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Membrane topology of NS2B of dengue virus revealed by NMR spectroscopy

Yan Li [a,1], Qingxin Li [b,1], Ying Lei Wong [a], Lynette Sin Yee Liew [a], CongBao Kang [a,*]

[a] Experimental Therapeutics Centre, Agency for Science, Technology and Research (A*STAR), 31 Biopolis Way, Nanos, #03-01, 138669, Singapore
[b] Institute of Chemical & Engineering Sciences, Agency for Science, Technology and Research (A*STAR), 1 Pesek Road, Jurong Island, Singapore 627833, Singapore

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ABSTRACT

Non-structural (NS) proteins of dengue virus (DENV) are important for viral replication. There are four membrane proteins that are coded by viral genome. NS2B was shown to be one of the membrane proteins and its main function was confirmed to regulate viral protease activity. Its membrane topology is still not known because only few studies have been conducted to understand its structure. Here we report the determination of membrane topology of NS2B from DENV serotype 4 using NMR spectroscopy. NS2B of DENV4 was expressed and purified in detergent micelles. The secondary structure of NS2B was first defined based on backbone chemical resonance assignment. Four helices were identified in NS2B. The membrane topology of NS2B was defined based on relaxation analysis and paramagnetic relaxation enhancement experiments. The last three helices were shown to be more stable than the first helix. The NS3 protease cofactor region between α2 and α3 is highly dynamic. Our results will be useful for further structural and functional analysis of NS2B.

1. Introduction

Dengue virus (DENV) belongs to the *Flaviviridae* family and consists of four serotypes (DENV1–4) [1]. DENV has become a public health threat affecting people living in the tropical and sub-tropical regions [2]. DENV infection can cause serious diseases such as dengue fever, dengue hemorrhagic fever (DHF) or dengue shock fever (DSS). It is estimated that approximately 100 million people are infected by DENV annually and serious cases can cause death. Currently, there is still no efficient chemotherapy or vaccine available to treat or prevent DENV infection [3].

DENV RNA encodes a poly-protein that can be further processed into three structural and several non-structural (NS) proteins including NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. Viral protease NS3 and host proteases are responsible for the processing of the poly-peptide into functional proteins [4]. Four non-structural proteins including NS2A, NS2B, NS4A and NS4B are indentified to be membrane proteins [5–7]. Membrane topologies and functions of NS2A, NS4A and NS4B have been studied and these proteins are shown to be important for viral replication by forming complexes with other NS proteins [7–9]. For the NS2B, it was a membrane protein localizing on the cell membrane. It mainly functions as a co-factor of the NS3 protease activity [10,11]. Studies have been conducted on the cofactor region containing approximately 40 residues that are indispensible for the NS3 protease activity and folding [12–16]. Its membrane regions may be important for its location on the membrane [17].

Although the membrane topology of NS2B has been proposed in different studies based on amino acid sequence [17], the details of NS2B membrane topology are still needed for structural studies. Dengue NS3 is a validated drug target. NS2B plays import roles in NS3 protease activity. Thus understanding NS2B membrane topology will be helpful for drug discovery against DENV. Here we report the determination of the NS2B membrane topology based on the secondary structural information obtained from chemical shifts of backbone atoms, ^{15}N relaxation analysis, H–D exchange and paramagnetic relaxation enhancement (PRE) experiments.

2. Materials and methods

2.1. Sample preparation

The cDNA for coding the full-length NS2B of Dengue 4 was synthesized (Genscript) and cloned into NdeI and XhoI sites of pET29b. The plasmid encodes NS2B with a 6 × histidine tag at the C-terminus. Plasmid was transformed into *Escherichia coli* (BL21DE3) competent cells and grown on LB plates containing kanamycin. Several colonies were picked up and incubated in 50 ml of M9 medium or 10 ml of LB medium with antibiotics. The overnight culture was transferred into 1 l of LB medium or M9 medium supplied with antibiotics. When OD_{600} of the culture reached 0.8, NS2B protein was induced by adding β-D-1-thiogalactopyranoside (IPTG) to a 1 mM final concentration for 18 h at 18 °C. The *E. coli* cells were harvested by centrifugation at

* Corresponding author.
E-mail address: cbkang@etc.a-star.edu.sg (C. Kang).
[1] These authors contributed equally to this work.

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Fig. 1. Membrane topology prediction of NS2B of DENV4 using different web-based servers. The information of the web servers used in this study is listed in the Materials and methods section. Box indicates the predicted transmembrane region and line indicates a non-transmembrane region. The NS3 cofactor region is underlined. The four residues (SMPL) at NS2B N-terminus was replace with a Met.

10,000 ×g for 10 min at 4 °C. Cell pellets were re-suspended into a lysis buffer containing 20 mM sodium phosphate, pH 7.8, 300 mM NaCl, 1% Lyso-myristoyl phosphatidylglycerol (LMPG) and 2 mM β-mercaptoethanol. Cells were broken up by sonication on ice. The cell lysate was first cleared by centrifugation at 20,000 ×g for 20 min at 4 °C. Supernatant was mixed with Ni^{2+}-NTA and NS2B was eluted using an elution buffer containing 500 mM imidazole, 0.1% Lyso-myristoyl phosphatidylglycerol (LMPG) and 2 mM β-mercaptoethanol. Purified sample was further purified using gel filtration chromatography and concentrated for NMR studies. Selectively ^{15}N-Met labeled protein was prepared by growing *E. coli* cells in M9 medium containing 0.5 g/l NH_4Cl. During protein induction, ^{15}N-Met (0.1 g) and 19 unlabeled amino acids (0.2 g each) were added before the addition of IPTG. Protein was purified as described above.

2.2. Membrane topology analysis of NS2B using different servers

The following server-based bioinformatics tools were used to analyze NS2B sequence [7]. These servers include HMMTOP (http://www.enzim.hu/hmmtop/index.php), TMHMM2 (http://www.cbs.dtu.dk/services/TMHMM/), DAS (http://www.sbc.su.se/_miklos/DAS/maindas.html), TOPCONS (http://topcons.cbr.su.se/), Split (http://split.pmfst.hr/split/4), PRED-TMR (http://athina.biol.uoa.gr/PRED-TMR/) and TMpred (http://www.ch.embnet.org/software/TMPRED_form.html).

2.3. Backbone resonance assignment

Uniformly ^{13}C, ^{15}N- or ^{13}C, ^{15}N and ^{2}H-labeled NS2B proteins were concentrated to 0.8–1.0 mM in a buffer containing 20 mM sodium phosphate, 1 mM DTT and 1–2% LMPG. Backbone resonance assignment was obtained based on two- (2D) and conventional three-dimensional (3D) experiments and transverse relaxation-optimized spectroscopy (TROSY) [18,19] -based experiments including 2D-HSQC, 3D-HNCACB, 3D-HNCOCACB, 3D-HNCOCA, 3D-HNCA, 3D-HNCACO, 3D-HNCO and NOESY–TROSY (100 ms mixing time) experiments. All the spectra were collected on a Bruker Avance II 700 MHz or 600 MHz spectrometer. All the pulse sequences were from a standard Bruker pulse program library (Topspin 2.1). Spectra were processed with NMRPipe [20] or Topspin and analyzed using NMRView [21] and CARA (http://www.mol.biol.ethz.ch/groups/wuthrich_group).

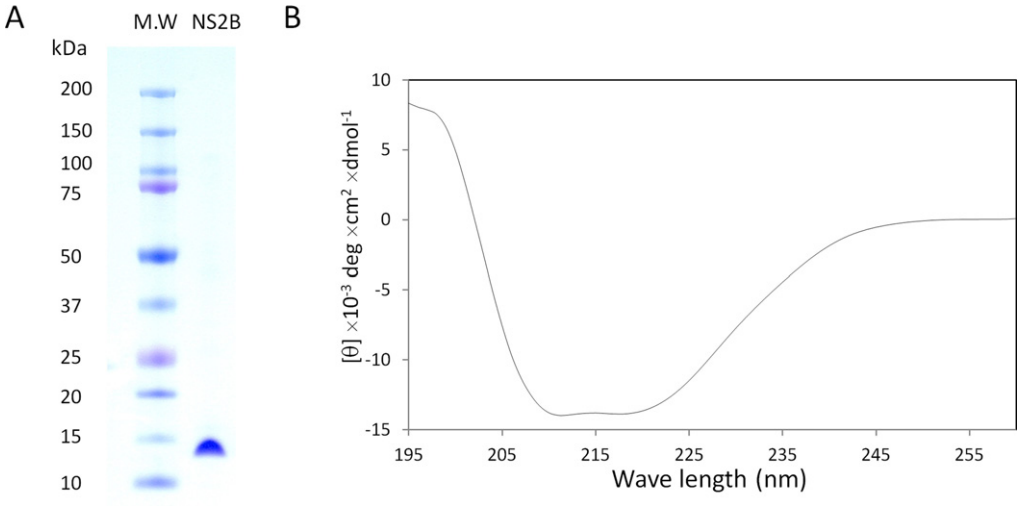

Fig. 2. Purification and structural analysis of NS2B. A. Purification of NS2B. Protein was purified in LMPG micelles. SDS-PAGE analysis of the purified protein is shown. B. CD spectrum of NS2B in LMPG micelles.

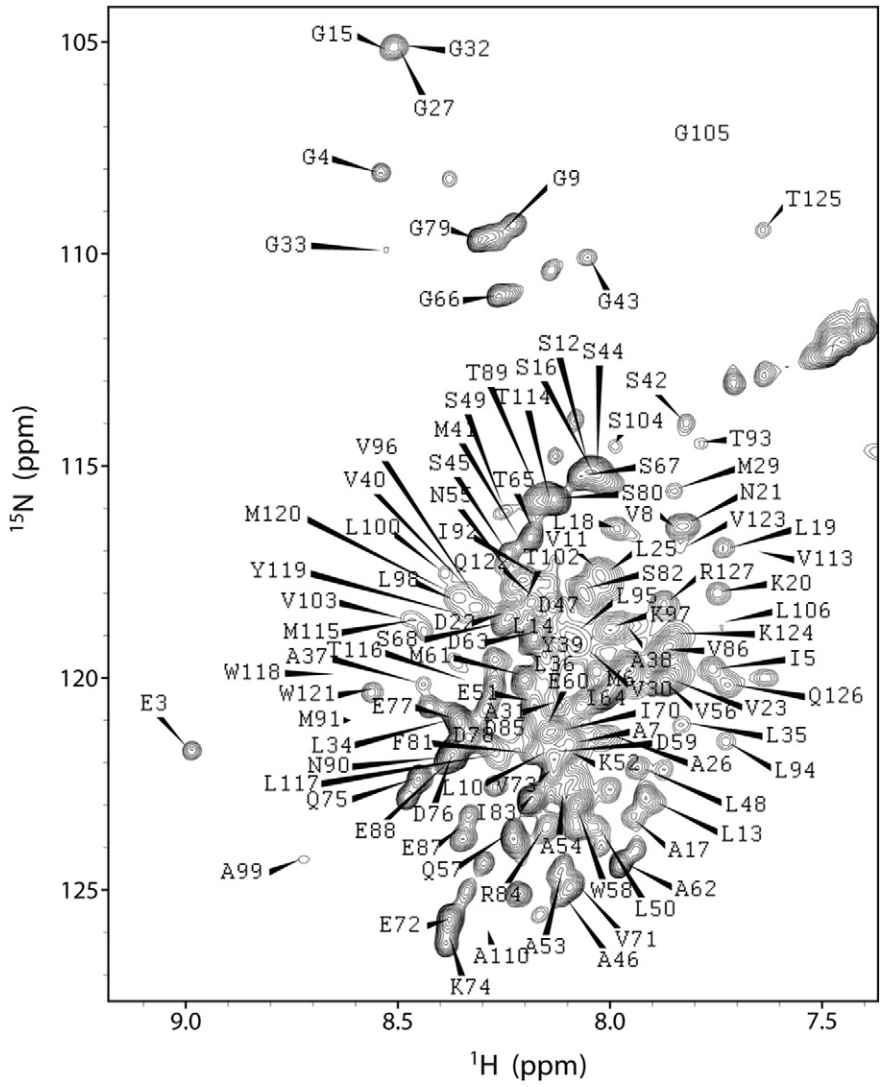

Fig. 3. ^{1}H–^{15}N-HSQC spectrum of [U-^{15}N, ^{13}C]-labeled NS2B at 40 °C in LMPG micelles. Peaks are labeled with residue name and sequence number.

Secondary structure was analyzed using TALOS + [22] and Cα chemical shifts [23]. For the T_1, T_2 and ^{15}N steady-state heteronuclear NOE (hetNOE) experiments [24], a ^{15}N-labeled NS2B and a ^{15}N-Met labeled NS2B were used. All the experiments were measured at 40 °C on a Bruker Avance 600 MHz spectrometer. For the T_1 measurement of NS2B, the data with relaxation delays of 10, 50, 100, 200, 400, 800, 1200, 1400, 1600 and 1800 ms were recorded and processed. For the T_2 measurement, the data were acquired with delays of 16.9, 34, 51, 68, 85, 102, 119, 136 and 153 ms. The spectra were processed and analyzed. The hetNOEs were obtained using two datasets that were collected with and without initial proton saturation for a period of 3 s [25].

2.4. H–D exchange experiment

The H–D exchange experiment was performed at 40 °C using the aforementioned sample. 200 μl of the sample was first frozen in liquid nitrogen and lyophilized under low pressure and temperatures. 200 μl of pure D_2O was then added into the sample and 2D ^{1}H–^{15}N-HSQC spectrum with 2048 × 128 complex points was collected after the powder was completely dissolved (~20 min). Cross-peaks appeared in the spectrum suggest that these residues are protected from exchanges and might be protected by micelles. Another method for the H–D exchange experiment was also conducted and compared [26].

2.5. Circular Dichroism (CD) analysis

The CD spectrum of NS2B was collected using a Chirascan™ Circular Dichroism Spectrometer at 25 °C. Protein at a concentration of 50 μg/ml was prepared in a buffer that contained 20 mM sodium phosphate at a pH of 6.5, 0.1% LMPG, and 2 mM β-mercaptoethanol. The CD data was acquired in the continuous mode with a 1-nm data pitch and a 1-nm bandwidth [27].

2.5.1. PRE experiment

To probe residues that are exposed to the solvent, the PRE experiment was conducted using the similar method described previously except gadolinium was used [28]. One ^{15}N-labeled NS2B was prepared in LMPG micelles. Freshly prepared gadolinium solution containing 50 mM $GdCl_3$ and 150 mM ethylenediaminetetraacetic acid (EDTA) was added into the protein sample. The ^{1}H–^{15}N-HSQC spectra of NS2B in the absence and presence of gadolinium were obtained and analyzed.

3. Results

3.1. Membrane topology analysis of NS2B based on sequence

Sequence analysis of NS2B revealed that there are three hydrophobic regions in NS2B that are possible transmembrane segments [17]. To

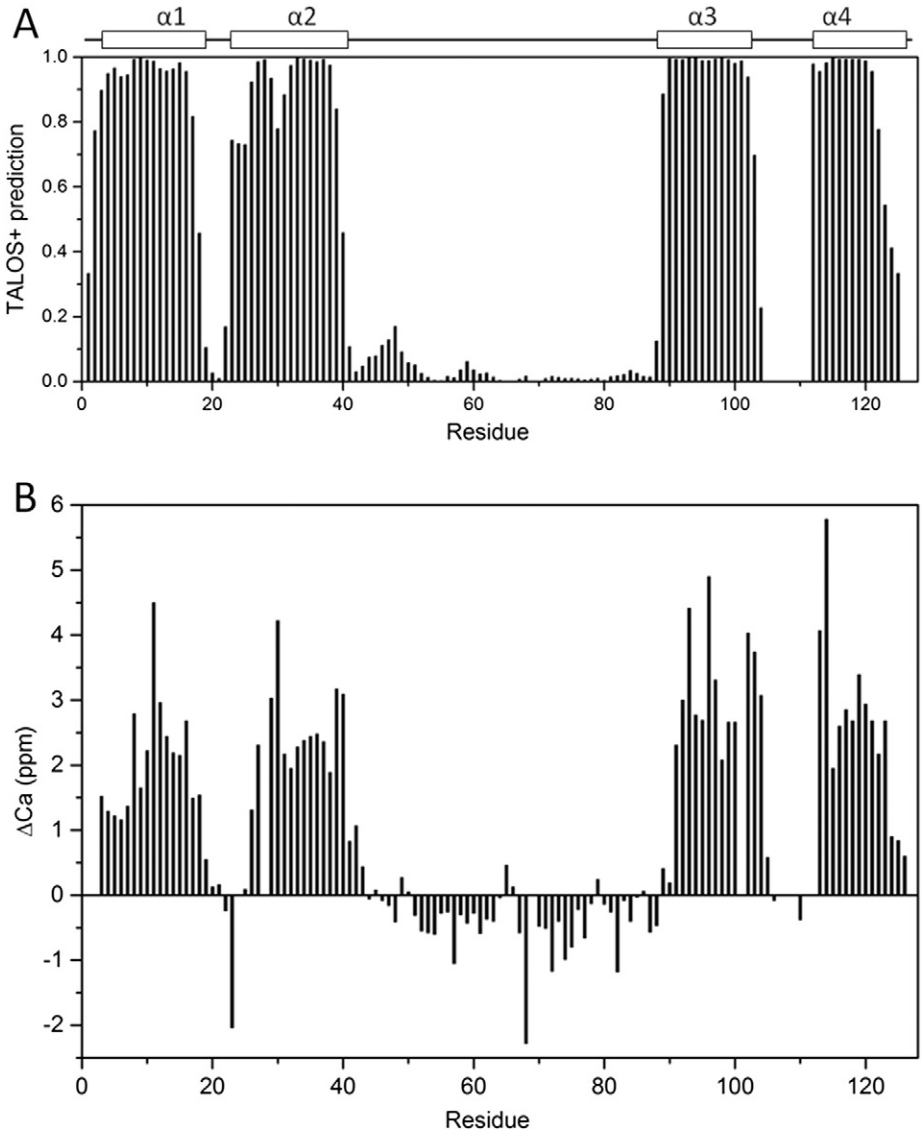

Fig. 4. Secondary structural analysis of NS2B. A. TALOS + prediction as a function of residue number is plotted. TALOS + prediction indicates the possibility of a residue to be helical. B. The Cα chemical shifts observed for NS2B subtracted from the Cα random-coil values (ΔCα) were plotted as a function of residue number. A residue with a positive value indicates that it may adopt a helical structure and with a negative value indicates that it may adopt a β-strand structure. The secondary structure prediction based ΔCα also suggested that the region between α2 and α3 also has a tendency to from β-strands. This region was shown to form strands in the presence of NS3 protein.

explore NS2B membrane topology, several membrane topology prediction severs were used to analyze NS2B sequence. Interestingly, several membrane topology prediction algorithms gave different topologies (Fig. 1). The number and length of transmembrane regions were different (Fig. 1). The only consistent result was that the cofactor region of NS3 protease was not a transmembrane region (Fig. 1). The differences observed from several servers might arise from their algorithms in transmembrane domain prediction and NS2B sequence. For example, TMHMM prediction showed that NS2B contained one transmembrane domain (Fig. 1). The N-terminal region of NS2B was also predicted by TMHMM to have two hydrophobic regions (data not shown). To further understand NS2B membrane topology, we expressed and purified NS2B and conducted membrane topology analysis using NMR spectroscopy.

3.2. Expression and purification of NS2B of DENV4

The procedure of expression and purification of NS2B of DENV2 was set up in our previous study [27]. We then expressed and purified NS2B of DENV4 using the same protocol as that of DENV2 (Fig. 2A). NS2B of DENV4 was expressed in *E. coli* and purified into in Lyso-myristoyl phosphatidylglycerol (LMPG) micelles. The folding of NS2B was first confirmed using CD spectroscopy, which showed that purified protein contained mainly helical structures (Fig. 2B). Our previous study showed that it is feasible to conduct structural study on NS2B using NMR spectroscopy [27]. A ^{1}H–^{15}N-heteronuclear single-quantum correlation (HSQC) spectrum of NS2B of DENV4 was collected and the dispersed peaks in the spectrum suggested that it is feasible to conduct further NMR experiments to understand its structure (Fig. 3).

3.3. Secondary structure analysis of NS2B

The backbone assignment of the NS2B of DENV4 in LMPG micelles was achieved using conventional 3D experiments and TROSY [18,19] - based experiments. Cross peaks were overlapped in the central part of the HSQC spectrum (Fig. 3), which is not surprising for such a helical membrane protein. With backbone assignment of NS2B (Table S1), the secondary structure for NS2B in LMPG micelles was obtained. The secondary structure of NS2B was determined using TALOS + based on the backbone resonance chemical shifts [22] and Cα chemical shift compared with random coil values [23]. Both methods gave similar

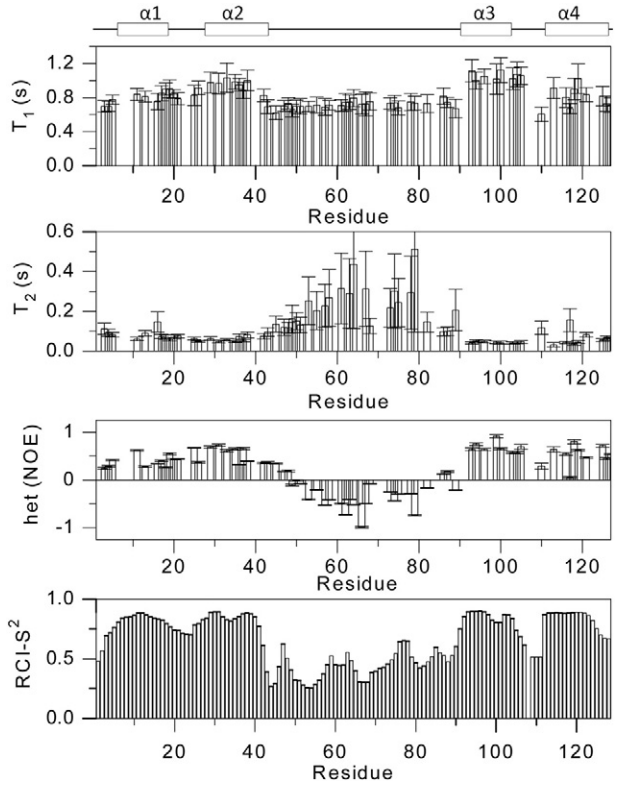

Fig. 5. Relaxation analysis of a uniformly ^{15}N-labeled NS2B. T_1, T_2 and hetNOE values were plotted as a function of residue number. Residues including prolines, unassigned and overlapped ones are not shown. RCS-S2 values were obtained using TALSON server (http://spin.niddk.nih.gov/bax/software/TALOS-N/) and were plotted against residue number.

results (Fig. 4). The secondary structural analysis showed that there are four helical segments in NS2B, namely α1 formed by residues G4 to L19, α2 formed by residues L25 to M41, α3 formed by residues N90 to G105 and α4 formed by residues P112 to T125 (Fig. 4). There is a break observed between α3 and α4, which might be the reason that the residues from Y107 to L109 could not been assigned because of conformational exchanges. The prolines residues, P108 and P112 might be responsible for the break and conformation exchanges. Functional and structural studies have shown that NS3 follows the C-terminus of α4 and interacts with the region between α2 and α3, which suggests that there must be a break present between α3 and α4 so that the C-terminus of α4 can point to the same direction as the cofactor region. Secondary structure analysis also showed that the NS3 protease cofactor region between α2 and α3 was not helical and had a tendency to form β-strands (Fig. 4B). In the NS2B–NS3 complex structure solved in previous studies, this region can form β-strand structures when it formed a complex with NS3 [11,29–31]. This region might not form a stable structure in the absence of NS3 protein.

3.4. Dynamic analysis of NS2B in LMPG micelles

With the secondary structural information obtained based on the backbone resonance assignment, relaxation experiments including T_1, T_2 and ^{15}N steady-state heteronuclear NOE (hetNOE) experiments were performed to understand protein dynamics in solution. It is challenging to analyze the relaxation data using a uniformly-^{15}N-labeled sample due to the signal overlap (Fig. 3). We analyzed 66 of the 127 residues and the unanalyzed residues included prolines, and overlapped and unassigned residues. The cofactor region is highly dynamic characterized by low and negative hetNOEs, and high T_2 and low T_1 values (Fig. 5). The α1 is more dynamic than other helices due to its slightly lower T_1 and hetNOE and higher T_2 values. Overall, the relaxation data is consistent with the RCI-S^2 values obtained using a TALOSN package [32,33] except that the α1 was shown to be more flexible than other residues. Due to the signal overlap, we then tested whether we could use a sample that is selectively labeled with a ^{15}N-labeled amino acid for relaxation analysis because such sample will have few peaks present in the HSQC spectrum. Although several types of residues can be selectively labeled for NMR studies, a ^{15}N-methionine (Met)-labeled sample was used for relaxation analysis because the eight Met residues present in NS2B sequence are localized in all the helices and the loop region of NS2B (Fig. 6A). This sample produced dispersed cross-peaks in the ^{1}H–^{15}N-HSQC spectrum (Fig. S1). Multiple cross peaks were observed for M6 and M61, which might arise from exchanges (Fig. S1). The ^{15}N T_1, T_2, T_1/T_2 and hetNOE for the eight Met residues were obtained (Fig. 6B). The result is consent with the one obtained from the uniformly ^{15}N-labeled sample (Fig. 5).

3.4.1. Membrane topology of NS2B

The N- and C-termini of NS2B are pointing to the cytoplasm direction. With the confirmed secondary structure based on backbone chemical shifts, the four helices are possible transmembrane helices (Fig. 6A). To further investigate its membrane topology, we first conducted the H–D exchange experiment (Fig. 6A, Fig. S2). The result showed that residues from these four helices were protected from exchanges, suggesting that these helices were forming stable structures in micelles. A few residues in the α1 were not protected from exchanges, suggesting that it might be flexible or behave like an amphiphilic helix. We then conducted helix wheel analysis for these four helices (Fig. 7A). Interestingly, all these helices contain small-XXX-small motifs [34] that are important for TM–TM interactions (Fig. 7A). Although the α1 was shown to be more dynamic than other helices, helix wheel presentation showed that it is not an amphiphilic helix (Fig. 7A). We also submitted the chemical shifts to the CS-ROSETTA server (https://csrosetta.bmrb.wisc.edu/csrosetta) and the result suggested that these four helices could form a helix bundle (Fig. 7B).

To confirm the membrane topology of NS2B, the PRE experiment was conducted (Fig. 8). The residues that are buried in the micelles will not be affected by the addition of gadolinium. The intensities of the cofactor region are significantly reduced in the presence of gadolinium (Fig. 8), suggesting that these residues are exposed to the solution (Fig. 8B). The intensities of most residues from the four helices were not strongly affected, suggesting that these helices are buried in the micelles. Based on all the results, the membrane topology of NS2B was proposed (Fig. 6A).

4. Discussion

Membrane proteins are predicted to constitute approximately one third of the genome and many of them are important drug targets [35,36]. Structural studies on membrane proteins will not only provide useful information to understand their functions, but also facilitate structure-based drug design [37]. Although X-ray crystallography is a powerful tool to determine structures of many membrane proteins such as G-protein coupled receptors, ion channels and transporters [38–41], NMR spectroscopy will also play an important role in determining membrane protein structure and understanding their dynamics under different conditions [42–45]. We carried out structural studies of NS2B of DENV4 to understand its membrane topology. We focused on NS2B of DENV4 instead of DENV2 because the assignment of NS2B of DENV2 was challenging due to signals from the affinity tag [27]. NS2B was shown to be a regulator of NS3 protease that was confirmed to be a validated drug target. Extensive studies have been carried out to develop potent inhibitors that are active against DENV protease, but there is still no potent dengue protease inhibitor available [46–49]. Most of the structural and functional studies of NS2B are using a NS2B construct without the hydrophobic regions [11,13,15,16]. The failure in developing DENV protease inhibitors might arise from the fact that little is known about the molecular interaction between full length

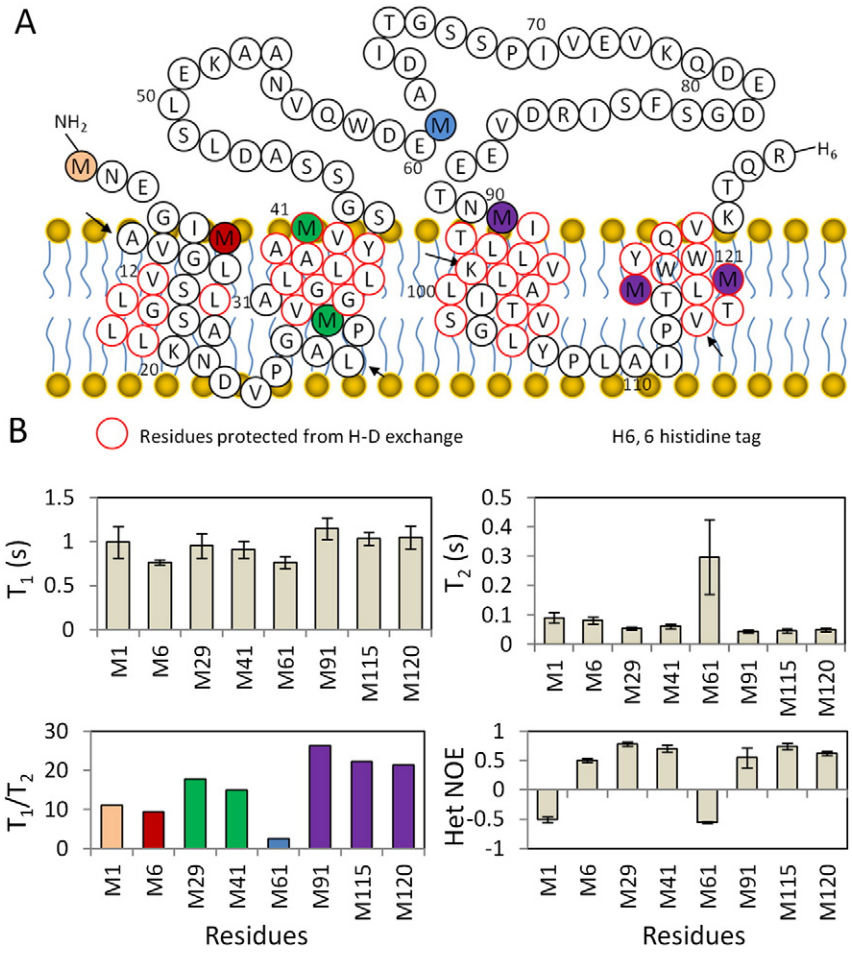

Fig. 6. Relaxation analysis of ^{15}N-Met-labeled NS2B. A. Predicted membrane topology of NS2B. Methionine residues having different relaxation parameters are classified into four groups shown in red, green, blue and pink, respectively. The first Met residue arisen from molecular cloning to replace the four residues (SMPL) at NS2B N-terminus is shown in orange. Relaxation rates T_1, T_2, T_1/T_2 and HetNOE are plotted as a function of residue number and shown in B. The color scheme is the same as A.

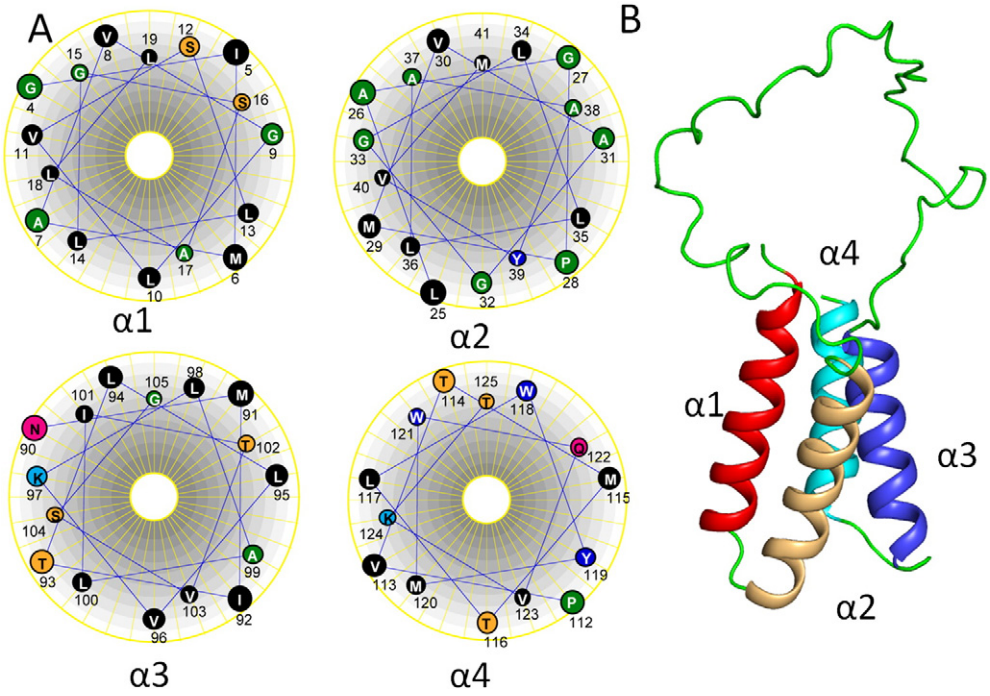

Fig. 7. Helix wheel representation of NS2B. A. Helix wheel analysis of the four helices in NS2B. B. One of the models of NS2B obtained from the CS-ROSETTA server. The four helices and the cofactor region are shown in different colors.

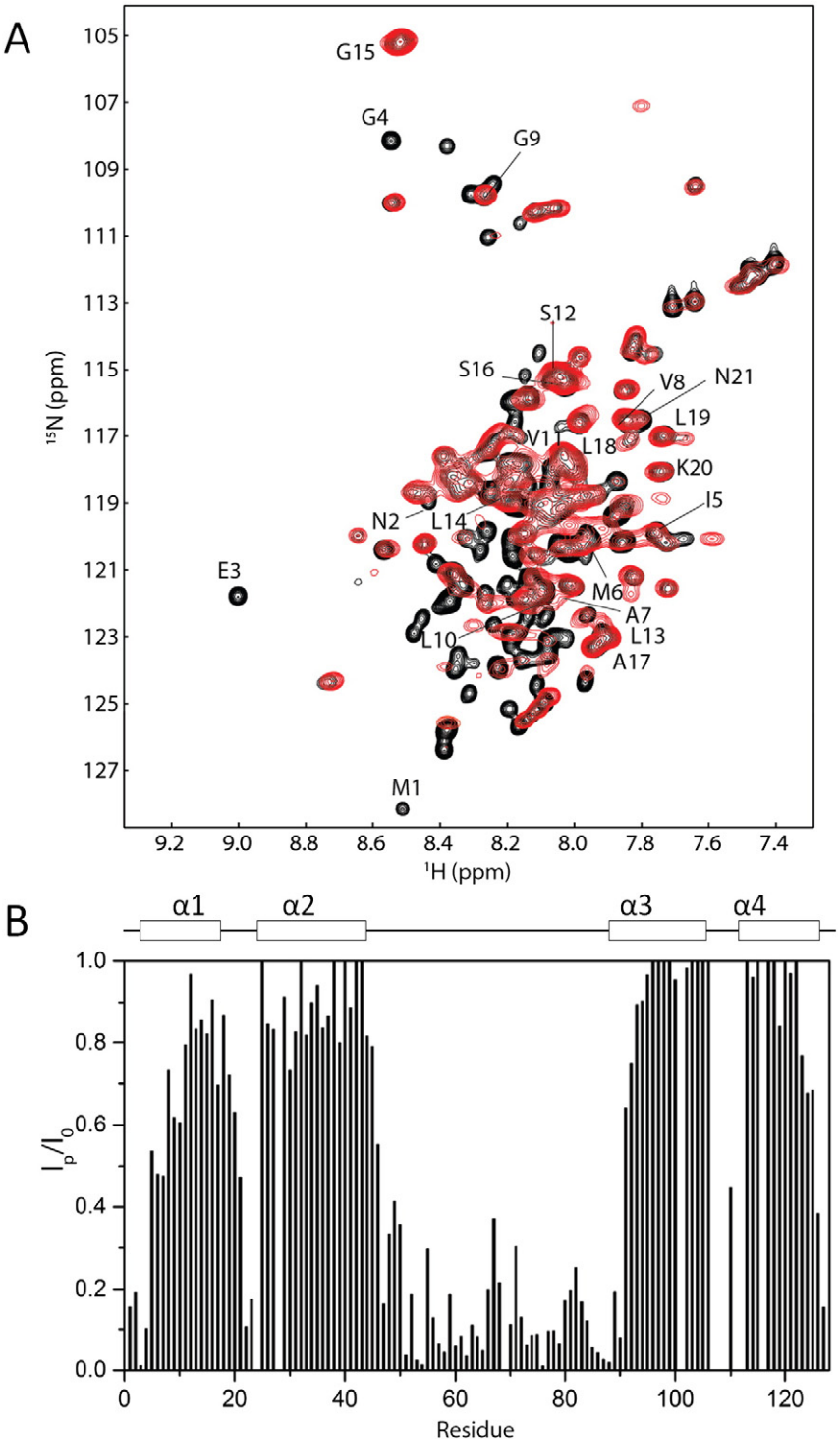

Fig. 8. PRE analysis of NS2B. A. ^{1}H–^{15}N-HSQC spectra of NS2B in the absence (black) and presence of 5 mM gadolinium (red). The assignment for some residues in α1 is shown. B. Changes of peak intensities in the absence of gadolinium. The relative ratio of intensities in the absence (I_0) and presence (I_p) of gadolinium was plotted. A value of 1 was given to some residues such as G105 whose intensity was increased after addition of gadolinium. The signals with reduced intensities suggest that the residue is exposed to the solvent.

NS2B and NS3 [50]. In this study, we have obtained NS2B of DENV4 in a large quantity for structural studies in LMPG micelles, which will make it possible to explore molecular interaction between full length NS2B and NS3 or other dengue membrane proteins. Although we were able to assign backbone resonances of NS2B in LMPG micelles (Fig. 3), line broadening of cross peaks and existence of multiple peaks for single residue were observed in the spectra (Fig. 3, Fig. S1). This might arise from the fact that NS2B contains regions with different dynamic natures and conformational exchanges (Fig. 5). Residues which exhibited broadened peaks might also arise from protein oligomerization. A previous study showed that NS2B could form oligomers [51].

NS2B can form a complex with NS3 that also interacts with other dengue membrane proteins such as NS4A and NS4B [5,6]. It is still unknown whether NS2B interacts directly with NS4A or NS4B. The only known function of the transmembrane segments of NS2B is that these regions might be important for membrane localization [17].

Using NMR spectroscopy, we analyzed the secondary structures and defined the membrane topology of NS2B (Fig. 6). There are four transmembrane helices present in NS2B (Fig. 6). The structural information of NS2B was obtained in LMPG micelles. It still debated whether micelles might or might not mimic the native membrane environment. We showed that detergent such as Lyso-myristoyl phosphatidylcholine (LMPC) can sustain the dengue protease activity [50]. Due to the similar structure between LMPG and LMPC, this secondary structural information obtained in this study is similar to its structure under physiological conditions. Both ^{15}N- and ^{15}N-Met-labeled NS2B showed the similar result that the α1 is more dynamic than other helices and the cofactor region is flexible in the absence of NS3 (Fig. 5). Signal overlap is always observed in the ^{1}H–^{15}N-HSQC spectrum for α-helical membrane proteins [52]. Residue-specific ^{15}N-labeling of a protein has been proven to be useful in structural and dynamic analysis of proteins [53]. Our result also showed that dynamic analysis of a ^{15}N-amino acid-specific labeled sample will provide useful information to understand protein dynamics. Further sequence analysis showed that all these four helices contain several hydrophilic residues and residues with short side chains such as Gly and Ser, which might explain the reason that several servers produced different membrane topologies (Figs. 1, 6). These hydrophilic residues in the helices might be important for helix–helix packing. We also submit the chemical shift values to CS-ROSETTA sever [54] and the result suggested that NS2B could form a helix bundle. These results will be useful for further functional analysis of NS2B, which will provide novel insight into drug discovery targeting DENV. It has been noted that the length of these four helices is shorter than a transmembrane domain (~21 amino acids) of other membrane proteins such as cytokine receptors [55]. A transmembrane domain with such length might have freedom to induce conformational changes in the cell membrane under different conditions. It is possible that NS2B can interact with other dengue membrane proteins through its transmembrane regions. Further functional studies will provide more information of NS2B in viral replication.

In summary, we have presented secondary structural analysis of NS2B of DENV4 base on backbone assignment. The membrane topology of NS2B was defined based on secondary structure determination, relaxation and PRE experiments. The results obtained in this study will facilitate structural study on NS2B, which will be useful to understand its role in NS3 protease activity and viral replication.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2015.06.010.

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