Biochimica et Biophysica Acta 1794 (2009) 50–60

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Metal ion-binding studies highlight important differences between flaviviral RNA polymerases

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ARTICLE INFO

Article history:
Received 9 April 2008
Received in revised form 4 September 2008
Accepted 11 September 2008
Available online 1 October 2008

Keywords:
West Nile virus
Magnesium ion
Denaturation study
Fluorescence spectroscopy
Circular dichroism

ABSTRACT

West Nile virus (WNV) is a member of the *Flaviviridae* family which includes a number of important human pathogens. The WNV NS5 protein harbors an RNA-dependent RNA polymerase activity which is required both for replication and transcription of the viral genome. To extend our studies on the role of metal ions in the activity of flaviviral polymerases, we have used fluorescence spectroscopy, circular dichroism, and a combination of chemical and thermal denaturation assays to monitor the consequences of metal ion binding to the enzyme. We demonstrate that the binding of magnesium is not critical for the structural stabilization of the enzyme. Moreover, structural studies indicate that the protein does not undergo conformational change upon the binding of magnesium ions. Additional binding assays also indicate that the interaction of magnesium ions with the enzyme does not significantly stimulate the interaction with the RNA or NTP substrates. The inability of cobalt hexamine, an exchange-inert metal complex structurally analogous to magnesium hexahydrate, to support the catalytic activity also allowed us to demonstrate a direct role of magnesium ions in the catalytic activity of the enzyme. Finally, a three-dimensional structural model of the active center of the enzyme was generated which highlighted the importance of two aspartate residues involved in the coordination of two metal ions. Mutational analyses confirmed the importance of these two amino acids for the binding of magnesium ions. Our data provide further insight into the precise role of magnesium ions for the RNA polymerase activity of the protein, and more importantly, highlight key differences between the RNA polymerases of the *Flaviviridae* family.

1. Introduction

West Nile virus (WNV) is a mosquito-borne virus that primarily infects birds but can also occasionally infect 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], spreading rapidly throughout North America. The lack of vaccines or therapeutic treatments for WNV infections has therefore prompted the elaboration of extensive prevention strategies.

The *Flaviviridae* virus family includes more than 70 human pathogens, and comprises three genera: the flaviviruses (including West Nile, yellow fever, and Dengue fever viruses), the hepaciviruses (such as hepatitis C virus), and pestiviruses (including bovine viral diarrhea virus) [5,6]. The flavivirus genome is a single-stranded RNA of positive polarity that harbors a methylated cap structure at its 5′ end [7–9]. The genome encodes for a single open reading frame of about 11 kb in length that is translated into a single large polyprotein precursor which is processed into three structural (capsid C, membrane M, and envelope E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) by both viral and cellular proteases [8,10,11].

The NS5 protein is the largest non-structural protein of flaviviruses. It harbors the RNA-dependent RNA polymerase activity which is required both for replication and transcription of the viral genome. As such, it represents an attractive target for the development of potent antiviral drugs. The crystal structures of the RNA polymerases of four members of the *Flaviviridae* family have been elucidated [12–17]. Analysis of the crystal structure of the RNA polymerases from hepatitis C virus (HCV), bovine viral diarrhea virus (BVDV), Dengue fever virus, and West Nile virus has revealed that the protein is folded into characteristic fingers, palm, and thumb subdomains [12–17]. The particular fold adopted by the palm subdomain is also shared by many proteins that bind nucleotides and/or nucleic acids [18]. The palm domain contains the catalytic center of the enzyme and displays the greatest structural conservation among all polymerases. However, analysis of the crystal structures of the four related flaviviral RNA polymerases has also revealed important structural differences [12–17]. For instance, a significant difference exists between the thumb domains of the BVDV and HCV polymerase. This region (termed β-hairpin in HCV and

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doi:10.1016/j.bbapap.2008.09.009

β-thumb in BVDV) is thought to play an important role during initiation of RNA synthesis. Moreover, important differences also exist between the fingers domain of the WNV enzyme and the corresponding domains of the HCV and BVDV RNA polymerases. It has been suggested that these differences may have mechanistic implications for the WNV enzyme, such as a requirement for conformational changes to promote efficient catalysis [16]. In addition, mounting evidences indicate that important differences exist at the molecular level between the RNA polymerases of the *Flaviviridiae* family. For instance, the enzyme from flaviviruses (Dengue fever virus and West Nile virus) also harbors a nucleoside-2′-O-methyltransferase activity which is involved in the addition of the RNA cap structure found at the 5′ end of the viral RNAs [19,20]. The RNA polymerases of both the pestiviruses (Bovine viral diarrhea virus) and hepaciviruses (Hepatitis C virus) do not harbor this methyltransferase activity. Kinetic assays also demonstrated that the various enzymes display differences upon the transition from the initiation to elongation of de novo RNA synthesis [21].

Although analysis of the crystal structure of the WNV RNA polymerase provided no information on the coordination geometry of metal ions in the active site of the enzyme [16], the crystal structure of the closely related HCV RNA polymerase revealed the presence of two metal ions in the active site of the enzyme [22]. These two metal ions are about 3.6 Å apart in the active site of the protein [22]. Structural studies indicated that the binding of metal ions to the HCV RNA polymerase induces a conformational change that is characterized by an increased exposure of hydrophobic regions [23,24]. These ion-induced conformational changes result in an increased structural stability of the enzyme, and are a prerequisite for catalytic activity, by correctly positioning the side chains of residues located in the active site of the enzyme [23,24]. Both crystallographic and fluorescence spectroscopy assays performed with the HCV RNA polymerase indicated that metal ion binding seems to be limited to the active site region, and does not involve other subdomains of the protein [22,23].

As a first step toward elucidating the nature and the role(s) of metal ions in the RNA polymerase reaction of flaviviral RNA polymerases, we had initially characterized the binding of metal ions to the HCV RNA polymerase [23,24]. Metal ions were shown to be critical for the structural stability of the enzyme [24]. Structural studies also demonstrated that the enzyme undergoes conformational change upon the binding of metal ions [23]. Here, we extend our analysis on the role of metal ions in the RNA polymerase reaction by evaluating the interaction of magnesium ions with the WNV RNA polymerase. Using a combination of fluorescence spectroscopy, circular dichroism, and denaturation assays, we analyzed both the functional and structural consequences of magnesium binding to the enzyme. Mutational studies were also performed and confirmed the importance of specific glutamate residues located in the active site of the enzyme for the binding of magnesium ions. These data shed light on the precise role of magnesium ions in the RNA polymerase activity of the protein, and more importantly, highlight important differences in the role of metal ions in the reactions catalyzed by the *Flaviviridae* RNA polymerases.

2. Materials and methods

2.1. cDNA synthesis and cloning

A cDNA corresponding to the WNV (strain NY99) NS5 gene was synthesized from genomic WNV RNA through reverse-transcription (RT)-PCR according to the manufacturer's instructions (Qiagen) using the primers 5′-GGCTCATACATAAGCGCGATA CATATGGGTGAAAG-GATGGATGAGCCA-3′ and 5′-CAGAACCTCAATGAGCC CGGATCCT-TAACGTTTTCCCGAGGCGAAGTC-3′. These primers were used to produce the NdeI and BamHI sites flanking the WNV NS5 gene.

2.2. Expression and purification of the recombinant NS5 protein

A plasmid for the expression of a full-length WNV NN5 protein was generated by inserting the WNV NS5 gene between the NdeI and BamHI cloning sites of the pET28a expression plasmid (Novagen). In this context, the 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-NS5) was transformed into *Escherichia coli* BL21(DE3) and a 1000 mL culture of *E. coli* BL21 (DE3)/pET-NS5 was grown at 37 °C in Luria-Bertani medium containing 30 μg/mL kanamycin until the A_{600} reached 0.5. The culture was adjusted to 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 2% ethanol, and the incubation continued at 18 °C for 20 h. 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 50 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 13,000 rpm for 45 min. The soluble extract was applied to a 5-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 the same buffer, 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 protein was retained on the column and recovered in the 200 and 500 mM imidazole fractions. Following dialysis against buffer C, buffer C (50 mM Tris–HCl, pH 8.0, 50 mM NaCl, 2 mM dithiothreitol, 10% glycerol, and 0.05% Triton X-100) the purified preparation was stored at −80 °C. Protein concentration was determined by the Bio-Rad dye binding method, based on the method of Bradford [25], using bovine serum albumin as the standard. Absorbance was measured at 595 nm in a final volume of 1 mL.

2.3. Polymerization assay

The standard RNA polymerase reaction (50 μl) was performed as described previously [26,27] in 20 mM Tris–HCl (pH 7.0), 1 mM DTT, 5 mM $MgCl_2$, 10 U of RNAguard (Amersham Biosciences), 50 mM NaCl, with 0.2 μCi [α-P^{32}]UTP, 10 μg/mL polyA/oligo(dT)$_{20}$, 0.2 μM of the purified enzyme, and 1 mM cold UTP. The reactions were incubated at 37 °C for 5 min, followed by phenol/chloroform extraction and ethanol precipitation. The precipitable radioactivity was quantitated by liquid scintillation counting.

2.4. Fluorescence measurements

Fluorescence was measured using a 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.

2.5. Circular dichroism spectroscopy measurements

Circular dichroism (CD) measurements were performed with a Jasco J-810 spectropolarimeter. The samples were analyzed in quartz cells with pathlengths of 1 mm. Far-UV and near-UV wavelength scans were recorded from 200 to 250 nm and from 250 to 340 nm, respectively. All the CD spectra were corrected by subtraction of the background for the spectrum obtained with either buffer alone or