have one gene/haploid genome. These include hypoxanthine-guanine phosphoribosyl-transferase (38) and glucosephosphate isomerase (39). Restriction endonuclease digestion analysis and Southern blotting with partial and complete digestion indicate the existence of one gene/haploid genome in this T. b. brucei clone. In contrast, T. vivax DNA digested with Pst I produces at least five hybridization bands greater than 2 kilobase pairs, suggesting that the "iagnh gene" in T. vivax is present in at least two copies per haploid genome (Fig. 1, panel C).

Chromosome-sized molecules of T. b. brucei DNA were separated using pulse-field gradient electrophoresis, blotted, and hybridized with the iagnh probe. The two chromosome-sized bands that hybridized with the iagnh probe consist of the band containing chromosomes III, IV, V, and VI and that containing only chromosomes III and IV. Because the iagnh probe hybridizes with equal intensity to both chromosome bands, the data support the location of the iagnh gene on chromosome III or IV. Although the mechanism underlying chromosome size polymorphism is not yet known, telomere lengthening and collapse or genetic exchange during cyclical transmission in the vector could generate size variability in T. b. brucei (40, 41).

Strong hybridization signals against the iagnh probe establishes the presence of an N-ribohydrolase gene in all African trypanosomes tested. However, the absence of a strong hybridization signal using the iagnh probe does not preclude the existence of N-ribohydrolase isozymes. N-Ribohydrolase isozymes have been identified in T. cruzi (9) and a cDNA-derived N-terminal amino acid sequence, which shows a high level of homology with the IU-nucleoside hydrolase, has been identified in Leishmania major (6). In contrast, there was only a weak hybridization signal with T. b. brucei, T. congolense, or T. vivax genomic DNA, when using the iunh probe from C. fasciculata (data not shown). Therefore, the above data suggest that the N-ribohydrolases are ubiquitous enzymes for purine salvage in protozoan parasites.

**Fig. 3.** Sequence analysis of iagnh nucleoside hydrolase from T. b. brucei and similar deduced protein sequences. The data were collected from the On-Line National Center for Biotechnology Information, (http://www.ncbi.nlm.nih.gov). The search was conducted using the default parameters of the BLAST X algorithm. Only the deduced amino acid sequences from the IU-nucleoside hydrolase and the intergenic reading frame of YIEK E. coli (YIEK_Ecoli) were obtained using these search parameters. The remaining sequences were obtained from Ref. 6 and aligned manually. Only the regions showing substantial similarity to the IAG-nucleoside hydrolase are shown. Abbreviations are defined at bottom of figure.

b. brucei, including tubulins (36), calmodulin (37), and glycer-
reinitiate an infection in the tsetse fly (5). Northern blots show that the iagnh gene is expressed in all developmental stages of the parasite that were tested. Although the iagnh mRNA is produced at higher levels in the actively dividing bloodstream forms and the in vitro cultured procyclic form when compared with the non-dividing short stumpy form, expression of iagnh mRNA is substantial in all actively dividing forms. In comparison, another gene that encodes a purine salvage enzyme from T. b. brucei, the hgprt gene, which encodes hypoxanthine-guanine phosphoribosyltransferase, is not developmentally regulated. This enzyme is also encoded in one gene/haploid genome (38). In contrast, the cysteine proteases from T. b. brucei are expressed at higher levels in the non-dividing short stumpy bloodstream form and exist as multiple tandemly repeated arrays (31, 42, 43).

Biological Role of IAG-Nucleoside Hydrolase—Berens et al. (1) have proposed that the salvage pathways utilized by parasitic organisms respond to the purine composition of the parasite’s environment. The equal expression of the iagnh and hgprt genes, which encode two vital purine salvage enzymes, in the actively dividing life-cycle stages of T. b. brucei support this hypothesis. Fig. 7 summarizes the processes by which a purine nucleoside or base is converted to IMP in T. b. brucei. An important component of purine salvage is the transport mechanisms for purine nucleosides and bases. T. b. brucei has efficient adenosine and inosine transporter systems with \( K_m \) values in the submicromolar range (44). Adenosine, released during tissue damage, has been proposed as the most important purine source for T. b. brucei (45). Another proposal is that the constitutive expression of the hypoxanthine-guanine phosphoribosyltransferase enzymes is due to hypoxanthine as the major source of purines (38, 46).

The concentration of available purine nucleotides in the bloodstream of both humans and domestic animals is still in question. Estimates of the concentration of free hypoxanthine in the blood of humans varies over 250-fold from 0.092 \( \mu M \) to 24 \( \mu M \) (47–50). The concentrations of hypoxanthine vary by as much as 20-fold depending on the method of collection and storage (51). The levels of adenosine, inosine, and hypoxanthine are reported to be 2, 0.2, and 0.7 \( \mu M \), respectively, when plasma was prepared in the presence of the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl) adenine (50). Free concentrations of adenosine, inosine, and hypoxanthine in the bloodstream of livestock have not been well documented. The continuous expression of both the iagnh and hgprt genes in African trypanosomes suggests that both the hypoxanthine-guanine phosphoribosyltransferase and N-ribohydrolases are required for the biosynthesis of IMP, with adenosine and/or inosine being the major source of purines in the bloodstream of the mammalian host.

Amino Acid Sequence Comparison—The IU- and IAG-nucleoside N-ribohydrolase isozyymes have distinct amino acid sequences with only two relatively conserved regions. The x-ray crystal structure of the IU-nucleoside hydrolase from C. fasciculata reveals that the N-terminal Asp residues are clustered near the catalytic site (16). The site contains a tightly bound metal ion, and the ion is held in place by ionic interactions with amino acids Asp-10 and Asp-15 (16). The conserved Asp-8 and Asp-14 residues are in the second sphere of amino acids, which stabilize the metal ion-binding amino acids. Recent results establish that the ion is a tightly bound \( Ca^{2+} \) present in both
the IU- and IAG-nucleoside hydrolase. The second group of conserved amino acids from 158 to 196 contains the AE amino acids essential for catalysis, pKₐ 8.8 (7), making amino acids of the corresponding region 254 YYAWD 258 of IAG-nucleoside hydrolase the most likely candidates for involvement in leaving group activation.

Purification and Characterization of the Recombinant IAG-Nucleoside Hydrolase—The recombinant enzyme has experimentally indistinguishable kinetic and chromatographic characteristics relative to the native IAG-nucleoside hydrolase. The kinetic parameters of the naturally occurring purines and the synthetic substrate p-nitrophenylriboside are identical. The observed molecular weight of 72,000 for the rIAG nucleoside hydrolase corresponds to a homodimer of 35,000 M₉. The similarity of the kinetic parameters, alignment of both the N-terminal amino acids and CNBr-generated fragments, and the comparison of calculated and observed molecular weights establish that the cDNA encodes a protein with the same kinetic and structural properties as the native IAG-nucleoside hydrolase. The coding region for the rIAG-nucleoside hydrolase provides access to structural, mechanistic, and metabolic studies of the purine salvage pathway in African trypanosomes.

Conclusion—Protozoan parasites are known to use nucleoside hydrolase enzymes in the essential pathways of purine salvage. The IAG-nucleoside hydrolase is the first purine-specific N-ribohydrolase to be characterized by molecular analysis, and is shown to be a common enzyme of purine salvage in African trypanosomes. Sequence data banks reveal no corresponding enzymes in the mammalian hosts. The IAG-isozyme differs substantially from the nonspecific IU-nucleoside hydrolase in amino acid sequences, subunit structure, and kinetic and chemical mechanisms. Amino acid sequences that are involved in the binding of Ca²⁺ to the catalytic site and those proposed for binding the ribose are part of the conserved sequences. Amino acids involved in the purine base-binding region are different, reflecting the amino acid sequences that are essential for catalysis, pKₐ 8.8 (7), making amino acids of the corresponding region 254 YYAWD 258 of IAG-nucleoside hydrolase the most likely candidates for involvement in leaving group activation.

TABLE I
Purification of recombinant IAG-nucleoside hydrolase from E. coli

<table>
<thead>
<tr>
<th>Purification</th>
<th>Volume</th>
<th>Protein</th>
<th>Units</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>mg</td>
<td>μmol min⁻¹</td>
<td>μmol min⁻¹ mg⁻¹</td>
</tr>
<tr>
<td>Initial extract</td>
<td>300</td>
<td>2940</td>
<td>17,000</td>
<td>5</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>110</td>
<td>2090</td>
<td>14,000</td>
<td>9</td>
</tr>
<tr>
<td>Q-Sepharose</td>
<td>75</td>
<td>480</td>
<td>16,000</td>
<td>31</td>
</tr>
<tr>
<td>Superdex 200</td>
<td>84</td>
<td>298</td>
<td>14,600</td>
<td>41</td>
</tr>
<tr>
<td>Red A and Superdex 75</td>
<td>5</td>
<td>208</td>
<td>9600</td>
<td>46</td>
</tr>
</tbody>
</table>

structural, and kinetic properties of N-ribohydrolase isozymes support their use as targets for chemotherapeutic agents and as diagnostic probes.

Acknowledgments—We thank Dr. Ruth Angeletti, Edward Nies, and staff of the Laboratory of Macromolecular Analysis, Albert Einstein College of Medicine, for providing the protein fragmentation, sequencing, and mass analysis. We also thank Drs. Onesmo ole-MoiYoi and Noel Murphy for their support and critical evaluation of the manuscript.

REFERENCES

Molecular Cloning and Expression of a Purine-specific N-Ribohydrolase from *Trypanosoma brucei brucei*: SEQUENCE, EXPRESSION, AND MOLECULAR ANALYSIS

Roger Pellé, Vern L. Schramm and David W. Parkin

doi: 10.1074/jbc.273.4.2118

Access the most updated version of this article at http://www.jbc.org/content/273/4/2118

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 46 references, 7 of which can be accessed free at http://www.jbc.org/content/273/4/2118.full.html#ref-list-1