^{1}H, ^{15}N and ^{13}C backbone resonance assignment of the N-terminal region of Zika virus NS4B protein in detergent micelles

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Abstract

Zika virus has raised global concerns due to its link to microcephaly and Guillain–Barré syndrome in adults. One of viral nonstructural proteins-NS4B, an integral membrane protein, plays crucial roles in viral replication by interacting with both viral and host proteins, rendering it an attractive drug target for antiviral development. We purified the N-terminal region of ZIKV NS4B (NS4B NTD) and reconstituted it into detergent micelles. Here, we report the assignments of the backbone resonances of NS4B NTD in detergent micelles. The available assignment is useful for understanding its structure and ligand binding to provide useful information for developing NS4B inhibitors.

Key word: zika virus; membrane protein; structure; NMR; drug discovery; NS4B

Biological Content

Zika virus (ZIKV) belongs to the genus *Flavivirus*, comprising over 70 members, including other important human pathogens like dengue virus (DENV), West Nile virus, yellow fever virus, tick-borne encephalitis virus, and Japanese encephalitis virus. The 2015 ZIKV outbreak led to global epidemics, prompting the World Health Organization to declare it a global emergency. While ZIKV infection typically results in mild symptoms, accumulated evidence links it to Guillain–Barré syndrome in adults and microcephaly in newborns (Cao-Lormeau et al. 2016, Schuler-Faccini et al. 2016). Despite a decline in case numbers, understanding the viral pathogenic mechanism and development of antiviral are still needed to prevent this type of virus (Vogel 2016). Structural biology is critical in drug discovery and development. Understanding the structures of viral proteins will shed light on antiviral develop.

The genome of ZIKV encodes a polypeptide that undergoes cleavage to result in three structural proteins (C, prM/M, and E) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The nonstructural proteins play important roles in viral replication by participating in a replication complex on the membrane. While NS3 and NS5 harbor enzymatic activities such as protease, nucleotide triphosphatase, RNA triphosphatase, helicase, methyltransferase, RNA-dependent RNA polymerase, other NS proteins lack enzymatic activities but are essential for viral replication and assembly. NS4B , a membrane protein with five transmembrane regions, (Li et al. 2016, Miller, Sparacio and Bartenschlager 2006) is an important drug target due to its roles in viral replication (Zmurko, Neyts and Dallmeier 2015). Previous studies on DENV NS4B have indicated that NS4B accumulates at or near the endoplasmic reticulum (ER) membrane or ER-derived membranes, where NS proteins and double-stranded viral RNA are concentrated, suggesting its involvement in viral replication (Xie et al. 2011, Welsch et al. 2009). Mutations in NS4B has been linked to increase of DENV replication kinetics (Grant et al. 2011). In addition, a study showed that ZIKV NS4B affects neurogenesis and induces autophagy through inhibiting the Akt/mTOR signaling pathway (Liang et al. 2016).

DENV NS4B has been observed to form dimers and associate with other NS proteins, a critical step for viral replication (Zou et al. 2015b, Zou et al. 2014). For instance, the cytoplasmic loop region spanning residues 129 to 165 of DENV NS2B has been identified as an important region for interaction with NS3 (Zou et al. 2015a). NS4B has demonstrated the ability to inhibit the interferon (INF) response, a host defense mechanism against viral infection (Sarratea et al. 2023). The first 125 residues of DENV2 NS4B are sufficient to suppress IFN-α/β signaling (Munoz-Jordan et al. 2005). Additionally, NS4B plays a role in suppressing host RNA interference response (Kakumani et al. 2013). Several small-molecule inhibitors targeting ZIKV NS4B gave been developed, although the protein-inhibitor complex is not yet fully elucidated (Xie et al. 2011, Xie et al. 2015, Patkar et al. 2009, Wang et al. 2015). While structural studies on DENV NS4B have provided insight into its secondary structures, membrane topology, and structural information for ZIKV NS4B remain essential to comprehend its function and facilitate inhibitor development.

To gain insight into the structure and dynamism of ZIKV NS4B, we expressed and purified the N-termina region containing residues 1-139, referred to as NTD, from *E. coli*. Subsequently, NS4B NTD was reconstituted into detergent micelles for solution NMR studies. Conventional NMR experiments were employed for backbone resonance assignment, and secondary structural analysis revealed the presence of four helices in ZIKV NS4B NTD in detergent micelles. These findings facilitate our understanding of the overall protein folding and provide a foundation for exploring protein-protein or protein-ligand interactions.

Methods and experiments

Protein purification

The cDNA to encode N-terminal region (NTD) containing residues 1-139 of ZIKV NS4B of ZIKV was synthesized (Genscript) and cloned into the Nde I and Xho I sites of pET15b (Li et al. 2015). The resulting

plasmid encodes a poly protein with an N-terminal tag, a thrombin cleavage site and N-terminal region of NS4B. To prepare isotopically labelled NS4B sample for NMR studies, bacterial cells harboring the plasmid were grown in M9 medium that contained 1 g/L ^{15}NH4Cl, 2 g/L ^{13}C-glucose and D_2O to replace normal water. Recombinant protein was induced at 18 °C, 200 rpm for overnight by adding β-D-1-thiogalactopyranoside (IPTG) to the culture with a final concentration of 1 mM when the cell optical density at 600 nm (OD_{600}) reached 0.6-0.8. The cells were harvested and the recombinant NS4B NTD was purified in the presence of into lyso-myristoyl phosphatidylglycerol (LMPG) micelles as described previously (Li et al. 2016). The cells were first broken in the presence 1-2% LMPG in an ice bath by sonication. The solution was cleared, and the supernatant was loaded in Ni^{2+}-NTA resin in a gravity column. Resin was then washed with a washing buffer. Recombinant NS4B NTD was then eluted with an elution buffer containing 300 mM imidazole, pH6.5, 0.1% LMPG (w/v), and 1.4 mM β-mercaptoethanol. Gel filtration chromatography was used for further purification and a buffer that contained 20 mM sodium phosphate, pH6.5, 0.1% LMPG (w/v), and 1 mM DTT was used. Purified protein was concentrated to 0.5-1.0 mM for NMR studies (Li et al. 2015).

NMR experiments for resonance assignments

A ^{13}C/^{15}N/^{2}H-labeled NTD was prepared for data acquisition for the backbone resonance assignments of ZIKV NS4B NTD (Li et al. 2023). Transverse relaxation-optimized spectroscopy (TROSY) (Pervushin et al. 1998, Salzmann et al. 1998)-based experiments including ^{1}H-^{15}N-HSQC, 3D-HNCACB, 3D-HNCOCACB, 3D-HNCOCA, 3D-HNCA, 3D-HNCACO, and 3D-HNCO experiments were collected at 313K on a Bruker Avance II 700 MHz or 600 MHz magnet which is equipped with a triple-resonance cryoprobe. The data were processed with TOPSPIN 2.1 (Bruker) and NMRPipe. Processed spectra were further analyzed using both NMRView (Johnson 2004), and CARA (http://www.mol.biol.ethz.ch/groups/wuthrich_group). Protein secondary structural elements were defined by using TALOSN (Shen et al. 2009) through analyzing the obtained chemical shifts.

Extent of assignments and data deposition

Analyzing the sequence of a membrane protein allows for the prediction of potential transmembrane regions. The transmembrane helix predictor-TMHMM (Krogh et al. 2001) was employed to assess the sequence of ZIKV NS4B NTD. The analysis shows three helical transmembrane regions in NS4B NTD, namely amino acids 37-54, amino acids 67-89, and amino acids 104-126 (**Fig. 1**). To obtain the protein for structural studies, detergent micelles, known for their membrane-mimetic properties, are commonly employed (Kang and Li 2011). Lyso-myristoyl phosphatidylglycerol (LMPG) was proven effective in the folding of membrane proteins of DENV and ZIKV for solution NMR studies (Li et al. 2015, Li and Kang 2020). LMPG was also utilized to support the folding of ZIKV NS4B NTD. The narrow dispersion range of amide protons (7.0-8.6 ppm) in the ^{1}H-^{15}N-HSQC spectrum indicates that the recombinant protein in micelles consists predominantly of helical and unstructured regions (**Fig. 2**).

As the protein-micelle complex behaves like a large molecular weight protein, a tiple-labeled sample was prepared and utilized for data collection. Transverse relaxation-optimized spectroscopy (TROSY)-based heteronuclear experiments were conducted and processed for the backbone resonance assignment. To enhance sensitivity, all the experiment were carried out at 40 °C. For the backbone amide resonance, 119 out of 133 residues (89%) excluding the fusion tags and six proline residues were assigned (**Fig. 2**). The residues with unassigned amide and amide protons include A39, A41-A44, T47, I50 and I113-L116. Approximately 88% Cα (122 out of 139), 82% Cβ (105 out of 128) and 75% C’ (105 out of 139) were assigned. The chemical shifts were analyzed to obtain the secondary structures of NS4B using TALOSN. NS4B NTD in LMPG micelles exhibits six helical structures namely α1 (E2-M17), α2 (A37-F49), α3 (A53-N63), α4 (S66-M80), α5 (L95-I122) and α6 (G126-R138) (**Fig. 3**). The presence of proline residues in the sequence might be important for the breakage of the helices. Further structural investigation will be crucial for elucidating detailed structural aspects. Nonetheless, our experimental findings have successfully defined the secondary structure of this region. In summary, we obtained ZIKV NS4B NTD in

LMPG micelles. Secondary structures and dynamics of NTD were explored using solution NMR spectroscopy. The availability of the recombinant protein and resonance assignment will be useful for probing its interactions with proteins or ligands.

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Author contributions Conceptualization: Y.L., Q.L. D.L. and C.K.; Methodology Y.L., Y.R.L. and C.K.; formal analysis and investigation: Y.L. and C.K.; Writing-original draft preparation: C.K.; Writing-review and editing: all authors.

Data availability The chemical shift assignments have been deposited to and are available from the RMRB data bank.

Declaration

Ethical approval Not applicable.

Consent to participate Not applicable

Competing interests The authors declare no competing interests.

Figure Legends

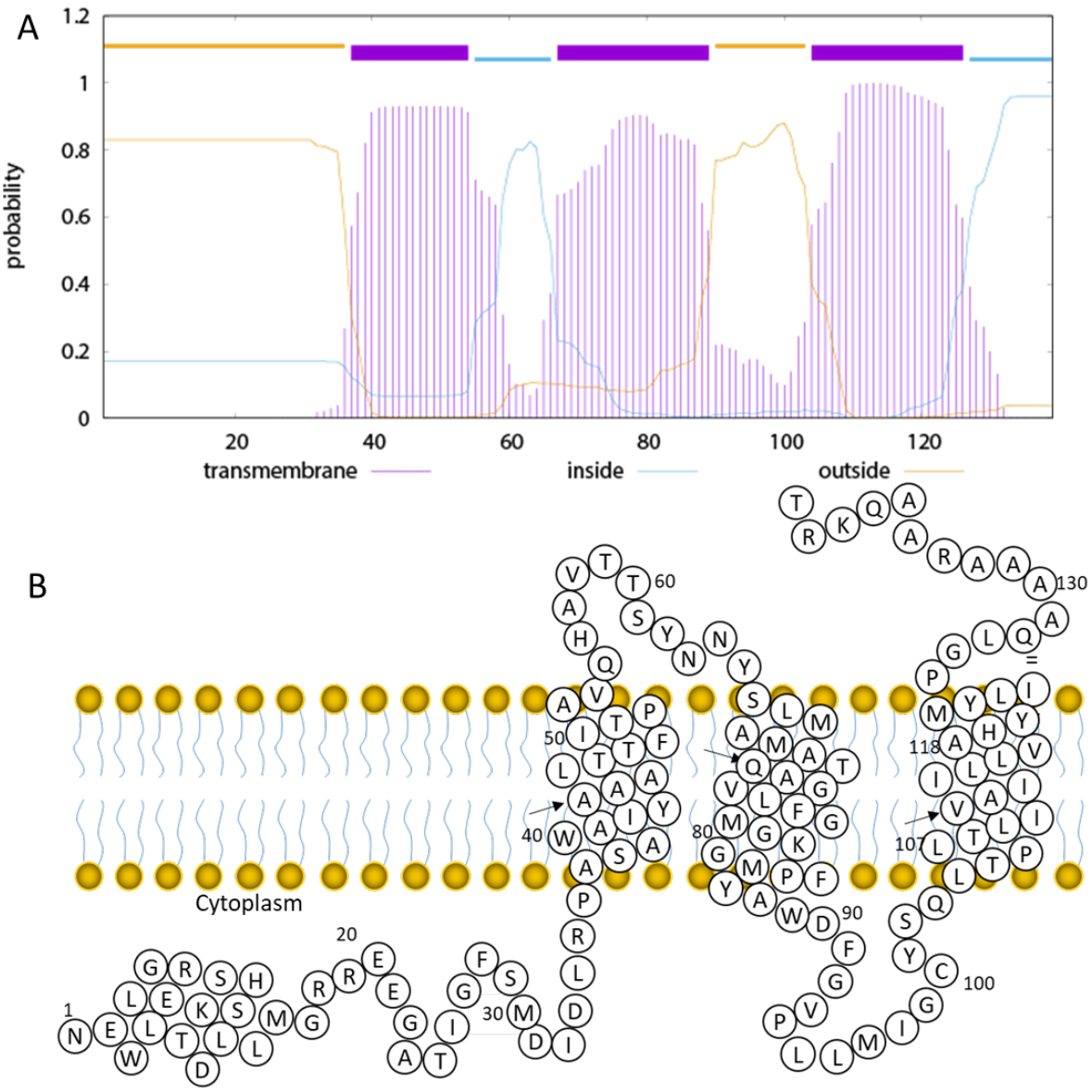

Figure 1. Sequence analysis of ZIKV NS4B NTD. A. Prediction of transmembrane helices by TMHMM. The predicted result is shown. B. Sequence presentation of transmembrane regions of ZIKV NS4B.

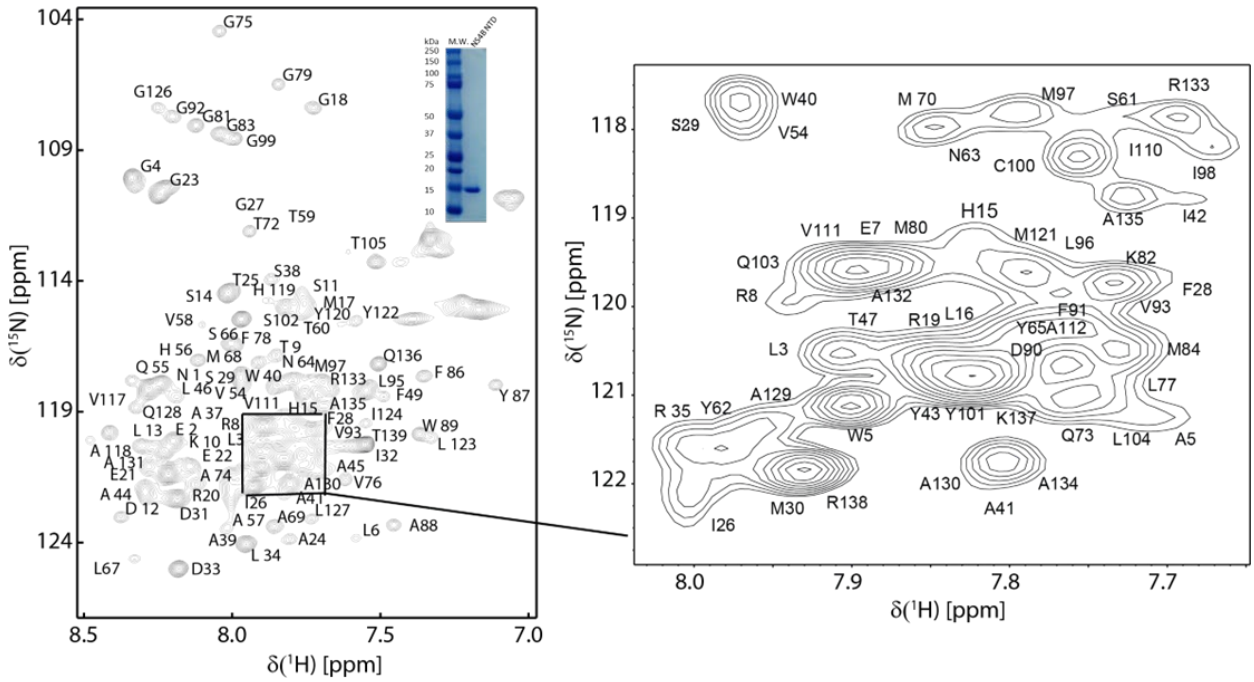

Figure 2. ^{1}H-^{15}N-HSQC spectrum NS4B NTD in LMPG micelles. Inset shows the purified NS4B NTD in LMPG micelles.

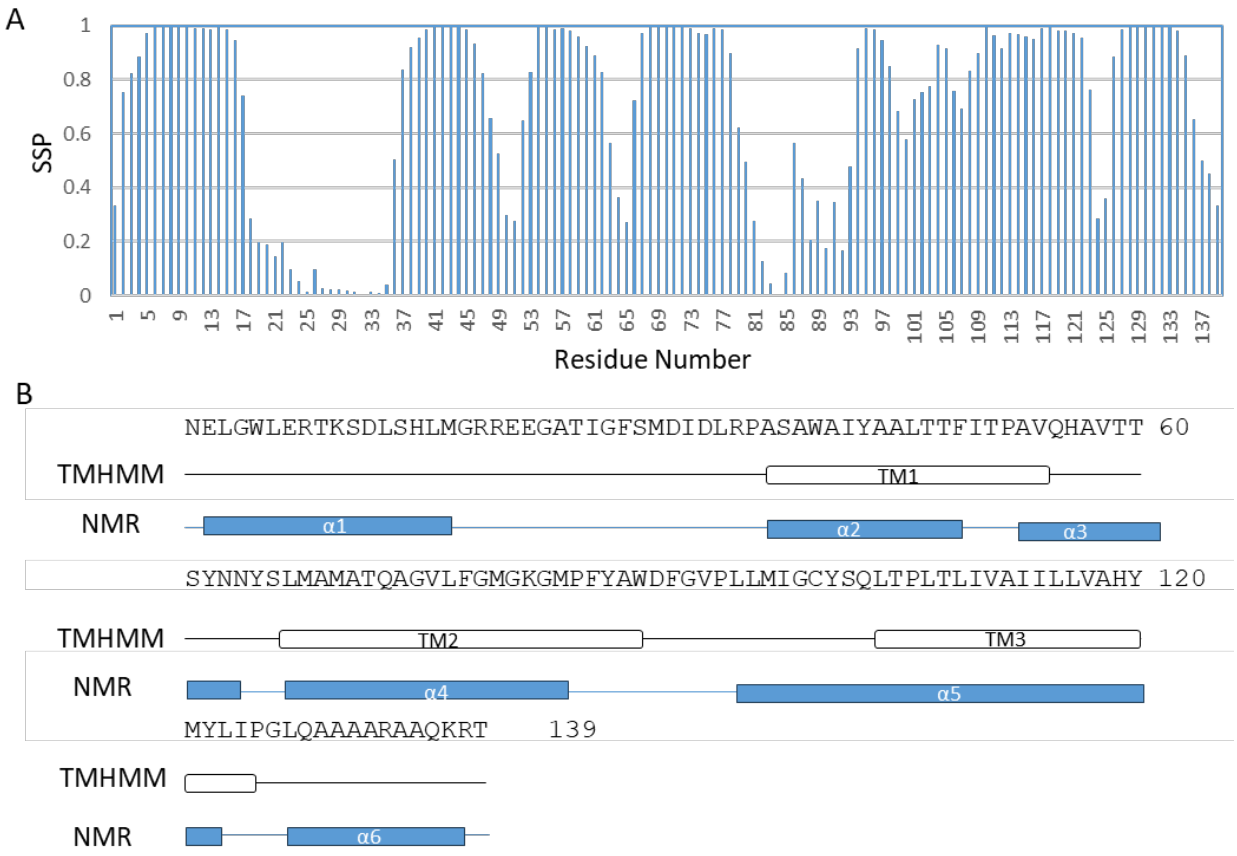

Figure 3. Secondary structures of ZIKV NS4B NTD in LMPG micelles. A. Secondary structures of prediction of ZIKV NS4B NTD based on the analysis of backbone assignments using TALOSN. The secondary structure prediction (SSP) is plotted against the residue number. B. The secondary structure of NS4B NTD. The location of three transmembrane helices (TM1-3) obtained from TMHMM was obtained from the server (https://services.healthtech.dtu.dk/services/TMHMM-2.0/). The secondary structures (α1-6) obtained from backbone resonances are shown.

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