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Cryptosporidium parvum has an active hypusine biosynthesis pathway

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ABSTRACT

The protozoan parasite *Cryptosporidium parvum* causes severe enteric infection and diarrheal disease with substantial morbidity and mortality in untreated AIDS patients and children in developing or resource-limited countries. No fully effective treatment is available. Hypusination of eIF5A is an important post-translational modification essential for cell proliferation. This modification occurs in a two step process catalyzed by deoxyhypusine synthase (DHS) followed by deoxyhypusine hydroxylase. An ORF of 1086 bp was identified in the *C. parvum* (Cp) genome which encodes for a putative polypeptide of 362 amino acids. The recombinant CpDHS protein was purified to homogeneity and used to probe the enzyme's mechanism, structure, and inhibition profile in a series of kinetic experiments. Sequence analysis and structural modeling of CpDHS were performed to probe differences with respect to the DHS of other species. Unlike *Leishmania*, *Trypanosomes* and *Entamoeba*, *Cryptosporidium* contains only a single gene for DHS. Phylogenetic analysis shows that CpDHS is more closely related to apicomplexan DHS than kinetoplastid DHS. Important residues that are essential for the functioning of the enzyme including NAD^+ binding residues, spermidine binding residues and the active site lysine are conserved between CpDHS and human DHS. N^1-guanyl-1,7-diaminoheptane (GC7), a potent inhibitor of DHS caused an effective inhibition of infection and growth of *C. parvum* in HCT-8 cells.

1. Introduction

Cryptosporidium parvum is an opportunistic protozoan parasite responsible for enteric infection and severe diarrheal disease in various mammals, including humans [1]. The importance of *C. parvum* as a human pathogen became evident with the emergence of the AIDS epidemic and to date it remains a leading cause of death in untreated AIDS patients in developing or resource-limited countries [2,3]. Several major outbreaks of *C. parvum* infections associated with contaminated water supplies have been reported [4]. *C. parvum* has a multistage life cycle during which the merozoites develop within a specialized vacuole which has an intracellular but extra-cytoplasmic location within the host cell requiring drug candidates to cross both host and parasite membranes and presenting unique challenges for drugdevelopment. *Cryptosporidium* sporozoite antigens have been tested as vaccine candidates; however, a suitable vaccine is not yet available [7–9]. Nitazoxanide (NTZ), paromomycin, and azithromycin are the most commonly used drugs against cryptosporidiosis but they are only partially effective [5,6]. Nitazoxanide is effective in the immunocompetent but is ineffective in the immunocompromised patients [6].

Hypusine [N^{ε}-(4-amino-2-hydroxybutyl) lysine] is formed by a post-translational modification of a lysine residue of the eukaryotic initiation factor 5A (eIF5A) [10,11]. Hypusine modification is important for cell proliferation and tumorigenesis [12,13]. The hypusine residue is also important in the binding of eIF5A to RNA and in its interaction with exportin 4, which was reported to facilitate the nucleo-cytoplasmic shuttle function of eIF5A [14–16]. Hypusine biosynthesis occurs in two steps. First, deoxyhypusine synthase (DHS) synthesizes deoxyhypusine (N^{ε}-(4-aminobutyl) lysine) by transferring the butyl amine moiety of spermidine to a specific lysine residue in NAD^+-dependent reaction. Second, deoxyhypusine is hydroxylated by deoxyhypusine hydroxylase (DOHH) to form hypusine. eIF5A, DHS, and DOHH are highly conserved in all eukaryotes, indicating an important function of this modification [17,18].

Abbreviations: eIF5A, eukaryotic initiation factor 5A; DHS, deoxyhypusine synthase; DOHH, deoxyhypusine hydroxylase; (Cp), *C. parvum*.

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Hypusine modification is essential in eukaryotic organisms as deletion of eIF5A or DHS in yeast or in mouse causes lethality [18–21]. However, a deletion mutant of DOHH is viable in yeast. DOHH is essential in higher eukaryotes [25,26]. Human DHS is a 41 kDa protein and forms a homo-tetramer of two identical dimers [25–28]. The crystal structure of human recombinant DHS shows that it has four active sites that bind four molecules of NAD^+ and this binding site is present near the spermidine binding pocket [27]. Normally, in the complete reaction mixture containing DHS, cofactor NAD^+, donor substrate spermidine, and acceptor substrate eIF5A, deoxyhypusine is formed in eIF5A. However, in the absence of acceptor substrate, only half the reaction occurs [31].

DHS is present as a single copy gene in yeast and human but two copies of DHS, DHSL20 (DHS-like gene from chromosome 20) and DHS34 are present in the *Leishmania* parasite. DHS34 is a catalytically active enzyme form whereas DHSL20 is inactive as its lacks the active site lysine residue. The origin and significance of the two forms of DHS in the *Leishmania* parasite is unknown [21]. *Trypanosoma brucei* also encodes two deoxyhypusine synthase paralogs, one that is catalytically functional but grossly impaired, and the other is inactive. In *T. brucei*, both homologs are required for optimal enzyme activity [22]. In the human malaria parasites, *P. falciparum* and *P. vivax* a single copy of DHS is present and has been evaluated as a potential drug target [23]. Recent experiments show that down regulation by silencing the eIF5A, DHS and DOHH genes with short hairpin RNAs lead to impaired hypusine biosynthesis and growth retardation of the parasite [24].

In the present study we have characterized a functional DHS from *C. parvum*. The results indicate that *C. parvum* has a single DHS gene which, based on neighbor joining bootstrap analysis, has close similarity to other apicomplexan DHS sequences. The ability of several guanyldiamines to inhibit the enzyme, and infection and growth of *C. parvum* was examined.

2. Materials and methods

2.1. Chemicals.

Radiolabeled spermidine trihydrochloride [1,8-^{3}H] spermidine (16.6-32.2 Ci/mmol) was purchased from PerkinElmer Life Sciences. All restriction enzymes and DNA-modifying enzymes were obtained from MBI Fermentas (Germany). N^1-guanyl-1, 7-diaminoheptane (GC7), N^1-guanyldiaminooctane (GC8), N^1,N^{7-}bisguanyl-1,7-diaminoheptane (GC7G), and N^1,N^8-bisguanyl-l,8-diaminooctane (GC8G) were synthesized as previously described [32]. Other materials used in this study were of analytical grade and were commercially available.

2.2. Parasite and culture conditions

C. parvum oocysts were obtained from G. & S. Pritchard (Bunch Grass Farm, Deary, ID 83823). Oocysts were passaged in 2–5 day old calves, collected, and purified on CsCl gradients as described [33,34]. Oocysts were surface sterilized using 10% (v/v) Clorox®, washed, and shipped in potassium dichromate. Prior to use, oocysts were washed with dH_2O to rinse them free of potassium dichromate, and washed in 10% (v/v) Clorox® before being suspended in minimal essential medium (MEM) containing 10% horse serum [35,36].

2.3. Inhibition of C. parvum growth and development by amine analogs

Human adenocarcinoma cells (HCT-8, ATCC CCL-244) were grown to confluence in 12 well plates (3.8 cm^2) containing MEM supplemented with 10% horse serum. *C. parvum* oocysts (3×10^4) were inoculated into HCT-8 containing plates and incubated for 1.5 h after which time the media was removed and replaced with fresh MEM + 10% horse serum and test compound (GC7, GC7G, diaminooctane, diaminononane, agmatine) and incubated for an additional 24 h. The media was removed and centrifuged ($14{,}000 \times g$ for 2 min) to obtain oocysts, which were stained using merifluor (Meridian Bioscience, OH) and counted using a plate reader with an excitation wavelength of 488 nm and emission wavelength of 518 nm (Spectromax, Molecular Devices, CA). The number of oocysts was determined by reference to a standard curve of fluorescence *versus* oocyst number. The ability of GC7 to prevent infection of HCT-8 cells was determined by pre-incubation of oocysts or sporozoites (3×10^4) with varying amounts of test compound for 30 min, followed by centrifugation at ($14{,}000 \times g$ for 2 min) and resuspension in MEM containing 10% horse serum. The GC7 pre-incubated oocysts or sporozoites were used to infect a confluent layer of HCT-8 cells and processed as described above.

2.4. Cloning of putative DHS genes from C. parvum and construction of CpDHS expression vector

Total RNA was isolated from *C. parvum* cells using RNAeasy kit (Qiagen, CA) and was treated with DNAse (Fermentas, MD) to remove gDNA contamination. Reverse transcription was performed using ThermoScript reverse transcription-PCR kit (Invitrogen, NY) in a 20 μl reaction containing 500 ng of purified RNA, according to the manufacturer's instructions. The open reading frames of the *C. parvum* DHS were PCR-amplified using cDNA as a template and forward and reverse primers 5′-GGAAGATCTGATGCATTCTTTAGGGAATT-3′ containing *Bgl*II site and 5′CCAAGCTTCTATGTATCAAGAAAAGTAGAATAA-3′ containing *Hind*III site, respectively (in which the restriction sites are underlined). PCR was performed using Jump Start REDTaq Ready Mix (Sigma, MO) and the program as follows: Initial denaturation at 95 °C for 5 min followed by 30 cycles at 95 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min and final extension at 72 °C for 10 min. The PCR product was digested and inserted at the *Bgl*II and *Hind*III sites of the pET30a (Novagen, Germany) vector. The recombinant plasmid CpDHS-pET30a was sequenced using both T7 promoter forward and reverse primers to confirm the accuracy of PCR amplification and correct insertion of the CpDHS open reading frames in the vector.

2.5. Sequence analysis

A comparative sequence analysis of DHS sequences from *Cryptosporodium parvum Iowa II* with its homologs from other eukaryotes was performed. The DHS homolog sequences were derived from Swissprot/UniprotKB [37], EupathDB [38] and GeneDB [39] databases. Multiple sequence alignment of these sequences was generated using CLUSTALW with default parameters. Phylogenetic analysis was performed using the neighbor joining tree generated using CLUSTALW [40]. MEGA v5 [41] was used both for visualization and analysis of the phylogenetic tree. The tree was annotated with bootstrap values (100 iterations).

2.6. Expression and purification of the recombinant CpDHS protein

The recombinant construct of CpDHS-pET30a was transformed into the BL21-DE3 strain of *E. coli* and protein expression was induced at 0.6 OD_{600nm} with 0.5 mM isopropyl-1-D-galactopyranoside (IPTG) at 14 °C for 18 h. The cell lysate containing His-tagged CpDHS, was loaded onto pre-equilibrated Ni^{+2} nitrilotriacetic agarose resin (Qiagen). The recombinant CpDHS protein was eluted with increasing concentrations of imidazole. The purified

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