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

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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. PfNMNAT 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; PfNMNAT; 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 (PfNMNAT) 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 PfNMNAT coding region

For the amplification of the PfNMNAT coding region, the sequences of the forward and reverse primers used were 5'-GGATCCATGCATAAGAATATATGT-3' and 5'-CTAATTAAAATCATATAAGTT, 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_2O, 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 45s and a final extension at 72°C for 10 min (Bio-Rad thermal cycler).

PfNMNAT 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 production of 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.

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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 Mg^{2+}, pH = 7.5) were incubated at 37°C for 30 minutes, at which point the reaction was stopped with 1.2 M $HClO_4$ 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 K_2CO_3, 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 used were 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, 10mM dNTPs, DEPC H_2O, 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 cycle 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).

Name of primer	Sequence 5'– 3'
N163D_Forward	CCCAAATTATCTTTTATCGATTTTATTTCATCAAGTG
N163D_Reverse	CACTTGATGAAATAAAATCGATAAAAGATAATTTGGG
N163Y_Forward	CCCAAATTATCTTTTATCTATTTTATTTCATCAAGTG
N163Y_Reverse	CACTTGATGAAATAAAATAGATAAAAGATAATTTGGG

Table 1: Primers used to produce PfNMNAT mutants.

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 $MgCl_2$, 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.

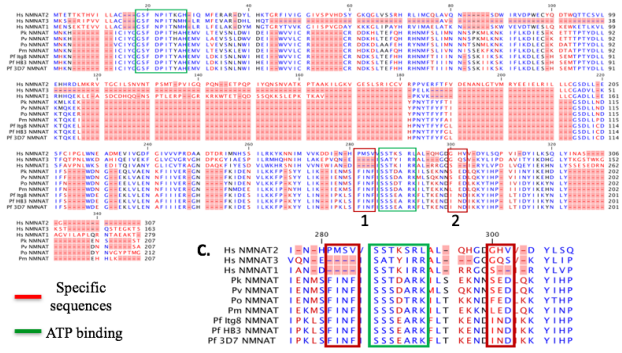
Production of MBP-PfNMNAT mutants

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.

A.

SEQUENCES USED IN MULTIPLE ALIGNMENT		
Protein code	**Organism**	**Description**
PF3D7_1327600	*Plasmodium falciparum*	PfNMNAT 3D7
KOB62901.1		PfNMNAT HB3
PFIT_1326800		PfNMNAT ITG8
PVX_116500	*Plasmodium vivax*	PvNMNAT
PocGH01_12034800	*Plasmodium ovale*	PoNMNAT
PmUG01_12036900	*Plasmodium malariae*	PmNMNAT
PKNH_1201000	*Plasmodium knowlesi*	PkNMNAT
NP_001284707.1	*Homo sapiens*	HsNMNAT1
NP_055854.1		HsNMNAT2
NP_835471.1		HsNMNAT3

B.



C.

Figure 1: Identification of specific PfNMNAT sequences.

A. NMNAT sequences in humans and *Plasmodium falciparum* (strains) used to perform the search for specific sequences. B. Multiple sequence alignment of *Plasmodium falciparum* NMNATs (strains) and HsNMNATs. 1, 2. PfNMNAT-specific sequence insertions. C. Amplification of PfNMNAT-specific sequences (Clustal W and CLC Main Workbench).

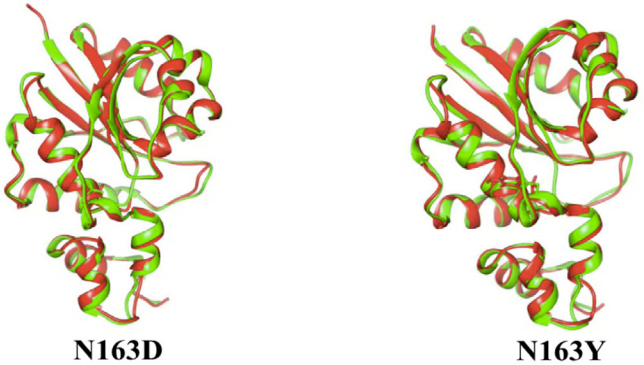


Figure 2: Comparison of PfNMNAT structural models (mutant vs. wild-type protein).

N163D (RMSD of 0.656 Å between 204 pairs of atoms), N163Y (RMSD of 0.721 Å between 204 pairs of atoms). Green: WT, and Red: Mutant. I-TASSER and UCSF Chimera software.

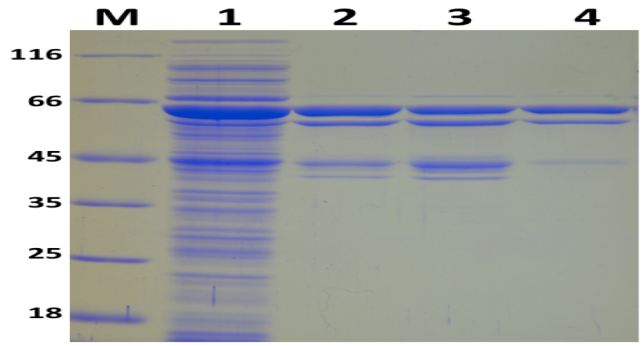


Figure 3: Purification of ΔMBP-PfNMNATs.

1. Soluble expression of wild-type MBP-PfNMNAT in *E. coli* BL21 (DE3). 2. Eluate N163D/MBP-PfNMNAT. 3. Eluate from the purification of N163Y/MBP-PfNMNAT. 4. Eluate WT/MBP-PfNMNAT. M. Molecular weight pattern (Thermo). 10% SDS-PAGE stained with Coomassie blue R-250.

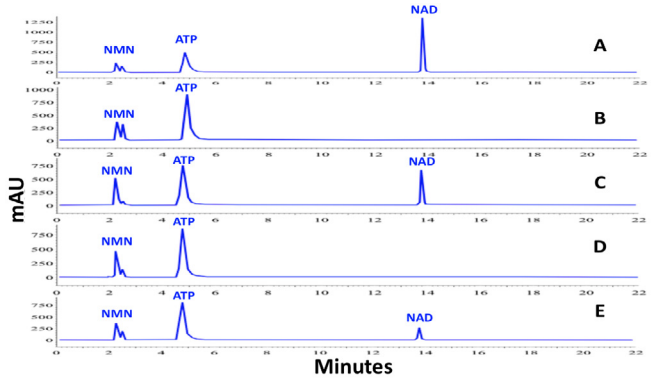


Figure 4: Evaluation of the catalytic activity of recombinant ΔMBP-PfNMNATs quantified by RP-HPLC.

A. HsNMNAT-3 (Control +), B. Elution buffer (Control -), C. MBP-PfNMNAT, D. N163Y/MBP-PfNMNAT, E. N163D/MBP-PfNMNAT. Phenomenex Luna C18 column. Mobile phases: 0.12 M potassium phosphate buffer at pH 6.0 and methanol. Constant flow rate of 1.5 mL/min, for a run time of 20 min. Spectrophotometric detection at 254 nm.

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, PfNMNAT 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, PfNMNAT 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 NMN 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 NMN, and the synthesis of NAD+ cannot proceed with the same effectiveness as with the wild-type protein (Figure 5).

Fraction	Total protein (mg)	Total activity (mU*)	Specific activity (mU/mg)	Recovery (%)	Purification (times)
S WT	6.96	185.18	26.60	100	-
E WT	0.29104	142.71	490.36	77.06	18.4
S N163D	20.8	61.72	2.96	100	-
E N163D	0.64	49.38	77.16	80	26
S N163Y	21.6	-	-	100	-
E N163Y	0.64	-	-	-	70-80[a]

*One unit of enzyme activity represents the amount of enzyme that catalyzes the synthesis of 1 µmol NAD^+ per minute at 37°C.
[a]Arbitrary value assuming 100 is complete purification.
S: Soluble fraction, E: Eluate

Table 2: Purification of MBP-PfNMNATs.

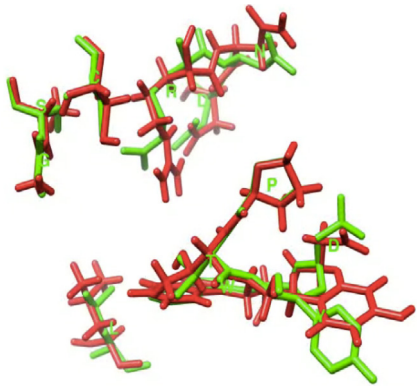


Figure 5: Spatial arrangement of the side chains of residues involved in NMN binding of N163Y/MBP-PFNMNATS. (Green: WT, and Red: Mutant).

Proteins	Substrate	K_m (µM)		V_o (µmol/min-mg)	
		Linear	Nonlinear	Linear	Nonlinear
MBP-PfNMNAT	NMN	158.4	149.2	2.207	2.252
	ATP	826.0	926.4	1.612	1.668
MBP-PfNMNAT N163D	NMN	258.2	282.6	0.3931	0.4057

Table 3: Kinetic parameters of MBP-PfNMNATs.

To test this hypothesis experimentally, enzyme kinetic parameters (Km and Vmax) were identified for the NMN 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-PfNMNAT has a ~5.2-fold higher affinity for NMN than for ATP, and the Km values obtained are within the values reported for other NMNATs. This result is similar to that reported for ScNMNAT2, whose affinity for NMN is ~10.7-fold higher compared to its affinity for ATP. With regard to the NMN Km for MBP-ΔPfNMNAT 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 NMN 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 NMNATs 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 (HsNMNAT) [17]. Comparing the functional effects generated by PfNMNAT 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-PfNMNAT. These results, in addition to ours, show that the insertions in NMNATs 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.

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