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# Analysis of the nucleoside triphosphatase, RNA triphosphatase, and unwinding activities of the helicase domain of dengue virus NS3 protein

Chun-Chung Wang [a,b], Zhi-Shun Huang [a], Pei-Ling Chiang [a,c], Chien-Tsun Chen [a,d], Huey-Nan Wu [a,*]

[a] Institute of Molecular Biology, Academia Sinica, Nankang, 115 Taipei, Taiwan
[b] Graduate Institute of Life Science, Medical College, National Defense University, Taipei, Taiwan
[c] Graduate Institute of Life Science, National Central University, Chung-Li, Taiwan
[d] Graduate Institute of Cell and Molecular and Biology, Taipei Medical University, Taipei, Taiwan

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ABSTRACT

**The helicase domain of dengue virus NS3 protein (DENV NS3H) contains RNA-stimulated nucleoside triphosphatase (NTPase), ATPase/helicase, and RNA 5′-triphosphatase (RTPase) activities that are essential for viral RNA replication and capping. Here, we show that DENV NS3H unwinds 3′-tailed duplex with an RNA but not a DNA loading strand, and the helicase activity is poorly processive. The substrate of the divalent cation-dependent RTPase activity is not restricted to viral RNA 5′-terminus, a protruding 5′-terminus made the RNA 5′-triphosphate readily accessible to DENV NS3H. DENV NS3H preferentially binds RNA to DNA, and the functional interaction with RNA is sensitive to ionic strength.**


## 1. Introduction

The mosquito-transmitted dengue virus (DENV) belongs to the *Flavivirus* genus of the *Flaviviridae* family. The *Flavivirus* genus includes other human pathogens such as Japanese encephalitis virus (JEV), yellow fever virus (YFV), and West Nile virus (WNV). The *Flaviviridae* family contains another two genera, *Pestivirus* and *Hepacivirus*, where hepatitis C virus (HCV) is the only characterized hepacivirus [1]. Flaviviruses are small, enveloped viruses that have an ~11 kb positive-stranded RNA genome containing a 5′ cap but lacking a 3′-poly(A) tail. The flavivirus genome encodes a polyprotein of 370 kDa that is processed into three structural proteins, C, prM, and E, and seven non-structural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5, by host and viral proteases [1]. Replication of flaviviruse RNA is initiated at the 3′-terminus of the genome by a semi-conservative, asymmetric replication mechanism with the double-stranded replication form functions as a recycling template, and the replication intermediates contain, in addition, nascent genomic RNA [2].

DENV NS3 protein is a multifunctional protein in which the N-terminal 180 amino acids encode a protease that mediates viral polyprotein processing, while the C-terminal two-thirds of the protein contains the conserved motifs found in the DExD/H box helicases. DENV with impaired protease or helicase activity fails to replicate, belying the importance of NS3 in the virus life cycle [3]. The full-length NS3, as well as the helicase domain of DENV and other flavivirus NS3 proteins, are able to hydrolyze ATP and unwind dsRNA [4–6]. However, the NS3 helicase of the *Flavivirus* genus but not the *Hepacivirus* genus also exhibits an RNA triphosphatase (RTPase) activity that catalyzes the cleavage of the γ–β phosphoric anhydride bond of 5′-triphosphorylated RNA [4,5,7,8], and the RTPase activity is the first of three sequential enzymatic reactions of RNA 5′-capping. NS5 is the largest viral protein, comprising a 5′-RNA methyltransferase activity at the N-terminus and a conserved RNA-dependent RNA polymerase domain of RNA viruses at the C-terminus [9,10]. NS3 and NS5 proteins exist in a complex in DENV-infected cells, and the NTPase and RTPase activities of NS3 protein can be stimulated by NS5 protein in vitro [4,11]. These observations are consistent with a role of the complex formed by NS3 and NS5 in viral replication and RNA capping.

The DENV genome is highly structured. It is speculated that the NS3 helicase facilitates the adoption by the 5′- and 3′-untranslated regions of a structure suitable for replication complex assembly and assists the release of newly synthesized viral genome from replication intermediates. The NS3 protein may also alter the secondary structure of the nascent genomic RNA near the 5′-terminus to render the 5′-triphosphate more readily accessible for γ-phosphate

* Corresponding author. Fax: +8862 7826085.
*E-mail address:* hnwu@gate.sinica.edu.tw (H.-N. Wu).

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removal, the first step of the 5′-capping process, by the same or another NS3 protein. The ATPase/helicase activity of the HCV NS3 helicase has been well characterized [12,13]. However, relatively little is known about the unwinding mechanism of the flavivirus NS3 helicases, such as their polarity of unwinding, loading strand requirements, specificity for duplex substrate, or the processivity of the enzyme. Mutational analysis has disclosed that the ATPase/helicase and RTPase activities of DENV NS3 share a common active site [7,8], while published data concerning the substrate specificity of the RTPase activity is limiting.

In this study, we investigated further the biochemical properties and enzymatic activity of the helicase domain of DENV NS3 protein, and report here our functional characterization of this protein. The findings of this study may provide a basis for further study of the flavivirus replication mechanism.

## 2. Materials and methods

### 2.1. Proteins

The wild type and the mutant DENV NS3 helicase (serotype 2, PL046 strain) and the HCV NS3 helicase (a Taiwan genotype 1b strain, Ref. [12]) were expressed in *Escherichia coli* BL21 (DE3) cells and purified by Ni-NTA column chromatography to near homogeneity (Supplementary Fig. S1).

### 2.2. Nucleic acids

Short DNA and RNA oligos and poly(rU) were purchased from commercial sources. When applicable, these nucleic acids were 5′-end labeled by [$\gamma$-$^{32}$P] ATP and T4 polynucleotide kinase. DENV RNA fragments were synthesized by T7 RNA polymerase, and RNAs were 5′-end or internally labeled by incorporating [$\gamma$-$^{32}$P] GTP or [$\alpha$-$^{32}$P] UTP during their in vitro synthesis. All [$^{32}$P]-labeled nucleic acids were gel purified. Duplexes were made by heat-denaturation and renaturation of complementary molecules at a 1:1 or 1:1.5 molar ratio, and with a final concentration of 5 nM duplex.

### 2.3. ATPase and RTPase assays

ATPase assay was performed with 0.25 mM [$\gamma$-$^{32}$P] ATP and 50 nM of NS3H protein in reaction buffer containing 40 mM HEPES (pH 7.5), 10 mM NaCl, 2 mM DTT, 0.1 mM EDTA, 1.5 mM $MgCl_2$ or $MnCl_2$, 0 or 0.1 mM nucleotide poly(rU) at 37 °C. RTPase assay was performed with 10 nM [$\gamma$-$^{32}$P] RNA and 50 nM of NS3H protein in reaction buffer containing 40 mM HEPES (pH 7.5), 10 mM NaCl, 2 mM DTT, 0.1 mM EDTA, 0.4 U/μL RNase inhibitor, and 2 mM $MgCl_2$ or $MnCl_2$ at 25 °C. Aliquots were taken and spotted onto a polyethyleneimine (PEI) cellulose thin-layer chromatography (TLC) plate, which was subsequently developed in 1 M formic acid and 0.5 M LiCl. Reaction products were visualized by autoradiography and quantified with a Phosphoimager and Image Quant software.

### 2.4. Unwinding reaction

The standard unwinding assay was performed with 0.5 nM duplex and 0–400 nM NS3H protein in the reaction buffer containing 20 mM HEPES (pH 7.0), 10 mM NaCl, 2 mM DTT, 0.1 mg/mL BSA plus 1.5 mM $MnCl_2$ and 2.5 mM ATP at 37 °C. The reaction was terminated by the treatment of protease K and nucleic acid molecules were separated by electrophoresis at 4 °C. Gels were dried and autoradiographed. The radiolabeled species were quantified with a Phosphoimager and Image Quant software.

### 2.5. Filter binding assay

The protein–nucleic acid complex was formed in binding buffer containing 40 mM HEPES (pH 7.5), 2 mM DTT, 0.1 mM EDTA, 10–410 mM NaCl, and 40 μg/mL BSA at 4 °C for 30 min. The reaction with a volume of 50 μL was filtered through a nitrocellulose membrane overlaid on a Hybond N+ nylon membrane in a slot blot apparatus. The complex was retained on a nitrocellulose membrane and the unbound nucleic acid was trapped by the Hybond N+ nylon membrane.

## 3. Results and discussion

### 3.1. The NS3 helicase proteins

We cloned cDNA encoding the NS3 ATPase/helicase from a DENV, expressed the protein in *E. coli*, and purified the C-terminal His-tag recombinant DENV NS3H protein. The DENV NS3 helicase motif I mutant containing the $^{199}$K$^{200}$T to $^{199}$A$^{200}$A substitution (DENV mNS3H) and the HCV NS3 helicase (HCV NS3H) were prepared for use as controls in the activity assays.

### 3.2. NTPase activity

DENV NS3H contains the Walker A and Walker B motifs (motif I, GK(S/T) and motif II, DExD/H) (Supplementary Fig. S1) that are present in many NTPases/helicases [14]. To test whether the recombinant DENV NS3H was active, we analyzed the ability of DENV NS3H to hydrolyze ATP. DENV NS3H catalyzed ATP hydrolysis in the presence of $MgCl_2$ or $MnCl_2$, and $MgCl_2$ was more effective than $MnCl_2$ at inducing ATPase activity at concentrations ranging from 0.1 mM to 5 mM (Fig. 1A and B, and data not shown). With $Mg^{2+}$ as the divalent cation, the rate of ATP hydrolysis increased slightly when the NaCl concentration was elevated in the range of 10 mM and 200 mM (Fig. 1C). Thus, DENV NS3H possesses an ATPase activity that requires a divalent cation cofactor to function but is not sensitive to high ionic strength.

Many NTPases/helicases require polynucleotide binding to turn on the NTPase activity [4,12,15]. To test the influence of polynucleotide on DENV NS3H, we assayed ATPase activity in the presence of poly(rU) (Fig. 1A). At saturating poly(rU) concentrations of around 0.1 mM nucleotides, the rate of ATP hydrolysis was enhanced approximately 10 times with $Mg^{2+}$ and more than 100 times over basal levels with $Mn^{2+}$ as divalent cation cofactors (Fig. 1B). These results show that DENV NS3H is a polyribonucleotide-stimulated ATPase. Presumably the poly(rU) binding triggers a conformational rearrangement of DENV NS3H that activates the catalytic core of the enzyme for ATP hydrolysis.

DENV NS3H also hydrolyzed other nucleoside 5′-triphosphates and the NTPase activity was stimulated by poly(rU) (data not shown). In the mutant protein, DENV mNS3H, the $^{199}$K$^{200}$T to $^{199}$A$^{200}$A substitution eliminated both the basal and the RNA-stimulated NTPase activity (Fig. 1A). Thus, DENV mNS3H is an NTPase-deficient mutant of DENV NS3H.

We performed the ATPase assay in the presence of poly(rU) and increasing concentrations of NaCl. The stimulatory effect of poly(rU) on ATP hydrolysis was significantly attenuated when NaCl concentration was 50 mM or higher (Fig. 1C, with MgCl textsubscript2). The hypersensitivity of the poly(rU)-stimulated ATPase activity but not the basal ATPase activity to high ionic strength confirms the speculation that the functional binding of poly(rU) to DENV NS3H is mainly through electrostatic interaction.

These findings of the NTPase activity of DENV NS3H are in accordance with the observed divalent cation-dependent and ionic