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Research paper

Identification of a nicotinamide/nicotinate mononucleotide adenylyltransferase in *Giardia lamblia* (GlNMNAT)

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Abstract

Giardia lamblia is an intestinal protozoan parasite that causes giardiasis, a disease of high prevalence in Latin America, Asia and Africa. Giardiasis leads to poor absorption of nutrients, severe electrolyte loss and growth retardation. In addition to its clinical importance, this parasite is of special biological interest due to its basal evolutionary position and simplified metabolism, which has not been studied thoroughly. One of the most important and conserved metabolic pathways is the biosynthesis of nicotinamide adenine dinucleotide (NAD). This molecule is widely known as a coenzyme in multiple redox reactions and as a substrate in cellular processes such as synthesis of Ca^{2+} mobilizing agents, DNA repair and gene expression regulation. There are two pathways for NAD biosynthesis, which converge at the step catalyzed by nicotinamide/nicotinate mononucleotide adenylyltransferase (NMNAT, EC 2.7.7.1/18). Using bioinformatics tools, we found two NMNAT sequences in *Giardia lamblia* (*glnmnat-a* and *glnmnat-b*). We first verified the identity of the sequences *in silico*. Subsequently, *glnmnat-a* was cloned into an expression vector. The recombinant protein (His-GlNMNAT) was purified by nickel-affinity binding and was used in direct *in vitro* enzyme assays assessed by C18-HPLC, verifying adenylyltransferase activity with both nicotinamide (NMN) and nicotinic acid (NAMN) mononucleotides. Optimal reaction pH and temperature were 7.3 and 26 °C. Michaelis–Menten kinetics were observed for NMN and ATP, but saturation was not accomplished with NAMN, implying low affinity yet detectable activity with this substrate. Double-reciprocal plots showed no cooperativity for this enzyme. This represents an advance in the study of NAD metabolism in *Giardia* spp.

Keywords: *Giardia lamblia*; NMNAT; NAD metabolism; Enzyme activity.

1. Introduction

Nicotinamide adenine dinucleotide (NAD) is a key molecule in cellular metabolism [1]. Its central role as a coenzyme in REDOX reactions has been known since its function as an acceptor and donor of hydride ions was established [2], which is an essential characteristic for the maintenance of cellular REDOX balance and energy production. In addition, NAD is used as substrate in non-REDOX reactions, e.g., deacetylation by sirtuins [3], mono and poly-ADP-ribosylation [4,5] and the synthesis of key second messengers in Ca^{2+} mobilization such as nicotinic acid-adenine dinucleotide phosphate (NAADP), cyclic ADP-ribose (cADP), ADP-ribose (ADPR) [6] and O-acetyl-ADP-ribose (OAADPR) [7]. These non-REDOX reactions are part of fundamental cellular processes, e.g., gene expression regulation, DNA repair, chromatin stability, calcium mobilization, circadian rhythm control and cell death [2,8]. When used as a coenzyme in oxidation–reduction reactions, NAD exists in its oxidized form NAD^+ and in its reduced form NADH, maintaining a constant concentration. However, in non-REDOX reactions

Abbreviations: QAPRT, quinolinic acid phosphoribosyltransferase; NAPRT, nicotinic acid phosphoribosyltransferase; NAMPRT, nicotinamide phosphoribosyltransferase; NRK, nicotinamide riboside kinase; NMNAT, nicotinamide/nicotinic acid mononucleotide adenylyltransferase; NAD synthetase, EC. 6.3.5.1; QA, quinolinic acid; NA, nicotinic acid; NAM, nicotinamide; NR, nicotinamide riboside; NAMN, nicotinic acid mononucleotide; NMN, nicotinamide mononucleotide; NAAD, nicotinic acid adenine dinucleotide; NAD, nicotinamide adenine dinucleotide.

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in which NAD undergoes chemical breakdown, it is consumed, reducing its concentration.

Given this scenario, the cell must have NAD biosynthetic pathways to maintain a constant source of this molecule. There are two main NAD biosynthetic pathways (Fig. 1): one *de novo* pathway from quinolinic acid (QA) and a salvage pathway from nicotinamide (NAM), nicotinic acid (NA) and nicotinamide riboside (NR). Both the *de novo* pathway and the salvage pathway converge in NMNAT catalytic activity, which involves the Mg^{2+}-dependent reversible condensation [9,10] of ATP with NMN (nicotinamide mononucleotide) or NAMN (nicotinate mononucleotide), producing NAD (nicotinamide adenine dinucleotide) or NAAD (nicotinate adenine dinucleotide), respectively [11]. Ubiquitous in nature, this enzyme has been found in all organisms from all three kingdoms of life (archaea, eubacteria, eukaryotes) in which it has been sought, justifying the importance of its function in the cell.

The present study aims to examine NAD metabolism in the parasitic protozoan pathogen *Giardia lamblia* (also called *Giardia intestinalis* or *Giardia duodenalis*) through the identification, cloning, expression, purification and enzymatic validation of nicotinamide/nicotinate mononucleotide adenylyltransferase (NMNAT), which was identified using bioinformatics tools.

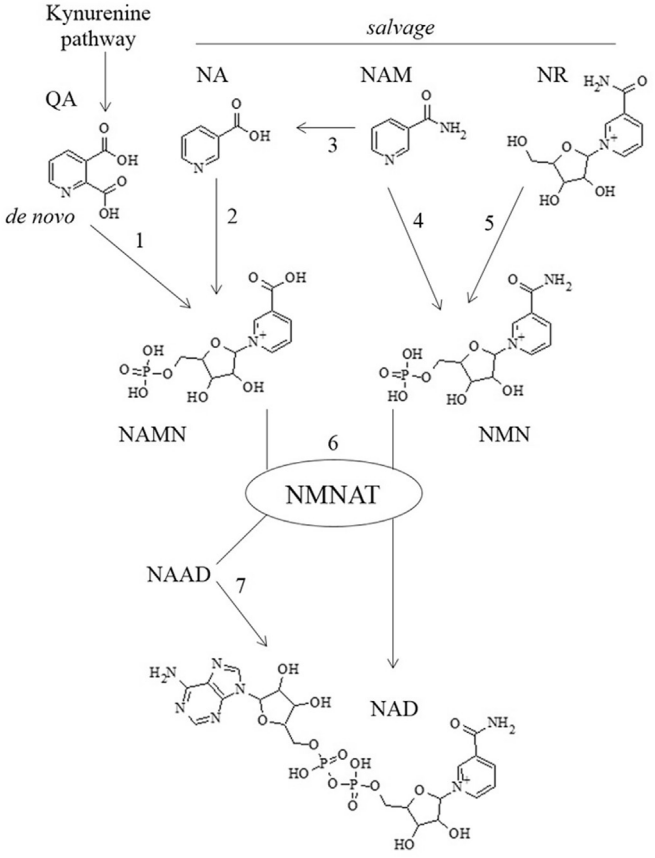

Fig. 1. **Representation of NAD biosynthesis**. Enzymes involved are denoted by numbers: 1. QAPRT (quinolinic acid phosphoribosyltransferase, EC. 2.4.2.19). 2. NAPRT (nicotinic acid phosphoribosyltransferase, EC. 2.4.2.11). 3. Nicotinamidase (EC. 3.5.1.19). 4. NAMPRT (nicotinamide phosphoribosyltransferase, EC. 2.4.2.12). 5. NRK (nicotinamide riboside kinase, EC. 2.7.1.22). 6. NMNAT (nicotinamide/nicotinic acid mononucleotide adenylyltransferase, EC. 2.7.7.1/18). 7. NAD synthetase (EC. 6.3.5.1). Images drawn with ChemSketch Freeware (ACD/Labs v14.01).

2. Materials and methods

2.1. Identification and bioinformatic validation of the putative sequence

Multiple sequence alignment of 16 NMNAT sequences reported in the UniProtKB database (http://www.uniprot.org/) was performed using the MUSCLE [12,13] algorithm in the CLC Sequence Viewer v6.9 (Additional Alignments plugin v.1.4.5) program. The resulting consensus sequence of 244 aa was used to search the GiardiaDB [14] (http://giardiadb.org/giardiadb/) using the BLAST algorithm. The sequences with the highest score and lowest E-value were subsequently validated by finding conserved domains and their putative function. Additionally, a 3-dimensional model of the putative protein was obtained (I-TASSER server [15,16]) to compare it with the structure of crystallized and reported NMNATs.

2.2. Culture of Giardia lamblia

Parasites (WB clone C6) were cultured in borosilicate tubes at 37 °C in TYI-S-33 medium (Diamond, 1978). Subsequently, the parasites were separated from the medium by centrifugation (1500 rpm, 10 min) and washed three times with cold sterile PBS. The washed parasites were resuspended in 6 mL of sterile PBS and counted in a Neubauer chamber. The parasites were centrifuged again, and the resultant pellet was stored at −20 °C to subsequently extract genomic DNA [17].

2.3. Genomic DNA extraction

Cells (7.5×10^7) were resuspended in 200 μL of sterile PBS. From these cells, genomic DNA was extracted using an Easy-DNA™ kit (Invitrogen). The extracted DNA was resuspended in 100 μL of TE buffer and stored at 4 °C. Purity and concentration were evaluated by a UV–Vis spectrophotometer (Spectronic Genesys 5) at 260 and 280 nm.

2.4. PCR amplification of the coding region

The NMNAT coding region of *Giardia lamblia* was amplified from genomic DNA using the following primers: 5'-caccATGCCCTGTCCGCCGGT-3′(forward) and 5′-TTATTGGAAACTAGGGGGTG-3 (reverse). The amplification conditions were as follows: 3 mM $MgCl_2$, 0.5 μM primers, 0.2 mM dNTPs, 2.5 U of Taq polymerase (Amplitaq) and 100 ng of gDNA template. PCR was performed in a final volume of 25 μL in a GeneAmp® PCR system 2400 Perkin Elmer™ thermal cycler. The optimal thermal cycle used was