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Functional identification and subcellular localization of NAD kinase in the protozoan parasite *Giardia intestinalis*

Abstract

Nicotinamide adenine dinucleotide phosphate (NADP) is an essential biomolecule that participates in the redox homeostasis and synthesis of signaling compounds. NAD kinase (NADK) (EC 2.7.1.23 / 2.7.1.86) is the only enzyme capable of synthesizing NADP. This study offers an approach to the NADP metabolism in the parasite *Giardia intestinalis*, the etiological agent of giardiasis, a disease of high prevalence in America, Asia and Africa. Through bioinformatics tools a NADK enzyme candidate was identified, whose tertiary structure modeling demonstrated distinctive and universal motifs of characterized NADKs. The corresponding recombinant protein (His-GINADK) was expressed in *Escherichia coli* BL21 (DE3) and its partial purification was achieved by nickel affinity chromatography. Functional identification, which showed NADP synthesis, was completed through enzymatic assays evaluated by RP-HPLC. A cytosolic localization of the endogenous GINADK enzyme was observed in trophozoites throughout indirect immunofluorescence analysis, using polyclonal antibodies produced in mice by its immunization with the His-GINADK protein, purified from inclusion bodies. Taken together, our results contribute to the understanding of the NADP metabolism and the physiological role of NADK in the *Giardia* model.

Keywords: cytosolic localization; NADP synthesis; recombinant protein.

Identificación funcional y localización subcelular de la NAD quinasa en el parásito protozoario *Giardia intestinalis*

Resumen

El dinucleótido de adenina y nicotinamida fosfato (NADP) es una biomolécula esencial que participa en la homeostasis redox y en la síntesis de compuestos de señalización. La única enzima capaz de sintetizar NADP es la NAD Quinasa (NADK, EC 2.7.1.23 / 2.7.1.86). En este estudio se presenta un acercamiento al metabolismo del NADP en el parásito *Giardia intestinalis*, agente etiológico de la giardiasis, una enfermedad de alta prevalencia en América, África y Asia. Mediante herramientas bioinformáticas se identificó un candidato a NADK, cuya predicción a nivel de estructura terciaria mostró motivos característicos y universales de NADKs previamente caracterizadas. La proteína recombinante correspondiente (His-GINADK) se expresó en *Escherichia coli* BL21 (DE3) y se purificó parcialmente mediante cromatografía de afinidad a níquel. La síntesis de NADP por parte de la proteína His-GINADK se comprobó mediante ensayos enzimáticos evaluados por RP-HPLC. Adicionalmente, se determinó una localización subcelular citosólica en trofozoítos del parásito, empleando inmunofluorescencia indirecta y anticuerpos policlonales producidos en modelos murinos inmunizados con la proteína His-GINADK purificada a partir de cuerpos de inclusión. Los resultados obtenidos representan un avance en el entendimiento del metabolismo del NADP y de la importancia fisiológica de la NADK en el modelo de *Giardia*.

Palabras clave: localización citosólica; síntesis de NADP; proteína recombinante.

Identificação funcional e localização subcelular da NAD quinase no parasita protozoário *Giardia intestinalis*

Resumo

A nicotinamida adenina dinucleótido fosfato (NADP) é uma biomolécula essencial que participa na homeostase redox e na síntese de importantes compostos de sinalização. A NAD quinase (NADK) (EC 2.7.1.23 / 2.7.1.86) é a única enzima capaz de sintetizar o NADP. Este estudo apresenta uma abordagem do metabolismo do NADP no parasita *Giardia intestinalis* que causa giardíase, uma doença de alta prevalência na América, Ásia e África. Através de ferramentas de bioinformática, um candidato a enzima NADK foi identificado no parasita, cuja modelagem de estrutura terciária, demonstra motivos distintos e universais de NADKs caracterizadas. A correspondente proteína recombinante (His-GINADK) foi expressa em *Escherichia coli* BL21 (DE3) e a sua purificação parcial foi conseguida por cromatografia de afinidade com níquel. A identificação funcional, que mostrou a síntese de NADP, foi completada através de ensaios enzimáticos avaliados por RP-HPLC. Uma localização citosólica da enzima GINADK endógena foi observada em trofozoítos ao longo da análise de imunofluorescência indireta, utilizando anticorpos policlonais produzidos em camundongos, imunizados com a proteína His-GINADK purificada de corpos de inclusão. Em conjunto, nossos resultados contribuem para a compreensão do metabolismo do NADP e da importância fisiológica do NADK no modelo de *Giardia*.

Palavras-chave: localização citosólica; síntese de NADP; proteína recombinante.

Introduction

Giardia intestinalis (G. lamblia or G. duodenalis) is a gastrointestinal parasite of significant medical and veterinary importance that affects humans and several mammals [1]. The disease caused by the parasite is known as giardiasis, with approximately 200 million human cases worldwide [1], [2]. Although this disease has been treated with nitro-heterocyclic and benzimidazole compounds, the effectiveness of these drugs is not 100%. Furthermore, cases of recurrent reinfection and drug resistance have been reported in the last decades [3]–[6]. Consequently, the study and characterization of essential metabolic pathways and enzymes related to *G. intestinalis* survival and proliferation are imperative in the search for new therapeutic targets that may allow efficient disease control.

In addition to its clinical importance, *G. intestinalis* is considered a valuable cellular model. Analysis of ribosomal RNA sequences and conserved proteins have allowed its classification as one of the earliest diverging eukaryotes [1], [7]. The availability of its complete genome sequence and the fact that its entire life cycle can be reproduced *in vitro* make this parasite an ideal model to study different cellular processes [1], [8], [9]. One of these processes corresponds to the biosynthesis of nicotinamide adenine dinucleotide NAD(H) and its phosphorylated form NADP(H). These molecules are essential for living organisms, participating in more than 300 redox reactions from the energetic metabolism. In addition, they are used as substrates in protein modification and cell signaling processes [10]–[13]. NAD(H) is mainly involved in oxidative catabolic reactions, whereas its phosphorylated form, NADP(H), is considered the main reducing power within the cell. Therefore, NADP(H) is essential for reductive anabolic reactions and oxidative stress defense systems [12].

NADP(H) is used by glutamate dehydrogenase (GDH) from *G. intestinalis* to catalyze the interconversion between α-ketoglutarate and L-glutamate, playing an important role in carbohydrate metabolism and ammonia assimilation. Furthermore, *Giardia* uses the GDH enzyme along with alanine aminotransferase to dissipate the excess of reducing power to maintain an intracellular balance of NAD(P)/NAD(P)H through the conversion of pyruvate to alanine [14]. Moreover, the NADP(H) participates in the trophozoites' antioxidant system by reducing dissolved molecular oxygen (dO_2), nitric oxide and associated reactive oxygen, and nitrogen species (ROS and RNS) in the context of a natural infection [3].

Specifically, the NADH oxidase and NADH-dependent flavodiiron protein utilize NADPH as a source of electrons to reduce oxygen to water, which limits the production of superoxide and hydrogen peroxide ROS [15], [16]. The DT-diaphorase also uses NADPH as an electron donor to catalyze the partial reduction of dO_2 to superoxide and hydrogen peroxide [17]. The involvement of NADP(H) in the parasites antioxidant systems highlights the importance of the metabolic pathway of synthesis and regulation of this molecule. Even, it has previously been suggested that global regulators of resistance in *Giardia* may be identified through investigation of the transcription/activity of NAD(P)H synthetizing enzymes [3].

The only enzyme capable of synthesizing NADP(H) through the phosphorylation of NAD(H) is the NAD kinase (NADK, EC 2.7.1.23/86). This protein is responsible for regulating the intracellular balance of NAD(H) and NADP(H), which is indispensable for normal cell function [18], [19]. NADK has been studied in *Archaea*, *Bacteria*, and *Eukarya* and it has been determined that the deletion or mutation of the corresponding gene is lethal for some organisms, including pathogens such as *Mycobacterium tuberculosis* and *Salmonella enterica* [20], [21]. In eukaryotes, there are compartment-specific NADKs for different organelles: *Saccharomyces cerevisiae* has three NADKs, one of these located in the mitochondrial matrix and the other two in the cytoplasm [22], *Arabidopsis thaliana* has three NADKs located in cytosol, chloroplast, and peroxisome [23], whereas human has two NADKs located in cytosol and mitochondria [24], [25].

In protozoan parasites, the research on this enzyme has been limited to *Entamoeba histolytica* [26].

Consequently, the study of NADK in another pathogenic organism such as *G. intestinalis* may contribute to the identification of potential therapeutic targets by revealing structural and functional differences between the proteins of the host and the pathogen. This paper presents the identification of NADK from *G. intestinalis* (GINADK) and offers data on its catalytic activity and subcellular localization within the parasite.

Materials and methods

Bioinformatics approach

Identification of the NAD kinase gene and its encoded protein in *G. intestinalis*

A multiple sequence alignment was performed with 21 NAD kinases from the UniProtKB database, applying the MUSCLE algorithm [27], [28] and the CLC Sequence Viewer v8.0 program (QIAGEN, Aarhus, Denmark). The obtained amino acids consensus sequence was used as a query to search similar sequences in the genome of G. *intestinalis*, using the BLASTP algorithm from the GiardiaDB database [29]. The found sequence was validated by conserved domains analysis.

Sequence analysis and predictive modeling of *G. intestinalis* NAD kinase (GINADK)

Predictions of physicochemical properties, the existence of a signal peptide, and subcellular localization, were performed using the ExPASy Bioinformatics Resource Portal [30]. Secondary and tertiary structures were predicted by employing the GOR IV and I-TASSER servers [31]–[33], respectively. Visualization of the model and its superposition with the human NAD kinase structure was conducted with the *UCSF* chimera 1.10.1 program [34].

Experimental approach

In vitro culture of *G. intestinalis*

In vitro culture of trophozoites (WB clone C6) was performed following standardized protocols [35]. The parasites were used to prepare total protein extracts, as indicated below. Additionally, genomic DNA was prepared using the Easy-DNA™ Kit (Invitrogen™).

Construction of the PET100-GINADK recombinant vector

Genomic DNA from *G. intestinalis* WB was used as a template to amplify the *GINADK* gene using the following primers: Forward 5'-CACCATGGACCAGCTTGTGCT-3' and Reverse 5'-TTACTGATCGTCGCTGTCCGT-3'. The reaction was performed in a final volume of 25 µL in a MyCycler™ Thermal Cycler (Bio-Rad) applying the following conditions: 2.5 mM $MgSO_4$, 0.2 µM primers, 0.2 mM dNTPs, 1X PCR Buffer (20 mM Tris-HCl pH 8.8, 10 mM $(NH_4)2SO_4$, 10 mM KCl, 0.1% v/v Triton X-100, and 0.1 mg/mL BSA), 1.25 U *Pfu DNA polymerase* (Fermentas) and 100 ng of genomic DNA. The thermal cycle used was as follows: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 2 min, with a final extension at 72 °C for 15 min. The PCR product was cloned into the expression PET100/D-TOPO vector (Invitrogen) to obtain the PET100-GINADK recombinant plasmid, which enabled the expression of a recombinant protein fused to a hexa-histidine tag (6xHis) at the N-terminus. The identity of the PET100-GINADK was verified by DNA sequencing.

Expression and partial purification of the His-GINADK recombinant protein

The production of soluble protein to perform the functional identification was achieved by the implementation of a co-expression system with molecular chaperones.

Independent transformations of chemically competent *Escherichia coli* BL21 (DE3) cells with the PET100-GINADK and PG-KJE8 (Takara BIO, Inc.) plasmids were performed. Chaperone expression was induced in 1 L of cell culture in LB medium supplemented with ampicillin (100 µg/mL), chloramphenicol (50 µg/mL), and the chaperone-inducing agents' tetracycline (10 ng/mL) and arabinose (1 mg/mL). The cells were incubated at 37 °C under constant shaking until an OD of 0.6 at 600 nm was reached. Immediately, the His-GINADK protein was induced with 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) for 2 h at 25 °C with constant stirring. Cells were harvested by centrifugation at 4790 x g for 15 min at 4 °C. The cell pellet was resuspended in 10 mL of lysis buffer with the following composition: 50 mM NaH_2PO_4, pH 7.5, 250 mM NaCl, and 10 mM imidazole supplemented with a cocktail of protease inhibitors (Sigma P8340) and 1 mg/mL lysozyme.

The mixture was incubated for 30 min at 4 ºC under constant stirring. Subsequently, the extract was sonicated applying pulses of 15 s and pauses of 15 s for 10 min, using the Ultrasonic Processor (Sonics & Materials, Inc., vibra cell™), 130 Watt and a probe of 6 mm in diameter. The soluble and insoluble fractions were separated by centrifugation at 19000 x g for 20 min. The soluble protein fraction was subjected to purification using nickel affinity chromatography. Binding was allowed to proceed by incubation for 12 h on ice with Ni-NTA resin (Qiagen), previously equilibrated with lysis buffer. The supernatant with unbound proteins was collected by centrifugation at 3000 rpm for 3 min. Subsequently, washes were performed with buffer 50 mM NaH_2PO_4, pH 7.5, 250 mM NaCl, and 50 mM imidazole and elutions with buffer 50 mM NaH_2PO_4, pH 7.5, 250 mM NaCl, and 250 mM imidazole.

Protein visualization, detection and quantification

Recombinant protein expression and purification were analyzed by 10% SDS-PAGE electrophoresis followed by Coomassie blue R-250 staining and immunodetection by western blot. For immunodetection, proteins were electroblotted from the gel to polyvinylidene fluoride (PVDF) or nitrocellulose membranes using the wet electroblotting method of Towbin [36], applying 20 volts overnight (14 h) or 200 mA for 2 h. The membranes were blocked with TBST-milk 5% *w/v* solution. The primary antibody used was mouse anti-6xHis tag (Abcam) at a 1:3000 dilution; the secondary antibody was biotinylated anti-mouse IgG at a 1:2000 dilution (Sigma), and alkaline phosphatase was employed for developing (Promega). Visualization was made possible by the chromogenic substrates NBT (nitro blue tetrazolium) and BCIP (5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt). Protein quantification was performed using the Bradford method, with bovine serum albumin as the standard [37].

Enzymatic activity assays

Enzymatic assays were based on [35], [38], [39]. Approximately 2 µg of the His-GINADK recombinant protein were used per assay in reaction buffer (25 mM HEPES/KOH pH 7.5, 10 mM $MgCl_2$, 1.25 mM ATP, and 1.25 mM NAD^+) at 37 °C for 30 min. The reaction was stopped with 1.2 M $HClO_4$ and neutralized with 1 M K_2CO_3 [39]. The reactions were analyzed by RP-HPLC using a C18 column (25 cm long x 4.6 mm internal diameter and 5 mm particle size, Phenomenex), at room temperature with a flow of 1.5 mL/min, using the following elution gradient with buffer A (0.1 M potassium phosphate, pH 6.0) and buffer B (0.1 M potassium phosphate, pH 6.0, and 20% *v/v* methanol): 7 min buffer A (100%), 2 min buffer B (30%), 4 min buffer B (60%), 2 min buffer B (30%), and 2 min buffer A (100%). The analytes were detected at 254 nm using a diode array detector (DAD).

Isolation of the His-GINADK antigen from inclusion bodies

Previous studies have showed enhanced recovery of antigen and strong immune responses by isolating recombinant proteins from inclusion bodies [41]. Therefore, we applied the following protocol: overexpression of the recombinant protein was induced in an *E. coli* strain BL21 (DE3) cell culture transformed with the PET100-GINADK recombinant plasmid. The *E. coli* BL21 PET100/GINADK clone was diluted 1:50 in 200 mL of LB medium supplemented with ampicillin (100 µg/mL) and incubated at 37 °C under constant stirring until it reached an $OD_{600\,nm}$ of ~ 0.6.

Protein expression was induced with 1 mM IPTG for 14 h at 37 °C. The cell pellet was recovered by centrifugation at 4790 x*g* for 10 min. Cell lysis was performed following the previously described procedure.

Purification of inclusion bodies using denaturing agents followed a previously reported method [40]. In addition, purified samples were run in a preparative 16 x 19 cm 10% SDS-PAGE. Guided by a pre-stained molecular weight marker, the band containing the protein of interest was identified and excised. The band was pulverized, and samples were eluted with deionized water at 37 °C under constant stirring. Protein content in the supernatant was quantified with the Bradford method. Antigen purity was assessed by 10% SDS-PAGE.

Production of polyclonal anti-His-GINADK IgG antibodies

The His-GINADK recombinant protein purified from inclusion bodies was used as an antigen to generate polyclonal IgG sera in female 6-8-week-old BALB/c mice (*Mus musculus*). Three mice were injected intraperitoneally (one control mouse with PBS and two mice with the recombinant protein). The immunization scheme was as follows: mice were injected with antigen every 8 days for a total of four injections. The first inoculation consisted of a homogeneous mixture of antigen and Freund's complete adjuvant, while the three booster injections were homogeneous mixtures of antigen and incomplete Freund's adjuvant [42], [43].

Immunodetection of endogenous GINADK

Total protein extracts were prepared by parasites resuspension in Laemmli sample buffer and heating at 92 °C, 10 min. The protein extract was separated by 10% SDS-PAGE and a western blot protocol for immunodetection of endogenous GINADK was followed using different dilutions of the polyclonal anti-His-GlNADK IgG antibodies.

Immunofluorescence assays

G. intestinalis trophozoites resuspended in PBS (1500 parasites/µl) were fixed with 4% *w/v* paraformaldehyde for 10 min at room temperature. Permeabilization and blocking were performed with 0.5% Triton X-100 and 10% *v/v* fetal bovine serum in PBS for 30 min at 37 °C. Samples were incubated for 1 h at 37 °C with the primary IgG anti-His-GINADK antibody (1:50) or the anti-tubulin antibody (1:500). Incubation with the secondary anti-IgG antibody coupled to Alexa Fluor 488 (Abcam) (1:1000) was performed for 1 h in darkness at 37 °C. Nuclei were labeled with 4', 6-diamidino-2-phenylindole (DAPI) at 1 µg/mL. Images were observed and recorded with a Nikon C1 confocal plus ECLIPSE Ti fluorescence microscope and analyzed with the NIS elements AR software [44].

Results and Discussion

The bioinformatics approach allowed the identification of a putative GINADK sequence

NADP is a vital molecule involved in REDOX homeostasis of eukaryotes and prokaryotes. Therefore, the study of the enzymes involved in its synthesis is of great significance. NADKs exhibit noteworthy differences between eukaryotes and prokaryotes and have been postulated as therapeutic targets [45]. Identification of the enzyme responsible for producing NADP in *G. intestinalis* allows us to understand its function at the biochemical and molecular levels, providing the foundation to propose it as a therapeutic target that may facilitate control of giardiasis.

The search for the NADK candidate in the genome of *G. intestinalis* led to the identification of a genomic sequence of 1,662 bp (GL50803_17316) with a score value of 133 and an e-value of 4 x 10-35. This sequence is reported as encoding a putative protein (UniProt code: A8B3D3) of 553 aa with a theoretical molecular weight (MW) and an isoelectric point of 61,269 Da and 7,27, respectively.

Although the found open reading frame (ORF) was already annotated as NAD Kinase, there is no experimental evidence available to date. To confirm that this sequence effectively encodes a protein with the expected function, it was aligned against representative NADK sequences using BLASTP. In addition, a search for conserved functional domains was conducted. The results showed a 25 to 44% similarity between the found sequence and putative and previously characterized NADKs (Table 1).

Table 1. Identity percentages between GINADK, putative NAD kinases (*) and characterized enzymes.

Organism	% Identity	Score	E-value	Query cover
H. sapiens 1	34	215	$9x10^{-55}$	64
H. sapiens 2	25	130	0.002	25
M. musculus 1	31	196	$1x10^{-54}$	85
M. musculus 2	34	110	0.002	29
A. thaliana 1	34	209	$2x10^{-58}$	65
A. thaliana 2	36	236	$4x10^{-59}$	77
A. thaliana 3	44	30	$9x10^{-5}$	4
M. tuberculosis	31	78.6	$1x10^{-20}$	35
E. coli	25	134	$4x10^{-28}$	56
S. typhimurium	26	118	$2x10^{-28}$	53
L. monocytogenes	28	82	$7x10^{-16}$	31
A. fulgidus	30	76.6	$3x10^{-20}$	32
E. histolytica	24	71.6	$1x10^{-7}$	36
*T. cruzi**	23	63.5	$3x10^{-9}$	63.5
*L. braziliensis**	25	83.9	$7x10^{-10}$	36

The multiple sequence alignment showed that the GINADK hypothetical protein exhibits the characteristic universal motifs of NADKs (Figure 1, 2) including the GGDG motif involved in the binding of the donor phosphoryl group, as well as the NE/D motif and the glycine-rich conserved domain, involved in NAD(H) substrate binding. The CDD server revealed the presence of functional domains belonging to the diacylglycerol kinase catalytic domain superfamily. Specifically, a bifunctional NADH/NAD kinase domain and ATP NAD/inorganic polyphosphate NADK domains were found, possibly indicating that the protein has dual specificity toward phosphoryl acceptor and donor substrates. In general, the predicted presence of conserved functional domains agreed with the inferences made from the analysis of the GINADK primary structure (Table 2).

Table 2. Prediction of conserved functional domains in the putative GINADK sequence.

Domain Name	Access number	Description	Aminoacid position	E-value
PLN02935	PLN02935	Bifunctional NADH Kinase/NAD(+) Kinase	207-538	$2.53x10^{-65}$
nadF	COG0061	NAD Kinase [Coenzyme metabolism]	214-527	$2.53x10^{-63}$
NAD_kinase	Pfam01513	ATP-NAD Kinase	214-507	$6.01x10^{-47}$
ppnK	PRK01231	Inorganic polyphosphate/ATP-NAD Kinase	271-528	$3.20x10^{-35}$
PLN02727	PLN02727	NAD Kinase	208-534	$3.52x10^{-59}$
pnk	PRK14076	Inorganic polyphosphate/ATP-NAD Kinase	197-500	$1.07x10^{-35}$

Furthermore, the multiple sequence alignment allowed the identification of amino acids involved in NADK specificity toward the phosphoryl acceptor substrate (NAD+ and/or NADH).

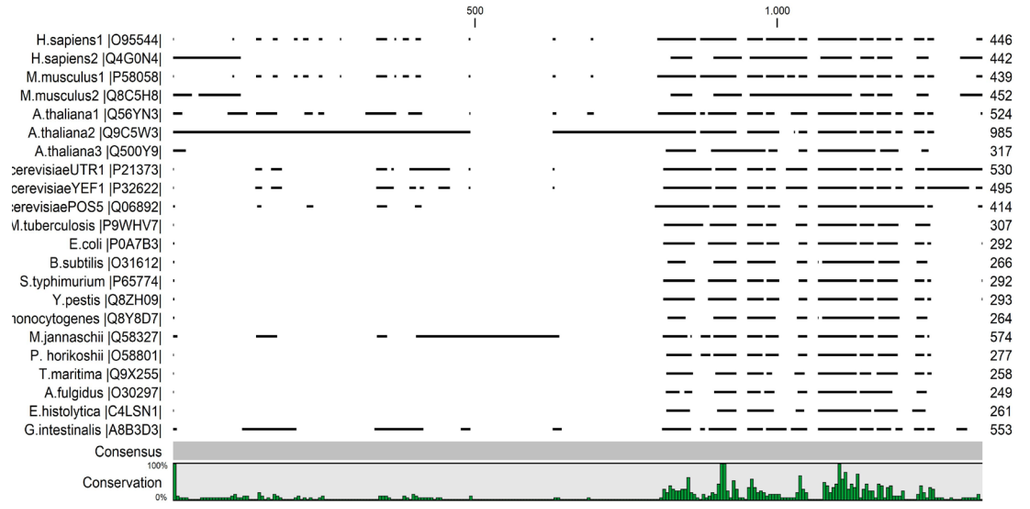

Figure 1. Multiple sequence alignment of 21 homologous NAD Kinase proteins from phylogenetically divergent organisms. The percentage of conservation is displayed throughout the sequence in bars. The numbers in brackets indicate UniProtKB codes of the NADK sequences.

The position corresponding to amino acid 187 of the NADK of M. tuberculosis has been previously identified as an essential residue in the specificity toward the phosphoryl acceptor substrate [46]. The presence of a polar amino acid or a glycine residue at this position, as opposed to a charged or hydrophobic residue, enables NADKs to have dual specificity. In the case of GINADK, the presence of glutamine (Q), a polar uncharged residue at this position (aa 410), suggests that GINADK may have the ability to phosphorylate the two types of substrates (NAD and NADH).

Additionally, the SignalP server was used to evaluate the possible presence of a signal peptide. The analysis did not reveal the presence of a signal sequence defining a possible localization. Different tools were used to predict the intracellular localization, but the results were ambiguous regarding nuclear or cytoplasmic localization (Table 3).

Table 3. Prediction of the subcellular localization of GINADK.

Server	Subcellular localization
SubLoc v1.0 [46]	Nuclear
Cello v. 2.5 [47]	Nuclear
ESLpred [48]	Cytoplasm
PSORTII [49]	52.2% Nuclear, 26.1% Cytoplasm
Euk-mPLoc 2.0 [50]	Cytoplasm

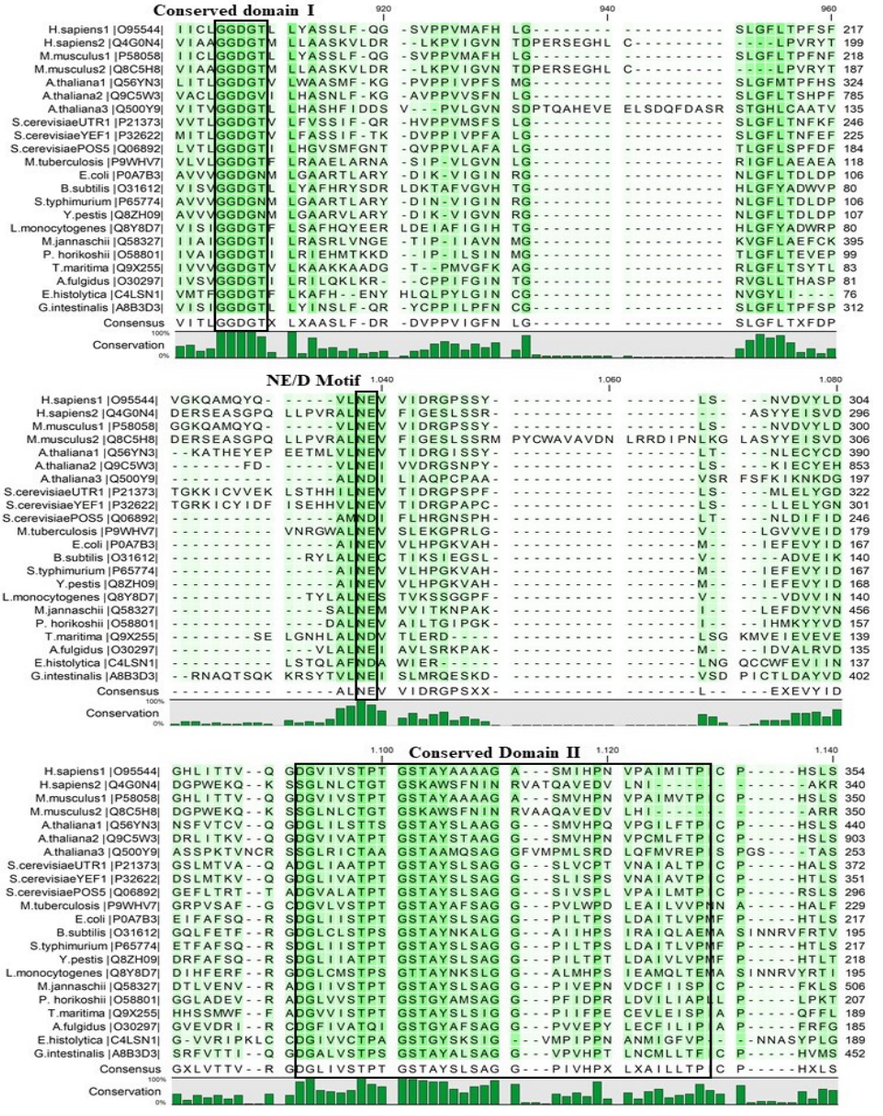

Figure 2. Identification of conserved motifs proposed as universal characteristics of NADKs in the putative sequence GINADK. The amino acids enclosed in black rectangles showed domain I (XGGDGXXL), domain II (DGXXXXTPXGSTXY), and the short NE/D domain. The arrow indicates the amino acid that determines the specificity toward the phosphate acceptor group (NAD/NADH). Amino acid conservation is indicated by the bars, the taller bars indicate a higher degree of conservation. The green, more intensely colored, areas show amino acids that are more than 87% conserved in all sequences.

The GINADK hypothetical protein displays typical structure of characterized NADKs

The secondary structure composition of the GINADK hypothetical protein was predicted to be 17.36% α helices, 24.77% β sheets, and 57.87% random coils. This secondary structure composition matches with the secondary structure obtained in the predictive model. Regarding the tertiary structure, the threading method was used to generate a three-dimensional (3D) predictive model of GINADK (Figure 3A), considering that its identity percentage with homologous proteins was approximately 30%. The results obtained from the I-TASSER server showed that the structural regions of two template proteins were mainly used: mitochondrial yeast NADK and cytosolic human NADK (PDB codes: 3afoA and 3pfnA, Figure 3B). The structural alignment performed between the GINADK predictive model and cytosolic human NADK (Figure 3C) revealed structural coincidences, with regions exhibiting extremely low RMSD values and a median RMSD value of 1.003 Å. There was also an additional region at the N-terminus of GINADK, absent in human NADK, characterized mainly by a helical configuration. This structural difference may be exploited with pharmacological proposes.

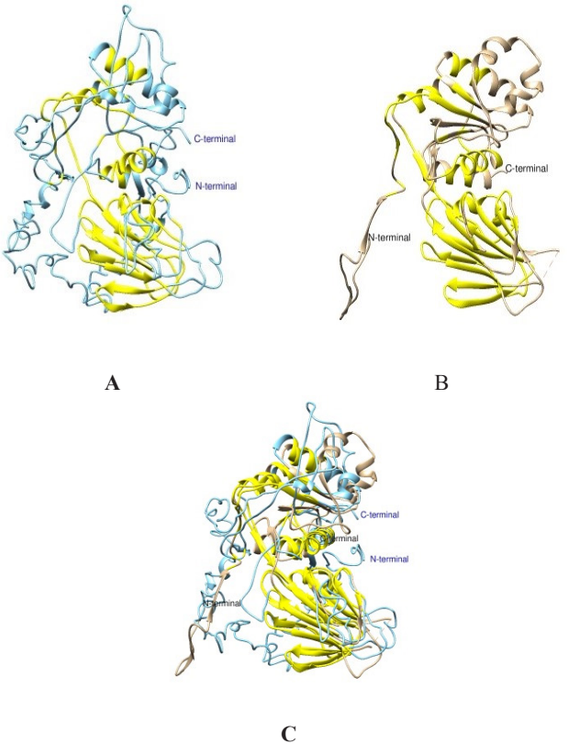

Figure 3. The putative GINADK protein displays the typical structure of characterized NADKs. A. Predictive model of the hypothetical GINADK protein. Statistical parameters of the predicted structure: C-score = -2.56, TM-score = 0.42 ± 0.14. B. Tertiary structure of human NAD kinase (PDB code: 3PFN). C. Structural superposition of the GINADK predictive model (blue) with the human cytosolic NADK (white). Regions with the lowest RMSD values between the two structures are showed in yellow. Images generated with *UCSF Chimera*.

The His-GINADK recombinant protein was partially purified by affinity chromatography

The His-GINADK protein was overexpressed in the soluble fraction of *E. coli* BL21 cells co-transformed with a plasmid encoding molecular chaperones. Protein expression was evidenced by the presence of an intense band of approximately 65 kDa, which matched the expected size, including the 6xHis tag. Western blot detection with the anti-6xHis antibody confirmed the identity of the recombinant protein (Figure 4). A purification protocol was undertaken from the soluble fraction using nickel affinity chromatography, which made it possible to isolate the His-GINADK recombinant protein, including the over-expressed molecular chaperones (Figure 4).

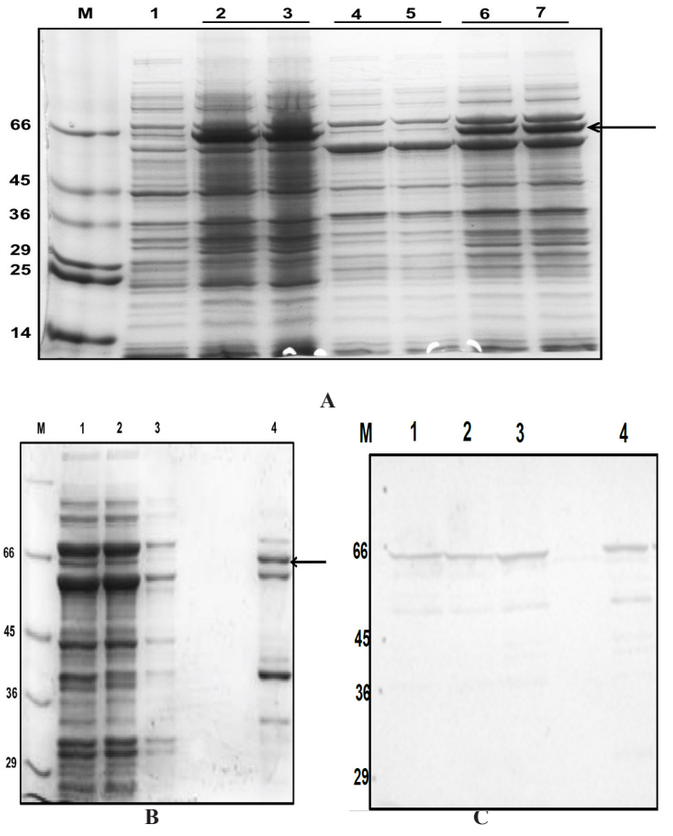

Figure 4. Expression and partial purification of the His-GINADK recombinant protein. A. Overexpression of the His-GINADK recombinant protein from two different clones (I and II) of *E. coli* BL21 (DE3). Lanes 1: Non-induced clone I. 2 and 3: induced clones with 1 mM IPTG. 4 and 5: induced clones with chaperone-inducers (arabinose and tetracycline). 6 and 7: induced clones with NADK and chaperone-inducers (IPTG, arabinose, and tetracycline). M: MW marker (kDa). Arrow indicates induction of the His-GINADK recombinant protein. 10% SDS-PAGE, Coomassie blue staining. B. The soluble fraction of a His-GINADK expression clone was applied to a nickel nitrilotriacetic acid (Ni-NTA) resin. Lanes 1: Soluble fraction. 2: Unbound fraction. 3: Wash fraction. 4: Eluate. M: MW marker (kDa). Arrow indicates the His-GINADK recombinant protein. 10% SDS-PAGE, Coomassie blue staining. C. Western blot performed with the primary anti-6xHis antibody and alkaline phosphatase developing system.

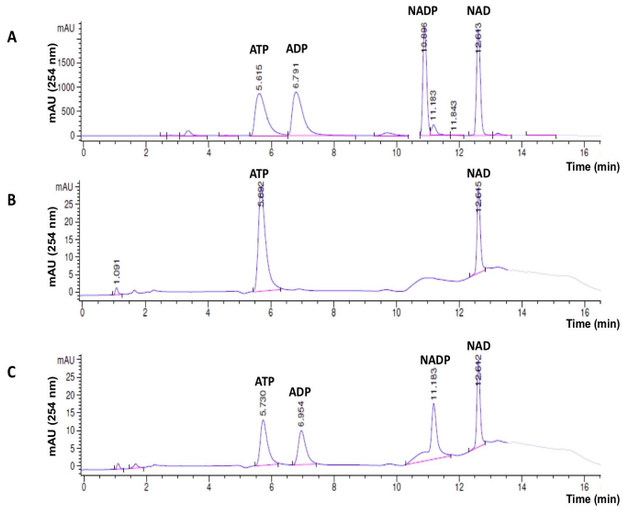

Figure 5. The His-GINADK recombinant protein synthesizes NADP. Registered chromatograms for: A. Mixture of NAD, NADP, ATP, and ADP reference standards (1 mM each). B. Negative Control: enzymatic assay performed with protein fraction from *E. coli* BL21 (DE3) culture induced for the chaperones and uninduced for GINADK instead of enzyme. C. His-GINADK recombinant protein assay: enzymatic assay performed with eluates from the purification procedure.

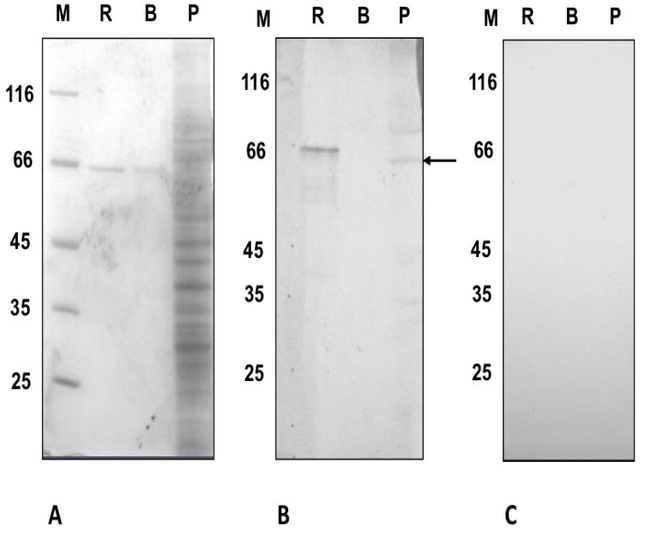

Figure 6. The polyclonal anti-His-GINADK antibodies detected the endogenous GINADK protein. A. Lanes R: His-GINADK recombinant protein (10 ng). B: Bovine serum albumin (10 ng). P: Total protein extract (from 2 x10^6 parasites). M: MW marker (kDa). 10% SDS-PAGE, Coomassie blue staining. B. Western blot performed on nitrocellulose membranes using the IgG anti-His-GINADK (1:200) (0.2 µg/µL) antibodies. The black arrow indicates the detected endogenous GINADK protein. C. Control serum (1:400) (0.2 µg/µL). Secondary antibody: biotinylated anti-IgG (1:2000). Alkaline phosphatase developing system.

The His-GINADK recombinant protein synthesizes NADP

To confirm the functionality of the recombinant protein, the partially purified His-GINADK enzyme was used to execute direct activity assays involving the production of $NADP^+$ from the substrates NAD^+ and ATP. The results were analyzed by reverse phase HPLC (Figure 5), assessing the presence of the chromatographic peak corresponding to NADP, which confirmed the catalytic activity of the His-GINADK recombinant protein. This result indicates the possible participation of the GlNADK protein in the parasite's energy metabolism and oxidative stress defense systems.

The endogenous GINADK was detected in parasites total protein extracts

The polyclonal IgG antibodies produced in this study against the His-GINADK recombinant protein constitutes an important immunological tool that was used to confirm the existence of the NADK protein in *G. intestinalis*.

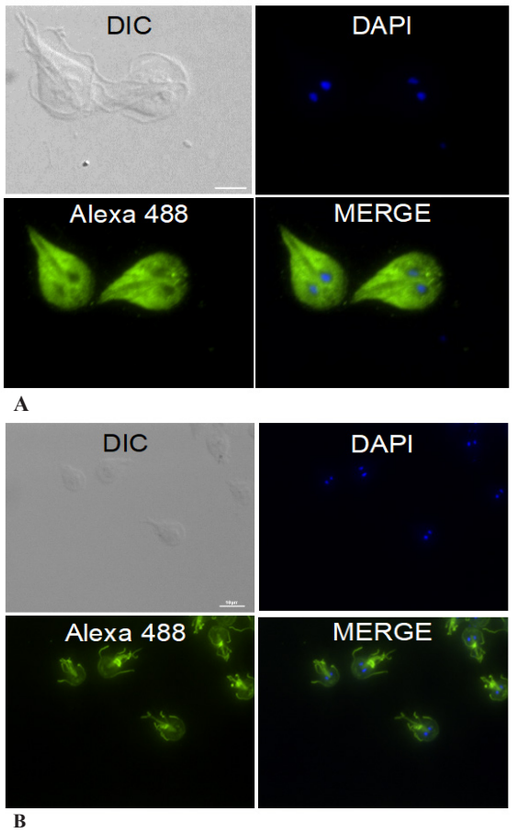

Figure 7. The endogenous GINADK protein shows a cytoplasmic location. Fixed trophozoites were labeled with the following primary antibodies: A. Polyclonal anti-His-GINADK (1:50). Bar scale 5 µm, 100X objective. B. Monoclonal anti-tubulin (1:500). Bar scale 10 µm, 60X objective. The secondary anti-IgG-ALEXA 488 antibody (1:1000) was used. Nuclear staining was carried out with DAPI (1 µg/mL). Microscopic analysis performed with a Nikon C1 confocal plus ECLIPSE Ti fluorescence microscope.

Immunodetection using trophozoite total protein extracts and the mentioned antibodies, clearly demonstrated the presence of a band of approximately 61 kDa in the extracts, corresponding to the endogenous GINADK, according with its expected theoretical MW (61,3 kDa) (Figure 6).

The endogenous GINADK exhibits a cytosolic location in trophozoites

The polyclonal anti-His-GINADK antibodies were used in immunofluorescence assays to determine the subcellular localization of the endogenous GINADK protein in the trophozoite stage of the parasite. The microscopic analysis revealed a cytosolic localization of the protein (Figure 7). The signal obtained was specific and, in contrast to DAPI signal (nucleus) and α-tubulin localization pattern (flagella and ventral disk), the GINADK location was clearly absent from nuclei, flagella, and ventral disk of the parasite.

In eukaryotic organisms, several NADK isoenzymes have been found, whose localization is restricted to the cytoplasm, mitochondria, or chloroplasts. For example, in *S. cerevisiae*, there are three isoenzymes: two localized in the cytoplasm and one in mitochondria [13]. Additionally, cytoplasmic and mitochondrial isoenzymes have been reported in humans [25], [52]. In the present study, a single cytoplasmic NADK was identified in G. intestinalis (Figure 7). This finding correlates with the fact that the metabolic and cellular signaling processes that require NADP mainly occur in this cellular compartment. Notably, *Giardia* sp. lack proper mitochondria, thus, it is not surprising that they do not possess a mitochondrial NADK.

Conclusion

Taken together, our results present the identification of the first NADK enzyme in *G. intestinalis*. The functional identity, expression, and subcellular location of GlNADK were confirmed through structural predicted models, enzymatic assays, and immunodetection, respectively.

Since NAD kinase is the unique enzyme able to synthesize NADP in prokaryotes and eukaryotes, it should play a pivotal role in NADP-dependent pathways of *Giardia*. Probably, this anaerobic human protozoan requires higher expression of this enzyme during colonization and invasion of the host, where it finds a high oxidizing environment. In this scenario, the parasite can employ this enzyme to synthesize the dinucleotide and carry out NADP(H)-dependent reactions to reduce reactive species (ROS and RNS). Our results represent an important advance to continue understanding the physiological role of the NADK protein in *G. intestinalis*.

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