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Characterization of soluble RNA-dependent RNA polymerase from dengue virus serotype 2: The polyhistidine tag compromises the polymerase activity

Maliwan Kamkaew, Sarin Chimnaronk *

Institute of Molecular Biosciences, Mahidol University, Salaya Campus, Phutthamonthon 4 Rd., Nakhon Pathom 73170, Thailand

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ABSTRACT

The viral RNA polymerase is an attractive target for inhibition in the treatment of viral infections. In the case of dengue virus (DENV), a member of the genus *Flavivirus*, the RNA-dependent RNA polymerase (RdRp) activity resides in the C-terminal two-thirds of non-structural protein (NS) 5 responsible for the *de novo* synthesis of the viral RNA genome. Among four distinct, but closely related dengue serotypes, serotype 2 (DENV-2) produces more severe diseases than other serotypes. It has been reported that bacterial production of the recombinant DENV-2 RdRp was difficult due to its low expression and solubility levels. To facilitate functional and structural analyses, we here demonstrate complete protocols for overexpression and purification of soluble DENV-2 RdRp, increasing protein yields by a remarkable 10 times compared to earlier reports. Three different forms of DENV-2 RdRp as either N- or C-terminally His-tagged fusions, or without tag, were purified to homogeneity. We show here that the presence of both the N- and C-terminal His-tag had a deleterious effect on polymerase activity and, in contrast to earlier studies, our non-tagged RdRp did not require manganese ions to activate RNA polymerization. We also determined an apparent K_d value of 53 nM for binding to the 5′-UTR RNA by surface plasmon resonance (SPR). Our work provide a more suitable material for basic research of viral RdRp and for drug development.

Introduction

Dengue fever has become one of the most important mosquito-borne disease worldwide, accounting for approximately 96 million patients per year presenting several degrees of symptoms, including life-threatening dengue hemorrhagic fever (DHF)[1] and dengue shock syndrome (DSS) [1]. Currently, there is no commercially available vaccine or specific medication. The causative dengue virus (DENV) belongs to the *Flavivirus* genus and is classified into four distinct serotypes, DENV-1–4, which share ~60% genomic sequence identity [2]. It was reported that severe dengue cases were significantly associated with circulating infection by DENV-2 [3]. While accurate mechanisms for dengue severe symptoms remain largely elusive, two viral non-structural (NS) proteins 3 and 5 were implicated in DHF as predominant T-cell antigens [4]. DENV possesses a single positive-sense stranded 10.7-kb RNA genome encoding a single long open reading frame (ORF) flanked by 5′ and 3′ untranslated regions (UTRs). The DENV ORF is translated into a pre-polyprotein having 3390 amino acids, which is subsequently proteolytically processed into three structural proteins: capsid (C), membrane (prM), and envelope (E), and seven non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 [5]. Of these, NS5 is the largest viral protein with the highest sequence conservation among serotypes [6]. NS5 is a multifunctional protein containing an N-terminal methyltransferase (MTase) and a C-terminal RNA-dependent RNA polymerase (RdRp), which play crucial roles in viral RNA synthesis in infected cells. Therefore, NS5 has been a primary target for antiviral drug development for a decade [7,8]. To date, crystal structures of the 73-kDa RdRp domain from DENV-3 have been determined to a high resolution of ~1.8 Å, revealing a classical polymerase right-hand conformation composed of fingers, thumb, and palm domains [6,9]. The fingers domain contains flexible loops that link together the fingers and thumb domains, and are likely to transmit conformational changes between these two

* Corresponding author. Tel.: +66 2 441 9004x1468; fax: +66 2 441 9906.
E-mail address: sarin.chi@mahidol.ac.th (S. Chimnaronk).

[1] *Abbreviations used:* DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome; DENV, dengue virus; NS, non-structural; ORF, open reading frame; UTRs, untranslated regions; MTase, methyltransferase; SLA, stem-loop A; IPTG, isopropyl-β-D-thiogalactopyranoside; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene fluoride; TAP, tandem affinity purification; CD, circular dichroism; EMSA, electrophoretic mobility shift assay; SPR, surface plasmon resonance.

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domains. The thumb domain is involved in shaping the RNA template tunnel. There are two zinc-binding motifs in the fingers and thumb domains, where the zinc ion is thought to play a role in regulating conformational switches within the domain [6,9]. RdRp harbors the catalytic active site in the palm domain, comprised of an invariant Gly662–Asp663–Asp664 (GDD) motif and a coordinated Asp533 that binds to a catalytic Mg^{2+} ion to locate incoming NTP substrates for RNA polymerization [6,9].

DENV RdRp is capable of *de novo* RNA polymerization without a primer, and is active on homopolymeric RNA template (for example, poly(rC)) *in vitro* in the presence of Mn^{2+} ions [10]. Considerable Mn^{2+} concentrations of 2–5 mM were also used in reactions with short genomic templates including the 3′-end of the positive strand [11]. Addition of Mn^{2+} apparently accelerated RNA synthesis by significantly decreasing K_m 30-fold [10,12]. However, the Mn^{2+} concentration exploited in the *in vitro* assay exceeded that in cytoplasm by three orders of magnitude [13]. On the other hand, it has been suggested that DENV RdRp required a specific interaction with the first 70-nt stem-loop A (SLA) RNA element in the 5′-UTR for initiation of RNA synthesis [14]. The RNA footprint experiments revealed the top and side loops of SLA as the RdRp-binding site; however, SLA alone did not bind to RdRp in an *in vitro* EMSA study [15]. These discrepancies imply substantial differences between *in vitro* studies and physiological conditions, and precise mechanisms for viral RNA synthesis still remain enigmatic.

Our interest is RdRp from DENV-2, for which the crystal structure is not available. This is due to difficulty in expression and solubilization of recombinant DENV-2 RdRp in *Escherichia coli* cells, with protein solubility only 10–20% in *E. coli* and protein yields was not higher than 0.3 mg per liter of culture [10,16]. Moreover, to our knowledge, RdRp has always been characterized in a form fused with a poly-histidine tag [10,12,14,15,17,18] that could affect the expression level, solubility, structure, and biological activity [19–23]. Here we describe preparation of the non-tagged DENV-2 RdRp with high quality and quantity. Characterization of our recombinant protein suggests intensive caution should be taken in interpretation of *in vitro* assays with the tagged protein.

Materials and methods

Cloning and expression of DENV-2 RdRps

The RdRp domain, residues 277–900, of NS5 was amplified from DENV serotype 2 cDNA with a forward primer (5′-GG*GCTAGC*CCAAACCTAGATATAATTGG-3′) and a reverse primer containing a stop codon (underline) (5′-GGG*CTCGAG*TTAACCACCCC ACAGAACTGGTGG-3′) for the N-terminal tagged construct (N_{His}-RdRp), or a tandem hexahistidine-FLAG tag (bold characters) (5′-GGG*CTCGAG*TTA**CTTATCATCATCATCCTTGTAATC**ACCACCACC**GT GGTGGTGGTGGTGGTG**ACCACCACCCCACAGAACTCCTGC-3′) for the C-terminal tag fusion (RdRp-$C_{HisFLAG}$). Resulting PCR products were digested with *Nhe*I and *Xho*I, gel-purified, and ligated into pET-28b and pET-21b vectors for N_{His}-RdRp and RdRp-$C_{HisFLAG}$, respectively. Resultant plasmids were transformed into *E. coli* BL21 (DE3) and Rosetta (DE3) strains, grown at 37 °C in LB medium including 1% glucose, and challenged with various conditions of expression induction: (i) final concentrations (50–500 μM) of isopropyl-β-D-thiogalactopyranoside (IPTG); (ii) the timing of IPTG induction (OD_{600} = 0.6–1.0); (iii) the induction temperatures (18–37 °C); (iv) the presence/absence of 10 μM $ZnSO_4$; and (v) the auto-induction media (0.5% glycerol, 0.05% glucose, and 0.2% lactose) [24]. Cells were harvested by centrifugation at 6000*g* for 10 min at 4 °C, and resuspended in lysis buffer containing 50 mM Tris pH 7.6, 500 mM KCl, 7 mM β-mercaptoethanol, 10% glycerol, 0.1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Proteins were extracted via sonication on ice, and fractionated by centrifugation at 15,000*g* for 1 h at 4 °C.

Western blotting

Total cell lysate, soluble and insoluble fractions were separated on 10% SDS–PAGE followed by electroblotting onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked in 5% (w/v) skim milk in phosphate-buffered saline containing 0.1% (w/v) Tween-20 (PBS-T) overnight at 4 °C. Rabbit anti-NS5 polyclonal antibody (GeneTex) was used at a dilution of 1/2000 and incubated with the membranes for 1 h at room temperature. After washing with PBS-T, the membrane was incubated with an anti-rabbit secondary antibody-horseradish peroxidase (Dako) at a dilution of 1/2000 for 30 min at room temperature, and DENV RdRp was visualized using ECL reagents (GE Healthcare) according to the manufacturer's instructions.

Protein purification

Expression of the N- and C-terminal tagged RdRps were induced in *E. coli* Rosetta (DE3) and BL21 (DE3) with 200 and 50 μM IPTG at the early log phase (OD_{600} = 0.6–0.7) at 18 °C and 25 °C, respectively, and proceeded for 16 h. Cells were harvested by centrifugation at 6000*g* for 10 min at 4 °C, and resuspended in 20 ml lysis buffer per approximately 3.6 g wet cell paste from 1 l culture. Proteins were extracted from cells by sonication in the presence of 1 mM PMSF and 50 μg/ml lysozyme, and fractionated by ultracentrifugation at 100,000*g* for 1 h at 4 °C to completely remove cell debris, membrane fraction, and insoluble proteins. The supernatants were loaded onto the nickel-affinity HisTrap HP column (GE Healthcare). Unbound proteins were washed twice with the lysis buffer supplemented with 50 mM and 88 mM imidazole. His-tagged RdRps were eluted by a linear gradient of imidazole from 88 to 350 mM. In some cases, the His-tag was removed from N_{His}-RdRp by addition of 2 units thrombin per 1 mg protein and dialysis against the lysis buffer for 2 days at 4 °C. The three different RdRp constructs were further purified via the anion exchange HiTrap Q column with a linear gradient of 50–900 mM KCl in solution containing 50 mM Tris–HCl pH 9.0, 0.2 mM EDTA, 2 mM DTT and 10% glycerol. The final purification process was performed with gel filtration chromatography using the Superdex 200 column (GE Healthcare) equilibrated with 50 mM Tris–HCl pH 7.6, 100 mM KCl, 2 mM DTT and 10% glycerol. Purified proteins were flash frozen by liquid nitrogen and stored at −80 °C until use.

Polymerase activity assay

We assayed the *in vitro* RdRp activity by measurement of accumulation of pyrophosphate (PPi) during the RNA polymerization reaction using a phosphate-based colorimetric method with malachite green-molybdate as the color-developing reagent. The reactions were prepared in 25 μl solutions containing 2 μM RdRp in 25 mM Tris–HCl pH 7.5, 2.5 mM $MgCl_2$, 4 mM DTT, 0.1 μM poly (rC) template, 1 mM GTP, 20 U/ml RNase inhibitor, and 3 μM thermostable pyrophosphatase (PPase), and conducted at 30 °C. The reaction mixtures were quenched by heating at 70 °C for 20 min, where PPi was simultaneously converted to two molecules of inorganic phosphate (Pi) by PPase. Detection of Pi was performed by mixing 10 μl of the reaction mixture with 30 μl Milli Q water and 100 μl of the malachite green-molybdate reagent [25,26]. The phosphomolybdate complexes were measured by the absorbance at 650 nm in a microplate reader. The amount of Pi was quantified using a phosphate standard curve obtained from 25 to 125 μM NaH_2PO_4 standard solution. An inactive GDD → GAA