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Conformational change study of dengue virus NS2B-NS3 protease using ^{19}F NMR spectroscopy

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ABSTRACT

The dengue virus NS2B-NS3 protease (NS2B-NS3p), an important antiviral target for drug development, has been reported to adopt an open or closed conformation in crystal structures with different NS2B C-terminus (NS2Bc) positioning. In solution, nevertheless, NS2B-NS3p forms a mixture of open, closed and maybe other intermediate conformations, which is difficult to characterize using conventional biophysical and biochemical techniques. In this study, we developed a new strategy to analyze these conformational changes using ^{19}F NMR spectroscopy. Low pH or bovine pancreatic trypsin inhibitor (BPTI) binding promote the conformation change from open to closed, showing the importance of charge forces in the interaction between NS2Bc and NS3p. The mutation H51A impairs the charge interaction and the pH dependence of the conformational changes. It stabilizes the open conformation, while the addition of BPTI still converts NS2B-NS3p from open to closed conformation.

1. Introduction

Dengue virus (DENV) belongs to the *Flaviviridae* virus family and is one of the most prevalent Mosquito-borne viruses in tropical and subtropical regions of the world. Infection by any of the four serotypes of DENV can lead to dengue fever, or the more severe dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [1]. There is currently no approved vaccine or drug available to treat DENV.

A primary target for anti-DENV drug development is the two component viral NS2B-NS3 protease (NS2B-NS3p), which is essential for the virus maturation and cleaves the polypeptide precursor into separated individual proteins [2]. NS2B-NS3p is composed of non-structural protein 2 B (NS2B) and the N-terminal segment of non-structural protein 3 (NS3p, residues 1–185). NS3p is a trypsin-like protein and requires NS2B as a cofactor [3]. NS3p activity increases 3300–7600 folds in the presence of NS2B [3].

Crystal structures of NS2B-NS3p were observed in two distinct conformations in the absence and presence of inhibitor. The protein construct contained a catalytically active linked NS2B-NS3p, in which the soluble portion of NS2B (residues 49*–95*; the residue numbers of NS2B are labeled with asterisks throughout this article) was fused to the N-terminus of NS3p (1–185) via an artificial Gly_4-Ser-Gly_4 linker [4]. In the free state, the C-terminal segment of NS2B (NS2Bc, residues 66*–95*) is located far from the substrate-binding pocket and adopts an open conformation (Fig. 1A) [5]. In the presence of the inhibitor, NS2Bc lines the substrate-binding pocket and adopts a closed conformation (Fig. 1B) [6]. Thus, it is assumed that the open and closed conformations are the inactive and active conformations, respectively.

In solution, NS2B-NS3p behavior is more complicated. The NMR spectra of NS2B-NS3p exhibit severe line broadening and signal overlap, indicating a flexible conformation [5]. It is likely to be a mixture of open, closed and maybe other intermediate conformations on a time scale of medium exchange [5,7,8]. However, upon inhibitor or substrate binding, the more stable closed conformation is induced [6,9,10]. Our previous results demonstrated that, in the presence of bovine pancreatic trypsin inhibitor (BPTI), the NS2B-NS3p/BPTI complex produced well dispersed cross-peaks enabling the assignment of chemical shifts of the backbone resonances [8]. So far, there is no report on a conformational change study for NS2B-NS3p.

In this study, ^{19}F NMR spectroscopy was used to investigate the conformational change of NS2B-NS3p. ^{19}F NMR is a powerful technique to provide insight into the protein conformation, topology, dynamics and the changes under biological conditions [11]. A

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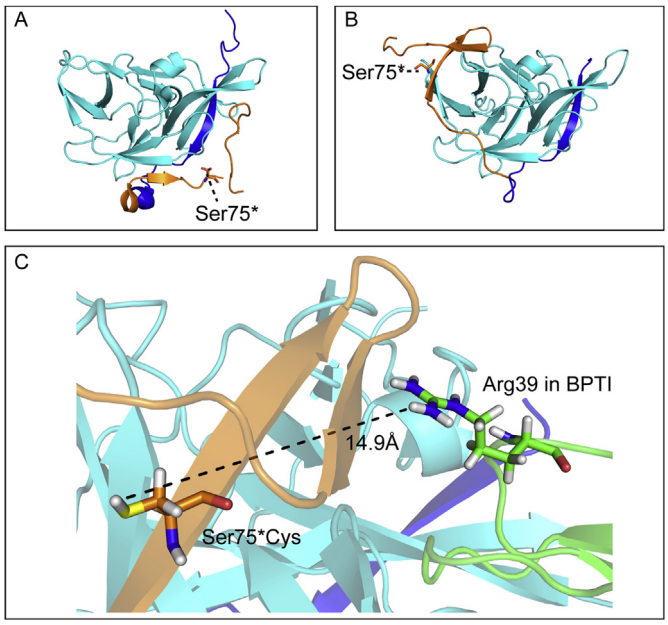

Fig. 1. Open and closed conformations of NS2B-NS3p. (**A**) Cartoon representation of the crystal structure of NS2B-NS3p (PDB ID: 2FOM), which adopts an open conformation. (**B**) and (**C**) Cartoon representation of a model of NS2B-NS3p bound with BPTI (BPTI is not shown in panel B), which adopts a closed conformation. The structure of WNV NS2B-NS3p bound with BPTI (PDB ID: 2IJO) was used as a modeling template. The mutation site for ^{19}F labeling and nearest residues in BPTI are shown in stick representation. NS3p is shown in cyan. The N-terminal part of NS2B is shown in blue, and NS2Bc is shown in orange. NS2Bc departs from NS3p in the open conformation, but it forms tight interactions with NS3p in the closed conformation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

^{19}F label in a trifluoroacetyl (TFA-) group was covalently linked to the cysteine thiol in the Ser75*Cys (S75*C) mutation. The conformational change of NS2B-NS3p was analyzed using ^{19}F NMR spectroscopy. The charge interactions play an important role in stabilizing the closed conformation. Lower pH promoted the conformation, changing it from open to closed. Site-directed mutation His51Ala (H51A) weakened the charge interaction, and NS2B-NS3p adopted a stable open conformation as expected. The bound of BPTI promotes the conformational change from open to closed in all conditions.

2. Materials and methods

2.1. Cloning, expression and purification

The gene encoding DENV2 NS2B (49*–95*)-GGGGSGGGG-NS3p (1–185) was amplified by PCR and subcloned into pET15b(+) to generate the linked NS2B-NS3p construct. The S75*C and H51A mutations were constructed using TaKaRa MutanBEST Kit. The proteins were expressed and purified as described previously [8,10]. Briefly, pET15b-NS2B-G_4SG_4-NS3p was transformed into *Escherichia coli* BL21 (DE3) cells. The proteins were expressed in ^{15}N-labled M9 medium and then purified by Ni-Sepharose affinity chromatography, anion-exchange chromatography (Resource Q) and gel filtration (Superdex-75).

2.2. Fluorine labeling

Cysteine thiol of the S75*C mutant protein was labeled with trifluoroacetyl groups by reaction with 3-bromo-1,1,1-trifluoroacetone (BTFA, purchased from Sigma–Aldrich). Proteins (at approximately 0.4 mM) were dialyzed against 100 mM sodium phosphate buffer, pH 7.0, and then stirred with 2 mM BTFA at 4 °C for 1 h. Excess BTFA was removed by gel-filtration using a Bio-Spin 6 Column (BIO-RAD). The extent of labeling was analyzed by detection of free thiols using Ellman's reagent, [5,5′-dithiobis(2-nitrobenzoic acid)] (DTNB, purchased from BIOSHARP). Proteins (at 2 μM) were added into the Ellman's reagent in 100 mM Tris. pH 8.0. The A412 was measured to monitor the free thiols.

2.3. NMR spectroscopy

For NMR experiments, protein samples were diluted to ~0.2 mM in a 10% D_2O/90% H_2O buffer containing 1 mM trifluoroacetic acid (TFA), 50 mM NaCl, 20 mM Tris (pH 8.5, 8.0 or 7.5) or Bis-Tris (pH 7.0 or 6.5). For NS2B-NS3p/BPTI samples, BPTI was dissolved in the NMR buffer and added into the protease at a molar ratio of 2:1. The ^{1}H–^{15}N HSQC spectra were acquired on a Bruker UltraShield™ Plus 600 MHz spectrometer equipped with a triple-resonance cryoprobe with pulsed field gradients at 298 K. The spectra were processed using nmrPipe [12] and analyzed using SPARKY (T.D. Goddard and D.G. Kneller, SPARKY 3, University of California, San Francisco). The ^{19}F NMR spectra were acquired on Bruker Ascend WB 600 MHz spectrometer using a Bruker Micro 5 imaging probe at 298 K. 20 ppm spectral width and 1024 scans with a relaxation delay (D1) of 1.5 s were used. The ^{19}F chemical shifts were referenced to the internal trifluoroacetic acid (TFA) at 0 ppm, and the data were processed and analyzed using TopSpin 3.0 (Bruker).

3. Results and discussion

The main difference between the open and closed conformations is the location of NS2Bc. Ser75* in NS2Bc is located more than 14.9 Å away from BPTI in the BPTI-bound model (Fig. 1C). Thus, it is assumed that in the BPTI bound state, the chemical environment of Ser75* is not directly affected by BPTI. Therefore Ser75* was selected to be mutated to Cys for ^{19}F labeling. The cysteine thiol was labeled with a TFA-group by reaction with the reagent 3-bromo-1,1,1-trifluoroacetone (BTFA) (Fig. S1) [13,14]. The extent of labeling was determined by the detection of free thiols by Ellman's reagent [15]. The changes in absorbance at 412 nm indicated that greater than 95% NS2B-NS3p was labeled. The ^{1}H–^{15}N HSQC of TFA-NS2B-NS3p showed no significant conformational change from wild type in the absence and presence of BPTI (Fig. S2).

The ^{19}F NMR spectra of TFA-NS2B-NS3p in the absence and presence of BPTI were obtained under different buffer conditions ranging from pH 6.5 to 8.5, respectively (Fig. 2A and B). Two major peaks were observed at −8.98 ppm (O) and −9.18 ppm (C). Peak volumes for the individual components in the signals of Peak O and C were obtained by fitting to a Lorentz/Gauss function (Table 1). The population of Peak C is much more prominent than that of Peak O upon BPTI binding (Fig. 2B), suggesting that Peak O and Peak C represent the open and closed conformation, respectively. In the absence of BPTI, The pH dramatically affects the conformations. The population of the closed conformation is comparable to that of the open conformation at pH 8.5 (42.4%) and pH 8.0 (54.1%). However, at pH 7.5 (67.6%), pH 7.0 (74.2%) and pH 6.5 (80.3%), the population of the closed conformation increases significantly, indicating that lowering of the pH induced a change from open to closed conformation. In the presence of BPTI, the closed conformation is the dominant component (>78%). Interestingly, the conformation exchanges equilibrium also shifts to closed conformation at low pH, but at a much less scale.

The conformational exchange of equilibrium shifts caused by pH is likely due to the effects of pH on the charge interactions between NS2Bc and NS3p. According to the structure of the closed

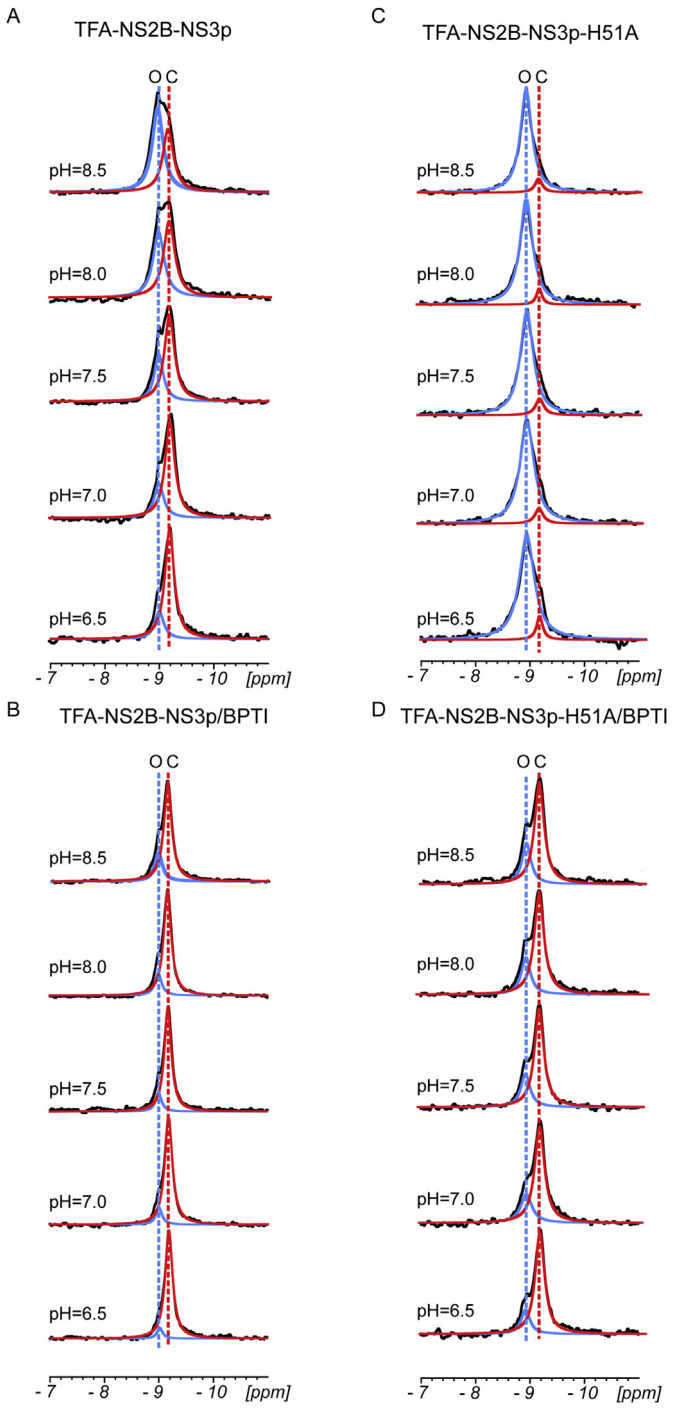

Fig. 2. One-dimensional ^{19}F NMR spectra of different TFA-labeled NS2B-NS3p under variable solution conditions. ^{19}F spectra of the following constructs were recorded: TFA-NS2B-NS3p (**A**), TFA-NS2B-NS3p/BPTI (**B**), TFA-NS2B-NS3p-H51A (**C**), TFA-NS2B-NS3p-H51A/BPTI (**D**). Peak volumes for the open (O, blue) and closed (C, red) conformations in the ^{19}F NMR signals were obtained by fitting to a Lorentz/Gauss function. The experimental data are indicated by black lines. The fit used the chemical shift positions of peaks O and C are indicated by the blue and red vertical dash lines, respectively. The ^{19}F chemical shifts are referenced to internal TFA at 0 ppm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

conformation, NS2Bc has a highly negatively charged region containing residues Glu80*, Asp81* and four additional glutamates at the tail of NS2B. The NS3p counterpart interacting with NS2Bc contains Arg54, Lys73, Lys74, and one of the catalytic triad residues

Table 1
The relative peak volumes of the open and closed conformation.

Peaks	Relative peak volumes (%)									
	TFA-NS2B-NS3p					TFA-NS2B-NS3p/BPTI				
	pH	8.5	8.0	7.5	7.0	6.5	8.5	8.0	7.5	7.0	6.5
O		57.6	45.9	32.4	25.8	19.7	21.6	16.3	13.5	11.2	6.9
C		42.4	54.1	67.6	74.2	80.3	78.4	83.7	86.5	88.8	93.1
	TFA-NS2B-NS3p-H51A						TFA-NS2B-NS3p-H51A/BPTI				
	pH	8.5	8.0	7.5	7.0	6.5	8.5	8.0	7.5	7.0	6.5
O		92.4	93.8	91.1	92.1	91.6	27.1	25.3	24.9	25.2	18.4
C		7.6	6.2	8.9	7.9	8.4	72.9	74.6	75.1	74.8	81.6

His51, which all provide positive charges that attract NS2Bc (Fig. 3). At high pH, the reduction of the positive charge of Arg54, Lys73, Lys74 and His51 in the active site probably weakens the charge interactions between NS2Bc and NS3p, destabilizing the closed conformation. Among these residues, His51 is the most likely residue to be responsible for the observed changes in the studied pH range (6.5–8.5), while lysine (side chain pKa = 10.53) and arginine (side chain pKa = 12.48) residues are not expected to play a significant role. The strong binding of BPTI provides an additional positively charged arginine (Arg39 in BPTI) and perhaps other interaction pattern such as H-bond interaction [16] help stabilize the closed conformation and is less affected by the change of pH. In a previous study [17], this pH effect was reported and exchange broadening of $^{1}H-^{15}N$ HSQC has been observed at high pH. Nevertheless, the population change between the open and closed conformation have not been characterized because of the dynamics and the influence of amide proton exchange at basic pH.

To verify our hypothesis, we mutated His51 to Ala, which should affect the pH dependent conformational change. ^{19}F NMR spectra of TFA-NS2B-NS3p-H51A were obtained under different buffer conditions ranging from pH 6.5 to 8.5. As shown in Fig. 2C and D, Peak O is the dominant component in the absence of BPTI (~92%) and the population does not vary with pH changing. In the presence of BPTI, the strong stabilizing effect still manages to push the conformation changing from the open to the dominantly closed conformation, but at a population less than that of the wild type. These results

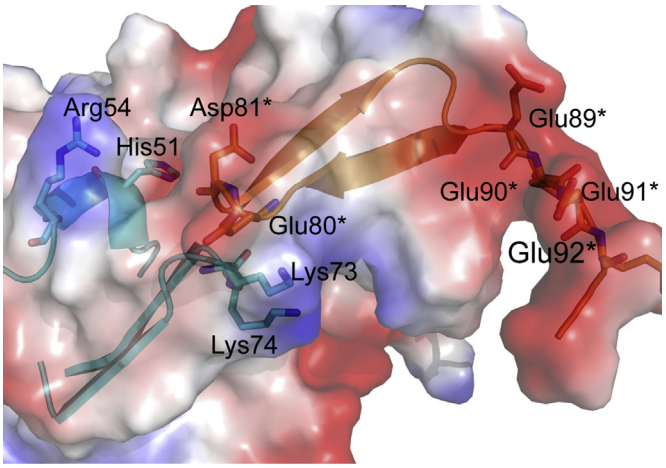

Fig. 3. Charge interaction between NS2Bc and NS3p. The surface electrostatic potential representation shows the main residues contributing to the charges for NS2Bc and NS3p interaction. Positive and negative charges are shown in blue and red, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

indicate that His51 plays a crucial role in the pH dependent conformational exchange by charge interacting with NS2Bc.

Hence, present study provides a simple and useful method for the conformation studies of NS2B-NS3p, and demonstrated that the pH-dependent conformation exchange is due to the charge interactions between NS2Bc and NS3p. Our results suggest that the ^{19}F NMR spectroscopy can be used as a potential drug screening tool for NS2B-NS3p targeting drug design. And it is possible to design chemical compounds or peptides to destabilize the closed conformation by competing with NS2Bc.

Conflict of interest

None declared.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.090.

Transparency document

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