NMR Analysis of a Novel Enzymatically Active Unlinked Dengue NS2B-NS3 Protease Complex *

Received for publication, December 5, 2012, and in revised form, March 7, 2013. Published, JBC Papers in Press, March 19, 2013, DOI 10.1074/jbc.M112.442723


From the *Experimental Therapeutics Centre, Agency for Science, Technology and Research (A*STAR), Singapore 138669 and the §Novartis Institute for Tropical Diseases, Singapore 138670

Background: Dengue protease is a two-component protease that is important for viral replication.
Results: An unlinked protease complex containing the NS2B regulatory region and the NS3 protease domain was obtained.
Conclusion: The unlinked protease complex produces dispersed cross-peaks in NMR spectra and exists predominantly in a closed conformation in solution.
Significance: This new construct will be a useful tool for drug discovery against the dengue virus.

The dengue virus (DENV) is a mosquito-borne pathogen responsible for an estimated 100 million human infections annually. The viral genome encodes a two-component trypsin-like protease that contains the cofactor region from the non-structural protein NS2B and the protease domain from NS3 (NS3pro). The NS2B-NS3pro complex plays a crucial role in viral maturation and has been identified as a potential drug target. Using a DENV protease construct containing NS2B covalently linked to NS3pro via a Gly4-Ser-Gly4 linker (“linked protease”), previous x-ray crystal structures show that the C-terminal fragment of NS2B is remote from NS3pro and exists in an open state in the absence of an inhibitor; however, in the presence of an inhibitor, NS2B complexes with NS3pro to form a closed state. This linked enzyme produced NMR spectra with severe signal overlap and line broadening. To obtain a protease construct with a resolved NMR spectrum, we expressed and purified an unlinked protease complex containing a 50-residue segment of the NS2B cofactor region and NS3pro without the glycine linker using a coexpression system. This unlinked protease complex was catalytically active at neutral pH in the absence of glycerol and produced dispersed cross-peaks in a 1H-15N heteronuclear single quantum correlation spectrum that enabled us to conduct backbone assignments using conventional techniques. In addition, titration with an active-site peptide aldehyde inhibitor and paramagnetic relaxation enhancement studies demonstrated that the unlinked DENV protease exists predominantly in a closed conformation in solution. This protease complex can serve as a useful tool for drug discovery against DENV.

The dengue virus (DENV) exists in four serotypes (DENV1–4) and belongs to the Flaviviridae family, which also includes several human pathogens such as the West Nile virus (WNV), Japanese encephalitis virus, and yellow fever virus (1). DENV causes a range of diseases from a self-limiting dengue fever to the more severe, sometimes life-threatening dengue hemorrhagic fever and dengue shock syndrome (2). Globally, an estimated 100 million people are infected yearly, including 500,000 dengue hemorrhagic fever cases, resulting in 22,000 deaths, mostly among children (3). There are currently no clinically approved antivirals or prophylactic vaccines for treating and/or preventing dengue fever (4).

The DENV genome is an 11-kb single-stranded positive-sense RNA. The genomic RNA encodes a polyprotein precursor that is processed proteolytically upon translation to 10 proteins, including three structural proteins (capsid, premembrane, and envelope) and seven nonstructural proteins (NS1, NS2A/B, NS3, NS4A/B, and NS5). The structural proteins form the viral particle. The nonstructural proteins participate in the replication of the RNA genome, virion assembly, and attenuation of the host antiviral response. Correct processing of these proteins is essential for viral replication, and this requires a host signal peptidase and a virus-encoded protease, NS2B-NS3. The N-terminal ~180-amino acid region of NS3 (NS3pro) is a serine protease that requires association with at least 40 amino acids of NS2B for activity (5). Virus-encoded proteases have been successfully targeted for HIV and hepatitis C virus, which makes the dengue NS2B-NS3 protease an attractive target for therapeutic intervention.

Knowledge of the three-dimensional structure of the target protein is a prerequisite for structure-based rational drug

* This work was supported in part by A*STAR.
[This article contains supplemental Figs. S1–S9.]
1 Supported by an A*STAR investigatorship. To whom correspondence may be addressed: Experimental Therapeutics Centre, A*STAR, Nanos 03-01, 31 Biopolis Way, Singapore 138669. Tel.: 65-6407-0352; Fax: 65–6478-8768; E-mail: cbkang@etc.a-star.edu.sg.
2 To whom correspondence may be addressed. E-mail: qing_yin.wang@novatis.com.
3 To whom correspondence may be addressed. E-mail: thkeller@etc.a-star.edu.sg.
4 The abbreviations used are: DENV, dengue virus; WNV, West Nile virus; NS3pro, NS3 protease domain; H5QC, heteronuclear single quantum correlation; PRE, paramagnetic relaxation enhancement; Ni-NTA, nickel-nitrilotriacetic acid, Bz-nKRR-AMC, benzoyl-Nle-Lys-Arg-Arg-amomethyl-ylcumarin; MTSL, Bz-nKRR-H, benzoyl-Nle-Lys-Arg-Arg-H; 1-oxyl-2,2,5,5-tetramethyl-Δ3-pyrroline-3-methylmethanethiosulfonate; NTF, N-terminal fragment; CTF, C-terminal fragment.
NMR Study of the Dengue Protease

design. Crystal structures of the DENV protease in the absence and presence of inhibitors have been determined recently (6, 7). The NS2B fragment in these structures adopts very different conformations, i.e., without an inhibitor, the C-terminal segment of the NS2B cofactor is dissociated from the active site (“open state”) (see Fig. 1A), whereas in the presence of inhibitors, it wraps around NS3pro and forms an active closed conformation, similar to that of the WNV protease (“closed state”) (see Fig. 1B). However, in solution, low population of the DENV protease assumes the active closed conformation (8), which might suggest an explanation for the lack of success in finding a low nanomolar inhibitor despite intensive efforts (9). All of the proteins used for the aforementioned structural studies have the ~40-amino acid region of NS2B covalently linked to NS3pro via a Gly4-Ser-Gly4 linker (see Fig. 1C), whereas in nature, the two proteins form the active enzyme through intermolecular association. It is currently unknown whether this linked construct bears any resemblance to the protein complex that is formed to process the viral polyprotein. If it is assumed that the closed form is the appropriate model for the protease, then the linked enzyme is not a suitable model for drug discovery.

In this study, we expressed and purified the NS2B cofactor region and NS3pro from Escherichia coli using a coexpression system in which NS2B and NS3pro were expressed as individual proteins (without any linker) to closely mimic the natural condition (see Fig. 1D). A hexahistidine tag was introduced at the N terminus of NS3pro to aid in protein purification. We refer to this construct as the unlinked protease complex (see Fig. 1D). This unlinked protease complex was catalytically active at neutral pH in the absence of glycerol and produced dispersed cross-peaks in the heteronuclear single quantum correlation (HSQC) spectrum, which enabled us to do a backbone assignment using conventional techniques. More importantly, a paramagnetic relaxation enhancement (PRE) study demonstrated that the NS2B cofactor region of the unlinked DENV protease complex adopts a paramagnetic conformation (see Fig. 1D), whereas in nature, the two proteins form the active enzyme through intermolecular association. It is currently unknown whether this linked construct bears any resemblance to the protein complex that is formed to process the viral polyprotein. If it is assumed that the closed form is the appropriate model for the protease, then the linked enzyme is not a suitable model for drug discovery.

EXPERIMENTAL PROCEDURES

Materials—The DNA polymerase and restriction enzymes were purchased from New England Biolabs. The FPLC system, gel filtration column, ion exchange columns, and PD-10 desalting column were purchased from GE Healthcare. The SDS-PAGE system, NuPAGE® gels (4–12%), and molecular weight standards were obtained from Invitrogen or Bio-Rad. The protein staining and destaining solutions, protein sample loading buffer, and nitrocellulose membrane were purchased from Bio-Rad. Ni-NTA and plasmid extraction and DNA purification kits were obtained from Qiagen. [15N]NH4Cl, [13C]glucose, deuterated methanol, and D2O were purchased from Cambridge Isotope Laboratories. Isopropyl 1-thio-β-D-galactopyranoside, DTT, and other chemicals were purchased from Sigma-Aldrich.

Expression Plasmid Construction—The gene encoding the DENV2 NS2B cofactor region (amino acids 48–100) was amplified by PCR using full-length NS2B as a template (8). The resulting PCR product was inserted into the NdeI/XhoI sites of pACYDuet to generate plasmid pACY-NS2B, encoding the NS2B cofactor region without any fusion tags. A hexahistidine tag was introduced at the N terminus of NS3pro to aid in protein purification. The gene encoding NS3pro(amino acids 1–185) was synthesized by GenScript (Piscataway, NJ) and inserted into the NdeI and XhoI sites of pETDuet to generate plasmid pET-NS3pro, encoding NS3pro with an N-terminal hexahistidine tag for purification. Different constructs of NS3pro (amino acids 14–175 and 14–185) were made in a similar fashion. All plasmid insertions were confirmed by DNA sequencing. The linked protease construct with NS2B(amino acids 48–95) linked to NS3pro by a Gly4-Ser-Gly4 linker was same as that used for crystallography (6).

Expression and Purification of the Unlinked Protease Complex—The plasmids encoding the NS2B cofactor region and NS3pro were cotransformed into E. coli BL21(DE3) competent cells, and cells harboring plasmids were selected on LB plates containing both 34 μg/ml chloramphenicol and 100 μg/ml ampicillin. Protein expression and purification were similar to those described previously (11, 12). One to three colonies were picked and incubated in 50 ml of M9 medium with antibiotics at 37 °C. The overnight culture was transferred into 1 liter of M9 medium with antibiotics. and induction was performed by adding isopropyl 1-thio-β-D-galactopyranoside to a final concentration of 1 mM with additional shaking at 240 rpm for 3 h at 25 °C when A600 reached 0.6. To purify the recombinant protein, E. coli cells were harvested by centrifugation at 9000 × g for 10 min at 4 °C. The resulting cell pellets were resuspended in lysis buffer containing 20 mM sodium phosphate (pH 7.8), 500 mM NaCl, and 2 mM β-mercaptoethanol. The cells were broken by sonication in an ice bath. Cell lysates were cleared by centrifugation at 40,000 × g for 20 min, and the supernatant was mixed with Ni-NTA resin at 4 °C for 1–2 h before being passed through a gravity column. Resin with protein was washed with at least 10 column volumes of wash buffer containing 50 mM sodium phosphate (pH 7.2), 1 mM NaCl, 10 mM imidazole, and 2 mM β-mercaptoethanol to remove nonspecific proteins bound to the resin. Protein was eluted with elution buffer containing 500 mM imidazole (pH 6.5), 500 mM NaCl, and 2 mM β-mercaptoethanol. [15N]−, [13C]−, or [13C]2H15N-labeled protease for the NMR study was prepared as described above, except that [15N]−, [13C]−, and 2H-labeled [15NH4Cl, [13C]−glucose, and D2O were used in M9 medium. Proteases selectively labeled with [15N]-amino acids was prepared by growing E. coli cells in M9 medium containing 0.5 g/liter unlabeled NH4Cl. During protein induction, [15N]-labeled amino acid (0.1 g/liter) and 19 unlabeled amino acids (0.1–0.4 g/liter) were added with isopropyl 1-thio-β-D-galactopyranoside (13). Protease was then purified as described above and analyzed by NMR spectroscopy.

Ion Exchange and Gel Filtration Chromatography—Purified protease from the Ni-NTA column was loaded onto a Superdex 200 column for further purification. The gel filtration buffer contained 20 mM Tris-HCl (pH of 7.8), 150 mM NaCl, and 2 mM DTT. Gel filtration was performed at 8 °C at a flow rate of 0.5 ml/min. The fractions containing protease were buffer-ex-
changed to buffer A (20 mM sodium phosphate (pH 6.5), 10 mM NaCl, and 1 mM DTT) using a PD-10 desalting column. The sample was loaded onto a HiTrap SP HP column equilibrated with buffer A. Protease was eluted with increasing concentrations of NaCl to 1 M. Some contaminating protein from E. coli that migrated to a position similar to NS3pro was removed in this step (14). The purity of the protease was analyzed by SDS-PAGE. All of the proteins were >90% purity as confirmed by SDS-PAGE and mass spectrometry and were used for further studies.

**Protease Activity Assay**—DENV protease activity assays were performed in 384-well plates. The enzyme activity was measured in assay buffer that contained 10 mM Tris-HCl (pH 8.5), 20% glycerol, and 1 mM CHAPS in a final volume of 50 µl as described previously (15). The protease-specific, fluorophore-tagged substrate benzoyl-Nle-Lys-Arg-aminomethylcoumarin (Bz-nKRR-AMC) was added in the protease buffer to 20 µM. Substrate cleavage was monitored after addition of protease. The increase in fluorescence (excitation at 380 nm and emission at 450 nm) was continuously monitored on a Tecan Safire2 microplate reader at room temperature. Kinetic studies using TTNTTRR-AMC peptide substrate were performed as described previously (15). Briefly, 20 nM unlinked protease complex was incubated with various concentrations of substrate at 37 °C in buffer containing 50 mM Tris-HCl (pH 7.5), 0.001% Triton X-100, and 0.5 mM EGTA. The proteolytic reaction was monitored as described above. Initial fluorescence velocities (relative fluorescence units/min) were converted to m⁻¹ s⁻¹ from a standard AMC calibration curve. The progression curves were fitted to the Michaelis-Menten equation by nonlinear regression using GraphPad Prism.

**Peptide Aldehyde Inhibitor Synthesis and Purification**—The peptide Benzoyl-Nle-Lys-Arg-H (Bz-nKRR-H) with a C-terminal aldehyde moiety was synthesized according to a previous report (16) and purified by reverse-phase HPLC (Shimadzu, Japan) using acetonitrile and water solvent.

**NMR Data Acquisition**—Purified protease was buffer-exchanged to NMR buffer containing 20 mM HEPES (pH 7.3) and 2 mM DTT. The sample was concentrated to 0.2–1.0 mM and transferred to a 3- or 5-mm NMR tube for NMR data acquisition. All spectra were acquired at 298 K on a Bruker AVANCE II 600- or 700-MHz spectrometer equipped with a triple-resonance CryoProbe (Bruker). Spectra were processed using TopSpin 2.1 (Bruker) and NMRPipe (17) and visualized with NMRView (18) or Sparky. The backbone ¹HN, ¹Ha, ¹⁵N, ¹³Cα, and ¹³C' resonances were assigned using two- and three-dimensional experiments, including ¹H-¹⁵N HSQC, three-dimensional HNCO, HN(CA)CO, HN(CA)CB, HN(CO)CA, HNCA, and ¹⁵N-edited NOESY experiments (19). All pulse programs were from the Bruker standard library. Protein secondary structure was analyzed using TALOS+ (20) and the chemical shift index (21). To facilitate backbone assignment, proteases selectively labeled with ¹⁵N-labeled Ala, His, Met, Arg, Thr, Ser, Phe, and Leu were prepared, and ¹H-¹⁵N HSQC spectra were collected for analysis. To probe protease and inhibitor interaction, 0.2 mM ¹⁵N-labeled protease was prepared in NMR buffer. The tetrapeptide inhibitor was dissolved in deuterated Me₂SO to 30 mM. ¹H-¹⁵N HSQC spectra of the protease in the absence and presence of 1 mM inhibitor were collected. The ¹⁵N chemical shift changes were measured, and superimposed spectra are shown.

**Relaxation Analysis of the Protease**—The steady-state ¹H-¹⁵N NOE, ¹⁵N longitudinal R₁, and transverse R₂ relaxation rates were measured at 298 K using a ¹⁵N-labeled sample in NMR buffer using the Bruker AVANCE II 700-MHz spectrometer. For R₁ measurements, relaxation delays of 5, 50, 210, 330, 470, 630, 800, 900, 1000, 1200, 1400, 1600, and 1800 ms were recorded as described previously (22). For R₂ measurements, the data were acquired with delays of 16, 9, 34, 51, 68, 85, 102, 119, 136, and 153 ms. Steady-state ¹H-¹⁵N NOEs were obtained using two data sets that were collected with and without initial proton saturation for a period of 3 s. The spectra were processed and analyzed as described previously (22).

**Spin Labeling**—Unlinked proteases with serine-to-cysteine mutations (S171C and S75C (NS2B)) were made by site-directed mutagenesis and purified in the presence of 2 mM β-mercaptoethanol. 1-Oxyl-2,2,5,5-tetramethyl-Δ₃-pyrroline-3-methylmethanethiosulfonate (MTSL) was used for the spin labeling. A 0.1 mM solution of sample was prepared in NMR buffer. MTSL was dissolved in methanol to make a 100 mM stock solution. MTSL was slowly added to the protein solution to 2 mM. The mixture was gently stirred at 37 °C for 1 h and left at room temperature overnight. The mixture was buffer-exchanged to NMR buffer using a PD-10 column to remove excess MTSL. The ¹⁵N HSQC spectra of spin-labeled samples in the absence and presence ascorbic acid were acquired and compared as described above.

**Thermal Shift Assay**—The thermal stability of the linked and unlinked proteases was assessed using the Thermostfluor assay as described previously (22). Assay samples consisting of 5 µM protein and SYPRO Orange dye in phosphate buffer (20 mM sodium phosphate (pH 7.0), 150 mM NaCl, and 2 mM DTT) were subjected to an increase in temperature from 20 to 95 °C in 0.5 °C increments. The experiment was performed on a real-time PCR machine with fluorescence readings taken at each temperature increment. The fluorescence measurements were plotted using GraphPad Prism version 5.03, and melting curves for the two proteins were obtained.

**RESULTS**

**Purification of Linked and Unlinked Proteases**—The standard linked protease as shown in Fig. 1C was overexpressed and purified for our NMR studies (supplemental Fig. S1). Previous attempts to study the solution structure of this protein by conventional NMR techniques were unsuccessful due to the broad NMR signals and overlapping (23). A recent NMR study of this linked protease using pseudocontact shifts with introduction of paramagnetic lanthanide tags was reported, and only assignments for the protease in complex with an inhibitor were achieved (23, 24). To the best of our knowledge, assignments for the linked inhibitor-free DENV protease have so far not been achieved. Due to the significant chemical shift difference observed for the linked protease in the absence and presence of an inhibitor (23), it is hard to use this construct to screen potential protease inhibitors in HSQC-type experiments by NMR. As NS2B and NS3 exist naturally as individual proteins after post-
translational processing in the host cell, we decided to coexpress the NS2B cofactor region and NS3pro in E. coli cells without a linker to determine if we could develop a construct more suitable for NMR studies.

Simple mixing of the purified NS2B cofactor region and NS3pro was not possible because NS3pro alone was not soluble when expressed in E. coli. Thus, we decided to use a coexpression system to obtain both fragments in E. coli (supplemental Figs. S1 and S2). The presence of the NS2B cofactor region in E. coli promoted the folding of NS3pro. An N-terminal hexahistidine tag was introduced into the N terminus of NS3pro, which enabled us to purify the unlinked protease complex by affinity chromatography. With this strategy, different constructs were made and purified from E. coli (supplemental Fig. S1). An additional ion exchange step was used to remove contaminants with molecular weights similar to that of NS3pro (supplemental Fig. S1). All constructs were stable at room temperature except one (amino acids 1–165), which precipitated after purification (supplemental Fig. S1). Purified proteins were confirmed by mass spectrometry analysis (supplemental Fig. S3).

Enzyme Activity of Unlinked Protease Complexes—The protease activities of the unlinked purified protease complexes were assayed using Bz-nKRR-AMC as a substrate and compared with the results obtained with the linked protein. The linked protease exhibited catalytic activity similar to that of unlinked protease complexes (Table 1). A previous study showed that the linked protease possessed the highest enzyme activity using small peptide substrates at pH 8.5 in the presence of glycerol and detergent (25).

The effects of pH and addition of salt, glycerol, and detergent on the purified unlinked protease complex were also explored to determine the optimal proteolytic conditions. We found that some detergents such as Brij 35® and CHAPS enhanced the protease activity of the unlinked protease (data not shown). The presence of salt (NaCl or KCl) in the assay buffer inhibited the protease activity, mirroring the results of the linked protease from DENV and WNV (25, 26). Interestingly, the unlinked protease complex maintained good protease activity at physiological pH (pH 7.3) in the absence of 20% glycerol, conditions that are suitable for NMR studies. We also tested the enzyme activity using the fluorogenic hexapeptide substrate TTNTRR-AMC, a natural enzyme cleavage site between NS4B and NS5. This substrate was efficiently cleaved in buffer containing 50 mM Tris·HCl (pH 7.5), 0.001% Triton X-100, and 0.5 mM EGTA with $K_m = 40.9 \pm 2.8 \mu M$, $k_{cat} = 6.03 \pm 0.4 s^{-1}$, and $k_{cat}/K_m = 147,910 \pm 12,931 M^{-1} s^{-1}$.

NMR Spectra of the Purified Protease—The linked protease at pH 6.5 produced a 15N HSQC spectrum with severe peak overlaps and line broadening, which were not caused by denaturation of the protease because the protease still possessed protease activity, and circular dichroism spectroscopy of this linked protease indicated that it was folded under these conditions (supplemental Fig. S4). The NMR spectrum of the linked protease showed fewer cross-peaks than predicted very likely due to the conformational flexibility of this protease construct (Fig. 2A), which was also observed in previous studies and is similar to WNV protease (8, 23, 27, 28). Surprisingly, removal of the Gly$_x$-Ser-Gly$_y$ linker from the constructs and coexpression of NS2B and NS3 as an unlinked protein complex greatly improved the quality of the 15N HSQC spectra (Fig. 2B). A 15N HSQC spectrum comparison of NS3 with different lengths revealed no observable chemical shift perturbations (supplemental Fig. S5), suggesting that all of the constructs had similar structures. Using the current data, the unlinked protease complex containing the NS2B cofactor region (amino acids 48–100) and NS3pro (amino acids 14–185) was selected for further study due to its higher yield during protein purification and shorter amino acid sequence.

Backbone Resonance Assignments and Secondary Structural Analysis—To aid in resonance assignment, proteases selectively labeled with 15N-labeled Ala, His, Met, Arg, Thr, Ser, Phe, and Leu were prepared (supplemental Fig. S6). The backbone

---

### TABLE 1

Activity of different protease constructs using Bz-nKRR-AMC as a substrate

<table>
<thead>
<tr>
<th>Protease construct</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ ($M^{-1} s^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS2B(48–100) + NS3(1–185)</td>
<td>33.69 ± 3.01</td>
<td>0.179 ± 0.003</td>
<td>330 ± 119</td>
</tr>
<tr>
<td>NS2B(48–100) + NS3(1–187)</td>
<td>69.54 ± 6.01</td>
<td>0.131 ± 0.004</td>
<td>1887 ± 134</td>
</tr>
<tr>
<td>NS2B(48–100) + NS3(1–185)</td>
<td>68 ± 4.02</td>
<td>0.33 ± 0.03</td>
<td>4850 ± 150</td>
</tr>
<tr>
<td>NS2B(48–100) + NS3(1–175)</td>
<td>56.94 ± 6.21</td>
<td>0.074 ± 0.004</td>
<td>1290 ± 160</td>
</tr>
<tr>
<td>NS2B(48–100) + NS3(1–175)</td>
<td>48.2 ± 6.04</td>
<td>0.027 ± 0.003</td>
<td>560 ± 17</td>
</tr>
<tr>
<td>NS2B(48–95) + NS3(1–181)</td>
<td>45.23 ± 5.73</td>
<td>0.64 ± 0.003</td>
<td>14140 ± 1517</td>
</tr>
<tr>
<td>NS2B + NS3(14–185)$^*$</td>
<td>49.27 ± 5.81</td>
<td>1.162 ± 0.003</td>
<td>23,580 ± 2364</td>
</tr>
<tr>
<td>NS2B + NS3(14–185)$^*$</td>
<td>51.93 ± 5.58</td>
<td>1.33 ± 0.044</td>
<td>25,610 ± 1463</td>
</tr>
<tr>
<td>NS2B(48–95) + NS3(1–185)</td>
<td>35.96 ± 5.12</td>
<td>0.162 ± 0.007</td>
<td>4500 ± 383</td>
</tr>
<tr>
<td>NS2B(48–95)–Gly$_x$-Ser-Gly$_y$-NS3pro</td>
<td>31.48 ± 4.01</td>
<td>0.188 ± 0.008</td>
<td>5970 ± 447</td>
</tr>
</tbody>
</table>

---

NMR Study of the Dengue Protease
FIGURE 2. NMR spectra of the DENV protease. A, ^1^H-^{15}N HSQC spectrum of the linked protease. B, ^1^H-^{15}N HSQC spectrum of the unlinked protease complex containing the NS2B cofactor region (amino acids 48–100) and NS3pro (amino acids 1–185). These two proteases were expressed and purified to 0.2 mM in buffer containing 20 mM sodium phosphate (pH 6.5), 150 mM NaCl, and 1 mM DTT.

FIGURE 3. Assignments for the ^1^H-^{15}N HSQC spectrum of the unlinked DENV protease complex. The residues from NS2B are labeled in red. To differentiate from NS3pro, the positions of NS2B are indicated by asterisks.
resonance assignments for the unlinked protease complex at pH 7.3 were achieved using conventional NMR methods. We obtained an ~80% assignment for backbone amide protons and amide nitrogen (Fig. 3). Other backbone assignments have been obtained for 85% of Cα, 81% of C', and 78% of Cβ. There were some cross-peaks in the 1H-15N HSQC spectrum that could not be unambiguously assigned (Fig. 2), which may due to the dynamic nature of these residues. Secondary structural analysis was carried out using TALOS+ (20) and the chemical shift index with Cα chemical shift deviations from random coil values (supplemental Fig. S7). For NS3pro, the secondary structure is similar to that determined by x-ray crystallography (6). For NS2B cofactor, the N-terminal fragment (NTF) adopts a β-sheet structure, consistent with the x-ray crystal result (6). In the solid state, the inhibitor-free NS2B C-terminal fragment (CTF) adopts a helical structure, which may have been caused by crystal packing effects (6). In our work with the unlinked protease complex, no α-helical structure was identified in NS2B (supplemental Fig. S6), but CTF residues of NS2B were observed to have a tendency to form β-sheet structures, indicating that NS2B may exist in a conformation similar to the closed form of the linked protease (supplemental Fig. S7).

Probing Protease-Inhibitor Interactions by NMR—For the linked protease, significant changes such as new cross-peaks appearing in the 1H-15N HSQC spectrum were observed in the presence of an inhibitor, which may due to stabilization of the closed form of the protease (23). We compared the 1H-15N HSQC spectra of the unlinked protease complex in the absence and presence of the Bz-nKRR-H inhibitor (7, 16). Chemical shift perturbation was observed, but no new peaks appeared (Fig. 4). The interaction between the unlinked protease complex and the peptide inhibitor was confirmed using a thermal shift assay (Fig. 5). The IC50 values of the peptide inhibitor for the linked and unlinked proteases were 6.49 ± 1.28 and 15.15 ± 4.36 μM, respectively (supplemental Fig. S8).
Relaxation Analysis of the Unlinked Protease

The flexibility of the unlinked protease complex was probed by measuring the $R_1$ and $R_2$ relaxation rates and the steady-state $^1H$-$^15N$ NOE using a uniformly $^{15}N$-labeled protease (29). For NS3pro, the N-terminal residues (amino acids 14–16) and C-terminal residues (amino acids 174–185) were found to be unstructured with lower $R_2$ and higher $R_1$ values (Fig. 6), consistent with the steady-state NOE result (Fig. 6). The correlation times for molecular reorientation based on $R_1$ and $R_2$ for NS3pro and the NS2B cofactor region were $\sim 12$ and $\sim 10$ ns, respectively. These values were in the expected range for a protein with a molecular mass of $\sim 20$ kDa, suggesting that the NS2B cofactor region and NS3pro form a monomeric complex in solution.

PRE Experiment—PRE has been widely used to probe conformational equilibriums (30, 31). PRE was used to understand the conformation of NS2B in the linked WNV protease (8). As wild-type DENV protease does not possess cysteines, two mutations (S171C and S75C (NS2B)) were made to enable spin labeling. The enzymatic assay indicated that these mutations were still active (Table 1). The $^1H$-$^15N$ HSQC spectra of the wild-type unlinked protease complex and the Ser-to-Cys mutants were found to be similar (supplemental Fig. S9), suggesting that the mutations did not alter the structure of the protease. The results obtained with S171C were ambiguous because Ser-171 is located in a flexible region (supplemental Fig. S9).

The DENV crystal structure shows that residues such as Gly-121, Gly-148, Gly-114, Thr-122, and Thr-156 are remote from Ser-75 (6) in the open linked form (Fig. 7, A and C) but are in proximity in the closed conformation (Fig. 7, B and D) (7). Analysis of the PRE data on our unlinked S75C complex revealed that Gly-121, Gly-148, Gly-114, Thr-122, and Gly-156 are in close proximity to Ser-75 (Fig. 7B), indicating that the unlinked protease complex adopts a conformation similar to the closed form of the linked protein in the presence of an inhibitor.

DISCUSSION

The DENV NS2B-NS3 complex is a trypsin-like serine protease involved in viral proteolytic processing and plays a crucial role in viral survival and replication. To date, efforts to make a catalytically active enzyme complex involved expressing the NS2B cofactor region ($\sim 40$ residues) tethered to NS3pro ($\sim 180$ residues) by a Gly$_4$-Ser-Gly$_4$ linker. This linker, although not found naturally in the protease complex, was introduced to stabilize the NS2B-NS3pro complex, without which the enzyme undergoes self-proteolysis after being expressed (5, 25, 32). NMR experiments conducted on the linked protease construct have met with little success due to severe peak broadening, hindering research efforts (23).

In this study, we coexpressed and purified several unlinked protease complexes from E. coli. The enzymatic assay indicated that the unlinked protease complexes exhibited enzyme activity similar to that of the linked protease (Table 1). In contrast to the
linked protease, which required basic pH and co-solvents, the optimal activity of the unlinked protease complex was achieved at pH 7.5 in the absence of glycerol. The unlinked protease complex in our study produced well dispersed NMR cross-peaks (Fig. 2B). It is surprising that the removal of such a short unstructured linker (8) improved the NMR spectrum significantly (Fig. 2B). One possible explanation is that the linker induces conformational dynamics that are absent in the unlinked complex. The removal of the Gly4-Ser-Gly4 linker may make the unlinked protease complex more stable, resulting in a well resolved NMR spectrum.

Much effort has been invested into screening for DENV protease inhibitors as potential drug targets (33–41). The linked DENV protease construct has been widely utilized for in vitro biochemical assays and in silico compound screening. Although the linked protease exhibited a resolved 1H-15N HSQC spectrum in the presence of small molecular inhibitors, significant chemical shift perturbations and new peaks were observed (23), making it difficult to map the protein-inhibitor interface. Using our novel unlinked protease complex, we were able to obtain dispersed cross-peaks in an HSQC spectrum, and chemical shift perturbations were observed only in the presence of a potent inhibitor (Fig. 5). This facilitates the mapping of the binding site for inhibitors and the generation of structure-activity relationships using NMR.

Using our unlinked protease construct, we successfully achieved backbone assignments using conventional NMR techniques (Fig. 3). The results from secondary structural analysis of NS3pro were in good agreement with structures determined by x-ray crystallography (supplemental Fig. S7) (6, 7). Relaxation analysis showed that the N-terminal residues (amino acids 14–18) and C-terminal residues (amino acids 174–185) of
NS3pro are flexible (Fig. 6). In the solution structure of the unlinked protease, the NTF of NS2B adopts a stable β-sheet that interacts with NS3pro. In contrast, the CTF of NS2B was found to be more flexible, with a tendency to form a β-sheet conformation (supplemental Fig. S6).

Previous structural studies suggested that proteases from DENV or WNV existed in an equilibrium between the open and closed forms and that this equilibrium was driven toward the closed form in the presence of an inhibitor (7, 8). In this work, we showed that the unlinked protein complex is similar to the closed form of the linked protease. Our hypothesis is supported by the following findings. (a) The appearance of cross-peaks from residues such as Gly-82 in NS2B was observed for the linked protease only in the presence of an inhibitor (Fig. 3) (8, 23, 24); (b) relaxation analysis showed that the tumbling time of NS2B CTF residues such as Gly-82 was close to that of NTF residues (Fig. 2), indicating NS2B forms a close contact with NS3pro (Fig. 8); and (c) in a PRE experiment, close contacts between Ser-75 from NS2B and residues such as Gly-114 and Gly-121 from NS3 were observed (Fig. 7) (7). In addition, in the presence of the potent active-site inhibitor Bz-nKRR-H were chemical shift perturbations observed.

In conclusion, we coexpressed and purified an enzymatically active unlinked DENV protease complex suitable for NMR structural studies. Our results reveal that the protease complex exists predominantly in a confirmation that is similar to the closed form of the linked protease. We believe that our unlinked construct provides a more realistic model for biochemicals, inhibitor screening, and modeling and will serve as a valuable tool in the development of DENV protease inhibitors.

Acknowledgments—We thank Prof. Ho Sup Yoon and Dr. Hong Ye (Nanyang Technological University) for help with the NMR experiments. We appreciate the helpful discussions with David Beer, Siew Pheng Lim, and Pornwaratt Niyomrattanakit (Novartis Institute for Tropical Diseases).

REFERENCES


FIGURE 8. Structure of the DENV protease. A, crystal structure of the linked protease from DENV2. The protein structure was obtained from Protein Data Bank code 2FOM (6). The CTF and NTF of NS2B are colored blue and cyan, respectively. B, crystal structure of the linked protease from DENV3. Residue 82 from NS2B is represented by a blue sphere.
NMR Study of the Dengue Protease


NMR Analysis of a Novel Enzymatically Active Unlinked Dengue NS2B-NS3 Protease Complex

Young Mee Kim, Shovanlal Gayen, CongBao Kang, Joma Joy, Qiwei Huang, Angela Shuyi Chen, John Liang Kuan Wee, Melgious Jin Yan Ang, Huichang Annie Lim, Alvin W. Hung, Rong Li, Christian G. Noble, Le Tian Lee, Andy Yip, Qing-Yin Wang, Cheng San Brian Chia, Jeffrey Hill, Pei-Yong Shi and Thomas H. Keller

doi: 10.1074/jbc.M112.442723 originally published online March 19, 2013

Access the most updated version of this article at doi: 10.1074/jbc.M112.442723

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2013/03/19/M112.442723.DC1

This article cites 42 references, 5 of which can be accessed free at
http://www.jbc.org/content/288/18/12891.full.html#ref-list-1