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Research article

Kinetic and oligomeric study of *Leishmania braziliensis* nicotinate/nicotinamide mononucleotide adenylyltransferase

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ABSTRACT

Nicotinamide adenine dinucleotide (NAD) is an essential coenzyme involved in REDOX reactions and oxidative stress defense systems. Furthermore, NAD is used as substrate by proteins that regulate essential cellular functions as DNA repair, genetic, and signal transduction, among many others. NAD biosynthesis can be completed through the *de novo* and *salvage* pathways, which converge at the common step catalyzed by the nicotinate/nicotinamide mononucleotide adenylyltransferase (NMNAT EC: 2.7.7.1/18). Here, we report the kinetic characterization of the NMNAT of *Leishmania braziliensis* (LbNMNAT), one of the etiological agents of leishmaniasis, a relevant parasitic disease. The expression and homogeneous purification of the recombinant 6xHis-LbNMNAT protein was carried out and its kinetic study, which included analysis of K_m, V_{max}, K_{cat} and the equilibrium constant (K_D) for both the forward and reverse reactions, was completed. The oligomeric state of the recombinant 6xHis-LbNMNAT protein was studied through size exclusion chromatography. Our results indicated the highest and lowest K_m values for ATP and NAD, respectively. According to the calculated K_D, the pyrophosphorolytic cleavage of NAD is favored *in vitro*. Moreover, the recombinant 6xHis-LbNMNAT protein showed a monomeric state, although it exhibits a structural element involved in potential subunits interaction. Altogether, our results denote notable differences of the LbNMNAT protein in relation to the human orthologs HsNMNAT1-3. These differences constitute initial findings that have to be continued to finally propose the NMNAT as a promissory pharmacological target in *L. braziliensis*.

1. Introduction

Leishmania braziliensis is one of the etiological agents of leishmaniasis, a set of relevant illnesses in tropical countries. Current control strategies depend on chemotherapy to treat infection and insecticides to reduce transmission [1]. Nowadays, effective vaccines for humans have not been developed [2]. The identification of new pharmacological targets and the implementation of rational therapeutic and long-lasting strategies, require research efforts focused on the molecular and biochemical characterization of the pathogen. In this sense, the study of the nicotinamide adenine dinucleotide (NAD) proves to be suitable given the essential functions that it performs.

NAD and its phosphorylated form (NADP) participate as coenzymes in energy metabolism and in oxidative stress defense systems. Additionally, NAD is used as a substrate by enzymes involved in cellular processes such as DNA repair and cellular death, calcium mobilization, circadian cycle and RNA stability, among others [3, 4]. In *Leishmania*, NAD participates in energy metabolism, anti-oxidative systems, proliferation, differentiation and cellular death processes through sirtuins [5, 6], whose characterization has been completed in *L. amazonensis* (LaSIR2) [7], *L. major* (LmSIR2) [8,9] and *L. infantum* (LiSIR2) [10].

The biosynthesis of NAD is carried out through the *de novo* and *salvage* pathways. Even though these routes employ different chemical precursors and intermediaries, both converge in the step catalyzed by the nicotinate/nicotinamide mononucleotide adenylyltransferase (NMNAT; EC: 2.7.7.1/18) [11], an essential enzyme [11, 12, 13, 14]. The NMNAT has been investigated in archaebacteria, bacteria, parasites, yeast, insects, plants and mammals. In humans three isoenzymes have been described (HsNMNAT1-3), which exhibit particular kinetic and oligomeric states [15].

A relevant aspect of the NMNATs refers to the preference for NAD precursors (NMN or NAMN). Even though the majority of NMNATs can employ both substrates, some of them possess a fixed selectivity. For instance, *Escherichia coli* has two isoenzymes: NadD and NadR, the former

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being 20 times faster when catalyzing the reaction employing NAMN as a substrate, while the NadR is 170 times faster adenylating NMN [16]. On their part, the NMNATs of archaebacteria prefer NMN as a substrate [17, 18, 19]. In *Saccharomyces cerevisiae* the isoenzymes ScNMNAT1-2 present differential selectivity, while in *Arabidopsis thaliana* (AtNMNAT) [20] and *Homo sapiens* (HsNMNAT1 and 3), NAD and NAAD are produced with similar efficiency [14].

NAD biosynthesis is considered as a therapeutic target against cancer, neurodegenerative diseases and pathogenic organisms [21, 22, 23, 24, 25, 26]. In this context, the characterization of the NMNAT of *L. braziliensis* (LbNMNAT) enzyme and the identification of differences in contrast to the human orthologs, comprise crucial points for the development of potential new control strategies of *Leishmania*.

The first experimental evidence on the NAD metabolism in *Leishmania* was reported by our research group through the identification, cloning and expression of the recombinant 6xHis-LbNMNAT protein [27]. In addition, protein-protein interaction (PPI) analysis based on the LbNMNAT enzyme revealed proteins involved in RNA binding, redox homeostasis, and translation [28].

In this study, the kinetic characterization for the forward and reverse reaction, the use of the NAMN as a substrate, as well as the evaluation of the oligomeric states of the 6xHis-LbNMNAT protein is shown. Our evidences denote differences among the LbNMNAT protein and the human orthologs HsNMNAT1-3, suggesting that the NAD biosynthesis in *Leishmania* could be manipulated as a pharmacological target.

2. Materials and methods

2.1. Expression of the recombinant 6xHis-LbNMNAT protein

The previously constructed pQE30-LbNMNAT plasmid [27] allows the expression of the LbNMNAT protein fussed with the 6xHis tag at the N-terminus, under the control of the T5 promoter. The recombinant plasmid was used to transform chemically competent *E. coli* M15 cells by a heat shock protocol. The resulting clones were pre-inoculated in LB media supplemented with 100 μg/mL of ampicillin and 50 μg/mL of kanamycin, ON at 37 °C with constant agitation. The pre-inoculums were diluted 1:50 in LB medium and incubated at 37 °C with constant agitation until an OD_{600} nm = 0.6–0.8. The induction of the recombinant 6xHis-LbNMNAT protein was carried out with IPTG (final concentration 0.5 mM) ON at 26 °C with constant agitation. The induced samples were analyzed by SDS-PAGE in discontinuous gels.

2.2. Preparation of soluble extracts of the recombinant 6xHis-LbNMNAT protein

The induced cells were collected by centrifugation at 8000 rpm for 10 min at 4 °C. The pellets were resuspended in lysis buffer (0.5 mg/mL lisozyme, protease inhibitor cocktail (Sigma P8340) (cocktail:extract, 1:400), 500 mM NaCl, 25 mM NaH_2PO_4 pH 8.0/NaOH, 10 mM imidazole, 2 mM $MgCl_2$, 1% V/V glycerol) adding 7 mL/g of wet cell weight. The resuspension was incubated 1 h at 4 °C with constant agitation, sonicated on ice (50 % amplitude, cycles: 15 s pulse, 45 s rest. Total sonication time: 3 min) and centrifuged at 14000 rpm for 30 min at 4 °C. Soluble and insoluble fractions were collected.

2.3. Purification of the recombinant 6xHis-LbNMNAT protein by IMAC

The recombinant protein was purified by immobilized metals affinity chromatography (IMAC). The soluble fraction (20 mL) was incubated with the Ni-NTA resin (Qiagen) (0.5 mL), previously equilibrated with binding buffer (500 mM NaCl, 25 mM NaH_2PO_4 pH 8.0/NaOH, 10 mM imidazole) for 1 h on ice with constant agitation (Qiagen). This mixture was centrifuged at 3000 rpm for 3 min at 4 °C and the supernatant (non binding proteins) separated from the precipitate, which was washed with 10 mL of wash buffer 1 (500 mM NaCl, 25 mM NaH_2PO_4 pH 8.0/NaOH, 10 mM imidazole). Then, a wash with 4 mL of wash buffer 2 was done (500 mM NaCl, 25 mM NaH_2PO_4 pH 8.0/NaOH, 30 mM imidazole). Then, the resin was resuspended with 2 mL of wash buffer 3 (500 mM NaCl, 25 mM NaH_2PO_4 pH 8.0/NaOH, 75 mM imidazole) and a chromatography column was packed by gravity, collecting the corresponding wash. Finally, 4 elutions with 0.5 mL of elution buffer were carried out (500 mM NaCl, 25 mM NaH_2PO_4 pH 8.0/NaOH, 300 mM imidazole). Each eluate was dialyzed against 1 L of dialysis buffer (500 mM NaCl, 25 mM NaH_2PO_4 pH 8.0/NaOH, 0,5 mM DTT) ON at 4 °C. The dialyzed samples were supplemented with 1 mM DTT and 10 % V/V glycerol and stored at -80 °C. The purification steps were analyzed by SDS-PAGE in discontinuous gels.

2.4. Purification of the recombinant 6xHis-LbNMNAT protein by SEC

The homogeneous purification of the recombinant 6xHis-LbNMNAT protein was achieved by size exclusion chromatography (SEC), injecting 500 μg of IMAC's eluates in a Superdex 200 10/300 GL column (GE Healthcare Life Sciences), previously equilibrated in equilibrium buffer (300 mM NaCl, 50 mM NaH_2PO_4 pH 8.0/NaOH, 0.5 mM DTT). The chromatography was carried out employing the Äkta Purifier system (GE Healthcare Life Sciences) at 4 °C with a flow of 0.5 mL/min. The collected fractions were monitored by absorbance at 280 nm, SDS-PAGE in discontinuous gels and coupled activity assays. Upon confirmation of the enzymatic activity of the eluted recombinant 6xHis-LbNMNAT protein, the chromatogram was used to calculate the molecular weight of the protein in order to infer its oligomeric organization. Consequently, a calibration curve was constructed using molecular weight standards, which were subjected to the same chromatographic protocol. Lysozyme (14 kDa), carbonic anhydrase (29 kDa), BSA (66 kDa) (GE Healthcare Life Sciences) and the recombinant 6xHis-HsNMNAT1 protein (32 kDa, which was purified in this study (Supplementary Figure 1)) were all mixed and applied into the SEC column. The void volume was determined with blue dextran (Sigma). The partition coefficient (K_{av}) of each standard was used to plot the corresponding calibration curve (K_{av} *vs* Log MW). The equation of the curve was used to determine the molecular weight of the recombinant 6xHis-LbNMNAT protein.

2.5. Coupled and direct enzymatic assays

The following protocols were standardized based on previous reports [15, 29]. In the coupled assays, a mixture of reaction that contained 40 mM ethanol, 25 mM HEPES/KOH pH 7.4, 10 mM $MgCl_2$, 1.25 mM ATP (Sigma), 1.25 mM NMN (Sigma) and 10 U of alcohol dehydrogenase (ADH) (Sigma) was used. This mixture was dispensed in plastic cuvettes and, upon adding 2.5 μg of the recombinant 6xHis-LbNMNAT protein; the reaction was initiated. The assays were carried out in a volume of 1 mL at 20 °C, measuring absorbance at 340 nm for 5 min. In the direct enzymatic assays, a mixture of reaction that contained 25 mM HEPES/KOH pH 7.4, 10 mM $MgCl_2$, 1.25 mM ATP and 1.25 mM NMN was used. Additionally, direct assays were carried out with 1.25 mM ATP and 1.25 mM NAMN (Sigma) to evaluate the mononucleotide specificity of the recombinant 6xHis-LbNMNAT protein. The reaction started upon adding 2.5 μg of the enzyme. The enzymatic reactions were incubated at 20 °C for 5 min and stopped by filtration through a Centricon device (Millipore, MWCO 30 kDa). The filtered samples were analyzed through RP-HPLC.

2.6. RP-HPLC analysis

The analysis was done using the Shimadzu Prominence System and the Eclipse XDB-C18 column (150 × 4.6 mm, 5 μm, Agilent). Solvents A 110 mM potassium phosphate buffer, pH 5.8, 8 mM tetra bromide-n-butyl ammonium (TBAB) and B (methanol) were employed to carry out the following elution gradient, varying the % of solvent B: 4%, 6.5 min, 30%, 7 min, 36%, 3 min, 4%, 3.5 min. A flow of 1 mL/min was used,

maintaining the compartment of the column at 26 °C during the analysis. 10 μL of the samples were injected and the analytes were detected at 254 nm. To carry out quantitative analysis, calibration curves of NAD, NMN and ATP were constructed.

2.7. Determination of kinetics parameters

The kinetic parameters Michaelis-Menten (K_m), maximum velocity (V_{max}) and turnover number (K_{cat}) of the recombinant 6xHis-LbNMNAT protein were determined maintaining saturating concentrations of one of the substrates while varying the concentration of the other. The exact concentrations for each substrate are indicated in the corresponding results (Figure 3). The coupled enzymatic assay was used to determine the parameters for the substrates NMN and ATP. The direct enzymatic assay was used to determine the parameters for NAD (Sigma) and PPi (Sigma). The initial reaction rate, expressed as μmol of NAD produced/min*mg protein, was determined from the lineal region of the corresponding progression curves. Data obtained were used to carry out non-linear (Michaelis-Menten) and linear (Hanes-Woolf) regression plots, using the GraphPad Prism version 6 program. The enzymatic reactions analyzed in the direct (I) and coupled (II) assays can be described as follows:

$$\text{I: } \mathrm{NMN} + \mathrm{ATP} \overset{\mathrm{NMNAT}}{\rightleftarrows} \mathrm{NAD^+} + \mathrm{PPi}$$

$$\text{II: } \mathrm{NAD^+} + \mathrm{ETHANOL} \overset{\mathrm{ADH}}{\rightarrow} \mathrm{NADH} + \mathrm{H^+} + \mathrm{ACETALDEHYDE}$$

2.8. Determination of the equilibrium constant (K_D)

Direct enzymatic assays were carried out for 2, 4 and 24 h using 500 μM of the substrates NMN and ATP or 1 mM of the substrates NAD and PPi. The respective quantity of the synthesized products was determined using appropriate calibration curves. The K_D was calculated with the formula K_D = [product 1] X [product 2]/[substrate 1] X [substrate 2] [15].

2.9. Protein determination

The protein concentration was determined by the Bradford method using BSA as a standard. Specifically, the Pierce BCA Protein Assay Kit (Thermo Scientific) was used.

3. Results

3.1. The recombinant 6xHis-LbNMNAT protein was purified to homogeneity and eluted as an active monomer from the SEC column

With the objective of carrying out a kinetic study of the LbNMNAT enzyme, the purification of the recombinant 6xHis-LbNMNAT protein (36 kDa) was undertaken in this study. Initially, an IMAC protocol was completed using the Ni-NTA resin. The collected eluates were subjected to SEC in a Superdex 200 10/300 GL column, which resulted in a pure preparation of the recombinant 6xHis-LbNMNAT protein, according to SDS-PAGE (Figure 1A).

The SEC chromatogram indicated a peak with an elution volume of 16.01 mL for the recombinant 6xHis-LbNMNAT protein (Figure 1B). Analysis of the fractions contained in the mentioned peak, through coupled enzymatic assays, confirmed the functionality of the 6xHis-LbNMNAT protein (Figure 1C). In addition, the SEC experiment was used to analyze the oligomeric organization of the recombinant 6xHis-LbNMNAT protein, plotting a calibration curve (K_{av} *vs* Log MW), whose R^2 value indicated linearity among the variables (Figure 1D).

A MW value of 41.98 kDa was obtained for the recombinant 6xHis-LbNMNAT protein, which is close to the theoretical MW of the monomeric recombinant protein (36 kDa). This result indicates that under the experimental conditions of this study, the 6xHis-LbNMNAT protein does not show a oligomeric assemblie, as observed for *E. coli* (EcNMNAT) [30], *Pseudomonas aeruginosa* (PaNMNAT) [31] and the human iso-enzyme HsNMNAT2 [32]. On the other hand, HsNMNAT1 and HsNMNAT3 display hexamers and tetramers, respectively [14].

NMNATs that exhibit quaternary structure contain a structural element involved in the interaction among subunits. This structural element, consisting of a loop or a loop attached to a β sheet, connects the Rossmann fold with the C-terminal domain of these NMNATs. In order to verify the existence of this structural element in the LbNMNAT protein, a predictive model of the tertiary structure of this enzyme was constructed; applying a described methodology [27]. The inspection of the model revealed the presence of the mentioned structural element (Figure 2). This bioinformatics evidence suggests that the LbNMNAT protein could establish oligomeric assemblies, although in our SEC protocol, the recombinant 6xHis-LbNMNAT protein eluted as an active monomer.

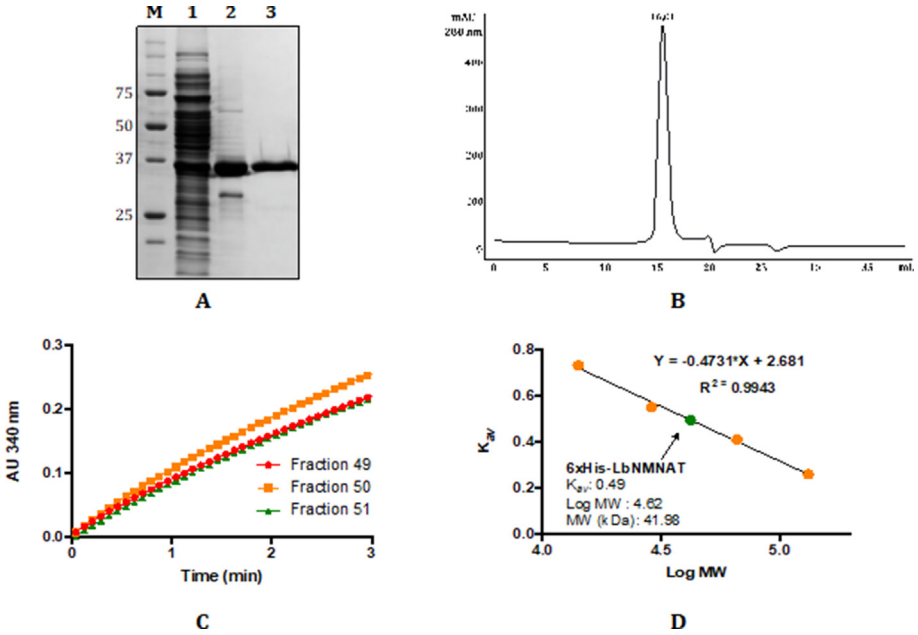

Figure 1. Purification of the recombinant 6xHis-LbNMNAT protein by IMAC-SEC. A. SDS-PAGE12%. Lanes: 1. Soluble starting fraction of *E. coli* M15; 2. Eluate from IMAC injected into the SEC column; 3. Fraction 50 obtained in the SEC protocol. Proteins were visualized with Coomassie R-250. M, molecular weight marker (kDa). B. The recombinant 6xHis-LbNMNAT protein eluated from the SEC column at an elution volume of 16.01 mL. The observed peak comprised fractions 49–51. C. The enzymatic activity of the recombinant 6xHis-LbNMNAT protein contained in fractions 49–51 was verified by enzymatic assays coupled to ADH. D. Calibration curve (*Kav* VS Log MW) used to analyze the oligomeric organization of the recombinant 6xHis-LbNMNAT protein. The orange circles represent protein standards lysozyme (14 kDa), carbonic anhydrase (29 kDa), BSA (66 kDa) and 6xHis-HsNMNAT1 (32 kDa, Supplementary Figure 1), which exhibits a hexameric organization in SEC (192 kDa).

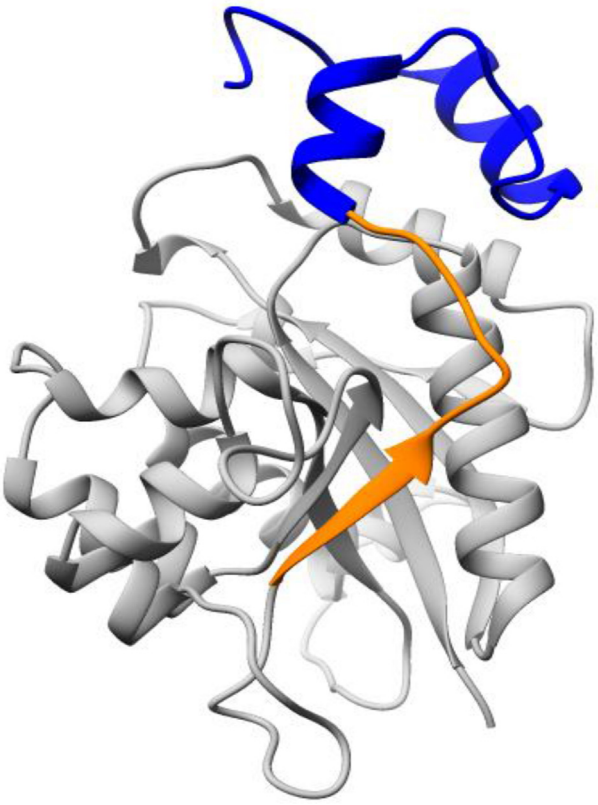

Figure 2. The predictive model of the LbNMNAT protein displays the structural element required to establish oligomers. The predictive model exhibits a structural element (orange) connecting the Rossmann fold (gray) with the C-terminal (blue). This element is common to the NMNATs showing quaternary structure [35]. Image generated with the UCSF Chimera program [43].

3.2. Enzymatic characterization of the recombinant 6xHis-LbNMNAT protein

In the enzymatic characterization of the recombinant 6xHis-LbNMNAT protein, preliminary data about the optimal experimental conditions of catalytic activity as buffer systems, pH, temperature and enzyme cofactors, were considered, under which the following kinetic parameters were determined: Michaelis Menten constant (K_m), maximum velocity (V_{max}) and turnover number (K_{cat}).

The determination of the K_m values for the NMN and ATP substrates was performed by coupled enzymatic assays to ADH, while for the NAD and PPi substrates direct enzymatic assays were carried out. The election of the type of assay was conducted considering that the determination of the kinetic parameters of the human iso-enzymes 6xHis-HsNMNAT1-3 has been performed in the same way [15, 29]. The data analysis was based on non-linear (Michaelis-Menten) and linear (Hanes-Woolf) regression methods, which generated consistent results with each other (Figure 3 and Table 1). The kinetic parameters of the 6xHis-LbNMNAT protein differ slightly among this study and a previous one [27]. This variation could be explained on the basis of different enzyme preparations; in this study, we describe a methodological advance to purify to homogeneity the 6xHis-LbNMNAT through a coupled IMAC-SEC protocol without contaminants that could interfere with the kinetic characterization of the enzyme.

Regarding the assessed substrates, the recombinant 6xHis-LbNMNAT protein exhibits the lowest and highest K_m values for ATP and NAD, respectively. Concerning NMN and PPi, their K_m values were determined within the range reported for other NMNATs. When comparing the K_m values for the substrates involved in the direct reaction (NMN and ATP), it was observed that the recombinant protein has a lower K_m value for ATP. This result is similar to those reported for *E. coli* (NadR) and *Sulfolobus solfataricus* NMNATs, which appears to be an exception in relation to other NMNATs characterized so far (http://www.brenda-enzymes.org).

The maximum velocity analysis revealed that the recombinant 6xHis-LbNMNAT protein is faster catalyzing the degradation of NAD, in contrast to its ability to synthesize it. Specifically, values of ~20 μmol/min*mg were obtained for the degradation of NAD in relation to ~0.2 μmol/min*mg for its synthesis (Table 1). The calculated K_{cat} confirmed this result, as the enzyme consumes ~16 molecules of NAD/s in the reverse reaction *VS* 0.2 molecules of NMN/s in the direct reaction. A similar trend has been reported for iso-enzymes HsNMNAT1-3, which are three times more efficient degrading NAD *in vitro* [15].

Due to the recombinant 6xHis-LbNMNAT protein can catalyze both forward and reverse reactions; the equilibrium constant (K_D) was determined for these reactions quantifying the synthesized products (Supplementary Figure 2). The K_D indicated that the reverse reaction is favored *in vitro* (Table 2). From a biological perspective, trypanosomatids

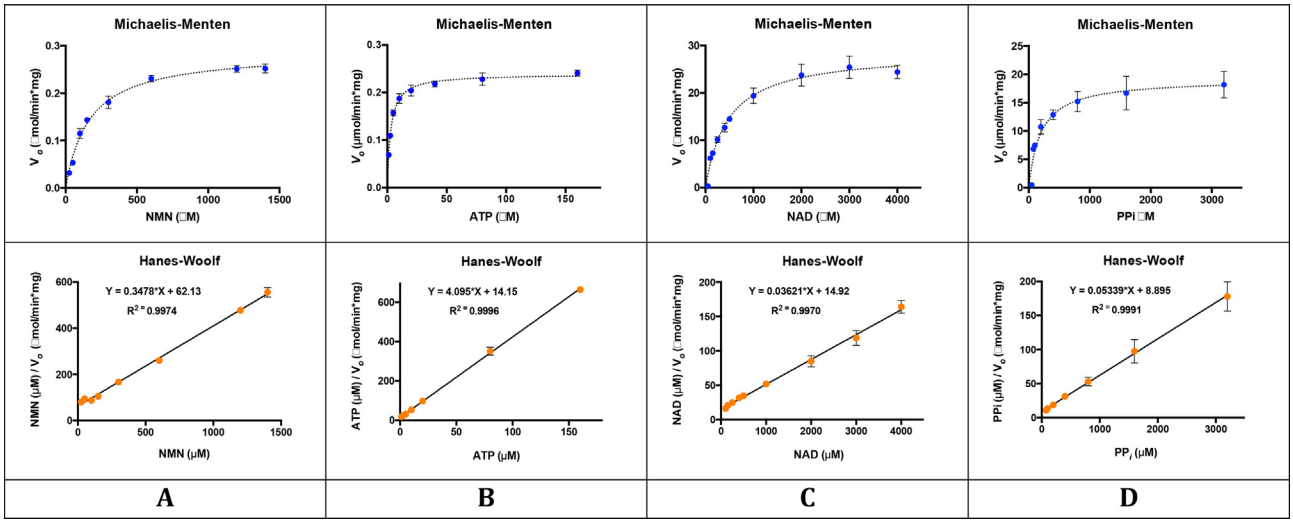

Figure 3. Kinetic parameters of the recombinant 6xHis-LbNMNAT protein. Using non-linear (Michaelis-Menten) and linear (Hanes-Woolf) regression methods, the values of *Km* and *Vmax* were determined. Saturating concentrations of the following substrates were used: A. ATP. B. NMN. C. PPi. D. NAD. Data presented as the mean + standard deviation (n = 3).

Table 1. Kinetic parameters of the 6xHis-LbNMNAT protein.

Substrate	K_m (μM)		V_{max} (μmol/min*mg)		Turnover number, K_{cat} (sec^{-1})	
	Linear	Non linear	Linear	Non linear	Linear	Non linear
NMN	178,7	166,7	0,3	0,3	0,2	0,2
ATP	3,5	2,9	0,2	0,2	0,1	0,1
NAD	412,2	470,2	27,6	28,7	16,6	17,2
PPi	166,6	196	18,7	19,2	11,2	11,5

such as *Leishmania* restrict PPi in acidocalcisomes, storage organelles that regulate its physiological availability [33, 34]. Low concentrations of free PPi would limit the reverse reaction of the NMNAT, which is why the adenylyltransferase activity would prevail *in vivo*.

Given that most NMNATs can employ NAMN as a substrate to synthesize NAAD, the ability of the recombinant 6xHis-LbNMNAT protein to catalyze this reaction, was evaluated. Analysis of direct enzymatic assays by RP-HPLC showed the synthesis of the product NAAD by the 6xHis-LbNMNAT enzyme, as it happened with the positive control (6xHis-HsNMNAT3) (Figure 4). This result agrees with previous observations in *L. infantum*, pathogen in which the existence of the nicotinamidase (EC: 3.5.1.19) was demonstrated, indicating the existing of the recycling pathway of NA (Preiss-Handler route) to synthesize NAMN. This mononucleotide is a substrate of the NMNAT, which then synthesizes NAAD [5], as showed herein.

4. Discussion

The NAD synthesis pathways converge in the step catalyzed by the NMNAT enzyme. For this reason and focused on identifying differences between the LbNMNAT protein and the human orthologs (HsNMNAT1-3), the kinetic characterization of the recombinant 6xHis-LbNMNAT protein was conducted in this study. The expression of that protein was carried out from the pQE30 vector as it has been done for the recombinant 6xHis-HsNMNAT1 and 3 proteins [15]. The homogeneous purification of the 6xHis-LbNMNAT protein established the starting point for future structural studies of the protein, such as X-ray crystallography, a technique that requires pure preparations.

The oligomeric state of the recombinant 6xHis-LbNMNAT protein was analyzed through structural and functional experiments, which revealed that the recombinant protein eluted as an active monomer from the SEC column (Figure 1). Similar observations have been reported for *E. coli* (NAdD), *P. aeruginosa* and *H. sapiens* (HsNMNAT2) NMNATs, monomeric enzymes capable of synthesizing NAD [30, 31]. This result confirms that oligomerization is not a mandatory requirement for the enzymatic functioning of all NMNATs. Similarly, the existence of the structural element involved in the oligomerization of NMNATs [35], does not imply that these proteins establish quaternary structure.

Nonetheless, our SEC assay was performed in the absence of substrates and cofactors. Other researchers have suggested that the NMNAT of *Bacillus subtilis* establishes tetramers in the absence of substrates and dimers in the presence of its product (NAAD) [36]. Consequently, it would be appropriate to repeat the SEC assay or to implement other analytical techniques in the presence of substrates and/or products, evaluating possible changes in the oligomeric organization of the recombinant protein. Dynamic Light Scattering (DLS) assays were initiated using the recombinant 6xHis-LbNMNAT protein in the absence or presence of ATP and NMN. Preliminary results have indicated that the hydrodynamic radius of the recombinant protein varies in the presence of ATP, suggesting that this substrate modulates the oligomerization state of the LbNMNAT protein.

Regarding the kinetic characterization of the recombinant 6xHis-LbNMNAT protein, an important aspect related to the K_m determined for ATP (~3 μM) (Table 1) was observed: This constant is up to 3 orders of minor magnitude with respect to other NMNATs characterized so far, except for NMNATs of prokaryotic organisms such as *E. coli* (NadR) and *S. solfataricus*, which also exhibit low K_m constants for ATP (K_m: 1.7 and 0.08 μM, respectively) [18, 37]. Assuming a limited availability of ATP in *Leishmania*, the low K_m value for ATP of the LbNMNAT protein would be reasonable, given the importance of NAD in oxidative stress defense systems and proliferation. Additionally, other enzymes of the parasite that also employ ATP as a substrate exhibit variable values of K_m for this molecule (hexokinase 300 μM, phosphoenolpyruvate carboxykinase 54 μM, Ecto-ATPase 980 μM and adenylate kinase 2104 μM) [38, 39, 40, 41]. From this viewpoint, low K_m for ATP is not a trend among *Leishmania* enzymes, but seems to be a particular case for its NMNAT, a key enzyme for energy metabolism and cell signaling.

The K_m for ATP of the recombinant 6xHis-LbNMNAT protein could be used to design potential inhibitors based on the structure of that substrate, contributing to the development of new strategies to control the parasite. Similarly, the importance of the NMNAT protein in *Plasmodium falciparum* has encouraged the development and implementation of specific enzymatic inhibitors, capable of negatively affecting its growth *in vivo* [42]. However, in the *Leishmania* model such inhibitors have not yet been reported.

Considering the K_m of the recombinant 6xHis-LbNMNAT enzyme for NAD and PPi, differences were observed with those constants reported for the iso-enzymes HsNMNAT1-3. For example, the K_m constants for NAD are lower in the three human iso-enzymes (K_m: 59, 70 and 130 *VS* 470 μM for the parasite) while the K_m for PPi is lower in *L. braziliensis* (K_m: 196 *VS* 937, 1119 and 309 μM for the human) (Table 1). Overall, the K_m

Table 2. Equilibrium constant (KD) of the 6xHis-LbNMNAT protein.

FORWARD REACTION (NMN + ATP)					
Time (h)	NAD (μM)	PPi (μM)	ATP (μM)	NMN (μM)	K_D = [NAD]*[PPi]/[NMN]*[ATP]
2	132,9	132,9	341,5	341,5	0,15
4	174,1	174,1	455,9	455,9	0,15
24	129,9	129,9	339,3	339,3	0,15
REVERSE REACTION (NAD + PPi)					
Time (h)	NAD (μM)	PPi (μM)	ATP (μM)	NMN (μM)	K_D = [NMN]*[ATP]/[NAD]*[PPi]
2	288,9	288,9	898,9	898,9	9,67
4	273,4	273,4	876,6	876,6	10,27
24	215,7	215,7	705,7	705,7	10,7

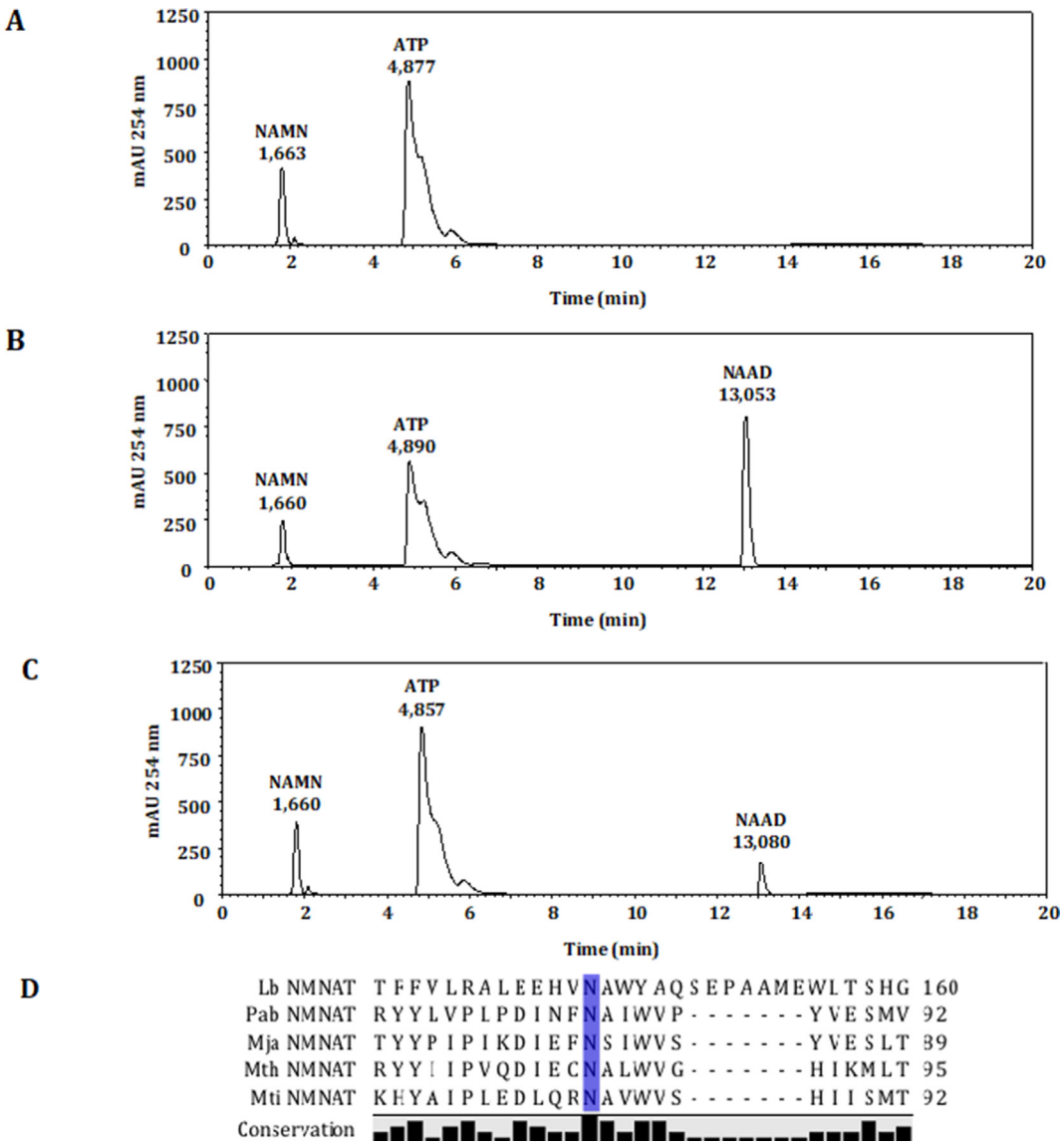

Figure 4. The recombinant 6xHis-LbNMNAT protein synthesizes NAAD. The synthesis of NAAD was verified by direct enzymatic assays analyzed by RP-HPLC using the following samples: A. Reaction buffer. B. Recombinant 6xHis-HsNMNAT3 protein (positive control). C. Recombinant 6xHis-LbNMNAT protein. 2.5 μg of each enzyme were used. The corresponding retention times for the NAMN and ATP substrates are indicated, as well as for the NAAD product. D. Multiple alignment of the primary structure of certain NMNATs that use NMN and NAMN as substrates. A section of the alignment is indicated, highlighting the conserved asparagine residue involved in the binding with both substrates. Alignment generated with the CLC Sequence Viewer program version 7.5 (ClustalW algorithm). *Pyrococcus abyssi* (Pab, WP_010868691.1), *Methanocaldococcus jannaschii* (Mja, AAB98533.1), *Methanothermobacter thermautotrophicus* (Mth, PDB 4YP7), *Methanolobus tindarius* (Mti, WP_048135196.1).

constants for NMN, ATP, NAD and PPi of the 6xHis-LbNMNAT protein and the human iso-enzymes, differ considerably.

The analysis of the V_{max}, the K_{cat} and the K_D of the recombinant 6xHis-LbNMNAT protein, indicated that this enzyme is faster, more efficient and promotes the catalysis of the reverse reaction in *in vitro* conditions (Tables 1 and 2). That is, the ATP and NMN synthesis from NAD and PPi, is the reaction that prevails under the experimental conditions reported in this study. In the parasite's *in vivo* context, is possible to suggest an explanation for this result: the PPi is limited to the acidocalcisomes, restricting the reverse reaction catalysis by the LbNMNAT. However, the possibility that the reverse reaction is catalyzed under energetic stress conditions, in order to synthesize ATP, is a scientific exploration route that has not yet been undertaken.

Depending on the organism, the NMNATs display differential selectivity for the NMN or NAMN substrates to synthesize NAD or NAAD, respectively. While archaebacteria prefer NMN, eubacteria use NAMN [13, 14]. Meanwhile, the human iso-enzymes HsNMNAT1-3 may synthesize both NAD and NAAD [15], as was observed for the recombinant 6xHis-LbNMNAT protein (Figure 4). This result is supported by previous studies that have reported the synthesis of NAAD in *L. infantum* [5]; the parasite depends on the activity of the nicotinamidase enzyme for NAD recycling and proliferation. This enzyme catalyzes conversion of nicotinamide to nicotinic acid, which can be converted to NAMN in the Preiss-Handler pathway. Moreover, NMNATs using both NMN and NAMN to synthesize NAD or NAAD, as those of archaebacteria, retain an asparagine residue around position 81 [19], residue also found in the LbNMNAT protein (Figure 4D).

5. Conclusion

A study on the metabolism of NAD in *L. braziliensis* was performed, focused on the central enzyme of its biosynthesis, the LbNMNAT. The

evidence obtained indicated differences among the parasite's NMNAT and the human orthologs HsNMNAT1-3 in terms of kinetic parameters and oligomeric organization. These findings have to be continued to finally propose the NMNAT as a promissory pharmacological target in *L. braziliensis*.

Declarations

Author contribution statement

Luis Ernesto Contreras Rodríguez: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper. Mathias Ziegler: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; María Helena Ramírez Hernández: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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