Purified NS2B/NS3 Serine Protease of Dengue Virus Type 2 Exhibits Cofactor NS2B Dependence for Cleavage of Substrates with Dibasic Amino Acids in Vitro*

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Dengue virus type 2 NS3, a multifunctional protein, has a serine protease domain (NS3pro) that requires the conserved hydrophilic domain of NS2B for protease activity in cleavage of the polyprotein precursor at sites following two basic amino acids. In this study, we report the expression of the NS2B-NS3pro precursor in Escherichia coli as a fusion protein with a histidine tag at the N terminus. The precursor was purified from insoluble inclusion bodies by Ni2+ affinity and gel filtration chromatography under denaturing conditions. The denatured precursor was refolded to yield a purified active protease complex. Biochemical analysis of the protease revealed that its activity toward either a natural substrate, NS4B-NS5 precursor, or the fluorogenic peptide substrates containing two basic residues at P1 and P2, was dependent on the presence of the NS2B domain. The peptide with a highly conserved Gly residue at P3 position was 3-fold more active as a substrate than a Gln residue at this position. The cleavage of a chromogenic substrate with a single Arg residue at P1 was NS2B-independent. These results suggest that heterodimerization of the NS3pro domain with NS2B generates additional specific interactions with the P2 and P3 residues of the substrates.

Dengue viruses, members of the family Flaviviridae, are transmitted by mosquitoes. There are four serotypes (types 1 to 4) that cause widespread human diseases such as dengue fever, dengue hemorrhagic fever, and dengue shock syndrome. About 40% of the world population living in tropical and subtropical regions of the world is at risk for infection. Of the 1 million cases of dengue hemorrhagic fever cases per year, about 5% are fatal. There is currently no effective vaccine or antiviral drug to protect against dengue diseases (1, 2). The virus has a single strand RNA genome of positive polarity. The viral RNA has a 3′ terminal region of flavivirus NS3 protease domain that contains conserved motifs that are found in several nucleoside triphosphatases (NTPase) and the DEXH family of RNA helicases (32–34). Many viral NTPases have been characterized and were shown to be stimulated by the addition of single stranded RNA (30, 35–41). RNA helicase activities have been arranged in the order C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5. Processing of the N-terminal region by the host signal peptidase associated with the endoplasmic reticulum yields three structural proteins that are assembled into the virion (C, prM, and E) (4–6). Processing of C-prM by signal peptidase occurs via a post-translational mechanism in which cleavage of the cytoplasmic domain of C in the C-prM precursor by the viral protease precedes the signal peptidase cleavage (7–9). prM undergoes further cleavage to M at a late step during virion morphogenesis mediated by a cellular protease in the post-Golgi acidic compartment (10). In addition, an endoplasmic reticulum membrane-bound host protease with the characteristics of the signalase mediates cleavages at the NS1-NS2A (11) and NS4A-NS4B junctions (12–14).

A trypsin-like serine protease domain within the N-terminal 180 amino acid residues of NS3 was identified by sequence comparisons (15, 16). Analysis of polyprotein processing in vivo established that the NS3 protease, as a heterodimeric complex with the viral activator protein NS2B (17–19), catalyzes the cis cleavage of 2A-2B and 2B-3, as well as the trans cleavage of 3–4A and 4B-5 sites in the polyprotein (20–26). These sites have in common Lys-Arg, Arg-Arg, Arg-Lys, and occasionally Gln-Arg at the P1 and P2 positions followed by a short chain amino acid Gly, Ala, or Ser at the P1′ position (see Ref. 27 for the nomenclature). In addition, the viral protease also cleaves internally within NS2A (28) and NS3 (18, 22, 29).

Mutational analysis of the NS3 protease domain revealed that the N-terminal 167 amino acid residues contain the active protease domain (30). Similar analysis of NS2B, which has three hydrophobic regions flanking a conserved hydrophilic domain of about 40 amino acid residues, revealed that this hydrophilic region is necessary and sufficient for activation of the NS3 protease domain in vivo (31) and in vitro (25). Although the hydrophobic regions of NS2B are dispensable for protease activity, they are required for cotranslational membrane insertion of full length NS2B and its efficient activation of the NS3 protease domain (25).

The C-terminal region of flavivirus NS3 protease domain contains conserved motifs that are found in several nucleoside triphosphatases (NTPase) and the DEXH family of RNA helicases (32–34). Many viral NTPases have been characterized and were shown to be stimulated by the addition of single stranded RNA (30, 35–41). RNA helicase activities have been...
shown for the hepatitis C virus (HCV) NS3, bovine viral diarrhea virus p80, and DEN2 NS3 (30, 42–44).

In this study, we expressed the precursor containing the hydrophilic domain of NS2B linked to the NS3 protease domain (NS2B-NS3pro). The precursor protein was purified under denaturing conditions. During refolding of the precursor protein, an active protease complex was produced by intramolecular cleavage of the 2B-3 site of the precursor (Den2 protease complex). We report the biochemical and kinetic analysis of the Den2 protease complex. The purified Den2 protease complex mediated trans cleavage of a natural substrate, NS4B-NS5 precursor, and several synthetic fluorogenic peptide substrates in vitro. Comparison of the activities of the purified NS3 protease domain alone and the Den2 protease complex revealed that the activity toward a chromogenic substrate containing an L-Arg residue at P1 and a benzoyl moeity in the place of a P2 residue was NS2B-independent. However, the trans cleavage of a natural substrate, or the fluorogenic peptide substrates containing dibasic residues at P1 and P2, was catalyzed optimally only by the Den2 protease complex. This in vitro protease assay with purified proteins will be of use to screen for inhibitors that could block viral polyprotein processing and hence viral replication.

**EXPERIMENTAL PROCEDURES**

**Materials**—The rabbit reticuloocyte-coupled transcription-translation system and canine pancreatic microsomal membranes were purchased from Promega (Madison, WI). Ni2+-nitrilotriacetic acid (NTA)-agarose resin was from Qiagen (Chatworth, CA). The Bradford protein assay kit was from Bio-Rad. trans-NS3-Labeled methionine (1,000 Ci/mmol) was purchased from ICN Pharmaceuticals (Costa Mesa, CA). Fluorogenic peptides linked to 7-amino-4-methyl-coumarin were purchased from Peninsula Laboratories Inc. (Belmont, CA). Chromogenic substrate Nα-benzoyl-L-arginine-p-nitroamide was obtained from Sigma. Expression Plasmids 6xHis-NS2B-NS3pro and 6xHis-NS2B-NS3*pro—5′-CGCGGATCCCTCGGCCGATTTGGAACTGGAG (underline) were used for polymerase chain reaction on the templates pTM1-NS2B(II)-NS3(Pro)-PPH and pTM1-NS2B(II)-NS3* (Pro)-PPH (45). The latter vector denotes a His6-Ala mutation in the catalytic triad (45).

**Expression and Purification of NS2B-NS3pro and NS2B-NS3*pro Precursors**—Competent Escherichia coli strain XLI-Blue MRF cultures were transformed with the NS2B-NS3pro or NS2B-NS3*pro expression plasmid and grown in 1 liter of LB medium containing ampicillin (100 μg/ml) at 37 °C until OD 600 nm reached approximately 0.6. Bacterial cells were induced for protein expression by addition of isopropyl-β-D-thiogalactopyranose (0.5 mM) for 2 h, collected by centrifugation, and stored at –70 °C until used. For protein purification, cells were resuspended in buffer A (100 mM Tris-HCl, pH 7.5, 300 mM NaCl) and lysed by French press. Cell lysates were centrifuged at 27,000 × g for 1 h at 4 °C. The pellet fraction was solubilized by resuspension in 20 ml of 10 M urea. The elution profile was monitored by using the Bio-Rad protein assay kit, and aliquots from the peak fractions were subjected to SDS-PAGE (46), then by Western blot analysis (47) using rabbit anti-α-NS3 polyclonal antibody (24) (1:3000), and finally by using goat anti-rabbit alkaline phosphatase conjugate (1:4000) (Life Technologies, Inc.) as described (48). The 6xHis-NS2B-NS3*pro precursor was expressed and purified in a similar manner except that the gel filtration column chromatography step was omitted. The 6xHis-NS3-pro domain alone was expressed and purified as described previously (45).

**Protease Assays using Fluorogenic Peptides**—Assays were carried out using a Perkin-Elmer 2045 spectrofluorometer. The standard reaction mixtures (100 μl) contained 200 mM Tris-HCl, pH 8.5, and 200 μM fluorogenic peptide substrate (except for kinetic analysis, indicated amounts were used). After enzyme addition, reaction mixtures were incubated at 37 °C for 30 min and terminated by addition of 1.9 ml of 125 mM ZnSO4. The precipitate was removed by centrifugation for 1 min in a microcentrifuge (15,000 × g), and the rate of product release was measured at 405 nm. The concentrations of the enzyme preparations were analyzed using SDS-PAGE (46), then by Western blot analysis (47) using 1:100 dilutions of antibody conjugate (1:4000) (Life Technologies, Inc.) as described (48). The 6xHis-NS2B-NS3pro precursor was expressed and purified in a similar manner except that the gel filtration column chromatography step was omitted. The 6xHis-NS3-pro domain alone was expressed and purified as described previously (45).

**Trans-cleavage of the NS4A-NS5 Precursor by the Active Den2 Protease Complex**—The labeled NS4B-NS5 precursor was synthesized in vitro as described previously (25). Microsomal membrane-associated precursor was isolated by centrifugation at 15,000 × g for 15 min. The pellet was resuspended in 20 μl of the buffer containing 50 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.25 mM cyclohexamide, 0.5% Triton X-100 and sonicated (4–10 s bursts on ice) using a cup-horn sonicator (model 185 F, Heat Systems-Ultrasonics, Inc., Farmingdale, NY) at a power setting of 7. For trans-cleavage assays, the purified Den2 protease complex (40 μl) was incubated with the labeled NS4B-NS5 precursor at 30 °C for 3 h. Reactions were terminated by addition of gel-loading buffer containing SDS. The products were analyzed by 12% SDS-PAGE and autoradiography.

**Protease Assay using Chromogenic Substrates**—Serine protease activity was assayed using the substrate, Nα-benzoyl-L-arginine-p-nitroamide in a spectrophotometer (Spectronic 3000 Array, Milton Roy Co., SLM-AMINCO, Huntley, IL or in a LKB Biochrom Ultraspec II). The standard reaction mixture was preincubated for 5 min and the final enzyme concentration in the assay was varied between 7.0 to 9.5. The substrate concentrations were varied from 0.2 mM to 3.4 mM. The final enzyme concentration in the assay was varied from 90 nm to 8 μM. Triplicate measurements of enzyme activity were recorded for each data point.
Expression and Purification of an Active Den2 Protease Complex—Previous studies showed that fusion of six histidine residues at the N terminus of NS3 allowed the expression of the protease domain in high levels that could conveniently be purified by Ni²⁺ affinity chromatography, followed by Sephadex G-75 gel filtration. Denatured proteins were refolded by successive dialysis to remove urea as described under “Experimental Procedures.” A, left panel, Coomassie Blue-stained gel of purified protein separated by SDS-PAGE; right panel, Western blot analysis using anti-DEN2 NS3 polyclonal antibody. Lanes: M, protein molecular weight markers; lane 1, supernatant after lysis using French press; lane 2, supernatant after sonication; lane 3, nonbinding fraction of Ni²⁺-NTA-agarose; lane 4, Fraction 4 eluted from the Ni²⁺-NTA column with 500 mM imidazole; lane 5, Fraction 21 eluted from the Sephadex G-75 column; lane 6, refolded precursor protein which underwent cleavage to generate NS2B (~8 kDa) and NS3pro (23 kDa) polypeptides. B, purification of NS2B-NS3pro precursor using a Ni²⁺-NTA affinity column. Left panel: SDS-PAGE followed by Coomassie Blue staining. Right panel: Western blot analysis of purified NS2B-NS3pro stained with anti-α-NS3 antibody. Lanes: M, molecular weight markers; lane 1, supernatant after lysis using French press; lane 2, supernatant after sonication; lane 3, nonbinding fraction on Ni²⁺-NTA resin; lane 4, Fraction 9 from the Ni²⁺-NTA column eluted with 500 mM imidazole; lane 5, refolded mutant precursor, NS2B-NS3pro, polypeptide. C, purification of the NS3pro domain. The 6xHis-NS3pro domain was expressed in E. coli XL1-Blue and purified using Ni²⁺-NTA-agarose affinity resin as described under “Experimental Procedures.” Protein fractions were analyzed by SDS-PAGE (12%) and stained with Coomassie Blue. Lane 1, molecular weight markers; lane 2, soluble bacterial proteins after expression induced with isopropyl-β-D-thiogalactopyranoside and lysis of bacterial cell pellet; lane 3, NS3pro protein eluted from Ni²⁺-NTA-agarose with 100 mM imidazole and dialyzed extensively to remove imidazole and urea and refold the protein as described under “Experimental Procedures.”

RESULTS

Expression of NS2B-NS3pro precursor polypeptides in E. coli. Recombinant NS2B-NS3pro and mutant NS2B-NS3pro* (α = His→Ala mutation) precursor polypeptides were expressed and purified from E. coli (XL1-Blue) cells by using Ni²⁺-NTA affinity column chromatography, followed by Sephadex G-75 gel filtration. Western blot analysis using anti-DEN2 NS3 polyclonal antibody. Lanes: M, protein molecular weight markers; lane 1, supernatant after lysis using French press; lane 2, supernatant after sonication; lane 3, nonbinding fraction of Ni²⁺-NTA-agarose; lane 4, Fraction 4 eluted from the Ni²⁺-NTA column with 500 mM imidazole; lane 5, Fraction 21 eluted from the Sephadex G-75 column; lane 6, refolded precursor protein which underwent cleavage to generate NS2B (~8 kDa) and NS3pro (23 kDa) polypeptides. B, purification of NS2B-NS3pro precursor using a Ni²⁺-NTA affinity column. Left panel: SDS-PAGE followed by Coomassie Blue staining. Right panel: Western blot analysis of purified NS2B-NS3pro stained with anti-α-NS3 antibody. Lanes: M, molecular weight markers; lane 1, supernatant after lysis using French press; lane 2, supernatant after sonication; lane 3, nonbinding fraction on Ni²⁺-NTA resin; lane 4, Fraction 9 from the Ni²⁺-NTA column eluted with 500 mM imidazole; lane 5, refolded mutant precursor, NS2B-NS3pro, polypeptide. C, purification of the NS3pro domain. The 6xHis-NS3pro domain was expressed in E. coli XL1-Blue and purified using Ni²⁺-NTA-agarose affinity resin as described under “Experimental Procedures.” Protein fractions were analyzed by SDS-PAGE (12%) and stained with Coomassie Blue. Lane 1, molecular weight markers; lane 2, soluble bacterial proteins after expression induced with isopropyl-β-D-thiogalactopyranoside and lysis of bacterial cell pellet; lane 3, NS3pro protein eluted from Ni²⁺-NTA-agarose with 100 mM imidazole and dialyzed extensively to remove imidazole and urea and refold the protein as described under “Experimental Procedures.”

Previous studies using a coupled transcription-translation system in vitro showed that cotranslational insertion of the NS2B-NS3 protease precursor into microsomal membranes was mediated by the hydrophobic regions of the NS2B and that this association with the membranes was required for cleavage of the 2B-3 site. However, the NS2B-NS3 protease precursor containing only the hydrophilic domain of 40 amino acid residues underwent cleavage readily in the absence of membranes (25). Consistent with this observation, the E. coli-expressed NS2B-NS3 protease precursor also was cleaved during refolding of the precursor (Fig. 1A). As a negative control, the expression plasmid to produce the mutant precursor protein, NS2B-NS3pro* (α refers to a His→Ala mutation in the catalytic triad of the protease domain) was also constructed. Purification of the mutant precursor was achieved in a similar manner except that the gel filtration chromatography was omitted (Fig. 1B). The His-tag-NS3pro domain was purified as described previously (45) (see Fig. 1C). The identity of the wild type or the mutant precursor protein and the 23-kDa NS3pro domain lib-
erated during refolding was established by Western blot analysis using rabbit polyclonal anti-NS3 antibody (Fig. 1, right panels). The smaller, NS2B polypeptide (8 kDa) was not immunoreactive to the anti-NS2B peptide antibody, although this antibody recognized the precursor protein (data not shown). The mutant precursor, NS2B-NS3*pro precursor, did not undergo cis cleavage as expected due to the catalytic His–Ala mutation, which is shown by both Coomassie Blue-stained gel and by Western blot analysis using the anti-NS3 antibody (Fig. 1B).

The Den2 Protease Complex Is Active in Cleaving the NS4B-NS5 Precursor Substrate in Trans—Because the NS2B-NS3pro precursor was proteolytically active in cis cleavage of the 2B-3 site during refolding, we sought to determine whether the resultant Den2 protease complex was active in trans cleavage of a natural substrate such as NS4B-NS5 precursor. [35S]Met-histidine-labeled substrate was synthesized by using the coupled in vitro transcription-translation system (25). The labeled substrate was incubated with the purified Den2 protease complex. The results shown in Fig. 2 indicated that the cleavage of the labeled substrate occurred with the Den2 protease complex (lane 3) but not with the NS3pro domain alone (lane 2) or with the uncleaved mutant NS2B-NS3*pro precursor containing the His51–Ala mutation within the catalytic triad (lane 4). The band that had a mobility similar to that of NS4B in lane 4 was shown to be a nonspecific band by carrying out the SDS-PAGE analysis under different conditions (Fig. 2, lanes 5–7). These experiments were repeated five times with similar results. These results clearly indicated that the purified Den2 protease complex is active in trans cleavage and that the trans cleavage of the NS4B-NS5 substrate was dependent on the presence of the 40-amino acid hydrophilic domain of NS2B.

The Activity and Specificity of Den2 Protease Complex with Fluorogenic Peptide Substrates—To develop a sensitive, rapid, and quantitative assay to determine the biochemical and kinetic parameters of the protease, a number of commercially available fluorogenic peptides were used as substrates. All of these peptides have two basic amino acid residues at P1 and P2. Some peptides contain residues at P3 and longer up to the P5 position. However, none of the peptides contains an amino acid residue at the P1’ position, but rather the P1’ residue is replaced by a fluorogenic moiety. Table I lists the substrates analyzed in this study. The results reveal that substrate 1, Gly-Arg-Arg-MCA, which contains a Gly residue at the P3 position, is the most active of the four substrates tested. Thus we used this substrate for determining the biochemical and kinetic parameters of the protease.

Biochemical and Kinetic Parameters of the Den2 Protease Complex—We determined the stability of the enzyme in various buffers between pH 7 to 9 at 37 °C using substrate 1 in Table I. The enzyme was incubated in the various buffers shown, and the activity was determined at 10-min intervals, and then at hourly intervals for 300 min as described under “Experimental Procedures.” The results shown in Fig. 3 indicate that the protease activity was fairly stable between pH 7.5 to 9. However, the enzyme was more active at pH 9 (Fig. 3). Fig. 3 also shows the stability in Tris-HCl in comparison with HEPES buffer at pH 8. The protease activity in Tris-HCl was more stable (t½ of 315 min) than in HEPES (t½ of 128 min) and the enzymatic activity in Tris-HCl at pH 8.0 was three times higher (Fig. 3). The Den2 protease activity fits into a sigmoidal pH titration curve comparable to Kex2 and other serine proteases (49). The activity increased sharply from pH 7 to 9 with a half-maximal activity at pH 8.0 and was constant between pH 9 and 9.5 (data not shown). The protease activity was also affected by ionic strength. Titration of protease activity under increasing NaCl concentration showed a 40% decrease in activity detectable at 100 mM NaCl compared with 0 mM NaCl, and then an additional gradual decrease of protease activity of up to 20% of the maximal value from 0.1 to 1 mM NaCl (data not shown).

The activity of the enzyme in cleavage of the Gly-Arg-Arg peptide substrate was dependent on the incubation time (data not shown) and the substrate concentration (Fig. 4), and it followed Michaelis-Menten kinetics. The Lineweaver-Burk plot was linear over the range of substrate concentration examined (data not shown).

### Table I

| Kinetic properties of NS2B(NS3(pro) and NS3(pro) proteases |
|-------------------------------------------------------------|
| The protease activity was assayed in 0.2 M Tris-HCl, pH 8.5 for 50 min at 37 °C. Protease activity with Bec-Gly-Arg-Arg-MCA substrate was taken as 100%. Substrate concentrations were in the range 10–300 μM. The reaction was stopped by the addition of 125 mM ZnSO4 and analyzed as described under “Experimental Procedures.” | |
| **Substrate** | **V_{max}** | **Activity** | **h_{cat}** | **K_{m}** | **h_{cat}K_{m}** | **K_{d}** | **K_{d}K_{m}** |
| | % | s⁻¹ | μM | s⁻¹μM | s⁻¹ | μM | s⁻¹μM |
| Bec-Gly-Arg-Arg-MCA | 0.14 | 100 | 0.031 | 180 | 172 | 4.3 x 10⁻⁶ | 823 | 0.052 |
| Bec-Gly-Arg-MCA | 0.046 | 33 | 0.01 | 93 | 107 | 1.8 x 10⁻⁶ | 128 | 0.014 |
| Z-Arg-Arg-MCA | 0.042 | 30 | 0.009 | 102 | 88 | 1.3 x 10⁻⁶ | 50 | 0.026 |
| pGlu-Arg-Thr-Lys-Arg-MCA | 0.061 | 44 | 0.013 | 134 | 97 | 2.7 x 10⁻⁶ | 85 | 0.03 |

The results shown in Fig. 3 indicated that the protease activity was fairly stable between pH 7.5 to 9. However, the enzyme was more active at pH 9 (Fig. 3). Fig. 3 also shows the stability in Tris-HCl in comparison with HEPES buffer at pH 8. The protease activity in Tris-HCl was more stable (t½ of 315 min) than in HEPES (t½ of 128 min) and the enzymatic activity in Tris-HCl at pH 8.0 was three times higher (Fig. 3). The Den2 protease activity fits into a sigmoidal pH titration curve comparable to Kex2 and other serine proteases (49). The activity increased sharply from pH 7 to 9 with a half-maximal activity at pH 8.0 and was constant between pH 9 and 9.5 (data not shown). The protease activity was also affected by ionic strength. Titration of protease activity under increasing NaCl concentration showed a 40% decrease in activity detectable at 100 mM NaCl compared with 0 mM NaCl, and then an additional gradual decrease of protease activity of up to 20% of the maximal value from 0.1 to 1 mM NaCl (data not shown).
As shown in Fig. 4, four different fluorogenic peptide substrates were all cleaved by the Den2 protease complex. The \( v_{\text{max}} \) and \( k_{\text{cat}}/K_m \) values for the four substrates are summarized in Table I. The presence of Gly at P3 in substrate 1 (Boc-Gly-Arg-Arg-MCA) increases its \( k_{\text{cat}} \) level 3-fold from substrate 3 without an amino acid residue (except the protecting group Z) at P3 and substrate 2 with Gln at P3 (Table I). The \( k_{\text{cat}}/K_m \) ratio of substrate 1 was also higher than that for substrates 2 and 4 and indicated that a small neutral amino acid is important at the P3 position. The \( k_{\text{cat}} \) values for substrate 4 with Lys at P2 and Arg at P1 are similar to substrates 2 and 3 with Arg at P2 and P1 and show that there is no strong preference for Lys or Arg at the P2 position. Interestingly, these fluorogenic peptide substrates were very poorly cleaved by the NS3pro domain alone even at high concentrations of the substrate (Table I). These results, taken together with those shown in Fig. 2, indicated that a substrate with even a single basic residue at P2 required the presence of the NS2B cofactor for optimal protease activity.

Activity of the NS3pro Domain and the Den2 Protease Complex with Chromogenic Substrates—The purified and refolded 6xHis-NS3pro domain was found to cleave the chromogenic substrate, which is the \( \text{N}^\alpha\text{-benzoyl-L-Arg-}\text{p-nitroanilide} \) substrate. These reactions were conducted at 23 °C, and the release of \( \text{p-nitroaniline} \) was measured at 405 nm as described under “Experimental Procedures.” The pH optimum for this reaction was about 8.0 in 50 mM Tris-HCl buffer (data not shown). The enzymatic cleavage was dependent on both substrate and enzyme concentrations (data not shown). Purified Den2 protease complex also cleaved this chromogenic substrate. However, when the activities of the two enzymes were compared under identical assay conditions, NS3pro showed much greater specific activity than the Den2 protease complex (Fig. 5). Therefore, it appears that the conformation of the substrate binding pocket in the Den2 protease complex is altered compared with the NS3pro domain alone, and it may interfere with binding of the chromogenic peptide compared with the fluorogenic peptide containing a P2 residue.

DISCUSSION

In this study, we report the bacterial expression, purification, and biochemical characterization of the Den2 protease complex in \textit{E. coli}. Our results, obtained by using purified Den2 protease, provide the first \textit{in vitro} evidence for the requirement of NS2B cofactor for the NS3 protease in cleavage of the natural precursor polypeptide, NS4B-NS5, and short fluorogenic peptides with two basic amino acid residues at P1 and P2. Comparison of the cleavage efficiency of the NS3pro domain and Den2 protease complex (\( k_{\text{cat}}/K_m \) values in Table I) revealed that the degree of activation of NS3pro domain by the NS2B cofactor peptide are in the range of 3300- to 7600-fold. This indicates that the protease activity of the NS3pro domain in the absence of NS2B is extremely low. This conclusion is supported by the result that Den2 protease complex is significantly active in \textit{trans} cleavage of the natural NS4B-NS5 precursor substrate, whereas this activity was not detectable with the NS3pro domain (Fig. 2). On the other hand, the activation of HCV NS3 protease domain by NS4A peptide occurs \textit{in vitro} to
different extents depending on the cleavage site: about 100-fold for the cleavage of NS4B-NS5A site but only 11- and 3-fold for the cleavages of 4A-4B and 5A-5B, respectively (51).

The dengue virus and HCV proteases also differ in their substrate specificities, although both are classified as serine proteases. HCV protease prefers a Cys residue at the P1 position at three of the sites cleaved by the enzyme with the exception of a Thr residue at the intramolecular cleavage of the 3–4A site. Moreover, the crystal structure of the NS3pro domain indicated that its conformation, in the absence of NS2B, is much closer to that of the HCV protease-NS4A complex (45). In contrast, the dengue virus protease, like any arthropod-borne flavivirus protease, prefers a basic residue (Lys or Arg) at the P1 position of all cleavage sites.

The activating polypeptide of the dengue virus NS3pro domain, NS2B, contains a central hydrophilic domain flanked by two hydrophobic domains at the N terminus (I and II) and a single hydrophobic domain (III) at the C terminus followed by a 10-amino acid region upstream of the 2B-3 cleavage site. The hydrophilic region consisting of 40 amino acids alone was most efficient in the activation of the NS3pro domain and the resultant cis cleavage of the 2B-3 site (25). However, the core region of HCV NS4A, required for activation of the HCV NS3 protease domain, consists of hydrophobic residues. From these studies, it appears that the mechanism of activation of the two viral serine proteases by their respective activating cofactors are likely to be different. Recently, based on the crystal structure coordinates of the HCV NS3pro in complex with the NS4A peptide, Brinkworth et al. (52) proposed a model for the Den2 protease interactions with both substrate and NS2B cofactor. According to this model, a central hydrophobic subdomain of NS2B, bearing some sequence similarity to HCV NS4A activating peptide, interacts with the NS3pro domain (52). It is essential to determine the structure of the Den2 protease complex in order to understand the nature of the interaction between NS2B and NS3pro and the mechanism of activation of the protease domain.

The crystal structure of the NS3pro domain predicts that the partially “open” conformation with the catalytic triad and the residues in the substrate binding pocket of the enzyme is likely to be accessible to at least some model substrates. Consistent with this prediction, we demonstrate in this study that the NS3pro domain is active in cleaving the chromogenic substrate, benzoyl-L-Arg-NS3pro domain alone is shown to interact with the P1 side chain. The NS3pro residues interact. In order to understand the nature of the interaction between NS2B and NS3pro and the mechanism of activation of the protease domain.

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It appears that the interaction of the Den2 protease complex with fluorogenic peptide substrates is mediated by electrostatic effects as shown by inhibition in high ionic strength buffers. Inhibition by increasing NaCl concentration was also observed with the HCV NS3 protease, but this sensitivity to NaCl inhibition was significantly reduced in the HCV NS3-NS4A peptide complex (51). These results suggest that the mechanism of activation of the protease domain by the respective cofactor is likely to be different.

Based on the crystal structure of the NS3 protease domain (45), the S2 pocket of Den2 protease has Asn-152 as one of the components which can provide hydrogen bonding interactions for Lys, Arg, or Gln side chains at P2. A number of carbonyl atoms in the vicinity provide an electrostatically favorable environment for a basic P2 side chain. From our results, the presence of a small amino acid, Gly, at P3 is also important and strongly increases the $k_{cat}$ level. The importance of the enzyme interaction with the P3 residue is underscored by the conservation of the Gly residue in the P3 position of the 3–4A site of the polyprotein among many flaviviruses. Substitution of the Gly with a Glu at P3 reduced the $k_{cat}$ by 3-fold. A previous study on the effect of substitution of P3 Gly to different amino acid residues indicated that Gly → Lys or Gly → Ala augmented the cis cleavage efficiency at the 2A-2B site of yellow fever virus polyprotein precursor in vivo. However, Gly → Glu had little effect on the cleavage efficiency of this site (28). P3 and P4 residues could affect cleavage efficiencies differentially depending on the cleavage site and mode of the cleavage (cis or trans) (28).

The Den2 protease has some resemblance to the yeast Kex2 protease, having a catalytic domain homologous to the subtilisin family of serine proteases (49) with substrate specificity of dibasic residues at P1 and P2. The Kex2 protease exhibits optimal activity toward substrates with Lys-Arg or Arg-Arg at P1 and P2 in contrast to Lys-Lys-containing substrates (53). The Den2 protease showed a rather low activity ($k_{cat}$) when compared with Kex2 protease ($k_{cat}$) but it is comparable with other viral proteases using different synthetic peptides. The proteolytic activity of human cytomegalovirus protease ($k_{cat}$) and herpes simplex virus protease ($k_{cat}$) was determined using synthetic peptides spanning at least the P5-P5′ positions surrounding the cleavage site (54, 55). HCV NS3 protease purified from insect cells has a $k_{cat}/K_m$ of 174 M$^{-1}$ s$^{-1}$ as determined by proteolysis of the 20-mer containing the NS4A/4B junction, and the same enzyme purified from E. coli has a $k_{cat}/K_m$ range of 0.4 to 650 M$^{-1}$ s$^{-1}$ as determined by proteolysis of decamer P6-P4′ peptides corresponding to all cleavage sites of HCV polyprotein (51). HCV NS3-NS4A protease complex expressed in insect cells has also been purified and characterized, and it has a $k_{cat}/K_m$ ratio of 3,700 M$^{-1}$ s$^{-1}$ using synthetic peptides of the NS3A-3B junction (56).

Although kinetic characterization of the Den2 protease com-
plex with peptidyl-MCA substrates provides useful information, it probes only one aspect of substrate specificity. It should be noted that native substrates may be cleaved with different kinetics than corresponding synthetic substrates due to substrate secondary structure and/or long range interactions with the C-terminal region of the cleavage site. From the crystal structure of the NS3pro domain, it was shown that NS3 protease has a substrate binding cleft that is small and shallow and does not appear capable of providing specific interactions with side chains beyond P2 and P2'.

As shown above the enzyme as a continuous line with side chains of P3 to P2' residues projecting out as different geometrical shapes. In the unactivated NS3pro domain (Fig. 6D), the substrate side chains except for the P1 residue do not fit into their respective binding pockets. This is consistent with experimental observations that NS3pro is capable of efficiently hydrolyzing the chromogenic substrate but not longer substrates. The binding of NS2B causes a conformational change in the NS3pro such that all the substrate side chains bind efficiently to their respective binding pockets, which results in the cleavage of the substrate. The difference between Fig. 6B and Fig. 6C is that the NS2B residues directly participate in substrate side chain interactions except with that of P1 residue (Fig. 6D), whereas in Fig. 6C all interactions with the substrate are provided by the NS3pro residues. Currently, there is no experimental evidence to support either of the two possible mechanisms of activation of the Den2 protease. Further analysis of the Den2 protease complex with native and synthetic substrates and inhibitors and determination of its crystal structure should help elucidate the importance of the elements of substrate recognition by the Den2 protease. The results of such studies will reveal the mechanistic differences and similarities between the Den2 protease and the HCV protease (57) and will be useful for the development of specific inhibitors with therapeutic potential for treatment of diseases caused by dengue virus.

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Purified NS2B/NS3 Serine Protease of Dengue Virus Type 2 Exhibits Cofactor NS2B Dependence for Cleavage of Substrates with Dibasic Amino Acids \textit{in Vitro}

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