



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

Philippe Lawton, Michel Pélandakis, Anne-Françoise Pétavy, Nadia Walchshofer

► To cite this version:

Philippe Lawton, Michel Pélandakis, Anne-Françoise Pétavy, Nadia Walchshofer. Overexpression, purification and characterization of a hexahistidine-tagged recombinant extended nucleotide-binding domain 1 (NBD1) of the *Cryptosporidium parvum* CpABC3 for rational drug design. *Molecular and Biochemical Parasitology*, 2007, 152 (1), pp.101-107. 10.1016/j.molbiopara.2006.12.004 . hal-02111014

HAL Id: hal-02111014

<https://univ-lyon1.hal.science/hal-02111014>

Submitted on 26 Apr 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

Philippe Lawton*, Michel Pélandakis, Anne-Françoise Pétavy, Nadia Walchshofer

Université Claude-Bernard Lyon 1, ISPB, Faculté de Pharmacie, EA 3741 Lyon, France

Received 19 July 2006; received in revised form 8 December 2006; accepted 12 December 2006

Available online 20 December 2006

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 ($K_m = 145.4 \pm 18.2 \mu\text{M}$), but ADP ($K_m = 4.3 \pm 0.6 \text{ mM}$) and AMP ($K_m = 5.4 \pm 1.5 \mu\text{M}$) were also substrates. TNP-ATP is a competitive inhibitor of the catalytic activity ($K_i = 36.6 \pm 4.5 \mu\text{M}$), 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 water-borne 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-heptylcarbonyl)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

* Corresponding author at: Laboratoire de Parasitologie, ISPB, 8 Avenue Rockefeller, F-69373 Lyon Cedex 08, France. Tel.: +33 4 78772831; fax: +33 4 78777158.

E-mail address: lawton@univ-lyon1.fr (P. Lawton).

nium dihydrogen phosphate ((NH₄)H₂PO₄) which came from Merck (Darmstadt, Germany). The oligonucleotide probes were purchased from Prologo (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'-TTTTTTGTCATTA-CTAACGC-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 denaturing 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 MgCl₂ 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 (NH₄)H₂PO₄ 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.

ATPase inhibition assays were carried out in the same buffer and conditions by serially diluting the analogue TNP-ATP in various ATP concentrations. The type of inhibition and K_i were computed by non-linear regression using the GraphPad Prism 4 software program.

3. Results

3.1. Overexpression and purification of H6-NBD1

Fig. 1 shows that H6-NBD1 was overexpressed upon IPTG induction as a major component of 45 kDa of the bacterial lysate. In order to obtain a soluble protein, it was necessary to use denaturing conditions followed by on-column renaturation and elution with 250 mM imidazole. About 1 mg of recombinant domain was routinely produced from 800 ml cultures.

3.2. Fluorescence studies

The H6-NBD1 protein displayed a maximum emission peak at 328 nm upon excitation at 295 nm. Denaturation by urea led to a considerable red shift to 370 nm, evidencing a correct refolding

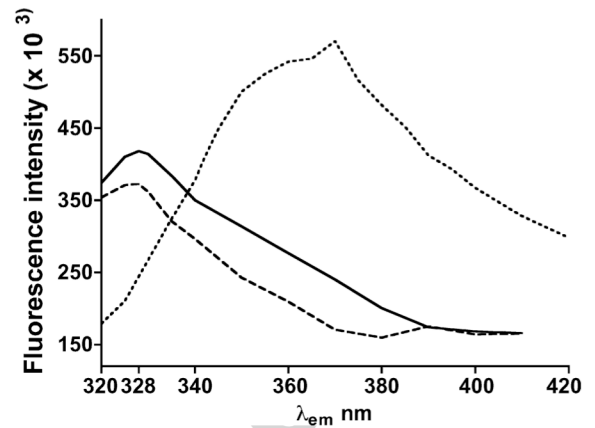
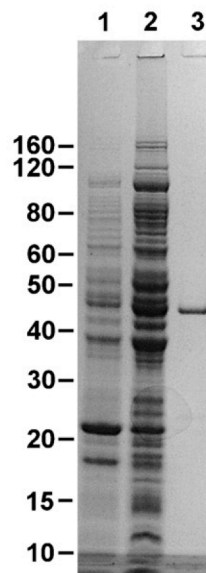


Fig. 2. Intrinsic fluorescence of H6-NBD1. The emission fluorescence spectra of 0.5 μ M H6-NBD1 were recorded after excitation at 295 nm (solid line), in the presence of 200 μ M ATP (broken line) and after dilution in 8 M urea (dotted line).

of the recombinant protein during preparation (Fig. 2). Addition of the substrate ATP produced only a low quenching in the millimolar range (Fig. 3), whereas the fluorescent analogue TNP-ATP strongly interacted with the recombinant protein and

MKPLEVEGAESHVHVVDNEDVEGNKTFEKSNDLGENDNIENEIKFEAKFSLHNFVKVIGFIMSVLSGISIPLFLLYFGLALQKIPLTPNYTEDQYNDFMRYIYYLIGVTLNSFFSGWLSIAIFESLAEFVKDIKRESFYILQMDWDHRD
 NDCGSVSSKIIANCALIREGYGIKFSQLISNVSQFLFGFIVGFYRGWKMALVMSASLPVAAAGFLITKIHRSWGTTRQVYSKSGALAFETFNNIKLVKSYCLESIMFNKYKVITEVENVGQKASVFSFGIGIVSLVIFSSYSLGFWYGGVL
 VADSMDSGCTFFDDLKCFIVANVFSIFFVITNASIALGQSTPSLSGLVKGSAFELGKLFKKPKVRNQKESVKFKINGEFKFDVFSYPGTNKKIILKDFNLELQSGKTALIGSGCGKSSILKILRLYDPDKGKILLDGLNIKKYDLAFL
 REQITIVDQESKLFNDTIRQNIYGNENATEKEIENALRLSQAWDFVNSFRDGLLETLVGNEGGLLSGGQRQRIARAIIRNSSVILDEATSGLDVKTESLFTQEFQKHISDRGITVIMIAHRLQITGFADQLVLDTPNNGIRVVEQGSSE
 LATLSNGIYSNLILKLNESGLDQWTONKPLLTSSSDKTFQKLATFFYSNNSSKSIFFRQSVSSLESNLQGLCIAHKDTRDYSVMVDFNLKALSMKLPKVSSFKLLLLKKDHFLLILGIISASVQGATFPLMGYLGKFTVSGVLPSELV
 RTETGKYSLYFLAAILIPLTFAQNSFLQISGERLIKRLRAECFYSMLYQDIKFHEQPNQSAIKLCEVLAEDTRLTKSLVGENIGLYTQNIIVTVLLGLTISFTSSLELTVILGFFIPLIPTGYAQSKVIGKSTNRDPENTNSEGSRKQSIHYL
 QEVLMHTIILKFLNQDPIKKYRRSTRYEYFKGTMDSHILGLCWGFGQAIQNTAQSGFLWYSGKMTVEKKIGLGEVLQVITLVILTAASVCRSQIYATDKKKAKVSANKIFSYIDRDPVIRNKKCFVSIINERCLKDFSLARDKRSKSIDGKMK
 TKLQRLGKFIKAREFATITPNKLDNLLARGDPNTYDSEEIELRKGEIVFKNVFFSYSDNIDNLVLSRVSAINSGEFVAVVGKSGSGKSTIFELLERFYPISSQFSEETRNEKIDQYFGERSSILIDSIDLNDLDVGKLRSSISYVQQNPILFSG
 TIQENILLGRMDASMEVEEATKMAQAYDFIMDLPEGFETKVGEGETELSVGQKQRINLARAFIKNSPVLILDEPTASLDIENEVFIKMSIYSYKKNSTILLITHRLQTIQNCDRIFLLGEDPENGRTISEQGTHEVSYKASVPEKILQE

(A)



(B)

Fig. 1. (A) Aminoacid sequence of CpABC3 with the expressed extended NBD1 domain. The lines indicate the primers, the two NBD are in boxes and the tryptophan residues involved in intrinsic fluorescence are printed in white. The A and B Walker's motifs are located in the ATP-binding site of each NBD. (B) Overexpression and purification of the extended H6-NBD1 from CpABC3. The production and purification of recombinant H6-NBD1 was visualized by electrophoresis. Lane 1: transformed *E. coli* crude lysate before induction; lane 2: same culture 4 h after induction with IPTG; lane 3: purified recombinant H6-NBD1. The migration of molecular weight markers is indicated at the left of the gel.

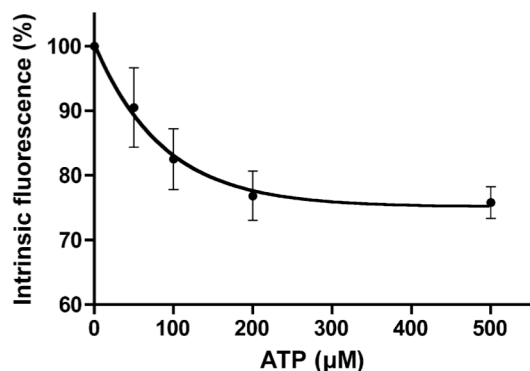


Fig. 3. Binding of ATP to H6-NBD1. The quenching of the intrinsic fluorescence of 0.25 μM recombinant domain was measured at 328 nm with increasing concentrations of ATP. The values are the mean ± S.D. of three separate experiments.

induced a 60% reduction at 100 μM (Fig. 4). Progesterone was less active and the flavonoid quercetin produced an equivalent quenching at 2 μM (Fig. 5).

The extrinsic fluorescence of TNP-ATP was monitored upon excitation at 408 nm. The binding of the analogue to the H6-NBD1 induced an increase in fluorescence with a maximum emission at 545 nm. The addition of ATP only minimally displaced the bound TNP-ATP (Fig. 6).

3.3. Enzyme activity

Incubation of the recombinant H6-NBD1 with ATP induced the formation of ADP, evidencing an ATPase activity with an optimum pH between 7.0 and 7.5 (not shown). Over time however, ADP was also hydrolyzed to produce AMP, which was eventually transformed into adenosine. The kinetic analysis showed that ADP ($K_m = 4.3 \pm 0.6$ mM) had a much lower affinity than ATP ($K_m = 145.4 \pm 18.2$ μM) and AMP ($K_m = 5.4 \pm 1.5$ μM), while the analogue TNP-ATP was a competitive ATPase inhibitor with a K_i of 36.6 ± 4.5 μM (Fig. 7).

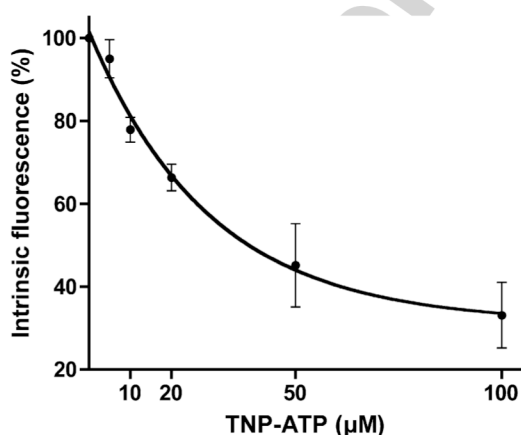


Fig. 4. Binding of TNP-ATP to H6-NBD1. The quenching of the intrinsic fluorescence of 0.25 μM H6-NBD1 was recorded at 328 nm with increasing concentrations of TNP-ATP. The values are the mean ± S.D. of three separate experiments.

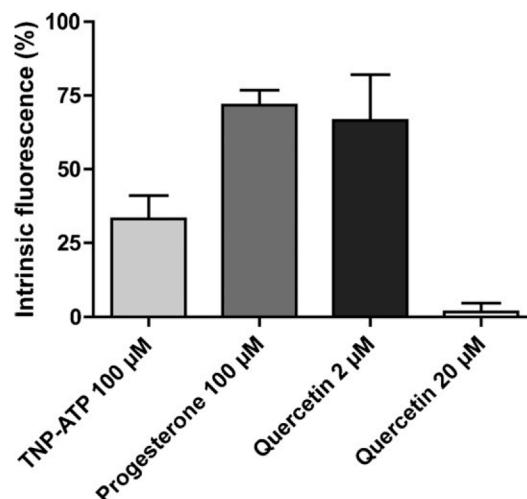


Fig. 5. Binding of TNP-ATP, progesterone and quercetin to H6-NBD1. The quenching of the intrinsic fluorescence of the recombinant protein was measured at 328 nm. The values are the mean ± S.D. of three separate experiments.

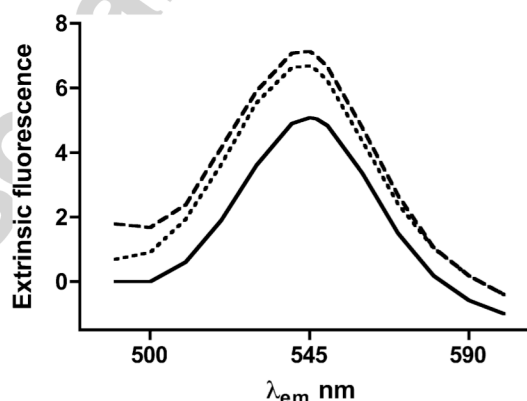


Fig. 6. Extrinsic fluorescence of TNP-ATP. The emission fluorescence spectra of 20 μM TNP-ATP were recorded after excitation at 408 nm (solid line), in the presence of 0.25 μM H6-NBD1 without (broken line) and with 1 mM ATP (dotted line).

4. Discussion

ABC transporters are members of a very large family of proteins involved in the efflux of xenobiotics across biological membranes. The natural resistance of *C. parvum* to most of the known antiprotozoal drugs is an obstacle to an efficient chemotherapy and it has been suggested that ABC transporters could be key effectors [1]. Although from the available evidence, it is likely that MRP proteins are more important than MDR proteins in mediating the active extrusion of xenobiotics in *C. parvum* [4], CpABC3 was chosen because the latter could be a target of cyclosporine, a drug with in vitro anticryptosporidial activity [1,5]. The production of the domain involved in the interaction with ATP should provide a useful tool for rational design of stereo-specific nucleotide inhibitors. The structural analysis of the *C. parvum* CpABC3 showed a typical structure of a P-glycoprotein with two TMD and two cytosolic NBD [3]. The extended NBD1 as defined from 337 to 673 contains two tryptophan residues: Trp509 located between the Walker A and the

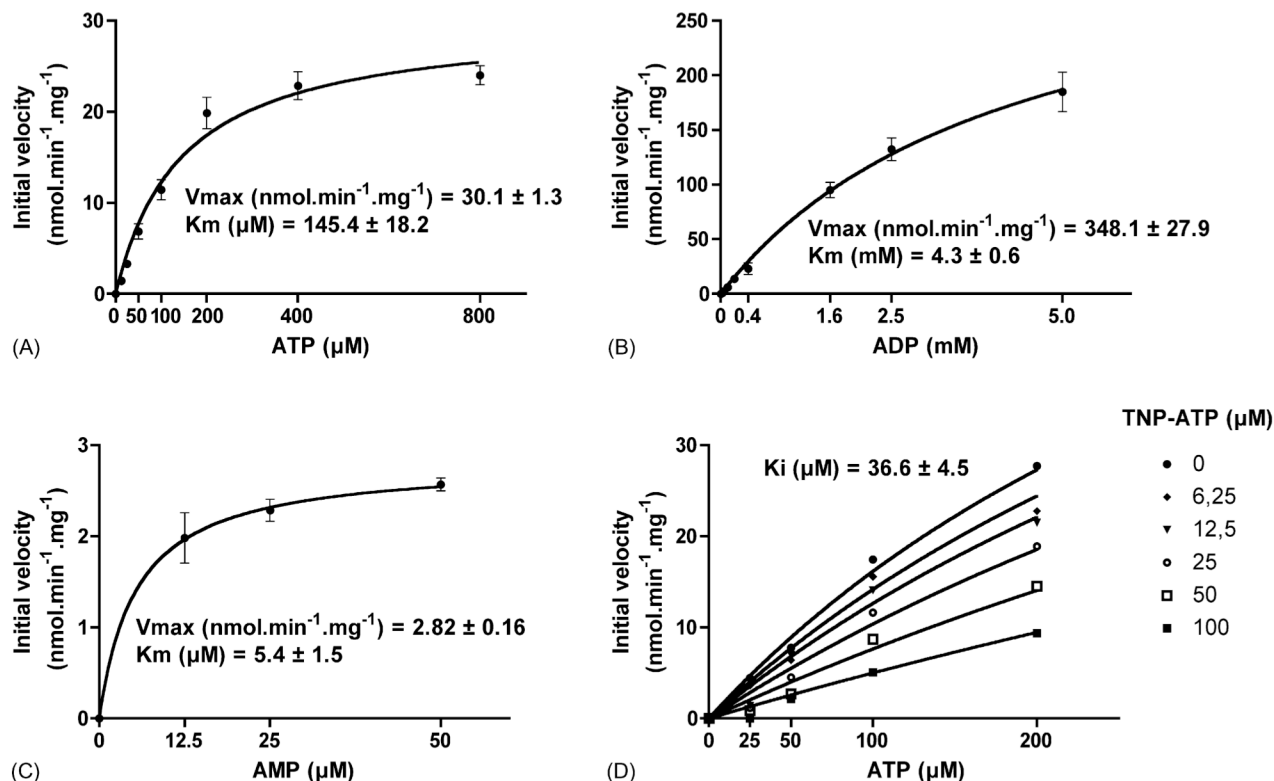


Fig. 7. Nucleotide hydrolysis by H6-NBD1 and inhibition by TNP-ATP. The recombinant protein was diluted at a final concentration of 2.5 μM H6-NBD1 and incubated at 37 °C, the enzyme kinetics were computed as described in Section 2. (A) Hydrolysis of ATP. The appearance of ADP was monitored with 1.8% methanol in the mobile phase. (B) Hydrolysis of ADP. The formation of AMP was measured with 10% methanol. (C) Hydrolysis of AMP. Adenosine was evaluated in the same conditions as panel (B). Results are the mean ± S.D. of triplicates from three separate experiments. (D) Competitive inhibition by TNP-ATP. Various analogue concentrations were made in each ATP concentration as described in Section 2 and the reaction was started by addition of the recombinant extended H6-NBD1 (v/v) at a final concentration of 2 μM. After a 10 min incubation at 37 °C, the samples were analyzed by HPLC. The results were computed from the mean ± S.D. of triplicates from three separate experiments.

ABC signature motifs, and Trp644 situated after the Walker B motif. NBD1 was preferred to NBD2, since the latter contains a small intron and hence was not usable for PCR amplification from genomic DNA. In order to facilitate its subsequent purification, the domain encompassing NBD1 was expressed and produced in *E. coli* as a N-terminal hexahistidine-tagged recombinant polypeptide.

The recombinant H6-NBD1 was successfully produced after induction with IPTG as a quite hydrophobic 44 kDa protein and it was thus necessary to use denaturing conditions to efficiently solubilize the recombinant domain. Refolding was successfully performed on-column using phosphate and Tris buffers and elution was achieved with imidazole. Dialysis was necessary to eliminate most of the imidazole, which interferes with the fluorescence tests. The protein was solubilized in the presence of the mild detergent HECAMEG and 20% glycerol, as previously reported [8–10]. Correct refolding was evidenced by the important red shift in the presence of urea, due to the unmasking of the buried Trp residues [9,11].

Intrinsic fluorescence upon excitation at 295 nm showed a characteristic emission peak at 328 nm due to buried Trp residues, similar to reported results on recombinant *Leishmania* NBD [9,11]. Addition of ATP produced a moderate quenching in the low millimolar range consistent with previous reports [9,12], whereas the fluorescent analogue TNP-ATP was about

50 times more efficient, as reported with the *Leishmania* NBD2 [11]. P-glycoproteins are known to bind steroid modulators [8] and progesterone exhibited a lower efficacy than TNP-ATP on intrinsic fluorescence. Flavonoids are also known to bind to various Pgp and are reported as efficient inhibitors of drug efflux [8–11,13–15], and isoflavones show some activity against *C. parvum* [16]. Indeed, quercetin was highly efficient, producing an almost complete quenching at 20 μM, and a 70% inhibition at 2 μM, consistent with the reported IC₅₀ of 7 μM [9]. These data suggest that the involved Trp residues are located near the binding sites of the flavones, rather than in the catalytic pocket, since the natural substrate ATP is a low quencher.

Extrinsic fluorescence measured with the fluorescent ATP analogue TNP-ATP was significantly enhanced at 545 nm in the presence of the recombinant H6-NBD1, a result consistent with the hydrophobic environment of the bound analogue [11], but without the blue shift observed by Baubichon-Cortay et al. [17], however. ATP lowered the fluorescence intensities at millimolar values, suggesting a stronger interaction of the TNP-analogue, probably due to the hydrophobicity of the TNP moiety [12]. No significant quenching was observed with progesterone and quercetin (not shown), confirming that these modulators do not directly interact with the catalytic site of the NBD.

The recombinant extended H6-NBD1 produced a measurable ATPase activity, comparable to the reported values for

similar expressed proteins [17–19], evidencing a correct refolding during preparation. Preliminary results had shown that the ADP concentration reached a maximum after 30 min, and that AMP and adenosine eventually appeared, indicating that ADP and AMP are also substrates for the recombinant protein. The kinetic studies showed that the K_m and V_{max} of the recombinant H6-NBD1 for ATP are comparable to the reported values for a yeast NBD1 [12,19] and that ADP has considerable less affinity towards the catalytic site than ATP or AMP, the latter being slowly hydrolyzed. It has been reported that ADP [17] and TNP-ADP [18] were also substrates for the catalytic site, but AMP hydrolysis was either not described or negative [19], even though the latter is a very poor inhibitor [18]. This is possibly due to the assay techniques, usually based on the production of phosphate from ATP [12,13,18] or on a cycling reaction [19]. TNP-ATP produced a dose-dependent competitive inhibition of ATP hydrolysis with a K_i about three times lower than reported for a purified Pgp [18], while progesterone and quercetin were ineffective (not shown). These results are consistent with published reports [8,9,11] where steroid modulators are thought to occupy vicinal binding sites and are not displaced by ATP. TNP-ATP binds in the ATP pocket, perhaps in a different orientation, thus stabilizing the ligand according to Rai et al. [12], and the TNP moiety could be responsible for the more efficient intrinsic fluorescence quenching of the analogue. Flavones only partially span the steroid and the ATP binding sites [8,9], thus explaining the high intrinsic quenching observed with quercetin, previously reported as a weak inhibitor of ATPase activity [13]. It is then highly possible that this modulator binds on another or many other sites on H6-NBD1, as reported with human MRP1 [20]. Flavonoids have been recently reported as inhibitors of *C. parvum* in vitro and quercetin had a moderate efficacy [21]. The inhibitory activity of these compounds against DNA topoisomerase and the synthesis of various HSPs, seems in favor of a broad or unspecific mode of action. The questions of the need for the TMD and the cooperativity between both NBD have aroused many times, the recombinant NBD having often extremely low ATPase activity [20,22]. Here, the recombinant H6-NBD1 displayed a similar catalytic activity, evidencing that both binding and hydrolysis of the adenine nucleotides occurred. These catalytic rates are however much lower than those reported with the purified native transporters [13,18], but correlation with the behavior of the native CpABC3 was impossible to assess, since activity was undetectable in oocyst crude extracts.

We report the production of a sensitive tool for the study and the rational design of specific inhibitors of the CpABC3 transporter. The recombinant H6-NBD1 can be used at low concentration for fluorescence quenching experiments either intrinsic or extrinsic as a preliminary screening and comparison with known reference molecules. Since it is fully functional, the activity of the recombinant protein on nucleotides and analogues may also be evaluated and should allow the synthesis and the selection of stereospecific inhibitors. Both kinds of assays will help to determine the interactions between the different parts of the catalytic site and permit to sort the molecules of interest according to their binding capabilities and their inhibitory activity. This efficient model will allow the selection of active

compounds before their further evaluation with time-consuming in vitro tests.

Acknowledgments

We wish to thank M. Naciri and R. Mancassola (INRA, Nouzilly, France) for providing the *C. parvum* oocysts, N. Alliolli for helpful discussions, J. Reynaud for providing quercetin and J.F. Sabot for technical facilities.

References

- [1] Perkins ME, Riojas YA, Wu TW, LeBlancq S. CpABC, a *Cryptosporidium parvum* ATP-binding cassette protein at the host–parasite boundary in intracellular stages. *Proc Natl Acad Sci USA* 1999;96:5734–9.
- [2] Chang G. Multidrug resistance ABC transporters. *FEBS Lett* 2003;555:102–5.
- [3] Klokouzas A, Shahi S, Hladky SB, Barrand MA, Van Veen HW. ABC transporters and drug resistance in parasitic protozoa. *Int J Antimicrob Agents* 2003;22:301–17.
- [4] Bonafonte MT, Romagnoli PA, McNair N, et al. *Cryptosporidium parvum*: effect of multi-drug reversing agents on the expression and function of ATP-binding cassette transporters. *Exp Parasitol* 2004;106:126–34.
- [5] Zapata F, Perkins ME, Riojas YA, Wu TW, LeBlancq SM. The *Cryptosporidium parvum* ABC protein family. *Mol Biochem Parasitol* 2002;120:157–61.
- [6] Lawton P, Mancassola R, Naciri M, Pétavy AF. Use of Percoll for the infection of cells in vitro with *Cryptosporidium parvum* oocysts. *J Microb Method* 2001;46:81–4.
- [7] Ghérardi A, Sarciron ME, Pétavy AF, Peyron F. Purine pathway enzymes in a cyst forming strain of *Toxoplasma gondii*. *Life Sci* 1999;65:1733–8.
- [8] Dayan G, Jault JM, Baubichon-Cortay H, et al. Binding of steroid modulators to recombinant cytosolic domain from mouse P-glycoprotein in close proximity to the ATP site. *Biochemistry* 1997;36:15208–15.
- [9] Conseil G, Baubichon-Cortay H, Dayan G, Jault JM, Barron D, Di Pietro A. Flavonoids: a class of modulators with bifunctional interactions at vicinal ATP and steroid-binding sites on mouse P-glycoprotein. *Proc Natl Acad Sci USA* 1998;95:9831–6.
- [10] Pérez-Victoria JM, Pérez-Victoria FJ, Conseil G, et al. High-affinity binding of silybin derivatives to the nucleotide-binding domain of a *Leishmania tropica* P-glycoprotein-like transporter and chemosensitization of a multidrug-resistant parasite to daunomycin. *Antimicrob Agents Chemother* 2001;45:439–46.
- [11] Perez-Victoria JM, Chiquero MJ, Conseil G, et al. Correlation between the affinity of flavonoids binding to the cytosolic site of *Leishmania tropica* multidrug transporter and their efficiency to revert parasite resistance to daunomycin. *Biochemistry* 1999;38:1736–43.
- [12] Rai V, Shukla S, Jha S, Komath SS, Prasad R. Functional characterization of N-terminal nucleotide binding domain (NBD-1) of a major ABC drug transporter Cdr1p of *Candida albicans*: uncommon but conserved Trp326 of Walker B is important for ATP binding. *Biochemistry* 2005;44:6650–61.
- [13] Conseil G, Decottignies A, Jault JM, et al. Prenyl-flavonoids as potent inhibitors of the Pdr5p multidrug ABC transporter from *Saccharomyces cerevisiae*. *Biochemistry* 2000;39:6910–7.
- [14] Comte G, Daskiewicz JB, Bayet C, et al. C-isoprenylation of flavonoids enhances binding affinity toward P-glycoprotein and modulation of cancer cell chemoresistance. *J Med Chem* 2001;44:763–8.
- [15] Tromprier D, Baubichon-Cortay H, Chang XB, et al. Multiple flavonoid-binding sites within multidrug resistance protein MRP1. *Cell Mol Life Sci* 2003;60:2164–77.
- [16] Stachulski AV, Berry NG, Low ACL, et al. Identification of isoflavone derivatives as effective anticryptosporidial agents in vitro and in vivo. *J Med Chem* 2006;49:1450–4.
- [17] Baubichon-Cortay H, Baggetto LG, Dayan G, Di Pietro A. Overexpression and purification of the carboxyl-terminal nucleotide-binding domain from mouse P-glycoprotein. *J Biol Chem* 1994;269:22983–9.

- [18] Liu R, Sharom FJ. Fluorescence studies on the nucleotide binding domains of the P-glycoprotein multidrug transporter. *Biochemistry* 1997;36:2836–43.
- [19] Jha S, Karnani N, Dhar SK, et al. Purification and characterization of the N-terminal nucleotide binding domain of an ABC drug transporter of *Candida albicans*: uncommon cysteine 193 of Walker A is critical for ATP hydrolysis. *Biochemistry* 2003;42:10822–32.
- [20] Berger AL, Ikuma M, Welsh MJ. Normal gating of CFTR requires ATP binding to both nucleotide-binding domains and hydrolysis at the second nucleotide-binding domain. *Proc Natl Acad Sci USA* 2005;102:455–60.
- [21] Mead JR, McNair N. Antiparasitic activity of flavonoids and isoflavones against *Cryptosporidium parvum* and *Encephalitozoon intestinalis*. *FEMS Microbiol Lett* 2006;259:153–7.
- [22] Sharom FJ, Liu R, Romsicki Y, Lu P. Insights into the structure and substrate interactions of the P-glycoprotein multidrug transporter from spectroscopic studies. *Biochim Biophys Acta* 1999;1461:327–45.