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ARTICLE

Backbone ^{1}H, ^{13}C and ^{15}N resonance assignments of dengue virus NS2B–NS3p in complex with aprotinin

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Abstract Dengue virus, belongs to *Flaviviridae,* is an arthropod transmitted virus that threatens millions of people's lives. As with other flaviviruses, a positive single-stranded 11-kilobases RNA in the dengue virus genome encodes three structural proteins (capsid protein C, membrane protein M, and envelope protein E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The two component protease NS2B–NS3p is essential for viral replication and is believed to be a potential antiviral drug target. Aprotinin, a native inhibitor, is proved to retard the activity of NS2B–NS3p. The backbone assignments of NS2B–NS3p will be essential for determining the high resolution solution structure of NS2B–NS3p and screening new antiviral drugs. Herein, we report the backbone ^{1}H, ^{15}N, ^{13}C resonance assignments of the N terminal fragment of NS2B (4.8 kDa) and NS3p (18.5 kDa) in complex with aprotinin (6.5 kDa) by high resolution NMR.

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Biological context

Dengue virus, a member of *Flaviviridae*, is one of the most prevalent Mosquito-borne viruses in tropical and subtropical regions of the world. It causes a range of clinical diseases, such as dengue fever, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (Jacobs and Young 1998). Lack of approved vaccine or effective antiviral therapy for these diseases puts millions of lives at risk. Like other flaviviruses, the genome of dengue virus contains a positive single-stranded 11-kilobases RNA, which encodes three structural proteins (capsid protein C, membrane protein M, and envelope protein E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Chambers et al. 1990). The N-terminus of NS3 (NS3p) functions as a serine protease, whose catalytic activity requires NS2B. A combination of NS2B–NS3p and the host proteases, cleaves the viral polyprotein into individual mature proteins. Therefore it is essential for viral replication (Falgout et al. 1991). Aprotinin, a classic inhibitor for trypsin and related proteolytic enzymes, was reported to inhibit the activity of NS2B–NS3p protease at submicromolar concentration (Leung et al. 2001). Here we report the backbone ^{1}H, ^{15}N, ^{13}C resonance assignments of the N terminal fragment of NS2B (4.8 kDa) and NS3p (18.5 kDa) in complex with aprotinin (6.5 kDa), which provides a requisite step for determining the solution structure of the complex. It is also important for screening and developing new antiviral drugs by structure-based designing strategies.

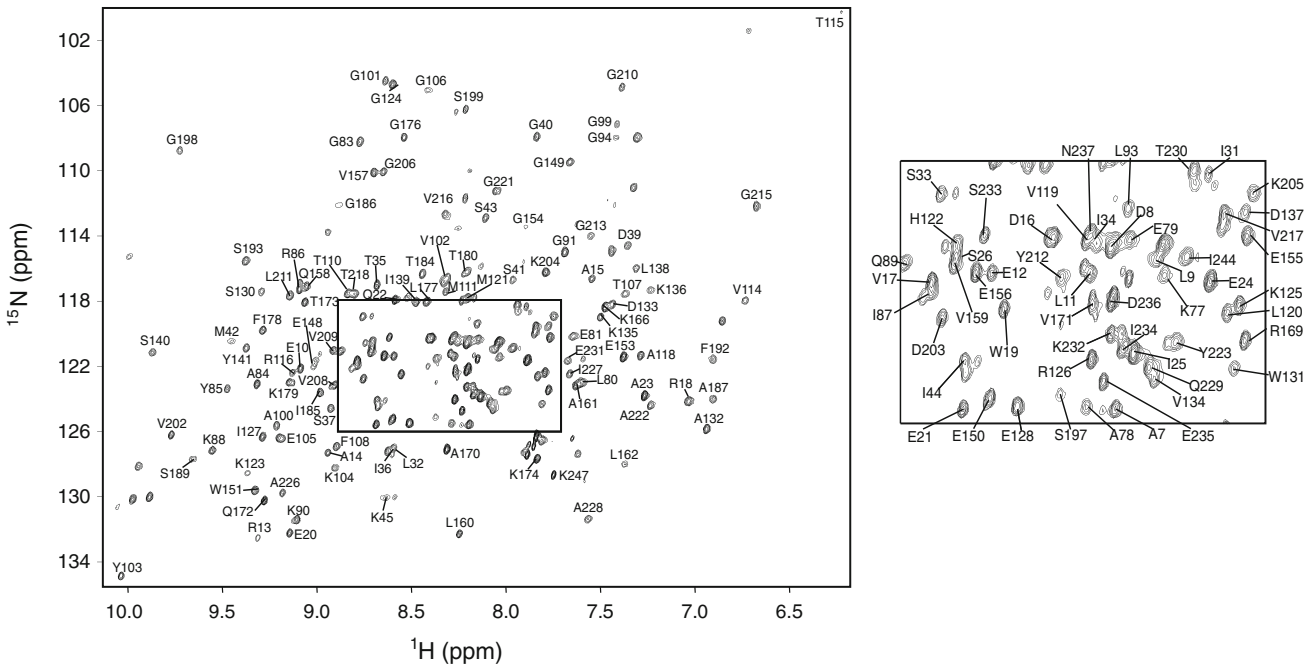

Fig. 1 The 2D TROSY HSQC spectrum of dengue virus NS2B-NS3p in complex with aprotinin (*left panel*) and an expanded portion (*right panel*) of the most congested region. The spectrum was collected on a Bruker Avance 850 MHz spectrometer (with a cryoprobe) at 30 °C. The assignments are annotated with the one-letter amino acid code and the sequence number

Methods and experiments

An NS2B–NS3p gene, comprising the 47-amino acids cofactor domain of NS2B connected to NS3p via a GGGGSGGGG flexible linker, was constructed and then cloned into the protein expression vector pET15b(+) (Novagen). The recombinant plasmid was transformed into *E. coli* BL21 (DE3) strain (Invitrogen) for protein expression. The *E. coli* cells were cultured overnight in 5 mL of Luria–Bertani (LB) broth medium containing 100 μg/mL of Ampicillin at 37 °C. The cells were centrifuged and transferred into ^{2}H/^{13}C/^{15}N-labeled M9 medium. The protein expression was induced by adding isopropyl-*β*-*D*-thiogalactoside (IPTG) at a final concentration of 0.5 mM when OD600 reached 0.8. The cells were cultured at 20 °C for 36 h and then harvested by centrifugation. The protein was purified by Ni-Sepharose affinity chromatography and the 6× HIS tag was removed by thrombin digestion overnight at 4 °C. Subsequently, the anion-exchange chromatography (Mono Q) and gel filtration (Superdex-75) by an ÄKTA FPLC system (Amersham Biosciences) were taken for further purification. The un-labeled aprotinin was purchased from Sigma.

The NMR sample of NS2B–NS3p/aprotinin complex was prepared in a buffer containing 20 mM Na_2HPO_4/NaH_2PO_4, 100 mM NaCl in 90 % H_2O/10 % D_2O (pH 7.0). The protein concentration was about 0.65 mM. Aprotinin was added to the triple-labeled NS2B–NS3p solution at the molar ratio of 2:1. The original NS2B–NS3p/aprotinin complex degraded over time, and a protein complex(30 kDa)containing the N terminal fragment of NS2B (4.8 kDa), NS3p (18.5 kDa) and aprotinin (6.5 kDa) was identified. The sample was incubated at 30 °C for 4 days and further purified with size exclusion chromatography to obtain the stable complex.

The NMR spectra were acquired at 30 °C on Bruker Avance 850 MHz (with a cryoprobe), equipped with four RF channels and a triple-resonance probe with pulsed field gradients. The TROSY (Pervushin et al. 1997) versions of the three-dimensional (3D) HNCA, HNCACB, HN(CO)CA, HNCO, HN(CA)CO experiments were carried out for the backbone assignments (Salzmann et al. 1998; Salzmann et al. 1999; Eletsky et al. 2001). The ^{1}H chemical shifts were referenced to internal 2,2-dimethyl-2-silapentanesulfonic acid (DSS), and ^{13}C and ^{15}N chemical shifts were referenced indirectly (Markley et al. 1998). All spectra were processed using the software package NMRPipe (Delaglio et al. 1995) and analyzed by the program Sparky (Goddard and Kneller, 2006, SPARKY3, University of California, San Francisco, CA).

Assignments and data deposition

A series of 2D ^{1}H-^{15}N HSQC spectra show that NS2B-NS3p/aprotinin complex experiences degradation, but

becomes stable after 4 days. Size exclusion chromatography and mass spectrum data identified a stable protein complex(30 kDa)containing the N terminal fragment of NS2B (1–45, 4.8 kDa), NS3p (77–247, 18.5 kDa) and aprotinin (6.5 kDa). The preserved residues from NS2B–NS3p/aprotinin complex show the almost identical resonances to those of the full length complex before degradation. The 2D TROSY ^{1}H-^{15}N HSQC spectrum of NS2B–NS3p/aprotinin complex is shown in Fig. 1. Except for 8 proline residues, we observed 187 resonances out of the remaining 208 NS2B–NS3p residues (1–45, 77–247). The triple resonance NMR experiments led to an assignment of 91 % (170 residues) of the observed signals. Among them, CO, Cα and Cβ chemical shifts have been determined for all residues except for K77, E148 and F245, which have Cα and Cβ but not CO assignments. Furthermore, CO, Cα and Cβ chemical shifts have been determined for 3 of the 8 prolines. For the remaining amino acids with unassigned and/or missing NH resonances, CO, Cα and Cβ chemical shifts were determined for E6, E38, Q97, H113, K152, G165, G183, D191, T196, S220 and D243, and only Cα and Cβ chemical shifts were assigned for I92, P129 and L147, by correlating them with the NH groups of the respective amino acids in the N + 1 position. In summary, we were able to assign 88, 88, and 83 % of all Cα, Cβ and CO chemical shifts, respectively. The absent resonances are mainly from residues within the flexible loops. The computer program TALOS (Cornilescu et al. 1999) shows that the structure of complex is mainly composed of β-strands, as shown in Fig. 2. The assigned backbone ^{1}H, ^{15}N and ^{13}C chemical shifts of NS2B–NS3p/aprotinin complex have been deposited in the BioMagResBank (http://www.bmrb.wisc.edu/) under the accession number 18266.

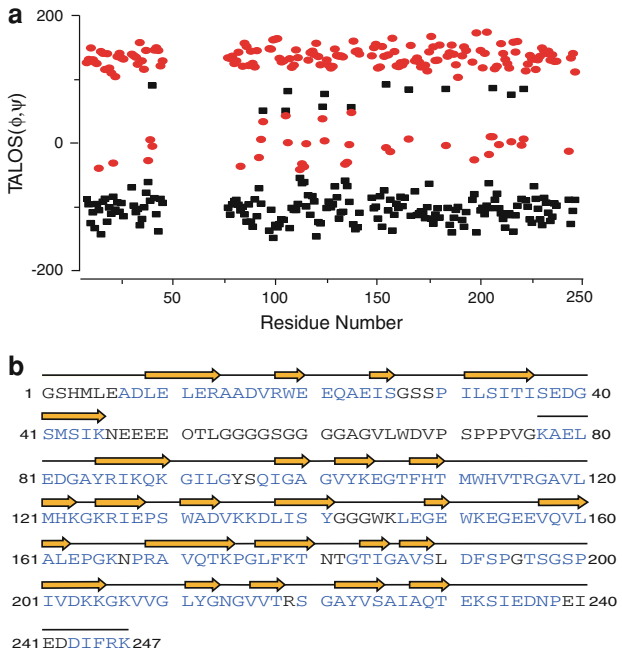

Fig. 2 TALOS plot of dengue virus NS2B-NS3p in complex with aprotinin. **a** Backbone dihedral angles (phi, psi) were calculated using TALOS (Cornilescu et al. 1999). Phi (φ) and psi (ψ) angles are shown in *black* and *red*, respectively. **b** Predicted secondary structure elements are indicated using *arrows* for β-strands. The residues used in TALOS prediction are colored in *blue*. The stable NS2B-NS3p/aprotinin complex contains an N terminal fragment of NS2B (1-45, 4.8 kDa), NS3p (77-247, 18.5 kDa) and aprotinin (6.5 kDa). Residues from the missing linker region (46-76) were labeled in *gray*

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