Adenosine Kinase Mediates High Affinity Adenosine Salvage in Trypanosoma brucei*

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African sleeping sickness is caused by Trypanosoma brucei. This extracellular parasite lacks de novo purine biosynthesis, and it is therefore dependent on exogenous purines, such as adenosine, that is taken up from the blood and other body fluids by high affinity transporters. The general belief is that adenosine needs to be cleaved to adenosine monophosphate (AMP) to enter the cell. However, we report here that adenosine is salvaged by adenosine kinase (AK), which has a higher affinity to adenosine than the cleavage-dependent pathway. The recombinant T. brucei AK (TbAK) preferentially uses ATP or GTP to phosphorylate both natural and synthetic nucleosides in the following order of catalytic efficiencies: adenosine > cordycepin > deoxyadenosine > adenosine arabinoside (Ara-A) > inosine > fludarabine (F-Ara-A). TbAK differed from the AK of the related intracellular parasite Leishmania donovani by having a high affinity to adenosine (0.04–0.08 μM depending on [phosphate]) and being negatively regulated by adenosine (K0.5 = 8–14 μM). These properties make the enzyme functionally related to the mammalian AKs, although a phylogenetic analysis grouped it together with the L. donovani enzyme. The combination of a high affinity AK and efficient adenosine transporters yields a strong salvage system in T. brucei, a potential Achilles’ heel making the parasites more sensitive than mammalian cells to adenosine analogs such as Ara-A. Studies of wild-type and AK knockdown trypanosomes showed that Ara-A inhibited parasite proliferation and survival in an AK-dependent manner by affecting nucleotide levels and by inhibiting nucleic acid biosynthesis.

Trypanosoma brucei is an extracellular parasite that is transmitted by tsetse flies and lives in the blood, lymph, and central nervous system of its mammalian hosts (1, 2). The parasite causes African sleeping sickness in humans and nagana in cattle. There are two variants of African sleeping sickness, a chronic form caused by the subspecies Trypanosoma brucei gambiense and an acute form caused by Trypanosoma brucei rhodesiense. Both variants are fatal, but the chronic form has a slower progress. Current treatment is unsatisfactory because of low efficacy and high toxicity. Therefore, there is a great need for new drugs to treat the disease, especially at later stages when the parasites infect the brain. Promising results with the adenosine analog cordycepin (3’-deoxyadenosine) on T. brucei-infected mice with brain infection suggest that adenosine analogs can be developed into new anti-trypanosomal agents (3).

Unlike mammalian cells, trypanosomes lack de novo purine biosynthesis, and they are therefore totally dependent on purine salvage (4). The major purine source in human blood is a matter of controversy; when the blood was directly mixed with an adenosine deaminase inhibitor to prevent purine degradation, adenosine was present at 2 μM concentration, whereas hypoxanthine (0.7 μM) and inosine (0.2 μM) were minor sources (5). However, other reports claim that the adenosine concentration is much lower and that hypoxanthine is the major source (6, 7). T. brucei has high affinity adenosine transporters driven by the proton motive force that are able to concentrate adenosine inside the cells (8, 9). Mammalian cells have both facilitated diffusion carriers and Na+/adenosine cotransporters (10), but the affinities for adenosine are 1–2 orders of magnitude lower than the adenosine affinities of the trypanosome transporters.

The current belief is that adenosine is salvaged by a two-step process in T. brucei where intracellular cleavage to adenosine is followed by phosphoribosylation to AMP (4). The adenosine cleavage is performed by inosine-adenosine-guanosine-nucleoside hydrolase (IAG-NH), which has a low adenosine affinity (K0.5 ~ 15 μM (11, 12)) as compared with the blood level. It is therefore important for the parasites to have efficient transporters that concentrate adenosine inside the cells. However, because of the great uncertainty about the blood adenosine concentration, it is not known whether this transport is efficient enough to saturate the enzyme activity. The AMP formed by the cleavage-dependent pathway can be used for the synthesis of all purines needed (GMP is made from AMP via IMP and XMP (4)).

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2 The abbreviations used are: IAG-NH, inosine-adenosine-guanosine-nucleoside hydrolase; AK, adenosine kinase; TbAK, T. brucei adenosine kinase; Ara-A, adenosine arabinoside (vidarabine); TxA, thiorodoxin A; TEV, tobacco etch virus; DTT, dithiothreitol; TBST, Tris-buffered saline Tween 20; BIP, homolog of immunoglobulin heavy-chain binding protein; EHHNA, 9-erythro-(2-hydroxy-3-nonyl) adenine; F-Ara-A, fludarabine; DFMO, difluoromethyl ornithine (efornithine); ADA, adenosine deaminase; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; HPLC, high pressure liquid chromatography; MIC, minimal inhibitory concentration.

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The cleavage-dependent pathway is probably not the only way to salvage adenosine because the published T. brucei genome (13) contains two putative adenosine kinase (AK) genes (Tb927.6.2300 and Tb927.6.2360). The encoded proteins are very similar to each other, differing by only four amino acids (see supplemental Fig. 1). Recently, it was shown that T. brucei becomes less sensitive to cordycepin when AK is silenced by RNA interference (14). We have cloned, expressed, and characterized the T. brucei AK (Tb927.6.2360) to understand its role in adenosine salvage and activation of nucleoside analogs. The T. brucei AK (TbAK) had ~2.5 orders of magnitude higher adenosine affinity (and catalytic efficiency) than IAG-NH (11, 12), which catalyzes the first step in the cleavage-dependent adenosine salvage and activation of nucleoside analogs. The T. brucei AK (TbAK) was taken up by the parasites and phosphorylated by the adenine arabinoside (Ara-A) was taken up by the parasites and phosphorylated by the parasite. The cleavage-resistant drug adenine (deoxyadenosine, or inosine) was taken up by the parasites and phosphorylated by the TbAK-dependent pathway. The resulting Ara-ATP inhibited trypanosome proliferation and survival by incorporation into nucleic acids and by affecting nucleotide levels in the parasite.

**EXPERIMENTAL PROCEDURES**

**Cloning and Expression of TbAK**—The AK gene was amplified from T. brucei TC221 genomic DNA. Primers used were 5′-AAG CGA GGC GAT CCC TGG TGA GCT TAT CAC G-3′ (TbAK forward) and 5′-AGC GGG GAT CCC TGC TGA GGG TTG T-3′ (TbAK reverse). The PCR product was cloned into the pETM-20 vector (European Molecular Biology Laboratory, Heidelberg, Germany) by using Ncol and Acc65I restriction sites. The cloning of the AK gene was confirmed by agarose gel analysis and DNA sequencing. The resulting plasmid (pETM20- Ak) contained a fusion construct with Escherichia coli thioredoxin A (TrxA) and TbAK. In addition, the construct also contained a hexahistidine site between the two proteins to facilitate purification and subsequent removal of the fusion protein.

**Expression and Purification of Recombinant TbAK**—The pETM20-TbAK vector was transformed into E. coli BL21(DE3) pllys cells (Merck) and expressed. The cells were grown in 1 liter of Luria-Bertani medium containing 60 μg/ml carbenicillin and 27.2 μg/ml chloramphenicol at 37 °C with shaking at 200 rpm (Forma Orbital Shaker, Thermo Electron Corp., Marietta, OH). At an optical density of 0.5 at 550 nm, isopropyl thio-β-d-galactoside was added to a final concentration of 1 mM, and the culture was grown for an additional 3 h at 37 °C. The cells were centrifuged (3,000 × g for 30 min) and resuspended in 25 ml of buffer A (0.3 M NaCl, 20 mM Tris-HCl, pH 7.8). The cells were lysed by freezing and thawing and centrifuged at 150,000 × g for 45 min. The supernatant was loaded onto a 1.25-ml His-Select™ cartridge (Sigma) equilibrated with 25 ml of buffer A. The column was washed with 30 ml of 5 mM imidazole in buffer A and subsequently with 5 ml of 10 mM imidazole in buffer A. The TrxA-tagged TbAK was eluted with 30 ml of 20 mM imidazole in buffer A. The protein-containing fractions were pooled and precipitated with 80% (0.52 g/ml) ammonium sulfate at 0 °C and centrifuged (30,000 × g for 20 min at 4 °C). The pellet was dissolved in 500 μl of buffer B (50 mM Tris-HCl pH 7.6 and 0.1 mM DTT) and desalted on a G-25 Sephadex column (6 ml) equilibrated with buffer B. The protein concentration was assessed by the Bio-Rad protein assay (Bio-Rad) and compared with a standard curve made from bovine serum albumin. The presence of 0.1 mM DTT was necessary to keep the protein stable during freezing and thawing.

**Removing the Fusion Partner from TbAK by TEV Proteolysis**—Approximately 0.7 mg of TrxA-TbAK protein in 2 ml of buffer B was cleaved by 40 μg of TEV protease for 1 h at room temperature. The cleaved protein mixture was loaded onto a 1.25-ml His-Select™ cartridge (Sigma) equilibrated with buffer B. Pure TbAK was collected in the flow-through, whereas the hexahistidine-tagged TrxA and TEV protease were bound to the column. The protein-containing fractions in the flow-through were pooled, concentrated by a 20-ml Vivaspin column (Vivascience, Hannover, Germany), frozen in liquid nitrogen, and stored at −80 °C. The TrxA tag had no obvious effect on enzyme activity (data not shown).

**TbAK Assays**—Assays were performed with 3H-labeled adenosine, 2′-deoxyadenosine, or inosine from Moravek Biochemicals Inc., Brea, CA. Prior to the enzyme activity analysis, TbAK was diluted in Dilution buffer, which consists of 50 mM Tris-HCl, pH 7.6 (10 mM Tris-HCl was used in the pH optimization experiment), 50 mM KCl, 0.05% (v/v) Tween 20, and 0.1 mM DTT. The diluted protein was mixed with assay components in a volume of 50 μl. The assay mixture contained 3H-labeled nucleoside (adenosine, 2′-deoxyadenosine, or inosine), 0.5 mM ATP, 5 mM MgCl2, 100 mM KCl, 5 mM potassium phosphate, and 50 mM Tris-HCl, pH 7.6. When high nucleoside substrate and enzyme concentrations were used, 0.05 mg/ml creatine kinase and 8 mM phosphocreatine were added to the assay mixture to avoid depletion of ATP (ADP inhibits the reaction). Phosphocreatine and creatine kinase had no effect on enzyme activity under conditions where ATP levels were stable (low enzyme and substrate concentration). The assay components were mixed on ice and stored for 10 min before the reactions were incubated at 37 °C for 30 min. After completion of the assays, the tubes were placed on ice, and 20 μl of the assay mixtures was spotted onto DE81 filters (Whatman). After drying, the filters were washed three times for 5 min by 1 mM ammonium formate under agitation. The radioactivity was eluted from the filters by 0.1 mM HCl/KCl before scintillation counting as described previously (15). The unit definition of enzyme activity from 1 mg of TbAK protein is in μmol/min.

**HPLC Enzyme Assay**—The standard AK assay was sometimes complemented by HPLC studies to check the exact nature of the products and to study TbAK activity with adenosine analogs that were not radioactive. After completion of the assay, the assay mixtures were diluted in water, and 20 μl was loaded onto a 4.6 × 100-mm ACE 3 AQ column (Advanced Chromatography Technologies, Aberdeen, Scotland, UK). The flow rate was 1 ml/min. Cordycepin and cordycepin-5′-monophosphate were separated with a running buffer consisting of...
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7.5% (v/v) methanol, 33 mM KH₂PO₄, and 61 mM KCl (the pH was adjusted to 6 with 5 M KOH). The same buffer was also used to separate 2'-deoxyadenosine from dAMP, whereas the methanol concentration was decreased to 5% (v/v) to separate adenosine from AMP, Ara-A from Ara-AMP, and F-Ara-A from F-Ara-AMP.

**Cultivation of Trypanosomes and Mammalian Cells**—Culture-adapted bloodstream forms of the *T. brucei brucei* cell line TC21 (16) were propagated in Hirumi’s modified Iscove’s medium-9 (17) lacking thymidine and Serum Plus but supplemented with 10% (v/v) fetal bovine serum at 37 °C in a humidified atmosphere containing 7% CO₂. This modification of the growth medium has no obvious effect on trypanosome proliferation (18). Mouse Balb/3T3 fibroblasts (ATCC no.CCL-163) or human WS1 fibroblasts (ATCC no.CRL-1502) were cultivated as monolayers in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with l-glutamine (0.584 g/liter) and 10% (v/v) fetal bovine serum at 37 °C in a humidified atmosphere containing 7% CO₂.

**Preparation of *T. brucei* Enzyme Extracts**—Trypanosomes (5 × 10⁸ cells) were collected by centrifugation at 3,000 × g at 4 °C for 10 min, and the pellet was washed twice in 50 mM Tris-HCl, pH 7.6, containing 0.1 mM DTT. The trypanosomes were lysed by sonication as described previously (19) and centrifuged at 21,000 × g at 4 °C for 10 min. The supernatant was quickly frozen in liquid nitrogen and placed at −80 °C. The assay was performed as before except that 2 mM ATP was used (instead of 0.5 mM) and that 50 mM NaF (phosphatase inhibitor) was included to prevent ATP degradation. Protein concentration was assessed by the Bio-Rad protein assay and compared with a standard curve made from bovine serum albumin.

**Western Blots**—Samples were prepared from noninduced and induced *Tb*AK knockdown *T. brucei* cells. Trypanosomes (4 × 10⁶ cells) were collected by centrifugation, washed twice with phosphate-buffered saline, and resuspended in 200 µl of phosphate-buffered saline. To break the cells, the suspensions were mixed with 300 mg of 0.5-mm zirconia/silica beads (Biodesic Products, Inc.) and vortexed three times for 15 s (the samples were chilled on ice between each vortex cycle). The cell extracts were centrifuged for 1 min (16,000 × g), and the supernatants were used for SDS-PAGE. Western analysis was performed with α-*Tb*AK immune serum diluted 33,000 times in Tris-buffered saline/Tween 20 (TBST) and 2% (w/v) skim milk. The blot was probed with an α-rabbit antibody (Amersham Biosciences), developed with the ECL system (Amersham Biosciences), and quantified by Kodak Image Station 2000R. The membrane was stripped, and a second Western blot was made with α-BiP antibody obtained from J. Bangs laboratory (University of Wisconsin Medical School, Madison). BiP is a *T. brucei* homolog of immunoglobulin heavy chain binding protein and is used as loading control in Western blots. The immune serum against *Tb*AK was produced by Agrissera AB (Vännäs, Sweden).

**Measurement of NTP and dNTP Pools**—Logarithmically growing trypanosomes (1–3 × 10⁸ cells) or 50–75% confluent mammalian cells from a 15-cm dish were collected, and their NTPs and dNTPs were extracted by a trichloroacetic acid-based procedure as described previously (18). The NTPs/dNTPs were quantified by HPLC (20) using a 100 × 4.6 mm, 3-µm 1500-Å PolyWAX A column from PolyLC Inc., Columbia, MD. The flow rate was 1 ml/min. The mobile phase was made up from 2% (v/v) acetonitrile and 0.3 mM KH₂PO₄ and was adjusted to pH 5 with 1 M K₂HPO₄. When necessary, dNTPs were purified from NTPs by boronate chromatography prior to the HPLC analysis (18). The nucleotides were quantified by comparing the peak heights to a standard mixture of nucleotides.

**Measurement of Trypanosome or Mammalian Cell Proliferation in the Presence of Drugs**—*T. brucei* cells (10,000 cells/well) were grown for 60 h in a 96-well microtiter plate in the presence of 200 µl of medium containing various concentrations of Ara-A (in combination with 1 µM 9-erythro-(2-hydroxy-3-nonyl) adenine (EHNA)) or F-Ara-A. Control trypanosomes that were incubated without drug (or with only EHNA) were still in the logarithmic growth phase at 60 h. The cells were quantified as previously described (21); after the addition of 20 µl of Alamar Blue® (BIOSOURCE) and a 2-h incubation period, the cell densities were quantified by fluorescence measurements (excitation at 540 nm and measurement at 583 nm). The equipment used consisted of an FP6500 spectrofluorometer and an FMP-963 microtiter plate reader (Jasco). Human WS1 fibroblasts and mouse Balb/3T3 cells (5,000 cells/well) were also grown for 60 h in the presence of drugs and quantified by the Alamar Blue® assay. Control cells incubated without drug (or with only EHNA) were 50–75% confluent at 60 h.

**Minimal Inhibitory Concentration (MIC) Determination**—Trypanosomes (500,000/ml) were treated with various concentrations of Ara-A (+1 µM EHNA) or F-Ara-A for 60 h. Control cells incubated without drug had reached a cell density of 2–3 million/ml at this time point. MIC values were determined by microscope inspection and defined as the lowest concentration of drug where no motile trypanosomes with normal morphology were detected (the detection limit was ~10,000 cells/ml). MIC₉₀ was defined as the lowest concentration where more than 90% of the cells were killed (less than 50,000 cells/ml remained).

**Measurement of ³H-Labeled Uracil/Thymidine Incorporation into Nucleic Acids**—Trypanosomes (2 × 10⁶ cells) were centrifuged at 1,800 × g for 2 min and resuspended in 10 ml of medium. After 30 min of preincubation with 1 µM EHNA, 10 µCi of [6-³H]uracil (39 Ci/mmol) or [methyl-³H]thymidine (15 Ci/mmol) was added together with various concentrations of drug where no motile trypanosomes with normal morphology were detected (the detection limit was ~10,000 cells/ml). Cell material precipitated with trichloroacetic acid was dissolved in 0.6 M NaOH and incubated at 37 °C overnight. Scintillation counting of 100 µl of this material revealed the total amount of radioactivity in nucleic acids (RNA + DNA). DNA could be recovered from the NaOH-treated nucleic acids by trichloroacetic acid precipitation of the material (100 µl) and collection on glass filters as described previously (22). The radioactivity in RNA was obtained by subtracting the DNA counts from the total counts. The incorporation of radioactivity into nucleic acid was linear during the 60-min incubation period (data not shown).
RESULTS

Expression and Purification of TbAK—The TbAK gene was cloned into pETM-20. The resulting plasmid encoded a hexahistidine-tagged TrxA protein fused to TbAK. Protein expression in the absence of this tag resulted in only small amounts of insoluble protein. The expressed TrxA-TbAK protein was purified to homogeneity by nickel-agarose chromatography (Fig. 1, lane 1). The fusion protein was digested by TEV protease and repurified by nickel-agarose. Because the TEV protease and the fusion partner had hexahistidine tags, they bound to the column, and pure TbAK could be collected in the flow-through (Fig. 1, lane 2) with a final yield of 6 mg from a starting material of 125 mg of protein (1-liter culture). The TrxA-TbAK and TbAK bands were in agreement with their expected molecular masses of 52 and 38 kDa, respectively.

Gel filtration analysis showed that, in agreement with the human, Leishmania donovani and Toxoplasma gondii AKs (23–25), the TbAK protein was primarily monomeric in the absence and presence of 3 mM ATP (data not shown).

Optimization of Enzyme Assay Conditions—TbAK activity was optimized using 0.5 μM adenosine (enzyme activity is inhibited at higher concentrations) and 0.5 mM ATP as substrates. Optimal enzyme activity was obtained at pH 6 – 8.5 (Fig. 2A).

The enzyme activity was stimulated by phosphate ions (Fig. 2B). The dependence on phosphate might be a general property of AKs because it has also been observed in enzymes from L. donovani, mammals, yeast, and plants (26). The human enzyme can also be activated by small phosphate-containing organic compounds such as phosphocreatine (27), but this compound had no effect on the trypanosome enzyme (Fig. 2B). TbAK activity was dependent on MgCl₂, whereas KCl (0 – 0.25 M) and DTT (0 – 1 mM) had no obvious effect on enzyme activity (data not shown). TbAK activity was linear with time (0 – 30 min) and protein concentration (data not shown). The specific activity of the enzyme was between 11 and 28 units/mg, depending on the phosphate concentration. This activity is higher than for the native and recombinant human AKs assayed under optimal conditions (3.7 and 3.4 units/mg, respectively (28)) but in the same range as for the mouse recombinant AK (16 units/mg (29)).

ATP and GTP Were Preferred Phosphate Donors for TbAK—The enzyme could use ATP, GTP, CTP, or UTP as phosphate donors. With ATP as phosphate donor, the enzyme activity had a Vₘₐₓ value of 11.6 units/mg (Table 1). The other phosphate donors gave higher Vₘₐₓ values, but they needed to be present at higher concentrations to saturate the enzyme as seen by their high Kₘ values. Comparing the catalytic efficiencies (kₐₐₖ/Kₘ) with different phosphate donors show that ATP and GTP are the preferred ones. Because ATP is present at higher concentrations than GTP in T. brucei cells (18), it is probably the major phosphate donor under physiological conditions.

TbAK Activity Was Regulated by Substrate Inhibition—TbAK activity measured at different adenosine concentrations showed an increasing enzyme activity up to a plateau starting at ~0.25 μM adenosine in the absence of phosphate (Fig. 3A). The 0 and 50 mM phosphate curves followed each other at low adenosine concentrations (<0.05 μM), but the 50 mM curve continued to increase when the 0 mM phosphate curve bent off, resulting in higher Kₘ and Vₘₐₓ values. At high adenosine concentrations, the enzyme was inhibited as seen in Fig. 3B where a wider range of substrate concentrations is plotted with a logarithmic abscissa. Substrate inhibition usually follows the rules of uncompetitive inhibition described by the Equation 1, which has also been used to calculate substrate inhibition constants for the mammalian adenosine kinases (30),

\[ v = V_{\text{max}} \times \frac{A}{(K_m + A + (A_0/K_i))} \]  

where A = [adenosine]. By using Equation 1, a good fit was obtained for all data points except when the substrate concentration was 100 μM or above. The kinetic constants calculated from this experiment are shown in the figure legend (0 and 50 mM phosphate curves) and Table 1 (5 mM phosphate).

Phylogenetic Analysis Grouped TbAK Separately from Other Regulated AKs—The inhibition at high adenosine concentrations makes TbAK similar to mammalian (26, 31), yeast, and plant AKs (26) and different from AKs of most protozoan parasites, including L. donovani (24), which belongs to the same family of organisms as trypanosomes (the Trypanosomatidae).
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A phylogenetic tree of AKs from different species. The enzymes that are known to be inhibited by adenosine are marked with + and enzymes lacking inhibition with - (ND, not determined). The amino acid sequences were aligned by ClustalW (Gonnet250 protein weight matrix), and the tree was constructed with the Molecular Evolutionary Genetic Analysis software version 3.1 using Neighbor Joining with 1000 bootstrap replicates (numbers are percent positives). The bar indicates 0.1 substitutions per site.

The enzymes from these experiments were as follows: 0 mM phosphate (using the same symbols as in Table 1), and 50 mM phosphate (see Table 1), whereas the inosine experiment was run at 5 mM phosphate. All experiments were run in duplicate with standard deviations shown by error bars. Calculated kinetic constants are as follows: inosine (see Table 1), deoxyadenosine with 0 mM phosphate (K_m = 0.041 ± 0.0037 μM, V_max = 11.3 ± 0.26 units/mg, K_s = 14 ± 1.3 μM), 5 μM phosphate (see Table 1), and 50 mM phosphate (K_m = 0.085 ± 0.0059 μM, V_max = 27.9 ± 0.61 units/mg, K_s = 8.2 ± 0.57 μM).

A phylogenetic tree of AKs from different species (Fig. 4) suggested that the T. brucei enzyme has developed the substrate inhibition property independently of the mammalian and yeast enzymes that belong to a separate branch. TbAK is instead grouped together with the L. donovani enzyme that lacks substrate inhibition. It was not obvious which amino acids or protein domains are responsible for the observed substrate inhibition in T. brucei (see supplemental Fig. 1).

Deoxyadenosine and Inosine Were Also Substrates of TbAK—T. brucei accumulates high intracellular levels of dATP when grown in the presence of deoxyadenosine in the culture medium (18). Therefore, we wanted to test whether TbAK is responsible for this high salvage activity. The recombinant AK was able to phosphorylate not only adenosine but also deoxyadenosine and inosine (Fig. 5A). However, the catalytic efficiencies with these substrates were several orders of magnitude lower than with adenosine (Table 1). Deoxyadenosine phosphorylation was affected by phosphate ions that increased the K_m and V_max values (Fig. 5A) as observed previously with adenosine as substrate (Fig. 3, A and B). Deoxyadenosine phosphorylation was also tested in T. brucei protein extracts (Fig. 5B) to find out whether AK is responsible for the high deoxyadenosine salvage activity in T. brucei cells (18). In addition to the two variants of TbAK, thymidine kinase (Tb10.70.7270) is the only nucleoside kinase known from the T. brucei genome, and we could not identify any more kinases than those three when making BLAST searches with sequences of known nucleoside kinases. Deoxyadenosine phosphorylation in T. brucei extracts was strongly inhibited by adenosine and AMP (Fig. 5B), which are the substrate and product of TbAK, respectively. TbAK seems therefore to be responsible for the phosphorylation of deoxyadenosine in these extracts. The enzyme activity was not affected by dTTP (allostERIC regulator of thymidine kinase) or thymidine (Fig. 5B).

The role of TbAK in intracellular deoxyadenosine phosphorylation was confirmed in knockdown trypanosomes where the TbAK level can be controlled by tetracycline induction (the knockdown cells were generated previously (14)). Western blot analysis showed that the TbAK protein level was reduced in cell samples taken from induced cells as compared with noninduced cells (Fig. 6A). By comparing the intensities of the bands to a standard curve made with the recombinant protein, it was possible to calculate that the noninduced cells contained 0.24 μg of TbAK per mg of the total cellular protein content and that the level decreased ~10-fold after 3 days of induction with 1 μg/ml tetracycline. No further reduction of the TbAK levels was observed at longer induction times (data not shown). Studies of deoxyadenosine salvage in the knockdown cells showed that the noninduced cells accumulated high intracellular levels

![Figure 3](image-url) Ak activity with adenosine as substrate. A, TbAK (0.004 ng) activity with a linear abscissa in the concentration range up to 1 μM adenosine. The experiments were run in the presence of 0 mM (○), 5 mM (■), or 50 mM phosphate (▲). B, TbAK activity measured at a wider adenosine concentration range (0.005–500 μM) in the presence of various concentrations of phosphate (using the same symbols as in A). The amount of TbAK used was 0.004 ng (0.005–1 μM adenosine), 0.04 ng (1–5 μM), 0.2 ng (5–25 μM), 1 ng (25–100 μM), and 50 ng (100–500 μM). The experiments were run in duplicate with standard deviations shown by error bars. Calculated kinetic constants from these experiments were as follows: 0 mM phosphate (K_m = 0.041 ± 0.0037 μM, V_max = 11.3 ± 0.26 units/mg, K_s = 14 ± 1.3 μM), 5 μM phosphate (see Table 1), and 50 mM phosphate (K_m = 0.085 ± 0.0059 μM, V_max = 27.9 ± 0.61 units/mg, K_s = 8.2 ± 0.57 μM).

![Figure 4](image-url) Phylogenetic tree of AKs from different species. The enzymes that are known to be inhibited by adenosine are marked with + and enzymes lacking inhibition with - (ND, not determined). The amino acid sequences were aligned by ClustalW (Gonnet250 protein weight matrix), and the tree was constructed with the Molecular Evolutionary Genetic Analysis software version 3.1 using Neighbor Joining with 1000 bootstrap replicates (numbers are percent positives). The bar indicates 0.1 substitutions per site.

![Figure 5](image-url) Nucleoside phosphorylation with TbAK or T. brucei extracts. A, enzyme activity with deoxyadenosine (○, ■) and inosine (▲) using 10 and 200 ng of TbAK, respectively. The deoxyadenosine experiments were run at three different phosphate concentrations, 0 mM (○), 5 mM (■), or 50 mM (▲), whereas the inosine experiment was run at 5 mM phosphate. All experiments were run in duplicate with standard deviations shown by error bars. Calculated kinetic constants are as follows: inosine (see Table 1), deoxyadenosine with 0 mM phosphate (K_m = 0.041 ± 0.0037 μM, V_max = 11.3 ± 0.26 units/mg, K_s = 14 ± 1.3 μM), 5 μM phosphate (see Table 1), and 50 mM phosphate (K_m = 190 ± 20 μM, V_max = 9.6 ± 0.35 units/mg). B, phosphorylation of 0.5 mM deoxyadenosine by a trypanosome extract (5 μg of protein) in the presence of various competing nucleosides/nucleotides at the indicated concentrations in mM.

TABLE 1
Kinetic parameters of TbAK incubated under standard assay conditions

| Substrate | K_m (μM) | V_max (units/mg) | K_s (μM) | V_max/K_m (s⁻¹ × μmol⁻¹ × mg⁻¹) |
|-----------|----------|------------------|----------|----------------------------------|
| ATP       | 26 ± 2.4 | 11.6 ± 0.16      | 7.3      | 0.28                             |
| GTP       | 82 ± 14  | 25 ± 0.81        | 16       | 0.19                             |
| UTP       | 340 ± 56 | 29 ± 1.5         | 18       | 0.052                            |
| CTP       | 610 ± 83 | 32 ± 1.7         | 20       | 0.032                            |
| dAdo      | 0.041 ± 0.0031 | 13.0 ± 0.25  | 0.037   | 10.21                            |
| dAdo      | 0.041 ± 0.0031 | 13.0 ± 0.25  | 0.037   | 10.21                            |
| dAdo      | 0.041 ± 0.0031 | 13.0 ± 0.25  | 0.037   | 10.21                            |
| dAdo      | 0.041 ± 0.0031 | 13.0 ± 0.25  | 0.037   | 10.21                            |
| dAdo      | 0.041 ± 0.0031 | 13.0 ± 0.25  | 0.037   | 10.21                            |
| dAdo      | 0.041 ± 0.0031 | 13.0 ± 0.25  | 0.037   | 10.21                            |
| dAdo      | 0.041 ± 0.0031 | 13.0 ± 0.25  | 0.037   | 10.21                            |
| dAdo      | 0.041 ± 0.0031 | 13.0 ± 0.25  | 0.037   | 10.21                            |
| dAdo      | 0.041 ± 0.0031 | 13.0 ± 0.25  | 0.037   | 10.21                            |
| dAdo      | 0.041 ± 0.0031 | 13.0 ± 0.25  | 0.037   | 10.21                            |
of dATP when grown in the presence of deoxycadenosine, whereas this ability was strongly reduced in the induced cells (Fig. 6B). EHNA (1 μM) was included in the growth medium in these experiments to prevent deamination of deoxycadenosine.

Cordycepin, Ara-A, and F-Ara-A Were Substrates of TbAK—The adenosine analogs, cordycepin and Ara-A, inhibit the growth and survival of T. brucei (3) and T. gondii (32), respectively. The inhibition is in both cases dependent on AK (14, 33). Studies of the recombinant TbAK showed that it could phosphorylate cordycepin (Fig. 7A), Ara-A, and F-Ara-A (Fig. 7B). The enzyme activity with cordycepin and Ara-A as substrates was higher in the presence of 50 mM phosphate than with 5 mM phosphate (A), whereas the F-Ara-A experiment was run at 5 mM phosphate. The experiments were run in duplicate with standard deviations shown by error bars. The calculated kinetic constants from most of these experiments are shown in Table 1 except the 50 mM phosphate experiments with cordycepin (Km = 32 ± 5.8 μM, Vmax = 9.8 ± 0.55 units/mg) and Ara-A (Km = 280 ± 48 μM, Vmax = 3.0 ± 0.20 units/mg).

TABLE 2
Inhibition of wild-type T. brucei (TC221), AK knockdown T. brucei (TbAK-RNA interference), and mammalian fibroblast growth with Ara-A (and 1 μM EHNA) or F-Ara-A

| Cell type          | Drug    | IC50  | 95% confidence interval | MIC     | MIC90    |
|--------------------|---------|-------|------------------------|---------|----------|
| T. brucei TC221    | Ara-A   | 0.071 | 0.064–0.077            | ~0.65   | ~0.35    |
| TbAK-RNAi − Tet    | Ara-A   | 0.12  | 0.11–0.14              |         |          |
| TbAK-RNAi + Tet    | Ara-A   | 1.3   | 1.0–1.6                | ~25     |          |
| T. brucei TC221    | F-Ara-A | 4.2   | 3.5–5.2                |         |          |
| TbAK-RNAi − Tet    | F-Ara-A | 2.3   | 1.2–4.3                |         |          |
| TbAK-RNAi + Tet    | F-Ara-A | 7.0   | 4.0–12                 |         |          |
| Balb/3T3 (mouse)   | Ara-A   | 1.8   | 1.3–2.4                |         |          |
| WS1 (human)        | Ara-A   | >10   |                        |         |          |

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FIGURE 6. TbAK protein levels and deoxycadenosine salvage activity in TbAK knockdown trypanosomes. A, recombinant TbAK and samples from knockdown trypanosomes were analyzed by Western blot using antibodies against TbAK and BIP. The cells were harvested before induction (NI, noninduced) and after 3 days of tetracycline induction of the RNA interference construct (I, induced). B, measurement of deoxycadenosine salvage in noninduced and tetracycline-induced knockdown trypanosomes (induced for 7 days). The cells were cultivated for 1 h in the presence of the indicated deoxycadenosine concentrations in mM (1 μM EHNA). The intracellular dATP levels were subsequently measured. The experiments were run in duplicates with standard deviations shown by error bars.

FIGURE 7. TbAK activity with nucleoside analogs. A, cordycepin phosphorylation in the presence of 5 mM (●) or 50 mM phosphate (▲) using 1 ng of TbAK. B, Ara-A (●, ▲) and F-Ara-A (□, ▼) phosphorylation using 25 and 200 ng of TbAK, respectively. The Ara-A experiment was run at 5 mM (●) and 50 mM phosphate (▲), whereas the F-Ara-A experiment was run at 5 mM phosphate. The experiments were run in duplicate with standard deviations shown by error bars. The calculated kinetic constants from most of these experiments are shown in Table 1 except the 50 mM phosphate experiments with cordycepin (Km = 32 ± 5.8 μM, Vmax = 9.8 ± 0.55 units/mg) and Ara-A (Km = 280 ± 48 μM, Vmax = 3.0 ± 0.20 units/mg).

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Ara-A Was More Efficiently Salvaged by T. brucei than Mammalian Cells and Affected Nucleotide Pools and Nucleic Acid Biosynthesis in the Parasites—Ara-A is only a moderate substrate of TbAK (see Table 1) but nucleoside transporters help to make its salvage efficient. Trypanosomes were incubated with various concentrations of Ara-A (±1 μM EHNA). After 1 h of incubation, the intracellular Ara-ATP pool was measured. The salvage of Ara-A was nearly saturated at 20 μM (Fig. 8A), and at this point Ara-ATP became one of the major nucleotides in the cell. In contrast, Ara-A salvage in mammalian cells was much lower and not saturated in the concentration range tested (Fig. 8B). The accumulated Ara-ATP level in the mammalian cells only made up 1–7% of the total NTP pool, although the concentration of Ara-A in the growth medium was increased up to 200 μM. Results from cell growth experiments showed the same tendency as the salvage experiments. T. brucei was more sensitive to Ara-A than mouse and human fibroblasts (Table 2). The sensitivity of the mouse fibroblasts was similar to what has been reported for mouse L1210 leukemia cells where the IC50 value...
was 2–3 μM in the presence of adenosine deaminase inhibitors (34). The Ara-A salvage in the human fibroblasts was even less efficient (Fig. 8B), and they were not sensitive to the drug in the concentration range tested (Table 2).

Ara-A treatment of *T. brucei* cells caused a general decrease in ribonucleotide levels, except GTP that was fairly constant (Fig. 8A). In contrast, the deoxyribonucleotide levels increased (Fig. 8C). The increased dNTP pools could be a result of a decreased usage of the nucleotides for DNA biosynthesis. In Fig. 8D, the incorporation of tritiated thymidine into DNA in Ara-A-treated trypanosomes was measured. The incorporation rate decreased with increasing Ara-A concentration. Similar conclusions were drawn from experiments with radiolabeled uracil. This nucleobase is converted to UTP, CTP, dTTP, and dCTP by cellular enzymes, and it can therefore be used to trace the newly characterized high affinity pathway catalyzed by *TbAK*. There is also a third pathway starting with the deamination of adenosine to inosine (35). Adenosine can be salvaged either by the previously known cleavage-dependent pathway that has a low affinity to adenosine or by the newly characterized high affinity pathway catalyzed by *TbAK*.

**FIGURE 8.** Ara-A treatment of *T. brucei* leads to altered nucleotide pools and inhibited nucleic acid biosynthesis. A, intracellular pools of Ara-ATP (■) and ATP (▲), GTP (●), and CTP (▲) in *T. brucei* treated with various concentrations of Ara-A. B, accumulation of Ara-ATP in mouse Balb/3T3 (●) and human WS1 (▲) fibroblasts treated with Ara-A. The Ara-ATP concentration was normalized to the total NTP pool (CTP + UTP + ATP + GTP), which was not affected by the treatment. C, effect of Ara-A treatment on the intracellular pools of dATP (▲), dTTP (♦), dCTP (●), and dGTP (●) in *T. brucei*. D, incorporation of [methyl-3H]thymidine into DNA in Ara-A-treated trypanosomes. E and F, incorporation of [6-3H]uracil into DNA (E) or RNA (F) in *T. brucei*-treated trypanosomes. In all experiments, the cells were treated with both Ara-A and 1 μM EHNA (and sometimes also radioactive thymidine or uracil) for 1 h before measurements of NTP/dNTP pools and radioactivity in nucleic acids. All experiments were run in duplicate with standard deviations shown by error bars.

**DISCUSSION**

*T. brucei* is an extracellular parasite that needs to salvage purines from the host in order to survive. The salvage of adenosine can either be performed by the cleavage-dependent pathway or by *TbAK* (Fig. 9). *TbAK* is probably not an essential enzyme in purine-rich environments because *T. brucei* is able to salvage adenosine through the cleavage-dependent pathway, and it also has a full set of enzymes to salvage hypoxanthine, inosine, and adenine (4). However, the total purine concentration is low in the blood and other body fluids, and the concentration might decrease even further when the purine demand increases at high parasitemia. IAG-NH, which participates in the cleavage-dependent pathway, has a low adenosine affinity (*K_m* ~ 15 μM (11, 12)), whereas the adenosine affinity of *TbAK* is ~2.5 orders of magnitude higher. Another advantage of having *TbAK* is to conserve energy. The high affinity pathway requires one ATP to synthesize AMP from adenosine, whereas the cleavage-dependent pathway (and salvage of other purines than adenosine) requires phosphoribosylpyrophosphate, which is made from ribose and three ATPs.

*TbAK* is able to efficiently use very low concentrations of adenosine but is inhibited when the concentration is high to prevent accumulation of too high intracellular purine nucleotide levels. The AK from the related parasite *L. donovani* is very different from *TbAK* by having a lower affinity to adenosine (*K_m* = 0.6–12.5 μM, depending on phosphate concentration (26)) and no substrate inhibition (24, 26). The properties of these enzymes might reflect the different environments of the parasites in their mammalian host; *L. donovani* is predominantly intracellular whereas *T. brucei* is extracellular. Similar to other adenosine kinases containing the NXXE motif (see supplemental Fig. 1), *TbAK* activity was stimulated by phosphate ions (*V_{max*} increased), but in contrast to the other enzymes (26) the affinity for adenosine was not increased. For comparison, the *L. donovani* AK gets ~20 times higher affinity to adenosine when phosphate is added to the reaction (26).

Previous experiments in *T. brucei* cell extracts have shown that AK activity is either absent (36) or very low (19, 37). Our experience is that it is very important to use the right extraction method to isolate AK from *T. brucei*. When proteins are extracted by freezing and thawing three times, no AK activity
was found, whereas sonication gave considerable activity (data not shown). In contrast, the isolation of IAG-NH was independent on extraction method. Because of the uncertainty of extraction efficiencies, it is difficult to judge whether AK or IAG-NH is the major activity from studies in cell extracts. However, the high accumulation of dATP (18) and Ara-ATP in dAdo/Ara-A-treated trypanosomes suggests that TbAK activity is high in trypanosomes. There is also a third alternative for adenosine salvage starting with deamination of adenosine (catalyzed by adenosine deaminase (ADA)) and subsequent cleavage of the formed inosine by IAG-NH (Fig. 9). It is known that the parasite has an ADA gene (Tb10.406.0070), but it is unclear whether adenosine deaminase is expressed in bloodstream trypanosomes. Studies of adenosine deaminase activity in T. brucei extracts showed conflicting results (19, 36, 37). We have not been able to detect any ADA activity in T. brucei cell extracts regardless of extraction method. The conflicting results may possibly be explained by contamination with mammalian adenosine deaminase, which is an abundant enzyme in serum.

The catalytic efficiency of TbAK was high in comparison with most of the other characterized AKs, except the mouse enzyme which has $K_m$ and $k_{cat}$ values in a similar range as TbAK (29). By having the combination of a high affinity AK and active transporters, T. brucei seems to be well adapted to compete with the mammalian cells for the scarce purine supplies. TbAK also phosphorylated drugs such as cordycepin, Ara-A, and F-Ara-A as shown by enzyme activity analyses and studies of TbAK knockdown cells. It is important that Ara-A is not recognized by the cleavage-dependent pathway because that would remove the modified sugar and only adenine (which is nontoxic) remains. Our studies in T. brucei extracts show that Ara-A is resistant to such cleavage (data not shown).

T. brucei was more sensitive to Ara-A than mammalian cells. Our results suggested that the selectivity of the drug against the trypanosomes was because of high salvage efficiency. The high salvage capacity can possibly be explained by both efficient nucleoside transport and by the Ara-A affinity to TbAK. In mammalian cells, Ara-A is phosphorylated by adenosine kinase and deoxycytidine kinase, which have 5–10-fold lower affinities to Ara-A than TbAK (the $K_m$ values of human deoxycytidine and adenosine kinases are in the 1–3 mM range (38–40)). The relative contribution of the two enzymes to Ara-A salvage in mammalian cells is cell type-dependent. In mouse L1210 leukemia cells and baby hamster kidney cells, adenosine kinase is responsible for Ara-A salvage (41, 42), whereas in human T-lymphoblastoid CCRF-CEM cells, the two enzymes are nearly equally responsible (38). In contrast, the deamination-resistant drug F-Ara-A is almost entirely phosphorylated by deoxycytidine kinase in mammalian cells (43).

The accumulated Ara-ATP in the Ara-A-treated trypanosomes caused inhibition of DNA (major effect) and RNA biosynthesis. The inhibition of RNA biosynthesis can also be an indirect effect caused by decreased NTP pools that are needed as substrates for RNA polymerization. We do not know why the NTP pools decreased. It could either be that Ara-A nucleotides affect the enzymes responsible for nucleotide biosynthesis in the parasites or that the energy charge in the cells (and thereby the NTP pools) is decreased because Ara-A phosphorylation requires ATP or other phosphate donors. In mammalian cells, Ara-ATP also inhibits ribonucleotide reductase by acting as an allosteric effector (44). However, the T. brucei ribonucleotide reductase lacks overall activity regulation (18, 45), and no inhibition of dNTP biosynthesis was observed.

Adenosine analogs have both disadvantages (some of them are toxic) and advantages for development into antitrypanosomal drugs. A major advantage is that many of them, including Ara-A (46) and cordycepin (3), seem to pass the blood-brain barrier that is a prerequisite to treat late stage African sleeping sickness. That is also true for the adenosine deaminase inhibitor deoxycoformycin that was readily transported into the cerebrospinal fluid in drug-treated monkeys (47). Today, there are only two drugs available that can be used against late stage sleeping sickness, melarsoprol and eflornithine (DFMO) (1, 2). Melarsoprol is highly toxic, and 10% of the treated patients get a life-threatening encephalopathy, whereas DFMO only works against one of the two subspecies causing the human disease (T. brucei gambiense). In addition, DFMO treatment is not always possible in rural areas of Africa because it requires 4 daily intravenous infusions for 14 days. Promising results with cordycepin (+deoxycoformycin) on T. brucei-infected mice with brain infection (3) indicate that adenosine analogs can be developed into future drugs against late stage African sleeping sickness. However, the toxicity of cordycepin must be investigated before it can be tried out as a drug on humans.

Ara-A, F-Ara, and deoxycoformycin have gone through extensive clinical trials when they were tested as drugs against various cancers and viral infections (43, 46). That is a great advantage because many of their pharmacological and toxicological properties are already known. Our preliminary results on T. brucei-infected mice treated with a drug combination of Ara-A and deoxycoformycin show that the trypanosome levels in the blood decreased below the detection limit within a few days (data not shown). Similar results were obtained with the deamination-resistant drug F-Ara-A. However, the dose regime needs to be optimized in both cases because the trypanosomes reappeared a few days after the treatment was completed.

In conclusion, the combined effect of an efficient TbAK and high affinity transporters makes the trypanosomes sensitive to AK substrate analogs that may be further developed into new medicines against African sleeping sickness. The selectivity of each analog is dependent on multiple factors such as transport efficiency, TbAK affinity, and the effect on downstream targets.

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