Purine-specific Nucleoside N-Ribohydrolase from Trypanosoma brucei brucei

PURIFICATION, SPECIFICITY, AND KINETIC MECHANISM*

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Trypanosomes have no de novo purine biosynthesis and thus depend upon salvage pathways to obtain purines for their metabolic pathways and for the biosynthesis of nucleic acids. An inosine-adenosine-guanosine preferring nucleoside hydrolase (IAG-nucleoside hydrolase) from the African trypanosome Trypanosoma brucei brucei represents >0.2% of the soluble protein in this organism. The enzyme has been purified over 400-fold to >95% homogeneity from the bloodstream form of this parasite. IAG-nucleoside hydrolase is a dimer of 95% homogeneity from the bloodstream form of this parasite. Its N-ribonucleoside hydrolase activity (Equation 1) has been identified in mammalian and trypanosome and mammalian hosts (Hammond and Gutteridge, 1984; Marr and Docampo, 1986). Mammals obtain purines primarily by de novo synthesis and/or the purine salvage pathways. In contrast, trypanosomes have no de novo purine synthesis and must depend upon the host to provide it with either purines or purine ribosides. Enzymes involved in the purine salvage pathways include phosphoribosyl transferases, adenine, and adenosine deaminases and purine nucleoside kinases (Gutteridge and Davies, 1981; Fish et al., 1982; Davies et al., 1983).

A group of trypanosome enzymes of the purine salvage pathways which catalyze the reactions

\[
\text{(Deoxy-nucleoside + H}_2\text{O}) \rightarrow \text{base + (deoxy-ribose)} \quad (\text{Eq. 1})
\]

or

\[
\text{(Deoxy-nucleoside + inorganic phosphate \rightarrow base + (deoxy-ribose) 1-phosphate} \quad (\text{Eq. 2})
\]

have been identified from crude parasite extracts. These enzymes include purine hydrolases (Koszalka and Krenitsky, 1979; Ogbonu and Ikediobi, 1983; Miller et al., 1984), purine phosphorylases (Miller et al., 1986), and deoxynucleoside hydrolases (Koszalka and Krenitsky, 1979; Miller et al., 1984; Steenkamp, 1991). In contrast, only purine nucleoside phosphorylase activity (Equation 2) and no purine nucleoside hydrolase activity (Equation 1) has been identified in mammalian cell lines or tissues (Hammond and Gutteridge, 1984).

To date, none of the purine salvage enzymes from T. brucei brucei or other African trypanosomes have been purified to homogeneity and characterized. However, the purification and extensive characterization of two nucleoside hydrolases has been reported from Cryptidia fasciculata, a trypanosome of the mosquito (Horenstein et al., 1991; Parkin et al., 1991; Estupiñan and Schramm, 1994). The nonspecific enzyme catalyzes the hydrolysis of both purine and pyrimidine nucleosides and has been named inosine-uridine nucleoside hydrolase. The more specific enzyme preferred purine nucleosides and was named guanosine-inosine (GI) nucleoside hydrolase.

The purine salvage pathways are targets for the development of a new generation of chemotherapy drugs, since they possess two important criteria. Foremost, the purine salvage pathway is essential for the viability of the parasite and second there are enzymes involved in the salvage pathway which differ from those of the host (Wang, 1984). With resistance developing to the currently available drugs and little prospect of new antiparasitic agents, a renewed effort to develop target specific drugs for the enzymes of the purine salvage pathway could be important in the control of parasitic...
Nucleoside Hydrolase from T. brucei brucei

Growth of Bloodstream Forms of T. brucei brucei (IL-Tat 1.1)—T. brucei brucei IL-Tat 1.1 is a cloned population of trypanosomes derived from an isolate collected from a naturally infected cow in Utembo, Kenya (Miller and Turner, 1981). Bloodstream forms of this clone were grown in sublethally irradiated (650 rads) Sprague-Dawley rats after inoculation with cryopreserved stably transformed parasites. The trypanosomes were then concentrated in phosphate-saline glucose, pH 8.0, containing protease inhibitors (5 mM 1,10-phenanthroline, 0.05% CuSO4 5H2O and 0.3 mM 2,9-dimethyl-1,10-phenanthroline, in a mixture of 100 mM Pipes, 1,4-piperazinediethanesulfonic acid. (pH 7.2), and antipain), then frozen in liquid nitrogen.

Colorimetric Assays for Nucleoside Hydrolase— Catalytic activity for the nucleoside hydrolase was determined by the formation of ribose (reducing sugar) from 2.5 mM inosine in 50 mM phosphate, pH 7.2. The assay volumes were 1 ml. The reaction was terminated by the addition of 0.3 ml of reagent containing 4% Na2CO3, 1.6% glycine, 0.045% CuSO4·SH2O and 0.3 ml of 0.12% 2,9-dimethyl-1-10-phenanthroline, in a total volume of 1.6 ml. The color was developed in boiling water for 10 min and the optical absorbance measured at 450 nm. The normal curve was used to determine the extinction coefficient under the assay conditions. A modified assay procedure, which removes excess protein, was used during the initial stages of the purification procedure. Protein solutions of up to 25 µl were added to 100 µl of the assay mixture and incubated for the desired time. The assay was terminated by the addition of 100 µl of 1 M ZnSO4. Excess Zn2+ was precipitated by the addition of 100 µl of 0.1 M NaOH. After centrifugation, 200 µl of supernatant was removed and assayed for ribose as described above.

Spectrometric Assay for Nucleoside Hydrolase—The 1-ml reaction mixture contained substrate at the desired concentration, 50 mM phosphate buffer, pH 7.2, and the desired concentration of inhibitor. For the pH profile studies a mixed buffer system consisting of 25 mM each of citrate, phosphate, and pyrophosphate, adjusted to the desired pH with 1 M HCl or NaOH, was used. A Millimolar Extinction Coefficients (δε)—The conversion of a specified concentration of substrate to product was accomplished by the addition of sufficient IAG-nucleoside hydrolase to completely convert substrate to product. The solution was scanned, before and after incubation with enzyme, to determine δε. All values of the starting substrates were determined at pH 7.2 unless otherwise stated. The δε (mM-1 cm-1) is: inosine, –0.92 at 280 nm; adenosine, –1.4 at 276 nm; guanosine, 0.16 at 308 nm; uridine, 4 at 281 nm; were fitted to their respective equations (Cleland, 1979), and the other proteins detected by

\[ \frac{V_{\text{max}}}{K_{\text{m,io}} + K_{\text{m,ad}} (\text{H}^+)} \]

where \( V_{\text{max}} \) is the initial rate without and with inhibitor, respectively. S is the substrate concentration, I is the inhibitor concentration, \( K_m \) is the Michaelis constant for the substrate, and \( K_i \) is the apparent dissociation constant for the inhibitor (deoxynucleosides). The \( V_{\text{max}} \) was then determined by setting the concentration of the deoxynucleoside to 10 \( \times K_i \) and measuring the initial rate.

Purification of IAG-nucleoside Hydrolase—Approximately 100 g of fresh T. brucei brucei IL-Tat 1.1 (bloodstream form) cells were thawed and suspended in 100 ml of 50 mM sodium phosphate, pH 7.5, containing 1 mM MgCl2, 0.1 mM EDTA, 0.1 mM dithiorthreitol, 1 mM benzamidine, 1 mM 1,10-phenanthroline, 0.1 mM phenylmethylsulfonyl fluoride, 50 µl/tube soybean trypsin inhibitor, 1.25 µl/tube leupeptin, and 6.5 µl/tube E-64. The suspension was sonicated, using a Branson Sonifier fitted with a 0.1-mm standard tip, 4 × 30 s on a continuous setting with cooling to 5°C between sonications. All the following steps were performed at 4°C. The disrupted cell suspension was then centrifuged for 2 h at 111,000 × g in a Ti-42.1 fixed angle rotor.

Soluble ammonium sulfate was added to the supernatant to give a 55% saturation at 4°C, stirred for 30 min, and centrifuged for 10 min at 20,000 × g. The resulting pellet was dissolved in a minimum volume of 50 mM Hepes, pH 7.5, and dialyzed exhaustively overnight against the same buffer.

The dialyzed fraction was applied to a Pharmacia Q-Sepharose Hi-Load 26/10 column, equilibrated with 50 mM Hepes, pH 7.5, connected to a Pharmacia FPLC system. The column was washed with 100 ml of buffer and the enzymes eluted with a 470-ml linear gradient of 0–1 M KC1 in the same buffer. IAG-nucleoside hydrolase elutes near 0.25 M KC1 and the IG-nucleoside hydrolase elutes near 0.5 M KC1. The fractions which contained the IAG-nucleoside hydrolase activity were pooled and concentrated to about 5 ml using a collodion bag concentrator which retained proteins with M, greater than 12,000. The fractions containing the IAG-nucleoside hydrolase activity were also pooled and concentrated, as described above, then stored at –70°C until further use.

The concentrated IAG-nucleoside hydrolase from Q-Sepharose was applied to a Hiload 26/10 Superdex-200 (Pharmacia Biotech Inc.) equilibrated with 50 mM Hepes, pH 7.5, containing 0.25 M KC1 and eluted at a flow rate of 2 ml/min using the same buffer. The resulting fractions which contained IAG-nucleoside hydrolase activity were pooled and then concentrated using a collodion bag as described above.

An Ado-agarose affinity gel was prepared from AMP-agarose (Sigma, A1271). The AMP-agarose was suspended in 50 mM Hepes, pH 9.2, containing 1 mM MgCl2 and 20 units/ml alkaline phosphatase. This mixture was incubated at 30°C overnight. Approximately 12 ml of the Ado-agarose was packed into a Pharmacia XK 16/20 column and attached to the Pharmacia FPLC column system. The column was equilibrated with 20 mM pyrophosphate, pH 8.5, and the IAG-nucleoside hydrolase applied to the column at a flow rate of 0.5 ml/min. The IAG-nucleoside hydrolase activity was retarded slightly and eluted after the contaminating proteins. The fractions containing the enzymatic activity were pooled and concentrated to about 1 ml using a collodion bag. Purity of the protein was estimated by electrophoresis in polyacrylamide gels containing SDS. The enzyme was then flash-frozen in dry ice/ethanol and stored at –70°C.

Molecular Weight of IAG-Nucleoside Hydrolase—The elution position of active IAG-nucleoside hydrolase relative to proteins of known molecular weight was determined with the Hiload 26/10 Superdex-200 column chromatography using the coelution positions of chymotrypsinogen A, ovalbumin, bovine serum albumin, aldolase, catalase, ferritin, and bovine thyroglobulin. Blue dextran 2000 was used to determine void volume (V0). The column was eluted with 50 mM Hepes, pH 7.5, containing 0.25 M KC1 at a flow rate of 2 ml/min. The IAG-nucleoside hydrolase was detected by and the molecular weight was detected by A280. The M, of the IAG-nucleoside hydrolase was determined from the elution profile of the Superdex 200 chromatography purification step. The ratio of elution position of the proteins (Ve/V0) relative to void volume (V0) was determined graphically, and the molecular weight for IAG-nucleoside hydrolase was estimated relative to the molecular weight

\[ V / (S + K_m + I + K_i) \]
The activity was based on the ΔA276 using 250 μM Ado in 50 mM phosphate buffer, pH 7.2. This was done in order to distinguish the IAG-nucleoside hydrolase from IG-nucleoside hydrolase. The purification of IAG-nucleoside hydrolase from bloodstream form Trypanosoma brucei brucei was dialyzed against 50 mM Hepes, pH 7.5, and assayed by spectrophotometry in a Sciex API-III mass spectrometer. Samples of 1–5 μg of IAG-nucleoside hydrolase were subjected to electrospray ionization mass spectrometry. IAG-nucleoside hydrolase gave a single band with an apparent subunit molecular weight of 41,000 (Fig. 2). Electron spray mass spectrometry of IAG-nucleoside hydrolase gave a relative mobility of IAG-nucleoside hydrolase relative to proteins of known molecular weight on SDS-polyacrylamide gel electrophoresis.

**Table I**

| Purification | Volume | Protein | Units | Specific Activity |
|--------------|--------|---------|-------|------------------|
| Initial extract | 130 | 4160 | 212 | 0.051 |
| (NH₄)₂SO₄ | 40 | 1800 | 184 | 0.10 |
| Q-Sepharose | 7 | 40 | 113 | 2.83 |
| Superdex 200 | 6 | 14 | 84 | 6.09 |
| Ado-agarose | 2 | 1.6 | 43 | 27 (50) |

* Specific activity using inosine as substrate.

The subunit molecular weight was estimated on denaturing SDS-polyacrylamide gel electrophoresis and mass spectroscopy. The purified IAG-nucleoside hydrolase was subjected to electrospray ionization analysis in a Sciex API-III mass spectrometer. Samples of 1–5 μg/ml were introduced through a 1 x 50-mm C-8 HPLC column equilibrated with 20% acetonitrile in H₂O containing 0.1% trifluoroacetic acid.

**RESULTS**

Purine Nucleoside Phosphorylase Activity—The cell-free extract was dialyzed against 50 mM Hepes, pH 7.5, and assayed for the presence of a purine nucleoside phosphorylase activity. The activity was determined using the spectrophotometric assay in either 50 mM Pipes, pH 7, or 50 mM phosphate, pH 7, containing one of the following substrates; 0.5 mM inosine, 0.2 mM adenosine, or 0.2 mM guanosine. The activity in the absence or presence of inorganic phosphate was, respectively, as follows: 5.2 ± 0.2 and 4.9 ± 0.1 units/ml using inosine as substrate, 2.0 ± 0.1 and 1.9 ± 0.1 units/ml using adenosine, and 4.7 ± 0.2 and 3.8 ± 0.1 units/ml using guanosine. These data show that the purine nucleoside phosphorylase activity in cell-free extracts of T. brucei brucei is negligible relative to the nucleoside hydrolase activity.

Purification of IAG-Nucleoside Hydrolase—Two distinct nucleoside hydrolase activities coprecipitate in the 0.55 saturated ammonium sulfate fractionation. These two activities were subsequently determined to be the inosine-adenosine-guanosine preferring nucleoside hydrolase (IAG-nucleoside hydrolase) and the inosine-guanosine preferring nucleoside hydrolase (IG-nucleoside hydrolase). The Q-Sepharose column step gave a 28-fold increase in specific activity of the IAG-nucleoside hydrolase and completely resolved the IAG-nucleoside hydrolase activity from the IG-nucleoside hydrolase. Superdex 200 size exclusion chromatography gave a 2-fold purification. The final Ado-agarose column provided a protein of >95% purity with a specific activity of 50 units/mg protein (Fig. 1). The enzyme did not bind tightly to the Ado-agarose column. However, the enzyme activity was retarded sufficiently enough to separate it from contaminating proteins. In total, the IAG-nucleoside hydrolase was purified over 400-fold from cell extracts with a 16% yield as summarized in Table I. IAG-nucleoside hydrolase represents about 0.2% of the total soluble protein of bloodstream form of T. brucei brucei.

**Molecular Weight and Subunit Structure**—The molecular weight of IAG-nucleoside hydrolase was estimated using Hi-Load 26/60 Superdex-200 gel chromatography, by denaturing SDS-gel electrophoresis using proteins of known molecular weights as standards, and by electrospray ionization mass spectroscopy. IAG-nucleoside hydrolase elutes with a Vₑ/Vₒ of 1.85 which corresponds to a Mₑ of 71,000 (Fig. 2). Denaturing polyacrylamide gel electrophoresis gave a single band with an apparent subunit molecular weight of 41,000 (Fig. 2). Electron spray mass spectrometry of IAG-nucleoside hydrolase gave a molecular mass of 35,759 ± 4. These values are consistent with the IAG-nucleoside hydrolase being composed of two subunits of identical, or similar, molecular weight.

**Substrate Specificity of IAG-Nucleoside Hydrolase**—The commonly occurring purine nucleosides are all substrates of IAG-nucleoside hydrolase with kcat/Km values from 0.83 to 1.9 x 10⁶ M⁻¹ s⁻¹ (Table II). Inosine is the best naturally occurring substrate for IAG-nucleoside hydrolase with a Vmax of 50 μmol min⁻¹ mg⁻¹, resulting in a turnover number of 34 s⁻¹ with a Km of 18 μM to give a kcat/Km catalytic efficiency of 1.9 x 10⁶ M⁻¹ s⁻¹. The Km values for the purines and pyrimi-
The $K_m$ or $K_{i(app)}$ value was calculated from the ratio of $-\text{inhibitor}/+\text{inhibitor}$ initial rates as described under “Results.” At least three concentrations of inhibitor was used to determine $K_{i(app)}$.

The inhibition constant $K_i$ was obtained from experiments using at least five substrate concentrations at each of three inhibitor concentrations. The inhibition constant was obtained by fitting the data to the equation for competitive inhibition using the computer programs of Cleland (1979).

dines are similar, ranging from 15 $\mu$M for adenosine to 106 $\mu$M for cytidine. The pyrimidines, however, are poorer substrates with $V_{\max}$ of 0.02 and 0.46 $\mu$mol min$^{-1}$ $\mu$g$^{-1}$ for uridine and cytidine, respectively. The $k_{cat}/K_m$ values for uridine and cytidine are 0.13 $\times$ 10$^3$ and 3.0 $\times$ 10$^3$ M$^{-1}$ s$^{-1}$, respectively. Based on $k_{cat}/K_m$ values, a 1000-fold decrease in catalytic efficiency exists between the purines and pyrimidines. Deoxyadenosines in the 2'- or 3'-position are poor substrates. When compared to adenosine, 2'-deoxyadenosine has a 1000-fold decreased $K_{cat(app)}$. 3'-deoxyadenosine has a 40-fold increased $K_{i(app)}$, and a 1000-fold decreased $K_{cat(app)}$. 5'-deoxyadenosine has a 600-fold increased $K_m$ and a 25-fold decreased $k_{cat}$. Thus the deoxyadenosines have a decreased catalytic efficiency of 4 to 5 orders of magnitude compared to adenosine or other purine nucleosides.

Additional nucleoside analogs were tested as substrates. Purine ribosides are almost as efficient a substrate as the other purine nucleosides, with an affinity the same as inosine but a slower turnover number resulting in a slightly less $k_{cat}/K_m$ value of 0.46 $\times$ 10$^6$ M$^{-1}$ s$^{-1}$. 5-Methyluridines has an affinity about the same as the purines. However, catalysis is significantly slower, lowering the catalytic efficiency to the same level as the other pyrimidines. When compared to inosine the synthetic substrate p-nitrophenylriboside has a 30-fold increased $K_m$ and a 20-fold decreased $k_{cat}$ resulting in a decrease in catalytic efficiency of 2 orders of magnitude compared to adenosine or other purine nucleosides.

Inhibitors of IAG-Nucleoside Hydrolase—Both ribose and hypoxanthine exhibit slope-linear competitive patterns using p-nitrophenylriboside as substrate (Table I). The $K_i$ is 1.35 and 19 $\mu$M for hypoxanthine and ribose, respectively. In the presence of 150 $\mu$M ribose and 612 $\mu$M p-nitrophenylriboside, the $K_i$ for hypoxanthine increased to 5.6 $\mu$M. 3-Deazaadenosine, formycin B, and 7-deazaadenosine (tubercidin) are competitive inhibitors of IAG-nucleoside hydrolase with a $K_i$ of 1.76, 13, and 59 $\mu$M, respectively.

Effect of pH on the Kinetic Constants of IAG-Nucleoside Hydrolase—IAG-nucleoside hydrolase is stable from approximately pH 5 to 10 during the assay. The $V_{\max}$ decreases between pH 5.2 to 9.7 with a plateau near pH 7.5 using inosine as the substrate (Fig. 3). The data are consistent with a requirement for two protonated groups essential for catalysis near pH 8.8 and a third protonated group, pK$a$, near 6.5, which increases the catalytic rate 10-fold. There is no effect of pH on $V_{max}/K_m$ between pH 5.2 and 8.2. However, the $V_{max}/K_m$ decreases as the pH increases from pH 8.2 to 9.4, indicating two groups on the free enzyme involved in the formation of the catalytically competent Michaelis complex, $pK_a$ 8.6. The $K_m$ decreases slightly from 150 to 25 $\mu$M between pH 5 and 7, with the $K_m$ remaining unchanged above pH 7. The effects of pH on $V_{max}$ with adenosine as substrate also indicate two protonated groups essential for catalysis at pH 8.8, and one protonated group which increases the catalytic rate at pH 6.7.

DISCUSSION

Kinetic Mechanism—The presence of two slope-linear competitive-product inhibition patterns establishes that the kinetic mechanism for IAG-nucleoside hydrolase, of the bloodstream form of T. brucei brucei, is rapid equilibrium random. Both the inhibition constants and the $K_m$ for inosine are dissociation constants in a rapid equilibrium system. Hypoxanthine binds over 14 times tighter than ribose, establishing the preferred release of ribose. Both ribose and hypoxanthine bind tighter to the free enzyme than the enzyme substrate ternary complex (Fig. 4). The rapid equilibrium condition holds only when substrate and product release are significantly faster than the catalytic step. The $k_{cat}/K_m$ value of 1.9 $\times$ 10$^6$ M$^{-1}$ s$^{-1}$ for inosine is approximately 2-3 orders of magnitude less efficient than a diffusion limited enzyme (Fersht, 1985). These data support a rate-limiting catalytic step at pH 7.2. The $k_{cat}$ in the reverse direction was calculated using the values for $K_{eq}$, $k_{cat}$ in the forward direction, and the kinetic constants for the enzyme-substrate complexes utilizing the Haldane relationship. The Haldane equation is given below.

$$
K_{eq} = \frac{k_{cat,forward} \cdot K_{hx,r}}{K_{hx,r} \cdot k_{cat,forward} - K_{hx,r} \cdot K_{eq}} (Eq. 7)
$$

where $K_{hx,r}$ is the dissociation constant for the ribose from the enzyme-hypoxanthine-ribose complex. The value for the $K_{cat,forward}$ is 34 s$^{-1}$ compared with 2 s$^{-1}$ for $k_{cat,reverse}$ (Fig. 4). The value used for the $K_{eq}$ was 106 (Parkin et al., 1991).

Substrate and Inhibitor Specificity of IAG-Nucleoside Hydrolase—The relatively tight binding of inosine depends on the relationship of both the ribose and hypoxanthine moieties, since both free ribose and hypoxanthine bind weakly to the enzyme. The product of the $K_i$ values for hypoxanthine and ribose is 25 $\mu$M, close to the observed $K_m$ for inosine of 18 $\mu$M. This relationship suggests that the enzymatic substrate recognition is the sum of the interactions of the groups on inosine.

| Substrate or inhibitor | $V_{max}$ | $K_m$ | $k_{cat}/K_m$ | $K_i$ or $K_{i(app)}$ |
|------------------------|-----------|-------|---------------|----------------------|
| Inosine                | 50 ± 0.8  | 18 ± 1| 1.9 $\times$ 10$^6$ | 19000 ± 1300 |
| Adenosine              | 27 ± 0.9  | 15 ± 2| 1.2 $\times$ 10$^6$ | 1350 ± 83 |
| Guanosine              | 56 ± 2.0  | 46 ± 5| 0.83 $\times$ 10$^6$| ND |
| Purine riboside        | 12 ± 0.4  | 19 ± 2| 0.46 $\times$ 10$^5$| 176 ± 0.02 |
| Cytidine               | 0.16 ± 0.010 | 106 ± 18 | 3.0 $\times$ 10$^3$ | 13 ± 1 |
| 5-Methyuridine         | 0.068 ± 0.004 | 26 ± 9 | $1.8 \times 10^3$ | 59 ± 5 |
| Uridine                | 0.02 ± 0.002 | 102 ± 5 | 0.13 $\times 10^3$ | ND |
| p-Nitrophenylriboside  | 1.2 ± 0.05 | 562 ± 51 | 1.5 $\times 10^5$ | ND |
| 2'-Adenosine           | 0.026 ± 0.005 | 42 ± 4 | 429 | ND |
| 3'-Adenosine           | 0.024 ± 0.007 | 600 ± 88 | 25 | ND |
| 5'-Adenosine           | 1.1 ± 0.3 | 9870 ± 310 | 77 | ND |
| AMP                    | <0.004 | ND | ND | ND |
| Ribose                 | ND | ND | ND | ND |
| Hypoxanthine           | ND | ND | ND | ND |
| 3-Deazaadenosine       | <0.002 | ND | ND | ND |
| Formycin B             | 0.05 | 106 | 34 | ND |
| 7-Deazaadenosine(Tubercidin) | 0.010 | 600 | 19 | ND |
The binding specificity of the ribose moiety involves the 3' - and 5'-hydroxyl groups of the ribose ring. The comparison of $V_{\text{max}}$ and $K_m$ values for adenosine to those from 2'-, 3',- or 5'-deoxyadenosine demonstrates that the ribose hydroxyl groups are important for binding and/or catalysis. The 2'-OH group of adenosine has a greater effect on $V_{\text{max}}$ (100-fold) than on $K_m$ (2-fold), indicating that it is important in the stabilization of the enzymatic transition state rather than the formation of the Michaelis complex. In contrast, the 3'-OH group is important both in the formation of the Michaelis complex (40-fold increase in $K_m$) and also the stabilization of the transition state, as indicated by the 1000-fold decrease in $V_{\text{max}}$. The 5'-OH contributes more to the formation of the Michaelis complex than catalysis since the $K_m$ increases 600-fold, whereas $V_{\text{max}}$ decreases only 25-fold. The binding specificity of the purine or pyrimidine-leaving group is much less stringent. The $K_m$ values differ only approximately 5-fold between the purines and pyrimidines, ranging from 15 to 106 $\mu M$. Other purine and pyrimidine analogs also bind with $K_m$ values in the 20 $\mu M$ range. Purine nucleosides, which is devoid of amino and/or hydroxyl groups attached to the heterocyclic ring, binds as tightly to IAG-nucleoside hydrolase as the other purines. Only p-nitrophenoxyriboside, which lacks the heterocyclic ring structure, has a significantly higher $K_m$.

The naturally occurring purine nucleosides are substrates of IAG-nucleoside hydrolase with $k_{\text{cat}}/K_m$ values around $10^6 M^{-1} s^{-1}$. The pyrimidines are much poorer substrates with $k_{\text{cat}}/K_m$ values around $10^3 M^{-1} s^{-1}$. The activation and binding energy difference ($\Delta \Delta G_{ab}$) between two substrates can be compared using the following equation,

$$\Delta \Delta G_{ab} = -RT \ln \left( \frac{k_{\text{cat}}}{k_{\text{cat}}(\text{purines})} \frac{K_m(\text{purines})}{K_m(\text{pyrimidines})} \right)$$

where $R$ is the gas constant (1.99 cal/T-mol) and $T$ is the absolute temperature, 303 K. $\Delta \Delta G_{ab}$ ranges from 3 to 6 kcal/mol for $k_{\text{cat}}/K_m$ between purines and pyrimidines. Substituting $K_m$ values for $k_{\text{cat}}/K_m$ indicates only about 1 kcal/mol binding energy difference between purines and pyrimidines. Therefore, approximately 3 to 5 kcal/mol of binding energy, about the energy of a hydrogen bond, is used to lower the energy of activation of the purines, compared to the pyrimidines (Fersht, 1985). These data support the hypothesis that protonation of the leaving group is an essential component in catalysis of purines and that IAG-nucleoside hydrolase is able to protonate the purine-leaving group more efficiently than the pyrimidine-leaving group.

I onizable Groups in Catalysis—The pH profile for hydrolysis of inosine indicates that three protonated groups contribute to the full catalytic capacity of IAG-nucleoside hydrolase. The presence of a plateau region around pH 7.5 indicates that two forms of the enzyme are catalytically active. One group with a $pK_a$ near 6.5 and two groups with $pK_a$ values near 8.8 are required for the most efficient catalysis. Loss of a proton from the most acidic group lowers the $V_{\text{max}}$ approximately 10-fold. However, the loss of the two protons on the amino acids, $pK_a$ 8.8, completely inactivates IAG-nucleoside hydrolase. The $V_{\text{max}}/K_m$ pH profile shows no change in catalytic efficiency between pH 5 and 8. However, above pH 8 the catalytic efficiency decreases significantly due to two ionizable groups on the free enzyme, $pK_a$ 8.6. The observed $K_m$ is lowered from 150 $\mu M$ at pH 5.2 to a constant value of 25 $\mu M$ above pH 7. There are two possible explanations for this observation: 1) there is one group on the enzyme with a $pK_a$ of 6.0 which aids in the formation of the Michaelis complex, since there are no titratable groups on the substrate over this pH range; or 2) catalysis is no longer rate-limiting, thus changing $K_m$ from a thermodynamic binding constant ($K_s$) to a kinetic constant. However, between pH 7 and 10 these data are consistent with inosine binding in rapid equilibrium with the enzyme since there is no
change in $K_m$ over this pH range and the kinetic mechanism of
the enzyme is rapid equilibrium with inosine as substrate at
pH 7.2 (Tipton and Dixon, 1979).

Protonation of the Leaving Group—Since proton donation to
the leaving group has been implicated in both chemical and
enzymatic depurination, the most likely positions are N-1, N-7,
and the oxo or amino group on C-6 of the purine (Garrett
and Mehta, 1972; Mentch et al., 1991). The enzymatic depurination
is most likely positions are N-1, N-7, the leaving group has been implicated in both chemical and
enzyme. The IAG-nucleoside hydrolase is most

| Substrate or inhibitor | IAG-nucleoside hydrolase$^a$ | GI-nucleoside hydrolase$^b$ | IU-nucleoside hydrolase$^c$ |
|------------------------|-------------------------------|-------------------------------|-------------------------------|
|                        | $k_{cat}/K_m$ | $K_i$ | $k_{cat}/K_m$ | $K_i$ | $k_{cat}/K_m$ | $K_i$ |
| Adenosine | 1.2 $\times$ 10$^6$ | 10 | 6.2 $\times$ 10$^6$ | 7.6 $\times$ 10$^6$ | 9.8 $\times$ 10$^3$ |
| Guanosine | 0.83 $\times$ 10$^6$ | 0.3 | 3.2 $\times$ 10$^6$ | 3.4 $\times$ 10$^6$ | 9.8 $\times$ 10$^3$ |
| Cytidine | 3.0 $\times$ 10$^3$ | 0.1 | 0.3 | 1.2 $\times$ 10$^3$ | 1.0 $\times$ 10$^3$ |
| Uridine | 0.13 $\times$ 10$^3$ | | | | |
| p-Nitrophenylriboside | 1.5 $\times$ 10$^3$ | 1.4 $\times$ 10$^3$ | | 4.1 $\times$ 10$^4$ |
| Ribose | 19000 | | | 1300000/630000 | 700 |
| Hypoxanthine | 1350 | | | 6100/6400 | 6200 |
| Cytidine | 1.78 | | | | |
| Formycin B | 13 | | | | |
| 7-Deazaadenosine (Tubercidin) | 59 | | | | |

| Enzyme | Native molecular weight ($M_r$) | Subunit molecular weight | Number of subunits |
|--------|--------------------------------|--------------------------|--------------------|
| IAG-nucleoside hydrolase | 71,000 | 36,000 | 2 |
| GI-nucleoside hydrolase$^b$ | 120,000 | 41,000 | 3 |
| IU-nucleoside hydrolase$^c$ | 143,000 | 34,000 | 4 |

$^a$ See Table II for standard errors.
$^b$ From Estupiñán and Schramm (1994).
$^c$ From Parkin et al. (1991), Horenstein and Schramm (1993), and Boutellier et al. (1994).
$^d$ From Mazzella et al. (1996).
$^e$ ND, not determined.

Chemical and Kinetic Mechanism of the Three Nucleoside
Hydrolases—The kinetic mechanism is rapid equilibrium random
for both IAG-nucleoside hydrolase and the IU-nucleoside
hydrolase. Ribose is released first from the IAG-nucleoside
hydrolase with hypoxanthine being released first from the IU-
nucleoside hydrolase. The chemical mechanism of the IAG-,
GI-, and IU-nucleoside hydrolases involves the formation of an
oxycarbonium ion in the transition state, characteristic of an
$S_{n1}$-like mechanism, as determined by kinetic isotope effects
(Horenstein et al., 1991). However, the magnitude of the oxycarbonium
ion formation and/or the reaction pathway by which the
transition state is reached differs for each of the nucleoside
hydrolases. The synthetic substrate p-nitrophenylriboside has
been used to determine the reaction pathway by which N-
ribosides reach their oxycarbonium-ion transition state. N-Ribosides which utilize protonation of the purine base
to achieve the oxycarbonium-ion transition state have a high
specificity for the aglycone and poor reaction rates with p-
nitrophenylriboside, whereas N-ribosides which utilize
activation of the ribose ring have much lower aglycone
specificity and high reaction rates with p-nitrophenylriboside (Mazzella et al., 1996).

The $k_{cat}/K_m$ for p-nitrophenylriboside hydrolysis is $4.1 \times 10^3$,$1.5 \times 10^2$, and $1.4 \times 10^2$ $M^{-1}$ s$^{-1}$ for IU-, IAG-, and GI-
nucleoside hydrolase, respectively (Table III). The $k_{cat}/K_m$ (p-
nitrophenylriboside) to $k_{cat}/K_m$ (purine substrate) ratio is $54,8 \times 10^{-4}$, and $4 \times 10^{-3}$ for the IU-, IAG-, and GI-nucleoside
hydrolase, respectively (Mazzella et al., 1996). These data show
that p-nitrophenylriboside is a better substrate than inosine for
the IU-nucleoside hydrolase. In contrast, p-nitrophenylriboside is
a much poorer substrate than the purine substrate for either the
IAG- or GI-nucleoside hydrolase.

The IU-nucleoside hydrolase has a much broader aglycone
specificity than the IAG- or GI-nucleoside hydrolase, as seen in
Table III. Whereas the IU-nucleoside hydrolase possesses the
ability to cleave both purines and pyrimidines, the IAG- and
GI-nucleoside hydrolases have a very strong preference for
purines. The role of the 5'-methoxy group in catalysis differs
between the hydrolases since 5'-deoxyadenosine is a substrate

2 D. W. Parkin and V. L. Schramm, unpublished results.
for the IAG-nucleoside hydrolase but not a substrate for the IU- or GI-nucleoside hydrolases (Parkin et al., 1991; Estupiñán and Schramm, 1994).

The pH profiles of the kinetic constants also establish fundamentally different catalytic mechanisms between the IAG- and GI-nucleoside hydrolase and the IU-nucleoside hydrolase. The pH profile studies show that protonation of the leaving group is essential in catalysis for the IAG- and GI-nucleoside hydrolases. The IAG- and GI-nucleoside hydrolases demonstrate an absolute requirement for two groups with pKₐ values around 9 to be protonated for catalytic activity. Protonation of a third group, pKₐ 6.5, on the IAG-nucleoside hydrolase increases the rate of catalysis but is not essential for activity. A similar pH profile pattern for V_max was obtained for the GI-nucleoside hydrolase (Estupiñán and Schramm, 1994). In contrast, the IU-nucleoside hydrolase requires one group to be unprotonated, pKₐ 7.1, and only one group to be protonated, pKₐ 9.1.

The above data support the hypothesis that the chemical mechanism of the IAG- and GI-nucleoside hydrolases is different from the IU-nucleoside hydrolase. These observations are consistent with the hypothesis that the oxycarbonium-ion transition state for the IAG- and GI-nucleoside hydrolases is reached via protonation of the purine ring, whereas the oxycarbonium-ion transition state for IU-nucleoside hydrolase is reached via the enzyme activating the ribosyl moiety.

Conclusion—The IAG-nucleoside hydrolase of T. brucei brucei is the first purine salvage enzyme to be purified and characterized from an African trypanosome. The enzyme exhibits a prominent role of the purine-specific enzyme has been established in C. fasciculata with over 80% of the inosine in C. fasciculata being metabolized via GI-nucleoside hydrolase (Estupiñán and Schramm, 1994). Kinetic isotope effects and subsequent transition state analysis of IU-nucleoside hydrolase has resulted in the design of several new transition state inhibitors (Horenstein et al., 1991; Boutilier et al., 1994). The determination of the Kₐ of these transition state inhibitors for both IU-nucleoside hydrolase and IAG-nucleoside hydrolase, and kinetic isotope effect studies for IAG-nucleoside hydrolase, are presently being conducted. The specificity, distribution, and mechanism of this family of unique salvage enzymes may eventually permit the targeting of the purine salvage pathways for novel antibiotic design. Using the above data and the subsequent transition state analysis of the kinetic isotope effects, novel transition state inhibitors could be designed for the IAG-nucleoside hydrolase as potential anti-parasitic agents.

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