Study of Specific Region of *Plasmodium falciparum* Nicotinamide/Nicotinate Mononucleotide Adenylyl Transferase (PfNMNAT): Characterizing a Possible Therapeutic Target

Nieto CA, Marin CY, Contreras LE and Ramirez MH*

Faculty of Sciences, Laboratory of Basic Research in Biochemistry (LIBBIQ), National University of Colombia, Bogotá, Colombia

**Abstract**

Nicotinamide mononucleotide adenylyl transferase (NMNAT) is a key enzyme in the biosynthesis of nicotinamide adenine dinucleotide (NAD\(^+\)), which is an essential molecule in cellular metabolism. Specific sequences have been described in other NMNATs, which are associated with the regulation of catalytic activity and intracellular localization. In addition, it has been observed that prokaryotic NMNATs have specific regions that could be used as possible therapeutic targets. By aligning *Plasmodium falciparum* (Pf) NMNAT sequences with their human orthologues (HsNMNAT), specific domains of the P. falciparum protein can be observed. PfinMNAT mutants were designed using bioinformatics software to obtain 2 mutants in order to evaluate the specific sequences of the P. falciparum enzyme. For mutant construction, directed mutagenesis was used to introduce changes in the wild-type maltose binding protein (MBP)-PfNMNAT clone previously obtained. The results were compared to those obtained with the wild-type protein. The experimental evidence indicates that the catalytic activity of the enzyme can be affected by transitional and transversional amino acid changes in charge and size. The study of these mutants allows an approach to studying the function and regulation of these proteins.

**Keywords:** *Plasmodium falciparum*; NAD\(^+\) biosynthesis; PfinMNAT; Mutagenesis; Recombinant protein

**Introduction**

*Plasmodium falciparum* causes malaria and is the leading parasitic cause of death worldwide [1]. Once a person has been infected, the only possible treatment is the administration of anti-malarials. Unfortunately, over the past 50 years, the parasite has become increasingly resistant to most drugs, making malaria one of the world’s major public health problems [2].

The identification of possible pharmaceutical targets and the development of strategies to control the parasite require research focused on the molecular and biochemical characterization of the parasite. In this regard, the study of nicotinamide adenine dinucleotide (NAD\(^+\)) is prominent and appropriate given the essential functions it performs, such as energy metabolism, defense against oxidative stress, and cellular regulation [3].

There are two pathways for NAD\(^+\) biosynthesis: de novo and recycling. Although these two pathways require different precursors and intermediates, both converge in the step catalyzed by nicotinamide/nicotinate mononucleotide adenylyl transferase (NMNAT; EC: 2.7.7.1/18) [4]. For this reason, the characterization of *P. falciparum* NMNAT (PfinMNAT) and the identification of possible structural differences compared with its human orthologues (HsNMNAT 1-3) are essential to developing new strategies to control the parasite.

*P. falciparum* NMNAT was recently identified in our laboratory [5], and possible enzyme inhibitors that are promising as antimalarial agents were found [6]. The tertiary structure of *P. falciparum* NMNAT was determined by X-ray analysis because of its importance [7].

**Materials and Methods**

**Amplification of the PfinMNAT coding region**

For the amplification of the PfinMNAT coding region, the sequences of the forward and reverse primers used were 5′-GGATCCCATG-CATAAGAATATATGT-3′ and 5′-CTAATTTAAATCTATATAAGTT, including a BamHI cleavage site. The amplification conditions were 1 U of Taq polymerase (Applied Biosystems), 2.5 mM MgCl\(_2\), 1x DNA polymerase buffer, 10 mM dNTPs, diethylpyrocarbonate (DEPC) H\(_2\)O, and approximately 50 ng of DNA from strain FCB-2 to a final volume of 15 µL. The following thermal profile was used: an initial denaturation cycle at 94°C for 10 min, followed by 30 cycles of 94°C for 45 s, 50°C for 1 min, and 72°C for 45 s and a final extension at 72°C for 10 min (Bio-Rad thermal cycler).

**PfinMNAT cloning and expression**

The amplified fragment was cloned into the pGEM-T Easy vector (PROMEGA) and subcloned into the pMal-c5X vector using BamHI and EcoRI enzymes. The recombinant plasmid was purified by alkaline lysis and verified by sequencing. E. coli BL21 (DE3) was transformed by heat shock. The transformed clones were inoculated in Luria Broth (LB) supplemented with 100 µg/ml ampicillin and 1% glucose, with overnight incubation at 37°C. The cultures were diluted (1:100) in the same medium, and when they reached an optical density of 0.6 measured at 600 nm, the recombinant protein was induced with 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37°C for 3h. Expression was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot by detection with anti-MBP (maltose binding protein) antibodies.

*Corresponding author: Dr. María H. Ramírez, Faculty of Sciences, Laboratory of Basic Research in Biochemistry (LIBBIQ), National University of Colombia, Bogotá, Colombia, Tel: +571316500; E-mail: mhramirez@unal.edu.co*

**Received** November 06, 2017; **Accepted** December 08, 2017; **Published** December 12, 2017

**Citation:** Nieto CA, Marin CY, Contreras LE, Ramirez MH (2017) Study of Specific Region of *Plasmodium falciparum* Nicotinamide/Nicotinate Mononucleotide Adenylyl Transferase (PfinMNAT): Characterizing a Possible Therapeutic Target. *J Mol Genet Med* 11: 311. doi:10.4172/1747-0862.1000311

**Copyright:** © 2017 Nieto CA, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.
Purification of recombinant MBP-PfNMNATs

After induction, bacteria were collected by centrifugation at 7500 × g for 15 min at 4°C and resuspended in lysis buffer (50 mM phosphate buffer at pH 7.8, 400 mM NaCl, 100 mM KCl, 10% glycerol, and 1 mM DTT). To the samples, 1 mg/ml lysozyme (Sigma) and protease inhibitor cocktail (Sigma P8340; 1 mM AEBSF, 14 μM E64, 15 μM Pepstatin A, 40 μM Bestatin, 20 μM Leupeptin, and 0.8 μM Aprotinin) were added. The lysate was incubated on ice for 30 min with shaking and sonicated for 5 min on ice (15 s pulse at 50% amplitude with 15 s rest). Finally, the lysate was centrifuged at 14500 × g for 20 min at 4°C to separate the soluble and insoluble fractions.

MBP-PfNMNAT was purified from the soluble fraction by amylose affinity chromatography (New England Biolabs). Prior to that, the protein was quantified densitometrically with SDS-PAGE using different bovine serum albumin (BSA) concentrations as the standards. MBP-PfNMNAT was eluted with elution buffer (lysis buffer +20 mM maltose), and 25 fractions of approximately 60 μL were collected and stored at -80°C. Purification was analyzed by SDS-PAGE. The protein in the soluble fraction and eluted fractions was quantified by the Bradford method [8].

Direct enzyme assay

An in vitro assay was performed to determine the activity of purified recombinant proteins. The reaction mixtures (1 mM nicotinamide mononucleotide (NMN) and 1.3 mM ATP in 100 mM HEPES buffer (Sigma) + 10 mM Mg2+, pH = 7.5) were incubated at 37°C for 30 minutes, at which point the reaction was stopped with 1.2 M HClO4, at 4°C. The protein was precipitated by centrifugation at 14,500 × g for 3 min at 4°C. The supernatant was neutralized with cold 1 M K2CO3, and 120 μL of supernatant was removed. The yield of the in vitro reaction was quantified by reverse phase HPLC (RP-HPLC). A Phenomenex Luna C18 column (250 mM × 4.60 mM, 5 μM) was used. The mobile phases were used 0.12 M potassium phosphate buffer at pH 6.0 and methanol.

Gradient separation was performed at a constant flow rate of 1.5 mL/min, for a run time of 20 min. The analytes, whose retention times were determined from standards, were detected spectrophotometrically at 254 nm with a diode array detector.

Design of PfNMNAT mutants

Several PfNMNAT mutants were analyzed by performing multiple sequence alignments of the primary protein with their human orthologues (HsNMNAT 1-3) using the ClustalW online server [9].

The specific regions of the P. falciparum protein that could be studied further were identified in this way. Finally, the tertiary structures of the wild-type and mutated proteins were predicted using the I-TASSER server [10] and solubility values were determined, with MBP-PfNMNAT as a control, using the ProSo II server [11].

Production of PfNMNAT mutants

To determine the differences in the PfNMNAT mutants compared to the wild-type protein, two mutants were studied. For this, the recombinant plasmid constructed as described above was used as the template for the production of mutants (pMal-c5X-PfNMNAT), using those designs that allowed the most remarkable differences in structural changes and solubility to be observed. Mutagenesis was performed using the "Phusion Site-Directed Mutagenesis Kit" (Thermo Scientific®). The amplification conditions were as follows: 1 U of Phusion Hot Start II DNA polymerase, 1x Phusion HF buffer, 10 mM dNTPs, DEPC H2O, 0.5 μM of primers (Table 1), and approximately 10 pg of template DNA, all to a final volume of 25 μL. The following thermal profile was used: an initial denaturation step at 98°C for 30 sec, followed by 25 cycles of 98°C for 10 s, 67°C for 30 s, and 72°C for 35s and a final extension at 72°C for 10 min (Bio-Rad thermal cycler).

Determination of the kinetic parameters (Km and Vmax) of recombinant MBP-PfNMNAT and N163D/MBP-PfNMNAT

A coupled enzyme assay was used to determine the parameters for NMN and ATP substrates using 10 mM ATP and 5 mM NMN as saturating concentrations. The initial reaction rate, expressed as μmol of NAD+ produced/min per mg of protein was determined from the linear range of enzyme activity. The data were used to perform Michaelis-Menten nonlinear and Hanes-Woolf linear regression analysis using GraphPad Prism 7 software.

Coupled enzyme assay

The catalytic activity of MBP-PfNMNAT was verified by coupled enzyme assays with alcohol dehydrogenase (ADH) and monitoring the increase in absorbance at 340 nm due to the reduction of NAD+ to NADH, as has been previously described [12].

A reaction mixture was prepared containing 40 mM ethanol, 25 mM HEPES/KOH at pH 7.4, 10 mM MgCl2, 1.25 mM ATP (Sigma), 1.25 mM NMN (Sigma), and 2 mU ADH (Sigma). This mixture was aliquoted into 96-well plates and incubated at 37°C for 5 min, and the reaction was started by adding 2.5 μg of the samples to be evaluated. The assays were performed in a 100 μL volume at 37°C under constant shaking in the GENios microplate reader (TECAN), with the absorbance recorded at 340 nm for 15 min.

Results

Expression, purification, and enzyme activity of MBP-PfNMNAT

The expression of recombinant MBP-PfNMNAT (~65.2 kDa) in the heterologous E. coli BL21 (DE3) system resulted in the presence of the protein of interest mostly in the soluble fraction, which allowed its purification using amylose affinity chromatography. The nucleotidyl transferase activity of the recombinant protein was evaluated by direct NMNAT assays and subsequent observation of the peak corresponding to synthesized NAD+ using reverse phase HPLC, which demonstrated that the fusion protein (MBP) does not affect enzyme activity. With respect to the activity found in the soluble fraction, the activity detected in the eluate from affinity purification was 18.4-fold higher, allowing good yields for the recombinant protein obtained from the pMal-c5X vector.

Design of MBP-PfNMNAT mutants

Analysis of the primary structure confirmed the presence of conserved ATP-binding motifs and the existence of two specific domains in the NMNATs of the parasites (Insertion 1: 161-164, genus-specific domain of Plasmodium, and insertion 2: 180-183, species-specific domain of P. falciparum), as can be observed in Figure 1.
Domain 1 is genus specific and was chosen to design several mutants. Structural prediction was performed for both deletion and substitution mutants using the I-TASSER server, and solubilities were obtained for each designed mutant using the ProSo II software. The most striking structure and solubility parameters, taking the wild-type protein as reference, were obtained for transition N163D (AAT/GAT) and transversion N163Y (AAT/TAT) (Figure 2). The two mutants produced allowed the effects of the increase in size and changes in charge on enzyme activity to be evaluated.

**Production of MBP-PfNMNAT mutants**

After verifying the identity of the processes by sequencing, E. coli BL21 (DE3) was transformed, and the production of the recombinant proteins was induced. There were no marked changes in expression between the different proteins.

The MBP-PfNMNATs expressed were purified by amylose affinity chromatography under the conditions evaluated in the first part. Direct enzyme assays were then performed to analyze the functional and structural relevance of the mutated residue and to evaluate changes in the catalytic activity compared to the wild-type MBP-PfNMNAT. Despite the fact that no significant changes were observed in the soluble expression of mutant proteins compared to wild-type, in the purification of MBP-PfNMNATs, there were significant changes in the degradation patterns of the 43 kDa product corresponding to the MBP tag compared to the wild-type protein (Figure 3).

Experimental evidence indicated that the catalytic activity of the enzyme can be affected by amino acid transitional and transversional substitutions in terms of charge and size. Small changes in the radical size (N163D), with charge modification, reduced the enzyme activity, while larger radicals (N163Y), with no charge modification, lacked enzyme activity (Figure 4 and Table 2).
Discussion

*Plasmodium falciparum* NMNAT has both genus- and species-specific regions. For the characterization of this promising therapeutic target, it must be obtained using the heterologous *E. coli* system. In previous studies, PNMNAT has been cloned into the pET100/d-TOPO vector (Thermo Scientific), but during its expression, large amounts of insoluble aggregates known as "inclusion bodies" (IBs) are produced. For this reason, PNMNAT was cloned into the pMal-c5x vector, which adds an MBP tag that does not interfere with the folding of the protein of interest and allows the production of active protein [13]. To date, the mechanism by which the fusion protein can modify the solubility of the protein of interest is still unclear; however, MBP is one of the most studied. MBP may function as a molecular chaperone, which, through its hydrophobic pocket, captures the protein-folding intermediate and enables it to have a second chance to fold. This allows the recombinant protein to retain native folding and thereby be expressed in the soluble fraction. On the other hand, if recombinant protein folding cannot be solved, it will form insoluble aggregates [14,15]. Another hypothesis about the mechanism of action of MBP is its role as a chaperone magnet, recruiting chaperones that normally associate with MBP that may help to solve the folding of the fused protein [16].

To evaluate if the loss of catalytic activity is due to a possible decrease in the affinity for some of the substrates due to alterations in the lateral disposition of the substrate binding sites, the MatchMaker tool of the UCSF Chimera software was used to perform structural alignments of mutant vs. wild-type protein, identifying site-specific changes affecting NMN and ATP binding. The results suggest that the possible loss of catalytic activity of mutant proteins may be due to changes in the spatial arrangement of residues involved in NNM binding, as the alteration in the disposition of the side chains of this residue is greater compared to those involved in ATP binding; that is, there is a loss of affinity for NNM, and the synthesis of NAD+ cannot proceed with the same effectiveness as with the wild-type protein (Figure 5).

To test this hypothesis experimentally, enzyme kinetic parameters (Km and Vmax) were identified for the NMM substrate using the wild-type and N163D proteins. The kinetic parameters for ATP were determined with the wild-type protein, as a control, with similar rates obtained for the two substrates (Table 3).

The results suggest that MBP-PNMNAT has a ~5.2-fold higher affinity for NMM than for ATP, and the Km values obtained are within the values reported for other NMMATs. This result is similar to that reported for ScNMNAT2, whose affinity for NMM is ~10.7-fold higher compared to its affinity for ATP. With regard to the NMM Km for MBP-ΔPNMNAT N163D, a ~1.7-fold loss of affinity is observed, and a ~2.5-fold reduced synthesis of NAD+ is also observed. This confirms the results obtained in direct enzyme assays predicted by the I-TASSER server, where the spatial arrangement of the side chains involved in NMM binding is possibly reflected in the reduction of NAD+ synthesis. These results then suggest that the catalytic activity of the protein can be balanced in terms of charge when size is maintained; however, inversely, there can be no balance, and there is a complete loss of catalytic activity.

Conclusion

When analyzing the primary structures of NMMATs of higher eukaryotes, specific insertions have been identified that are related to regulation by posttranslational modifications and in relation to subcellular localization. These specific insertions or sequences have been studied in the 3 human isoenzymes (H4NMMAT) [17]. Comparing the functional effects generated by PNMNAT insertions with those of the human orthologues, different results are obtained since in the latter, the changes of their specific domains do not affect the catalytic activity of the protein [18]. With regard to *Leishmania braziliensis* NMNAT, it was identified that these specific insertions are needed for the catalytic activity of the protein [19], similar to what occurs with MBP-PNMNAT. These results, in addition to ours, show that the insertions in NMMATs of these protozoan parasites can serve as possible pharmacological targets in the search for treatments directed specifically against the parasite protein since their alteration can cause the loss of catalytic activity without affecting the activity of human isoenzymes.

Acknowledgments

The authors thank Colciencias for funding project 110156935240, the National University of Colombia, and the Faculty of Sciences.

References

1. WHO (2016) World Malaria Report.
2. OMS (2015) Estrategia Técnica Mundial Contra La Malaria 2016–2030. Organización Mundial de la Salud.
3. Nikiforov A, Kulikova, V, Ziegler M (2015) The human NAD metabolome: Functions, metabolism and compartmentalization. Crit Rev Biochem Mol Biol 1:1–14.

4. Dolle C, Skoge RH, Vanlinden MR, Ziegler M (2013) NAD biosynthesis in humans - Enzymes, metabolites and therapeutic aspects. Curr Top Med Chem, 13: 2907–2917.

5. Marín C, Ramirez M (2009) Identificación, expresión y caracterización de la Nicotinamida/nicotinato mononucleotido adenililtransferasa de Plasmodium falciparum (PfNMNAT) 31: 129.

6. O’Hara JK, Kerwin LJ, Cobbold SA, Tai J, Bedell TA, et al. (2014) Targeting NAD+ metabolism in the human malaria parasite Plasmodium falciparum. PLoS One 9: 4.

7. Bathke J, Fritz-Wolf K, Brandstädtler C, Burkhardt A, Jortzik E, et al. (2016) Structural and functional characterization of Plasmodium falciparum nicotinic acid mononucleotide adenylyltransferase. J Mol Biol 428: 4946–4961.

8. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analy Biochem 72: 48–254.

9. Thompson JD, Higgins DG, Gibson TJ (1994) Clustal W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673–4680.

10. Zhang Y (2008) I-Tasser server for protein 3D structure prediction. BMC Bioinf 9: 40.

11. Smialowski P, Doose G, Torkler P, Kaufmann S, Frishman D (2012) PROSO II - A new method for protein solubility prediction. FEBS 279: 2192–2200.

12. Balducci E, Emanuelli M, Raffaelli N, Ruggieri S, Amici A, et al. (1995) Assay methods for nicotinamide mononucleotide adenylyltransferase of wide applicability. Analy Biochem.

13. Planson AG, Gujarro JI, Chauffotte AF (2013) New insights for native production of MSP119, the disulfide-Rich C-terminal fragment from Plasmodium falciparum merozoite surface protein 1. PLoS One 8: 2–9.

14. Fox JD, Kapust RB, Waugh DS (2001) Single amino acid substitutions on the surface of Escherichia coli maltose-binding protein can have a profound impact on the solubility of fusion proteins. Protein Sci 10: 622–630.

15. Kapust RB, Waugh DS (1999) Escherichia coli maltose-binding protein is uncommonly effective at promoting the solubility of polypeptides to which it is fused. Protein Sci 8: 1666–1674.

16. Randall LL, Topping TB, Smith VF, Diamond DL, Hardy SJS (1998) SecB: A chaperone from Escherichia coli. Methods Enzy 290: 444–459.

17. Lau C, Dölle C, Gossmann TI, Agledal L, Niere M, et al. (2010) Isoform-specific targeting and interaction domains in human nicotinamide mononucleotide adenylyltransferases. J Mol Biol 285: 19868–19876.

18. Brunetti L, Di-Stefano M, Ruggieri S, Cinadamoore F, Magni G (2010) Homology modeling and deletion mutants of human nicotinamide mononucleotide adenylyltransferase isozyme 2: New insights on structure and function relationship. Protein Sci 19: 2440–2450.

19. Contreras LE, Neme R, Ramírez MH (2015) Identification and functional evaluation of Leishmania braziliensis nicotinamide mononucleotide adenylyltransferase. Protein Expr Purif 115: 26–33.