Available online at www.sciencedirect.com
ScienceDirect
Molecular & Biochemical Parasitology 149 (2006) 223–230
MOLECULAR & BIOCHEMICAL PARASITOLOGY

Adenosine kinase from *Cryptosporidium parvum*

Jon Galazka [a], Boris Striepen [b], Buddy Ullman [a,*]

[a] *Department of Biochemistry and Molecular Biology, Oregon Health & Science University, OR 97239, USA*
[b] *Center for Tropical and Emerging Global Diseases & Department of Cellular Biology, University of Georgia, Athens, GA 30602, USA*

Received 20 April 2006; received in revised form 5 June 2006; accepted 6 June 2006
Available online 27 June 2006

Abstract

Analysis of the *Cryptosporidium parvum* genome demonstrates that the parasite cannot synthesize purines *de novo* and reveals that the sole route for purine salvage by the parasite is via adenosine kinase (CpAK). In order to initiate a biochemical characterization of CpAK and ultimately validate this apparently essential enzyme as a therapeutic target, the *CpAK* gene was redesigned for optimum codon usage, overexpressed in *Escherichia coli*, and the recombinant protein purified to homogeneity and characterized. CpAK appears to be specific for adenosine among the naturally occurring nucleosides but can utilize ATP, GTP, UTP and CTP as the phosphate donor. The enzyme exhibits K_m values of 1.4 μM for adenosine and 41 μM for ATP, has a pH optimum ∼7.0, and is dependent upon the presence of a divalent cation. Structure–activity data intimate that catalysis requires contacts between residues on CpAK with the six-position of the purine ring and the O2′ and O3′ hydroxyls of the ribose sugar. Additionally, 4-nitro-6-benzylthioinosine, a compound that demonstrates therapeutic promise against the related parasite *Toxoplasma gondii*, also inhibits adenosine phosphorylation by CpAK. The overproduction and purification of CpAK now enables a thorough evaluation of its potential as a drug target.

Keywords: *Cryptosporidium parvum*; Adenosine kinase; Purine salvage; Protozoa; Parasite

1. Introduction

Cryptosporidium parvum is the causative agent of cryptosporidiosis, an enteric disease afflicting millions of people worldwide [1]. *C. parvum* is present in 65–97% of surface waters [2–4], and cryptosporidiosis can be contracted through ingestion of as few as 30 infectious oocysts [5]. Because of the threat to public water supplies, *C. parvum* is listed as a Category B Biodefense Pathogen by the National Institutes of Health. In individuals with an intact immune system, cryptosporidiosis is probably mostly asymptomatic [6] but can present itself as an acute, self-limiting diarrhoea [7]. However, symptoms can be severe, unremitting, and life threatening among immunocompromised individuals [7,8]. A 1993 outbreak of cryptosporidiosis in Milwaukee caused by contaminated drinking water resulted in ∼400,000 cases of gastrointestinal disease [9] and 54 deaths [10]. Unfortunately, there are no fully effective chemotherapeutic treatments currently available for treating or preventing cryptosporidiosis, especially in AIDS patients [11].

Rational and selective chemotherapies for any infectious disease require the exploitation of fundamental biochemical or metabolic discrepancies between pathogen and host. Unfortunately, the identification of drug targets in *C. parvum* has been hampered by minimal knowledge of the basic biochemistry of the parasite, mostly because of the lack of a continuous *in vitro* propagation system. The completion of the *C. parvum* genome sequence [12] has finally allowed insight into some of these biochemical pathways, and *in silico* metabolic reconstruction has revealed adaptation to extreme parasitism; *C. parvum* lacks a Krebs cycle, much of the oxidative phosphorylation pathway, and is incapable of synthesizing amino acids, purines, and pyrimidines *de novo* [12,13].

The nutritional necessity for purines must be overcome through the obligatory salvage of host purines. The purine sal-

Abbreviations: AK, adenosine kinase; CpAK, *Cryptosporidium parvum* adenosine kinase; ORF, open reading frame; TgAK, *Toxoplasma gondii* adenosine kinase; HsAK, *Homo sapiens* adenosine kinase; IPTG, isopropyl-β-D-thiogalactopyranoside; NTP, nucleoside triphosphate; NBMPR, 4-nitro-6-benzylthioinosine

* Corresponding author at: Department of Biochemistry and Molecular Biology, L224, Oregon Health & Science University, 3181 S.W. Sam Jackson Park Road, Portland, OR 97239-3098, USA. Tel.: +1 503 494 2546; fax: +1 503 494 8393.
E-mail address: ullmanb@ohsu.edu (B. Ullman).

0166-6851/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.molbiopara.2006.06.001

vage pathway of *C. parvum*, compared to other protozoan parasites [6], is austere. The solitary route by which preformed host purines can be salvaged into the parasite nucleotide pool is via adenosine kinase (AK) [13]. AK (ATP: adenosine 5′-phosphotransferase, EC 2.7.1.20) catalyzes the ATP-dependent phosphorylation of the 5′-hydroxyl moiety of adenosine to form AMP and ADP [14–16]. The AMP product of the AK reaction can then be converted to guanylate nucleotides via the purine interconversion enzymes, AMP deaminase, IMP dehydrogenase, and GMP synthase (http://www.cryptodb.org). The *C. parvum* AK (CpAK) gene has been expressed in *Toxoplasma gondii*, a related apicomplexan parasite, and shown to encode a functional AK activity [13]. However, this heterologous expression system is not amenable to extensive biochemical investigations on the CpAK enzyme.

To overcome this limitation, a codon-optimized version of *CpAK* was synthesized and transformed into *Escherichia coli*. The CpAK protein was purified to homogeneity, and its basic kinetic properties are now reported. The properties of CpAK and its critical nutritional role in purine salvage by the parasite support further therapeutic validation of CpAK.

2. Materials and methods

2.1. Chemicals and reagents

[^{3}H]-adenosine (25 Ci mmol^{-1}), [^{14}C]-adenosine (50 mCi mmol^{-1}), [^{3}H]-inosine (15 Ci mmol^{-1}), and [^{3}H]-guanosine (6.7 Ci mmol^{-1}) were purchased from Moravek Biochemicals (Brea, CA) and American Radiolabeled Chemicals (St. Louis, MO). Unlabeled nucleosides, nucleoside analogs, and nucleotides were bought from Sigma–Aldrich (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA). Ni-NTA agarose beads were from Qiagen (Valencia, CA). Complete Mini EDTA free protease inhibitor was obtained from Roche Diagnostics (Indianapolis, IN). DE81 anion exchange filters were acquired from Whatman (Middlesex, UK). The pET200/D-TOPO® expression vector, and BL21 Star™ (DE3) One Shot® competent cells came from Invitrogen (Carlsbad, CA), while the Biosafe™ Coomassie, and Bio-Rad Protein Assay kits were procured from Bio-Rad (Hercules, CA).

2.2. Alignment of CpAK primary structure with T. gondii and human AK sequences

The open reading frames (ORFs) of CpAK (accession AAS47710), *T. gondii* AK (TgAK) (accession AAF01262), and *Homo sapiens* AK (HsAK) (accession AAA97893) were aligned using the ClustalW multisequence alignment algorithm [17], and the figure produced with BOXSHADE.

2.3. Codon-optimization and in vitro synthesis of the CpAK

The cloning of *CpAK* has been reported [13]. The *CpAK* gene was codon-optimized for expression in *E. coli* and synthesized by Epoch Biolabs (Sugar Land, TX) from codon usage tables supplied by the Kazusa DNA Research Institute [18]. A 6× histidine tag was introduced between the first and second codons of the *CpAK* ORF and the codon-optimized *CpAK* bracketed by an NdeI restriction site at the initiation codon and a SacI site located just 3′ of the termination codon.

2.4. Expression of the CpAK in E. coli

The codon-optimized His$_6$-*CpAK* ORF was excised from the pSK Bluescript II (Stratagene) vector and inserted into the bacterial expression vector pET200/D-TOPO® as an NdeI-SacI fragment and sequenced to ensure fidelity of the chemical synthesis. This construct was then transformed into BL21 Star™ (DE3) One Shot® *E. coli*, and after a 1 h recovery at 37 °C, transformed cells were expanded in 10 ml of LB containing 50 μg/ml kanamycin and incubated overnight at 37 °C. The following morning, a 500 ml culture containing 50 μg/ml kanamycin was inoculated with the confluent overnight culture and grown at 37 °C to an OD$_{600}$ = ∼0.5 at which point isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. The culture was incubated at 37 °C for an additional 4 h after which cells were harvested by centrifugation, washed in phosphate buffered saline, frozen in an ethanol/dry ice bath, and stored at −80 °C until further use.

2.5. Purification of recombinant CpAK

E. coli harboring the *CpAK* expression construct were thawed on ice and resuspended in phosphate buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) containing protease inhibitors (PB-PI buffer). Cells were lysed by two passages through a French press at 12,000 psi and the bacterial lysate was clarified by centrifugation at 10,000 × *g* for 20 min at 4 °C. The 10,000 × *g* supernatant was then incubated with Ni-NTA agarose beads for 1 h at 4 °C to allow binding of the His$_6$-tagged protein to the resin. The beads were loaded onto a column and washed twice with PB-PI buffer to which an additional 10 mM imidazole had been added. His$_6$-CpAK was ultimately eluted with PB-PI buffer supplemented to 250 mM imidazole. Purity was confirmed by fractionation on a 10% sodium dodecyl sulfate-polyacrylamide gel and subsequent staining with Biosafe™ Coomassie. Protein concentration was determined using the Bio-Rad Protein Assay method [19] with absorption at 600 nm determined on a Thermo Labsystems Multiskan Ascent plate reader. After the addition of 10% glycerol, purified CpAK was aliquoted, frozen in liquid nitrogen at protein concentrations of either 0.35 or 1.15 mg ml^{-1}, and stored at −80 °C.

2.6. AK measurements

CpAK activity was measured using a modification of previously published radiometric AK assay protocols [20,21]. Reactions were performed at 37 °C in TD100 buffer (100 mM Tris, pH 8.0, 5 mM $MgCl_2$, 200 μM dithiothreitol) unless otherwise noted and contained various concentrations of ATP, [^{3}H]-adenosine, [^{14}C]-adenosine, [^{3}H]-inosine, or [^{3}H]-guanosine, and unlabelled nucleoside or nucleoside analogs as indicated. Each reaction was initiated by the addition of CpAK enzyme to