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Energetics of RNA binding by the West Nile virus RNA triphosphatase

Ines Benzaghou[1], Isabelle Bougie[1], Frédéric Picard-Jean[1], Martin Bisaillon*

Département de Biochimie, Faculté de Médecine, Université de Sherbrooke, 3001 12e avenue, Sherbrooke, Québ., Canada J1H 5N4

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Abstract **The West Nile virus (WNV) RNA genome harbors the characteristic methylated cap structure present at the 5′ end of eukaryotic mRNAs. In the present study, we report a detailed study of the binding energetics and thermodynamic parameters involved in the interaction between RNA and the WNV RNA triphosphatase, an enzyme involved in the synthesis of the RNA cap structure. Fluorescence spectroscopy assays revealed that the initial interaction between RNA and the enzyme is characterized by a high enthalpy of association and that the minimal RNA binding site of NS3 is 13 nucleotides. In order to provide insight into the relationship between the enzyme structure and RNA binding, we also correlated the effect of RNA binding on protein structure using both circular dichroism and denaturation studies as structural indicators. Our data indicate that the protein undergoes structural modifications upon RNA binding, although the interaction does not significantly modify the stability of the protein.**

Keywords: RNA triphosphatase; West Nile virus; RNA binding; Thermodynamics; Fluorescence spectroscopy

1. Introduction

West Nile virus (WNV) is a mosquito-borne virus that primarily infects birds but occasionally also infects humans and horses [1,2]. In humans, the most serious manifestation of WNV infection is a fatal inflammation of the brain [3]. The frequency of WNV outbreaks in humans has increased in recent years, and the geographical distribution of WNV has expanded to the Western hemisphere in 1999 [4]. Since then, the virus has spread rapidly throughout North America and has recently reached the European continent [5]. The lack of vaccines or therapeutic treatments for WNV infections has therefore prompted the elaboration of extensive prevention strategies.

WNV is a member of the *Flaviviridae* family which includes more than 70 human pathogens such as hepatitis C, yellow fever, and Dengue fever viruses [6]. The flaviviral genome is a single-stranded RNA of positive polarity containing a single open reading frame of about 11 kb in length [7,8]. This RNA encodes for a large polyprotein precursor that is further processed into three structural and seven non-structural proteins [9,10]. The non-structural protein 3, NS3, is a multifunctional protein that displays several distinct enzymatic functions. The N-terminal domain of the protein harbors a serine protease activity which is involved in the proteolytic cleavage of the viral polyprotein precursor [11]. The C-terminal domain of NS3 has been shown to possess both nucleoside triphosphatase (NTPase) and helicase activities [12]. Finally, an RNA triphosphatase activity has previously been reported for a 50 kDa C-terminal region of the WNV NS3 protein released with subtilisin from the membranes of infected cells [13].

The WNV RNA genome harbors the characteristic methylated cap structure present at the 5′ end of eukaryotic mRNAs [14]. This structure, ^{m7}GpppN-, has been shown to play critical roles both in translation and mRNA stability [15,16]. The RNA cap structure is formed co-transcriptionally by three sequential enzymatic reactions catalyzed by an RNA 5′ triphosphate, an RNA guanylyltransferase, and an RNA (guanine-N7)methyltransferase. The importance of the cap structure for RNA metabolism is underscored by genetic analyses in *Saccharomyces cerevisiae* where it has been shown that the triphosphatase, guanylyltransferase and methyltransferase components of the capping apparatus are essential for cell viability [17–22].

Although the RNA cap structures originating from viral and cellular enzymes are often identical, the physical organization of the genes, subunit composition, structure, and catalytic mechanisms of the known virus-encoded mRNA capping enzymes are significantly different from those of metazoan host cells [16]. Of particular interest is the RNA triphosphatase component of the capping apparatus, which is both structurally and mechanistically different in mammalian cells. As a consequence these viral cap-forming enzymes, such as the WNV NS3 protein, are attractive targets for antiviral drugs that would interfere with the capping of pathogen mRNAs while leaving the host capping enzymes unaffected. Two distinct families of RNA triphosphatases have been clearly established so far. The first family includes enzymes from metazoan and plants which belong to the cysteine phosphatases superfamily. These proteins contain a characteristic HCXXXXXR(S/T) motif, and act via the formation of a covalent enzyme-(cysteinyl-*S*-)-phosphate intermediate [22,23]. Their enzymatic activity is independent from divalent cations. Moreover, crystal structure analyses suggest that they share a phosphate binding loop and a core tertiary structure with other cysteine phosphatases [24]. The second family comprises RNA triphosphatases from DNA viruses, fungus, and protozoan [22,25–28]. These enzymes require divalent cations for their enzymatic activity and share characteristic conserved elements. Furthermore, they also possess the ability to hydrolyze NTPs. Crystal structure analyses of the yeast RNA triphosphatase have revealed a un-

*Corresponding author. Fax: +1 819 564 5340.
E-mail address: martin.bisaillon@usherbrooke.ca (M. Bisaillon).

[1] These authors contributed equally to this work.

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ique active site architecture in which an eight-stranded β-barrel forms a topologically closed tunnel [29]. Interestingly, RNA triphosphatases from RNA viruses belonging to the *Alphaviridae*, *Reoviridae*, and *Flaviviridae* families appear to lack any resemblance to enzymes from the two classical RNA triphosphatase families [13,30–33]. It is thought that the active site of the RNA triphosphatases from RNA viruses might share elements with the helicase/NTPase catalytic center.

As a first step towards elucidating the nature of the specific interaction between RNA and the WNV RNA triphosphatase, we have utilized fluorescence spectroscopy to precisely monitor the energetics of RNA binding to the enzyme. We report a detailed study of the binding kinetics and thermodynamic parameters involved in the interaction between RNA and the NS3 protein. Using circular dichroism, we also investigated the effect of RNA binding on both the structure and stability of the protein. Finally, we developed a three-dimensional model of the WNV NS3 protein based on the crystal structure of related proteins from other flaviviruses. We believe that such quantitative analyses, and provide crucial insights on the interaction between RNA and RNA triphosphatases.

2. Materials and methods

2.1. cDNA synthesis and cloning

A cDNA fragment covering the C-terminal region of the WNV (strain NY99) NS3 gene was synthesized from genomic WNV RNA (a generous gift of Dr. Hugues Charest) through reverse-transcription (RT)-PCR according to the manufacturer's instructions (Qiagen) using the primers 5′-GGCTCATACATAAGCGCGATACATATGGGTGAAAGGA-TGGATGAGCCA-3′ and 5′-CAGAACCTCAATGAGCCCGGAT-CCTTAACGTTTTCCCGAGGCGAAGTC-3′. These primers were used to generate an *Nde*I site and a *Bam*HI site flanking the 3′ region of the WNV NS3 gene. The generated cDNA corresponds to the 5486–6495 nucleotide region segment of the WNV genome strain NY99.

2.2. Expression and purification of the recombinant NS3 protein

A plasmid for the expression of a truncated WNV NS3 protein was generated by inserting a truncated version of the WNV NS3 gene, encompassing amino acid residues 168–618, between the *Nde*I and *Bam*HI cloning sites of the pET28a expression plasmid (Novagen). In this context, the truncated NS3 protein is fused in frame with an N-terminal peptide containing 6 tandem histidine residues, and expression of the His-tagged protein is driven by a T7 RNA polymerase promoter. The resulting recombinant plasmid (pET-NS3Δ167) was transformed into *Escherichia coli* BL21(DE3) and a 1000-ml culture of *E. coli* BL21(DE3)/pET-NS3Δ166 was grown at 37 °C in Luria-Bertani medium containing 30 μg/ml of kanamycin until the A_{600} reached 0.5. The culture was adjusted to 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and the incubation continued at 18 °C for 20 h in the presence of 2% ethanol. The cells were then harvested by centrifugation, and the pellet stored at −80 °C. All subsequent procedures were performed at 4 °C. Thawed bacteria pellets were resuspended in 5 ml of lysis buffer A (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 10% sucrose) and cell lysis achieved by the addition of lysozyme and Triton X-100 to final concentrations of 50 μg/ml and 0.1%, respectively. The lysates were sonicated to reduce viscosity, and any insoluble material removed by centrifugation at 13000 rpm for 45 min. The soluble extract was applied to a 3-ml column of Ni-nitrilotriacetic acid-agarose (Qiagen) that had been equilibrated with buffer A containing 0.1% Triton X-100. The column was washed with buffer A containing 5 mM imidazole, and then eluted stepwise with buffer B (50 mM Tris–HCl, pH 8.0, 0.1 M NaCl, and 10% glycerol) containing 50, 100, 200, 500, and 1000 mM imidazole. The polypeptide composition of the column fractions was monitored by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE). The recombinant NS3 protein was retained on the column and recovered predominantly in the 200 mM imidazole eluate. The eluate was applied to a 3-ml column of phosphocellulose that had been equilibrated in buffer C (50 mM Tris–HCl, pH 8.0, 50 mM NaCl, and 10% glycerol). The column was washed with the same buffer, and then eluted stepwise with buffer C containing 0.1, 0.2, 0.3, 0.4, 0.5, and 1.0 M NaCl. The recombinant protein was retained on the column and was recovered in both the 0.5 M and 1.0 M NaCl fractions. Following dialysis against buffer C, the phosphocellulose preparation was stored at −80 °C. Protein concentration was determined by the Bradford dye binding method using bovine serum albumin as the standard.

2.3. Preparation of the RNA triphosphatase substrate

An RNA substrate of 84 nucleotides was synthesized with the MAXIscript kit (Ambion) using T7 RNA polymerase and [γ-^{32}P]GTP. The RNA transcript was synthesized from the pBS-KSII+ plasmid (Stratagene) that had been linearized with *Hin*dIII. The RNA substrate was purified on a denaturing 10% polyacrylamide gel and visualized by ultraviolet shadowing. The corresponding band was excised, and then eluted from the gel by an overnight incubation in 0.1% SDS/0.5 M ammonium acetate. The RNA was then precipitated with ethanol and quantitated by spectrophotometry.

2.4. RNA triphosphatase assay

Reaction mixtures (20 μl) containing 50 mM Tris–HCl, pH 7.5, 5 mM DTT, 0.9 μM of the RNA substrate radiolabeled at its terminal γ-phosphate, and various concentrations of enzyme (as indicated) were incubated for 60 min at 37 °C. The reactions were quenched by adding 2 μl of 5 M formic acid. Aliquots of the mixtures were applied to a polyethyleneimine-cellulose TLC plate, which was developed with 0.75 M potassium phosphate, pH 4.3. The release of ^{32}Pi was quantitated by scanning the TLC plate with a phosphorimager.

2.5. Synthetic RNAs

RNA molecules of various lengths were obtained from Integrated DNA Technologies (Coralville, Iowa). The 5′ hydroxy-terminated RNAs were quantified by spectrophotometry at 260 nm.

2.6. Fluorescence measurements

Fluorescence was measured using an Hitachi F-2500 fluorescence spectrophotometer. Excitation was performed at a wavelength of 290 nm. Background emission was eliminated by subtracting the signal from either buffer alone or buffer containing the appropriate quantity of substrate.

The extent to which RNA binds to the WNV NS3 protein was determined by monitoring the fluorescence emission of a fixed concentration of proteins and titrating with a given ligand. The binding can be described by

$$K_d = \frac{[NS3][RNA]}{[NS3 \cdot RNA]} \quad (1)$$

where K_d is the apparent dissociation constant, [NS3] is the concentration of the protein, [NS3 · RNA] is the concentration of complexed protein, and [RNA] is the concentration of unbound RNA.

The proportion of RNA-bound protein as described by Eq. (1) is related to measured fluorescence emission intensity by

$$\Delta F/\Delta F_{max} = [NS3 \cdot RNA]/[NS3]_{tot} \quad (2)$$

where ΔF is the magnitude of the difference between the observed fluorescence intensity at a given concentration of RNA and the fluorescence intensity in the absence of RNA, ΔF_{max} is the difference at infinite [RNA], and $[NS3]_{tot}$ is the total protein concentration.

If the total RNA concentration, $[RNA]_{tot}$, is in large molar excess relative to $[NS3]_{tot}$, then it can be assumed that [RNA] is approximately equal to $[RNA]_{tot}$. Eqs. (1) and (2) can then be combined to give

$$\Delta F/\Delta F_{max} = [RNA]_{tot}/(K_d + [RNA]_{tot}) \quad (3)$$

The K_d values were determined from a non-linear least square regression analysis of titration data by using Eq. (3).

2.7. Electrophoretic mobility shift assays

An electrophoretic mobility shift assay was used to measure the binding of the WNV NS3 protein to RNA. A radiolabeled RNA substrate of 84 nucleotides was synthesized by in vitro transcription using