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Overexpression, purification and characterization of a hexahistidine-tagged recombinant extended nucleotide-binding domain 1 (NBD1) of the *Cryptosporidium parvum* CpABC3 for rational drug design

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Abstract

Its natural resistance to antiprotozoal chemotherapy characterizes the intestinal protozoan parasite *Cryptosporidium parvum* and the P-glycoprotein-related multidrug resistance proteins such as CpABC3 could be involved. In order to design and study specific inhibitors of the CpABC3 nucleotide-binding domain, a hexahistidine-tagged recombinant protein encompassing the N-terminal cytosolic NBD1 domain was overexpressed in *E. coli* and purified. The 45 kDa H6-NBD1 displayed intrinsic fluorescent properties consistent with the presence of two Trp residues in a hydrophobic environment. The binding of ATP and the fluorescent analogue TNP-ATP produced a dose-dependent quenching as well as progesterone and the flavone quercetin. The extrinsic fluorescence of TNP-ATP was enhanced upon binding to H6-NBD1, which was only partially displaced by the natural substrate ATP. The recombinant protein hydrolyzed ATP ($K_m = 145.4 \pm 18.2$ μM), but ADP ($K_m = 4.3 \pm 0.6$ mM) and AMP ($K_m = 5.4 \pm 1.5$ μM) were also substrates. TNP-ATP is a competitive inhibitor of the catalytic activity ($K_i = 36.6 \pm 4.5$ μM), but quercetin and progesterone were not inhibitors, evidencing different binding sites. The recombinant *C. parvum* H6-NBD1 should be a valuable tool for rational drug design and will allow the discrimination between specific inhibitors of the catalytic site and molecules binding to other sites.

Keywords: *Cryptosporidium parvum*; Parasite; CpABC3; Nucleotide-binding domain; NBD1

1. Introduction

Cryptosporidium parvum is the causative agent of waterborne intestinal infections affecting mostly children especially in developing countries and immunosuppressed patients worldwide. This protozoan is remarkable by its intrinsic resistance to most of the available antiparasitic molecules and it has been suggested that one of the reasons was natural drug efflux involving the ATP-binding cassette transporters (ABC proteins) [1]. These transmembrane proteins mediate the ATP-dependent translocation of xenobiotics out of the cell [2] and many homologues have been described in parasites [3]. Three types of ABC proteins have been described in *C. parvum*, CpABC1, CpABC2 and CpABC3 and are produced in infected cell cultures [4]. While the two former are members of the MRP sub-family, the latter is related to the MDR sub-family and as such, also possibly involved in the efflux of xenobiotics [5]. The CpABC3 transporter has two membrane-spanning domains (TMD) and two cytosolic nucleotide-binding domains (NBD). Since biochemical purification of this potential drug target is hampered by the lack of continuous cultivation of this parasite, a heterologous expression system was used to overexpress and produce a histidine-tagged recombinant protein containing the N-terminal NBD1 of the CpABC3 transporter.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals were of analytical grade and purchased from Sigma–Aldrich (L'isle d'Abeau, France) except for ammo-

Abbreviations: H6-NBD1, hexahistidine-tagged NBD1; HECAMEG, 6-0-[(*N*-heptylcarbamoyl)methyl]-α-D-glucopyranoside; IPTG, isopropyl-1-thio-β-D-galactopyranoside; Pgp, P-glycoprotein; NBD, nucleotide-binding domain; TMD, transmembrane domain; TNP-ATP, 2′,3′-*O*-(2,4,6-trinitrophenyl)-ATP

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nium dihydrogen phosphate ($(NH_4)H_2PO_4$) which came from Merck (Darmstadt, Germany). The oligonucleotide probes were purchased from Proligo (Paris, France). The Taq polymerase was obtained from Eppendorf (Le Pecq, France). The pCRT7 NT-TOPO® expression vector, TOP10F′ and One Shot® BL21(DE3)pLysS competent cells came from Invitrogen (Cergy-Pontoise, France).

2.2. Parasites and DNA extraction

C. parvum oocysts obtained from calves experimentally infected were isolated as previously described [6] and stored at 4 °C in sterile water with penicillin–streptomycin. Prior to use, they were aseptized on ice with 5% sodium hypochlorite for 30 min, washed three times with sterile ultra pure water and the oocyst pellet was stored at −196 °C. The oocysts were thawed, the genomic DNA was extracted with a DNA purification kit (Promega, Charbonnières, France) and stored in aliquots at −20 °C.

2.3. Amplification

The DNA encoding a domain encompassing the CpABC3 (Gene bank accession no. AF315509) NBD1 was amplified using the following primers: forward 5′-TTTTTTGTCATTACTAACGC-3′ and reverse 5′-CTTTTGCTGGAATTATTAGA-3′. Amplification was performed with the following conditions: denaturation at 94 °C, annealing at 55 °C, elongation at 72 °C for 32 cycles, with a final elongation for 10 min.

2.4. Insertion into the expression vector

The PCR product with an expected size of 1013 bp was inserted into the linearized pCRT7 NT-TOPO expression vector. *E. coli* TOP10F′ cells were transformed with the ligation product and grown overnight on agar plates supplemented with ampicillin (100 μg/ml). The positive clones were grown in ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml)-containing Luria–Bertani (LB) medium and the plasmids were extracted with a Qiagen Miniprep kit (Courtabœuf, France). Correct recombinants were identified by restriction digests and sequencing.

2.5. Overexpression and protein purification

BL21(DE3)pLysS cells harboring the appropriate recombinant plasmid were grown at 37 °C in LB medium supplemented with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. Expression of the fusion protein was induced with 1 mM IPTG for 4 h. The cells were harvested by centrifugation at 5500 × *g* for 8 min, washed once in PBS in the same conditions and the cell pellet was stored at −80 °C until further use.

The pellet was thawed and lysed at 37 °C in denaturing conditions with 6 M guanidine HCl buffer for 30 min. After centrifugation 8 min at 5500 × *g*, the pellet was re-extracted in the same conditions. The pooled supernatants were sequentially applied to a Ni-NTA affinity column for 30 min on a rotating wheel. The column was washed twice with denaturating washing buffer (6 M urea in 50 mM phosphate buffer, pH 7.8) and the recombinant protein was renatured by two washes in 50 mM phosphate buffer pH 8 containing 0.5 M NaCl. The column was then washed three times with 50 mM Tris pH 8 containing 0.5 M NaCl, 20 mM imidazole and 0.02% (v/v) HECAMEG. The H6-NBD1 was eluted with the same buffer containing 250 mM imidazole and 1 ml fractions were collected in microtubes. The fractions containing the eluted protein were pooled and dialyzed overnight at 4 °C against 50 mM Tris pH 6.8 with 100 mM KCl, 0.02% HECAMEG and 20% glycerol with one buffer change. The purified H6-NBD1 was stored in aliquots at −80 °C. Protein fractions were analyzed by electrophoresis with 4–12% Nupage® polyacrylamide gels (Invitrogen, Cergy-Pontoise, France) and protein concentration was determined by a modified Bradford method with the Roti®-nanoquant protein assay kit (Carl Roth, Karlsruhe, Germany).

2.6. Fluorescence assays

Fluorescence experiments were performed in triplicates on a Perkin-Elmer LS-3B spectrofluorimeter in a 1 cm-path quartz microcuvette. The recombinant protein was diluted at a concentration of 0.25–0.5 μM in 390 μl of 50 mM Tris pH 6.8, 100 mM KCl, 0.02% HECAMEG. The ligands were added in 10 μl and the mixture was incubated for 30 min at 20 ± 2 °C. Intrinsic fluorescence emission was measured upon excitation at 295 nm and spectra were scanned between 310 and 400 nm. Extrinsic fluorescence of TNP-ATP was measured upon excitation at 408 nm and spectra were scanned between 520 and 600 nm. The binding of nucleotide analogues was assayed either by quenching of the intrinsic fluorescence at 328 nm or by enhancement of the extrinsic fluorescence at 545 nm. Controls were made in the same conditions with the last dialysis buffer to eliminate any interference due to the remaining imidazole. The hydrophobic modulators progesterone and quercetin (3,5,7,3′,4′-pentahydroxy flavone) were dissolved as stock solutions in DMSO and controls included the same solvent concentration, not exceeding 2.5%. Statistics and curve fitting were made with the Prism 4 software program from GraphPad (San Diego, CA).

2.7. Kinetic analysis of the recombinant H6-NBD1 and inhibition studies

The V_{max} and K_m values of the H6-NBD1 for ATP, ADP and AMP were measured by incubating at 37 °C the purified recombinant H6-NBD1 with various concentrations of substrates in 50 mM Tris pH 7.5, 100 mM KCl, 4 mM $MgCl_2$ and 0.02% HECAMEG. The reaction products were measured in triplicates by HPLC on a 5 μm reverse phase Hypersil column (Interchim, Montluçon, France) with either 1.8% (v/v) (ATP) or 10% (v/v) (ADP and AMP) methanol in 25 mM $(NH_4)H_2PO_4$ as the mobile phase [7]. The injection volume was 20 μl, the flow rate was 0.5 ml/min and the effluent was monitored at 254 nm.