Activity of Recombinant Dengue 2 Virus NS3 Protease in the Presence of a Truncated NS2B Co-factor, Small Peptide Substrates, and Inhibitors*

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Recombinant forms of the dengue 2 virus NS3 protease linked to a 40-residue co-factor, corresponding to part of NS2B, have been expressed in Escherichia coli and shown to be active against para-nitroanilide substrates comprising the P6-P1 residues of four substrate cleavage sequences. The enzyme is inactive alone or after the addition of a putative 13-residue co-factor peptide but is active when fused to the 40-residue co-factor, by either a cleavable or a noncleavable glycine linker. The NS4B/NS5 cleavage site was processed most readily, with optimal processing conditions being pH 9, I = 10 mM, 1 mM CHAPS, 20% glycerol. A longer 10-residue peptide corresponding to the NS2B/NS3 cleavage site (P6-P4) was a poorer substrate than the hexapeptide (P6-H11541P4) (2). In addition to host proteases, signalase and furin, act on the remaining carboxyl-terminal region encodes both nucleoside triphosphatase and helicase activities (4, 5).

The dengue virus NS3 protease shares with other flavivirus NS3 proteases a trypsin-like character with a classic serine protease catalytic triad (His51, Asp 75, and Ser 135) (6–8). Although unlike trypsin it has a marked preference for dibasic residues (e.g. Arg and Lys at P1 and P2) and requires the co-factor activity supplied by the nonstructural protein NS2B for efficient catalysis of the proteolytic cleavage of the dengue virus polyprotein (3). The NS2B-NS3 conjugate has been shown to cleave the precursor polyprotein at the NS2A/NS2B, NS2B/NS3, NS3/NS4A, and NS4B/NS5 junctions as well as at internal sites within C, NS2A, NS3, and NS4A (9–11), whereas the host cell proteases, signalase and furin, act on the remaining cleavage sites (Fig. 1A) (12–15). Deletion studies employing vaccinia recombinants have further shown that a central 40-amino acid conserved hydrophilic domain within NS2B is sufficient for co-factor activity (16). The flanking hydrophobic residues of NS2B are likely to function by promoting association between the protease complex and infected cell membranes (17). The essential nature of the central hydrophilic domain for proteolytic activity has been recently confirmed in vitro with Escherichia coli expressed and purified recombinant NS3pro complexed with this NS2B co-factor domain (18).

A therapeutic strategy based on inhibiting proteases (19–21) has precedent in the success of HIV-1 protease inhibitors (19–21) which prevent HIV replication by blocking the viral protease responsible for producing structural and functional HIV proteins in host cells. To mount a similar antiviral program for the dengue viruses, we describe in this paper the expression and purification of two recombinant forms of NS3 protease and the preliminary characterization of the enzymology, including limitations imposed by assay conditions, sub-

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180 amino acids of NS3 (NS3pro) is responsible for cleavage both in cis and in trans to generate viral proteins that are essential for viral replication and maturation of infectious dengue virions (3). NS3 is at least a trifunctional protein because the carboxyl-terminal region encodes both nucleoside triphosphatase and helicase activities (4, 5).

Dengue viruses belong to the flaviviridae family and are transmitted via mosquitoes to millions of people each year in tropical and subtropical regions of the world. They consist of a single-stranded RNA genome that encodes a proteolytic polyprotein (3). The NS2B-NS3 conjugate has been shown to cleave the precursor polyprotein at the NS2A/NS2B, NS2B/NS3, NS3/NS4A, and NS4B/NS5 junctions as well as at internal sites within C, NS2A, NS3, and NS4A (9–11), whereas the host cell proteases, signalase and furin, act on the remaining cleavage sites (Fig. 1A) (12–15). Deletion studies employing vaccinia recombinants have further shown that a central 40-amino acid conserved hydrophilic domain within NS2B is sufficient for co-factor activity (16). The flanking hydrophobic residues of NS2B are likely to function by promoting association between the protease complex and infected cell membranes (17). The essential nature of the central hydrophilic domain for proteolytic activity has been recently confirmed in vitro with Escherichia coli expressed and purified recombinant NS3pro complexed with this NS2B co-factor domain (18).

A therapeutic strategy based on inhibiting proteases (19–21) has precedent in the success of HIV-1 protease inhibitors (19, 22, 23), which prevent HIV replication by blocking the viral protease responsible for producing structural and functional HIV proteins in host cells. To mount a similar antiviral program for the dengue viruses, we describe in this paper the expression and purification of two recombinant forms of NS3 protease and the preliminary characterization of the enzymology, including limitations imposed by assay conditions, sub-

The abbreviations used are: HIV-1, human immunodeficiency virus, type 1; TFA, trifluoroacetic acid; DIPEA, N,N-diisopropylethylamine; HBTU, O-benzotriazole N,N,N,N-tetramethyluronium hexafluorophosphate; BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; DMF, N,N-dimethylformamide; EtOAc, ethyl acetate; DCM, dichloromethane; THF, tetrahydrofuran; ESMS, electrospray mass spectrometry; pNA, para-nitroanilide; HPLC, high pressure liquid chromatography; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; Fmoc, N-(9-fluorenylmethoxycarbonyl); MES, 2-(N-morpholino)ethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid; HCV, hepatitis C virus.
Fig. 1. Flavivirus polyprotein processing and dengue virus NS3 expression constructs. A shows sites on the flavivirus polyprotein cleaved by host-encoded proteases (black arrows below) and the virus-encoded protease complex NS2B.NS3 (open arrows above). The NS2B co-factor and the proteolytic domain of NS3 (NS3pro) are shaded. B, overview of the expression constructs used in this study. The shading in A corresponds to the first construct and is designated NS2B.NS3pro. Highlighted on this construct in B are key features of the protease complex, including the cleavage site (open arrow), the catalytic triad of NS3pro (asterisk), conserved hydrophobic domains of the NS2B co-factor (solid boxes), and the minimum 40-residue co-factor region required for catalytic activity of NS3pro (solid bar). The remaining four constructs comprise NS3pro alone (NS3pro), the central 40-amino acid NS2B co-factor domain fused to the 10 amino acids upstream of the NS2B/NS3 cleavage site, the first 184 amino acids of NS3 (CF40.NS3pro), a construct containing a Ser to Ala catalytic triad mutation (CF40.NS3pro mutant (CF40.NS3pro), and a construct comprising the 40-amino acid co-factor domain tethered to NS3pro via a flexible linker (CF40.gly.NS3pro).

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strates, and co-factors. We also describe the activity of the first substrate-based peptide inhibitors.

EXPERIMENTAL PROCEDURES

General Methods

Protected amino acids and resins were obtained from Novabiochem. TFA, piperidine, DIPEA, and DMF (peptide synthesis grade) were purchased from Auspep. All other materials were reagent grade unless otherwise stated. Crude peptides were purified by reversed-phase HPLC separations performed on a Waters Delta-Pak reversed-phase C18 preparative column, using a gradient mixture of solvent A (0.1% TFA with water) and solvent B (0.1% TFA, 10% water, and 90% acetonitrile). Analytical HPLC was performed on a Waters system equipped with a 717 plus autosampler, 660 controller, and a 996 photodiode array detector using a reversed-phase C18 (Vydac 201HS5415) column or a reversed-phase protein and peptide column (Vydac 218TP5415). Purified peptides were characterized by analytical HPLC (linear gradient 0–100% solvent B over 30 min) and mass spectrometry. The molecular mass of the peptides was determined by electrospray mass spectrometry recorded on a triple quadrupole (PE SCIEX API III) and electrospray TOF (Applied Biosystems Mariner) mass spectrometers. 1H NMR spectra were recorded on a Varian 300MHz or a Bruker 500MHz NMR spectrometer at 303 K. Proton assignments were determined by two-dimensional NMR experiments (double-quantum filtered correlation spectroscopy). In vitro activity of recombinant dengue viral NS3 protease was determined by gel electrophoresis and enhanced spectroscopy.

Plasmid Construction

Generation of NS2B.NS3pro PCR product for cloning. C6/36 cells were infected with dengue 2 virus strain NGC at a multiplicity of infection 1.0 and incubated at 32 °C for 3 days. Infected cell RNA was then extracted with RNazol B (Tel-Test Inc) according to the manufacturer’s instructions. cDNA was generated using the NS3proRXholI reverse primer (5’-TATACACTCGAGCTATCAGGAAGAATTGTATCTT-3’; restriction sites are underlined), which was then used as template in a PCR reaction employing this primer and NS2BFLncoI (5’-CAACCATGCGAAGACGCTAATCC-3’) as reverse primers. The resulting PCR product comprised the full NS2B sequence and the first 184 amino acids of NS3 (NS3pro) flanked by Ncol and Xhol sites for cloning into pTM1.

pTM Constructs

Four vectors were generated for eukaryotic in vitro translation analysis: pTM.NS2B.NS3pro (full-length NS2B.NS3pro), pTM.NS3pro (NS3pro alone), pTM.CF40.NS3pro (NS3pro fused to the 40 amino acid co-factor domain of NS2B), and pTM.CF40.NS3pro mutant (containing a mutation in one of the amino acids comprising the NS3pro catalytic triad Ser130 to Ala138) (Fig. 1B). The NS2B.NS3pro PCR product described above was digested with Ncol and Xhol and cloned into the pTM1 vector to generate pTM.NS2B.NS3pro. The primers NS3proF/Ncol (5’-CATGGCTGAAATGTGGTGGATGTG-3’/NcoI) and NS3proRS135A (5’-ATTAGGATCCGCCGATTTAGAACTG-3’/XhoI) were used to amplify the pTM.NS2B.NS3pro template with the primer pairs, CF40/NcoI (5’-AGTACTCAGGCGATTGGAAATGTGGTGGATGTG-3’/HindIII) and NS3proF/HindIII and NS3proRS135A (5’-ATTAGGATCCGCCGATTTAGAACTG-3’/XhoI). Both PCR products were digested with Ncol, gel-purified, and then ligated. The products of the ligation were then amplified with the outside primers CF40/NcoI and NS3proRXholI. The resulting PCR product, comprising the 40-amino acid co-factor domain of NS2B fused to the 10 amino acids immediately upstream of the NS2B cleavage site and the first 184 amino acids of NS3, was digested with Ncol and Xhol and cloned into the pTM1 vector. pTM.CF40.NS3pro mutant was generated by PCR mutagenesis using pTM.CF40.NS3pro as template and the complementary mutation primers NS3proF/NS3proR (5’-TCTCCTGAGACCGAGGATCTCCATC-3’) and NS3proRS135A (5’-ATTAGGATCCGCCGATTTAGAACTG-3’/NcoI) in separate PCR reactions with NS3proRXholI and CF40/NcoI, respectively. The nucleotide change leading to the S135A mutation is bold type.

pQE9 Constructs

Three vectors were generated for the expression in E. coli of amino-terminal His-tagged recombinant dengue viral NS3 protease: pQE9.NS3pro, pQE9.CF40.NS3pro, and pQE9.CF40.gly.NS3pro (CF40 fused to NS3pro via a GlySerGly linker). pTM.CF40.NS3pro was used as template for the generation of PCR products with the primer pairs NS3pro/RamH1 (5’-ATTAGGATCCGCCGATTTAGAACTG-3’/NcoI) and NS3pro/RamH1 (5’-ATTAGGATCCGCCGATTTAGAACTG-3’/HindIII) and NS3pro/RamH1 (5’-ATTAGGATCCGCCGATTTAGAACTG-3’/HindIII) as reverse primers. These PCR products were digested with BamHI and HindIII and cloned into pQE9 to generate pQE9.NS3pro and pQE9.CF40.gly.NS3pro. The resulting PCR product, comprising the 40-amino acid co-factor domain of NS2B fused to the 10 amino acids immediately upstream of the NS2B cleavage site and the first 184 amino acids of NS3, was digested with Ncol and Xhol and cloned into the pTM1 vector. pTM.CF40.NS3pro mutant was generated by PCR mutagenesis using pTM.CF40.NS3pro as template and the complementary mutation primers NS3proF/NS3proR (5’-TCTCCTGAGACCGAGGATCTCCATC-3’) and NS3proRS135A (5’-ATTAGGATCCGCCGATTTAGAACTG-3’/NcoI) in separate PCR reactions with NS3proRXholI and CF40/NcoI, respectively. The nucleotide change leading to the S135A mutation is bold type.
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**Expression and Purification of NS3pro, CF40.NS3pro, and CF40.gly.NS3pro**

The pQE9 vector was used for high level, inducible expression of amino-terminal hexahistidine-tagged recombinant proteins. Cultures of E. coli strain S13009, transformed with the corresponding expression plasmid were grown in 2 liters of LB medium containing 100 μg/ml ampicillin and 25 μg/ml kanamycin at 37°C until the absorbance at 600 nm reached 0.6. The cells were induced for expression by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM and incubated for an additional 3 h at 30°C. The cells were then harvested by centrifugation, and the pellets were stored at −80°C until used. For protein purification, the cells were thawed and resuspended in 1 ml of lysis buffer (50 mM HEPES, pH 7.5, 300 mM NaCl, 5% glycerol/10 mM of original culture. The resuspended cells were lysed by French press or subjected to probe sonication (five 30-s pulses) on ice and then centrifuged at 27,000 × g for 30 min at 4°C. The supernatant was stored at 4°C or purified immediately by passage through a 2-ml column of Ni2+-nitrilotriacetic acid-agarose (Qiagen) pre-equilibrated with 50 mM HEPES, pH 7.5, containing 300 mM NaCl. The column was extensively washed with buffer containing 20 mM imidazole, and protein was then eluted from the column in buffer containing 100 mM imidazole. Elution fractions were analyzed by 15% SDS-PAGE. Samples of pre- and post-induced cells as well as soluble and insoluble fractions following lysis were collected and also analyzed by 15% SDS-PAGE.

**In Vitro Transcription and Translation**

An in vitro rabbit reticulocyte-coupled transcription/translation system (TNT, Promega) was used to express recombinant protein from the pTM1 vector constructs. The reactions were performed according to the manufacturer’s instructions. Briefly, [15S]methionine-labeled protein was expressed by incubation of 0.5 μg of vector DNA with reticulocyte lysate, amino acid (minus methionine) mixture, RNasin, T7 RNA polymerase, and 10.0 μCi of [35S]methionine in a final volume of 25 μl at 30°C for 90 min. Reactions requiring membranes included 3 μl of canine pancreatic microsomal membranes. The reactions were terminated by the addition of SDS containing gel buffer, and the products were examined by 15% SDS-PAGE and autoradiography.

**Peptide Substrates**

**para-Nitroanilide (pNA) substrates** were synthesized by solid phase synthesis (24) using Fmoc protecting groups for the following amino acids: Arg (pme), Glu (OBut), Gln (ttrt), Lys (boc), Ser (bBu), and Thr (bBu). The Fmoc protecting group was removed by shaking the resin with two 1-min treatments with 50% piperidine in dimethylformamide. All amino acids (4 meq) were activated by HBTU (4 meq) and DIPEA (5 meq) in DMF. Couplings were monitored by the quantitative ninhydrin test. All peptides were acetylated with acetic anhydride (20 meq) and DIPEA (10 meq) after the final Fmoc deprotection. Peptide p-aminoanilides were assembled on monocharged trityl resin (substitution value = 0.96 mmol/g) and subsequently cleaved from the solid support with 95% TFA, 2.5% triisopropylsilane, and 2.5% water (15 ml/g resin) at −5°C for 1–2 h. Crude peptide was purified by reverse-phase HPLC and lyophilized overnight (30%). Stock solutions of peptides were prepared in water and kept at −80°C until use. Peptide substrates were Ac-RTSKKR-pNA, Ac-EVKKQR-pNA, Ac-FAAGGRK-pNA, Ac-EVKKKR-pNA, and Ac-EVKKQRAGVL-OLH, which correspond to pNA analogues of the 2A2B, 2B3A, and 4B5 cleavage sites and a 10-amino acid segment derived from the 2B3 site, respectively. Ac-RTSKKR-pNA, retention time (Rt) = 13.5 min, ESMS [M + H]+ = 907.6, [M + 2H]2+= 454.3; Ac-EVKKQR-pNA, Rt = 15.3 min, ESMS [M + H]+ = 949.7, [M + 2H]2+= 475.3; Ac-FAAGGRK-pNA, Rt = 16.7 min, ESMS [M + H]+ = 921.3; Ac-EVKKR-pNA, Rt = 17.5 min, ESMS [M + H]+ = 883.6, [M + 2H]2+= 442.3; and Ac-EVKKKRAGVL-OLH, Rt = 15.5 min, ESMS [M + H]+ = 1169.4, [M + 2H]2+= 585.5.

**Protease Assays**

A spectrophotometric assay was conducted, using peptide substrates appended to a pNA chromophore, in 96-well plates with each well containing a final volume of 200 μl, an enzyme concentration of 0.5 or 1 μM, and a reaction incubation temperature of 37°C. Each reaction was monitored continuously by following the increase in pNA at 500 nm on a Spectra Max 250 reader. Initial velocities and substrate concentrations were fitted by nonlinear regression to the Michaelis-Menten equation. pH dependence experiments were carried out with the following buffers: MES (pH 5.5–7.0), MOPS (pH 6.5–8.0), Tris (pH 7.5–9.0), and ethanolamine (pH 8.5–10.0), and CAPS (pH 10.0–11.5) together with an enzyme concentration of 1 μM, para-nitroanilide substrates at 500 μM, and a constant ionic strength (50 mM). For overlapping pH regions, the activity was shown to be unaffected by buffer composition. Buffers included NaCl, glycerol, and detergents varied to optimize assay conditions (see “Results”). Subsequent assays with pNA substrates were performed under optimal conditions that involved 50 mM Tris, pH 9.0, 10 mM NaCl, 20% glycerol, 1 mM CHAPS in a final volume of 200 μl in 96-well plate format. Clearing of the reaction mixture was performed by the addition of acetic acid, and substrate was determined by analytical HPLC using a Waters system equipped with a 717 plus autosampler, 660 controller, and a 996 photodiode array detector. The enzyme concentration was 1 μM, the incubation temperature was 37°C, the incubation time was 2 h (<10% substrate conversion), and the reaction was quenched by addition of 70 μl of 0.1% TFA. Samples of 22.5–90 μl were injected on a Vydac reverse-phase column, and fragments were separated using a 0–50% solvent B gradient at 5%/min. Peak detection was monitored by following A280 and quantified by integration of chromatograms with respect to the appropriate standard peptide. Initial velocities (v) were determined, and kinetic parameters were calculated from weighted nonlinear regression of initial velocities as a function of eight substrate concentrations [5] using GraphPad Prism® software. The IC50 values were calculated assuming a Michaelis-Menten kinetics, i.e., vmax/[S] + Km, in which KI values were determined, and IC50 values were determined for inhibitors from substrate titration experiments performed in the presence of increasing inhibitor concentration using Dixon plots (ln v versus [I]). Kinetic parameters were calculated from IC50 values according to the equation, KI = IC50/(1 + Km/[S]). Triplicate measurements were taken for each data point. The data are reported as the means ± S.E.

**Inhibitor Assay**

General protease inhibitors were used to confirm the presence of a serine protease. Inhibitors were assayed in a 96-well plate format using 50 μl Tris, pH 9.0, 10 mM NaCl, 20% glycerol, 1 mM CHAPS in a final volume of 200 μl. Typically, 5 μl of inhibitor was added, and reactions were carried out with various concentrations of test compounds at 37°C for 10 min. The reaction was initiated by the addition of substrate Ac-TTSTRR-pNA at 500 μM. On the other hand the peptidic inhibitors were assayed using CF40.gly.NS3pro (0.5 μM) in a 96-well plate format using 50 mM HEPES, pH 7.5, 50 mM NaCl, 20% glycerol, 1 mM CHAPS in a final volume of 200 μl. Typically these test compounds were incubated with the enzyme conjugate at 37°C for 50 min, and then substrate cleavage was initiated by the addition of the chromogenic substrate Ac-TTSTRR-pNA at 500 μM. Reaction progress was monitored continuously by following the increase in A280 on a Spectra Max 250 plate reader. IC50 values were determined for inhibitors from substrate titration experiments performed in the presence of increasing inhibitor concentration using Dixon plots (ln v versus [I]). Km values were calculated from IC50 values according to the equation, KI = IC50/(1 + Km/[S]). Triplicate measurements were taken for each data point. The data are reported as the means ± S.E.

**Peptide Inhibitors**

α-Keto Amide Inhibitors (1–3)—Inhibitors 1 (Ac-FAAGGR-[COONH]-SL-[CONH]2), 2 (Ac-TTSTRR-[COONH]-SL-[CONH]2), and 3 (Ac-TTSTRR-[COONH]-GQTN-[CONH]2) were synthesized using synthesis methodology reported earlier [5]. The peptides were synthesized as above and purified on a reverse-phase HPLC column. Peptide fragments were monitored by the quantitative ninhydrin test. Peptide fragments were analyzed by reverse-phase HPLC and lyophilized overnight (30%). Solution stock of peptides were prepared in water and kept at −80°C until use. Peptide substrates were Ac-RTSKKR-pNA, Ac-EVKKQR-pNA, Ac-FAAGGRK-pNA, Ac-EVKKKR-pNA, and Ac-EVKKQRAGVL-OLH, which correspond to pNA analogues of the 2A2B, 2B3A, and 4B5 cleavage sites and a 10-amino acid segment derived from the 2B3 site, respectively. Ac-RTSKKR-pNA, retention time (Rt) = 13.5 min, ESMS [M + H]+ = 907.6, [M + 2H]2+= 454.3; Ac-EVKKQR-pNA, Rt = 15.3 min, ESMS [M + H]+ = 949.7, [M + 2H]2+= 475.3; Ac-FAAGGRK-pNA, Rt = 16.7 min, ESMS [M + H]+ = 921.3; Ac-EVKKR-pNA, Rt = 17.5 min, ESMS [M + H]+ = 883.6, [M + 2H]2+= 442.3; and Ac-EVKKKRAGVL-OLH, Rt = 15.5 min, ESMS [M + H]+ = 1169.4, [M + 2H]2+= 585.5.
meq), which was activated with PyBOP (2 meq) and DIPEA (3 meq) in DMF. Couplings were monitored by the quantitative ninhydrin test. All peptides were acetylated using activated acetic acid with HBTU/DIPEA in DMF after the final Boc deprotection. The α-amidoxymoiety was oxidized on resin with DessMartin Periodinane (2 meq) in dry Me2SO for 2 h at 0 °C. The deprotected mixtures were precipitated in diethyl ether (30 ml). The aqueous layer was subsequently acidified with 10% KHSO4 (2 ml) and added 10% KHSO4 solution (20 ml), and the mixture was extracted with a small volume of ethyl acetate (20 ml) to give a white powder. The mixture of methanol and water (70 ml) and added to the aldehyde. The mixture was dissolved in EtOAc (65 ml). KCN (3.54 g) was dissolved in 1:1 mixture of methanol and water (70 ml) and added to the aldehyde. The homogenous reaction mixture was stirred overnight. The mixture was diluted with water (50 ml), and the organic layer was washed with water (3 × 100 ml). The EtOAc layer was dried with MgSO4 and concentrated in vacuo to yield product as a white solid (0.68 g, 66%). ESMS [M+H]+ = 459.3. H NMR (CDCl3) (the two diastereomeric products had coincident NMR spectra; only the two Boc signals at δ 1.40, 1.44 were observed). The mixture of Boc signals at δ 7.22 (m, 4H, Ar-H), 7.70 (m, 4H, CH=CH), 5.20 (2H, BocNH), 4.67 (2H, CH), 4.00 (2H, OCH), 3.18 (4H, CH2), 2.38 (8H, CH2), 2.77 (6H, OCH2COOH + NH), 1.54 (m, 8H, βCH2 + γCH3), 1.43 (s, 9H, Boc), 1.40 (s, 9H, Boc).

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Boc-Arg(Tos)-NMeOMe (15.0 g, 31.8 mmol) was dissolved in anhydrous THF (200 ml) and stirred under argon at 0 °C and treated with a mixture of hydrochloric acid (1 m, 2 × 200 ml), dried with MgSO4, and evaporated to yield the crude product as a yellow foam. Column chromatography (10% EtOAc/DCM) yielded pure product (2.1 g, 50%) as a white foam. ESMS [M+H]+ = 302.1. H NMR (CDCl3) δ 10.40 (bs, 1H, NH), 9.53 (bs, 1H, NH), 5.15 (m, 1H, BocNH), 4.65 (1H, 1H, 6CH), 4.46 (m, 1H, 6CH), 3.56 (m, 1H, OCH), 2.48 (m, 1H, OCH2), 1.95 (2H, OCH2), 1.63 (m, 1H, βCH2), 1.48 (s, 9H, Boc).

N-α-Boc-N-ω-nitroarginine Lactam—To a stirred suspension of N-α-Boc-N-ω-nitroarginine (6.38 g, 20 mmol) and BOP (9.72 g, 20 mmol) in THF (120 ml) was added DIPEA (10.4 ml, 60 mmol), and the solution was stirred at room temperature overnight. The solvent was removed under vacuum, and the residue was partitioned between EtOAc (400 ml) and hydrochloric acid (1 m, 200 ml). The organic extract was washed with hydrochloric acid (1 m, 2 × 200 ml), dried with MgSO4, and evaporated to yield the crude product as a yellow foam. Column chromatography (10% EtOAc/DCM) yielded pure product (5.01 g, 50%) as a white foam. ESMS [M+H]+ = 563.2936, found 563.2935. H 11NMR (MeSO-d6) δ 8.18 (d, 1H, AlnNH), 8.15 (t, 1H, GlyNH), 8.12 (d, 1H, PheNH), 8.03 (d, 1H, ArgNH), 8.02 (d, 1H, AlnNH), 7.79 (m, 4H, Ar-H), 7.17 (m, 1H, Ar-H), 4.50 (m, 1H, PheCH), 4.28 (m, 1H, AlaCH), 4.25 (m, 1H, AlaCH), 4.22 (m, 1H, ArgCH), 3.74 (m, 2H, GlyCH), 3.32 (bs, 1H, COOH), 3.10 (m, 2H, ArgCH2), 3.00 (m, 1H, PheCH), 2.73 (m, 1H, PheCH), 1.78–1.49 (m, 4H, Arg CH2+Arg CH3), 1.73 (s, 6H, CH3).

N-α-Boc-N-ω-nitroarginine Ethyl Cyclol—To a stirred solution of L-arginine ethyl cyclol (200 ml) containing an aqueous solution was added dropwise EtOAc (1.13 ml, 10 mmol) in THF (5 ml). The solution was stirred for 30 min at 0 °C and treated with a solution of N-α-Boc-N-ω-nitroarginine lactam (3.01 g, 10 mmol) in THF (15 ml). The mixture was stirred for 30 min at 0 °C and treated with a mixture of hydrogen chloride (1 m, 3 ml) and THF (3 ml) (dropwise, followed by hydrochloric acid (1 m, 60 ml) at room temperature. The solution was extracted with diethyl ether, washed with saturated NaHCO3 (3 × 100 ml), dried with MgSO4, and evaporated to yield the crude aldehyde as a white foam. ESMS [M+H]+ = 304.2. The crude aldehyde was dissolved in ethanol (15 ml), and concentrated HCl (50 μl) was added. The mixture was stirred at room temperature for 7 h, and the solvent was removed under vacuum. The crude product was purified by column chromatography (0–10% EtOAc/DCM to yield 1.72 (52%) of the title compound as a white foam. ESMS [M+H]+ = 332.2.

N-ω-Nitroargininal Ethyl Cyclol.TFA—N-α-Boc-N-ω-Nitroargininal ethyl cyclol (1.72 g, 5.2 mmol) was treated with 50% TFA/DCM (5.6 ml) for 10 min. The solution was added dropwise to diethyl ether (50 ml) with swirling. The product was collected at the pump, washed briefly with diethyl ether (3 × 100 ml), and dried under vacuum to yield pure product (1.13 g, 67%). ESMS [M+H]+ = 332.1. H NMR (MeSO-d6) δ 8.74 (bs, 1H, NH), 8.05 (bs, 1H, NH), 5.93 (m, 1H, CH), 3.74 (m, 1H, CH2), 3.51 (m, 1H, 1/2 of CH2), 3.45 (m, 1H, 1/2 of CH2), 3.32 (m, 3H, NH2), 3.27 (m, 1H, OCH2), 3.03 (t, 1H, CH), 1.76 (m, 3H, βCH2 + γCH3), 1.55 (m, 1H, γCH3), 1.19 (t, 3H, CH3).

Ac-FAAGR-OH—To a stirred solution of Ac-FAAGR-OH (31 mg, 0.09 mmol) in DMP (1.5 ml) was added DIPEA (39 μl, 5 meq). The mixture was stirred at room temperature for 3 h, and the residue was brought under reduced pressure. The residue was purified by preparative HPLC (gradient: 0–100% B over 50 min, Rf = 12.5 min) to yield the product as a white powder. ESMS [M+H]+ = 776.6.

Ac-FAAGR-Arg Ethyl Cyclol—To a solution of the above nitroarginal ethyl cyclol in a mixture of water (1 ml), ethanol (1 ml), and
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In Vitro Expression of Catalytically Active NS3pro—The first aim of this study was the expression and purification of a catalytically active recombinant dengue virus NS3 protease, initially for use in in vitro based assays. To confirm that the sequences we derived by reverse transcription-PCR from a dengue virus-infected cell RNA encoded an active protease, we first expressed these recombinant proteins in a rabbit reticulocyte-coupled translation/translation system (Fig. 2A). pTM.NS2B.NS3pro encodes the NS2B gene, which is an essential co-factor for proteolytic activity, fused to the first 184 amino acids of the NS3 protease (NS3pro). Similar constructs have previously been shown to exhibit cis cleavage at the NS2B-NS3 junction when expressed either by recombinant vaccinia viruses (3) or in in vitro reticulocyte lysate translation systems employing microsomal membranes (17). As shown in Fig. 2A, expression of pTM.NS2B.NS3pro in a reticulocyte lysate supplemented with microsomal membranes (lane 1) clearly demonstrates processing of the 34-kDa NS2B.NS3pro precursor product into the expected 20-kDa NS3pro. In the absence of microsomal membranes, processing is less efficient, indicating the likely misfolding of the highly hydrophobic NS2B (Fig. 2A, lane 2). Given the hydrophobic nature of NS2B, with three predicted membrane-spanning domains (29), expression in association with NS3pro in E. coli would likely generate an insoluble product. However, Clum et al. (17) have shown that expression of the central conserved 40-amino acid hydrophilic domain of NS2B fused to NS3pro was sufficient for efficient co-factor activity. We generated a similar construct, pTM.CF40.NS3pro, and showed highly efficient processing of the expressed product in vitro in the absence of microsomal membranes (Fig. 2A, lane 4). Indeed, processing appeared complete with no precursor product apparent in the SDS-PAGE profile. It is possible that cleavage was mediated not by the cis-activity of NS3pro but via trans-cleavage by a contaminating protease. This possibility was addressed by mutation of one of the amino acids comprising the catalytic triad of NS3pro, Ser<sup>135</sup>→Ala to generate pTM.CF40.NS3pro<sup>−</sup>. Expression of this construct yielded the expected 32-kDa CF40.NS3pro precursor with no detectable cleavage (Fig. 2A, lane 6), demonstrating that the processing of pTM.CF40.NS3pro was due to the cis-activity of a catalytically active NS3pro. The additional species at ~28 kDa for both pTM.CF40.NS3pro and pTM.CF40.NS3pro<sup>−</sup> (Fig. 2A, lanes 4 and 6) was likely the product of internal initiation of translation at a downstream start codon.

Expression and Purification of NS3pro and CF40.NS3pro—To facilitate purification of E. coli expressed recombinant proteins, we used the pQE9 vector, which incorporates an aminoterminal hexahistidine tag allowing convenient purification by Ni<sup>2+</sup> affinity chromatography. After the commencement of this project, Yusof et al. (18) reported a similar expression strategy and noted that although both NS3pro and an equivalent version of CF40.NS3pro were produced at high levels, the expressed proteins were found associated with insoluble inclusion bodies. Their solution was to purify urea-denatured recombinant protein from the insoluble pellet fraction of bacterial cell lysates and then refold the expressed products by successive dialysis. We investigated this approach but found that the refolding dialysis step consistently led to unacceptable levels of precipitation and poor recovery. Instead, we focused on optimizing expression conditions that favored the generation of soluble recombinant protein. Investigation of a range of parameters (data not shown) indicated that expression at 30 °C yielded low but acceptable levels of soluble protein (Fig. 2B, lanes 4 and 9) when purified by Ni<sup>2+</sup> column affinity chromatography (Fig. 2B, lanes 6 and 11). This was particularly apparent for the CF40.NS3pro recombinant, which is suggestive of a chaperone-like function for CF40.NS2B in mediating the correct folding of NS3pro. Furthermore, although the majority of expressed recombinant in the insoluble pellet was uncleaved precursor (Fig. 2B, arrowhead in lane 10), the correct folding of

RESULTS

In Vitro Activity of a Catalytically Active Recombinant Dengue Virus NS3 Protease

In Vitro Translation of NS2B/NS3 Constructs and Expression in E. coli. A, pTM constructs encoding NS2B.NS3pro (lanes 1 and 2), NS3pro (lanes 3 and 5), CF40.NS3pro (lane 4), and CF40.NS3pro<sup>−</sup> (lane 6) were expressed in vitro in a coupled transcription/translation reaction, TnT. Incubations were carried out with or without canine pancreatic microsomal membranes as indicated, and products of cis-mediated cleavage were examined by SDS-PAGE and autoradiography. B, pQE9 recombinant constructs NS3pro and CF40.NS3pro were expressed in E. coli, and cell lysates were examined by SDS-PAGE and Coomassie Blue staining. The samples analyzed included protein molecular mass markers (M, lane 1), uninduced cell lysates (U, lanes 2 and 3), 1 ml induced cell lysates (I, lanes 3 and 5), 1 ml induced cell lysates (I, lanes 3 and 5), and the insoluble pellet fraction (P, lanes 5 and 10). His-tagged recombinant products were purified by Ni<sup>2+</sup> affinity chromatography from the soluble fraction of cell lysates (lanes 6 and 11).

Fig. 2. In vitro translation of NS2B/NS3 constructs and expression in E. coli. A, pTM constructs encoding NS2B.NS3pro (lanes 1 and 2), NS3pro (lanes 3 and 5), CF40.NS3pro (lane 4), and CF40.NS3pro<sup>−</sup> (lane 6) were expressed in vitro in a coupled transcription/translation reaction, TnT. Incubations were carried out with or without canine pancreatic microsomal membranes as indicated, and products of cis-mediated cleavage were examined by SDS-PAGE and autoradiography. B, pQE9 recombinant constructs NS3pro and CF40.NS3pro were expressed in E. coli, and cell lysates were examined by SDS-PAGE and Coomassie Blue staining. The samples analyzed included protein molecular mass markers (M, lane 1), uninduced cell lysates (U, lanes 2 and 3), 1 ml induced cell lysates (I, lanes 3 and 5), and the insoluble pellet fraction (P, lanes 5 and 10). His-tagged recombinant products were purified by Ni<sup>2+</sup> affinity chromatography from the soluble fraction of cell lysates (lanes 6 and 11).
between pH 5.5 and 11.5. These were MES (pH 5.5–6.5) and HEPES, pH 7.5, 70 mM NaCl, 20% glycerol, 0.1% Triton X-100 (indicated by arrowheads in Fig. 2B, lane 11). Two additional intermediates were also consistently identified in varying levels (lane 11). These were amino-terminally sequenced and shown to be truncated amino-terminal CF40.NS3pro products of auto-catalytic cleavage by CF40.NS3pro.

**Characterization of Enzymatic Activity**—To investigate whether CF40.NS3pro had proteolytic activity against small peptide substrates, we initially used buffer conditions similar to those reported for the hepatitis C virus NS3 protease (50 mM HEPES, pH 7.5, 70 mM NaCl, 20% glycerol, 0.1% Triton X-100). Reversed-phase HPLC was used to examine cleavage of the 10-residue peptide Ac-EVKKKQRAGVL-COOH derived from the NS2B/NS3 site. Analytical HPLC (Fig. 3) showed the expected cleavage products Ac-EVKKKQR-COOH and NH2-AGVL-COOH, their identities being established by molecular mass determination through mass spectrometry and HPLC. The rate of cleavage of the decapeptide was slow. To optimize the enzyme processing conditions and to facilitate continuous monitoring of this reaction, we constructed the analogous chromogenic substrate cleavage mixture was assayed. This peptide did not activate the protease, and its complete processing to the 20-kDa NS3pro and the 8-kDa terminal CF40.NS3pro products of auto-catalytic cleavage by CF40.NS3pro.

**Comparison of Peptide Substrates**—The catalytic efficiency and specificity of the CF40.NS3pro protease was examined by the soluble form of the CF40.NS3pro complex was evidenced by its complete processing to the 20-kDa NS3pro and the 8-kDa co-factor domain (Fig. 2B, lane 11). Two additional intermediates were also consistently identified in varying levels (indicated by arrowheads in Fig. 2B, lane 11). These were amino-terminally sequenced and shown to be truncated amino-terminal CF40.NS3pro products of auto-catalytic cleavage by CF40.NS3pro.

**Effect of pH, Salt, Glycerol, and Detergents on Enzyme Activity**—Using 1 μM purified CF40.NS3pro, we found that the substrate Ac-TTSTRR-pNA was cleaved most rapidly and most efficiently at a concentration of 500 μM, which was therefore used to optimize assay conditions. The pH dependence for enzyme processing was determined at a constant ionic strength (I = 50 mM) using various buffers with overlapping pH ranges between pH 5.5 and 11.5. These were MES (pH 5.5–7.0), MOPS (pH 6.5–8.0), Tris (pH 7.5–9.0), ethanolamine (pH 8.5–10.0), and CAPS (pH 10.0–11.5). The optimum pH for proteolytic processing was found to be 9.2, but above pH 9.5 the pNA substrate itself begins to undergo base-catalyzed hydrolysis in the absence of enzyme (Fig. 4A). At pH 9.0, base-catalyzed cleavage of pNA was negligible, whereas the enzyme was about five times more active in processing substrate than at a pH of 7.5. We therefore chose a 50 mM Tris buffer at pH 9.0 for investigating substrate cleavage.

**Comparison of Peptide Substrates**—The catalytic efficiency and specificity of the CF40.NS3pro protease was examined by
measuring kinetic parameters for processing of the pNA substrate analogues of four hexapeptides corresponding to the P6-P1 residues amino-terminal to four cleavage sites (2A/B, 2B/3, 3/4A, 4B/5) within the polypeptide precursor. The rate of substrate hydrolysis and the Michaelis-Menten equilibrium constants are markedly sequence-dependent (Fig. 4D), with the sequences corresponding to the NS4B/NS5 and NS2B/3 cleavage sites being most rapidly cleaved. The rank order of these pNA substrates is 4B/5 > 2B/3 > 2A/2B > 3/4A (Table I). These residues have either Arg-Arg or Gln-Arg at the P2-P1 positions. On the other hand, Km values vary in the order 2B/3 > 4B/5 > 2A/2B > 3/4A leading to a rank order of substrate efficiency or fitness as substrates (kcat/Km): 4B/5 > 3/4A > 2A/2B > 2B/3 (Table I). Interestingly the decapeptide Ac-EVKKQRAGVL-OH, corresponding to the 2B/3 cleavage site, is processed less efficiently than the corresponding hexapeptide-pNA substrate Ac-EVKKQR-pNA (Table I).

Inhibition By Standard Serine Protease Inhibitors—The effect of standard protease inhibitors on substrate processing by CF40(NS3pro was also investigated. The percentage of cleavage in the presence of added inhibitors was determined as residual activity with respect to the control sample in the absence of inhibitors (Fig. 5). Aprotinin was shown to inhibit the enzyme (IC50 = 65 mM), whereas other standard serine protease inhibitors such as 4-(2-aminoethyl) benzzenesulfonfonyl fluoride hydrochloride and N-Tosyl-L-phenylalanine chloromethyl ketone showed only 20–30% inhibition at 500 μM and 1 mM, respectively. Other serine protease inhibitors for which no enzyme inhibition was found included soybean trypsin inhibitor (50 μM), 4-Amidinophenylmethanesulfonyl fluoride hydrochloride (0.5 mM), phenylmethylsulfonfonyl fluoride (0.5 mM), leupeptin (0.5 mM), pepstatin A (0.1 mM), benzamidine (1 mM), and N-Tosyl-L-lysine chloromethyl ketone hydrochloride (0.5 mM). The effect of divalent cations (Ca2+, Mg2+, and Mn2+) was also investigated with the addition of 20 mM CaCl2, 50 mM MgCl2, or 50 mM MnCl2 having no effect on dengue virus NS3 protease activity. The failure of 1 mM EDTA to inhibit the enzyme (data not shown), further confirmed that divalent cations are not critical for enzyme activity.

**Expression, Purification, and Enzymatic Characterization of CF40,gly.NS3pro—SDS-PAGE analysis of the purified CF40(NS3pro fractions eluted from the Ni2+ affinity column revealed that the products of cis cleavage (His-CF40 and gly.CF40) were not necessarily in equimolar proportions (Fig. 2B, lane 11) and therefore may not display optimal enzyme activity. As a consequence of cleavage within E. coli, the recombinant CF40(NS3pro complex was purified via binding of the His tag fused to the amino terminus of CF40. Varying levels of imidazole were shown, however, to preferentially elute the tightly but noncovalently bound NS3pro from the complex (data not shown). Mass spectrometry confirmed that we had not purified the CF40 and NS3pro components in equimolar quantities (data not shown). Our earlier modeling of the den-
Dengue virus NS3 protease complexed with its co-factor (29) indicated that the carboxyl terminus of the co-factor was likely to be in close proximity to the amino terminus of NS3pro, suggesting the possibility of incorporating a short linker between the two domains that would not disrupt the structural integrity of the complex. So, to remedy the problems associated with purifying a noncovalently bound complex, we engineered a new construct that expressed a catalytically active NS3pro fused to CF40 via a noncleavable, flexible nonapeptide (Gly4SerGly4) linker, the fusion protein designated as CF40.gly.NS3pro (Fig. 1B). Expression of this construct resulted in substantially higher levels of recombinant protein being found in the soluble fraction of the bacterial cell lysate (Fig. 6, lane 4) with yields of purified CF40.gly.NS3pro (Fig. 6A, lane 6) approaching 50 mg/L of culture.

Using 1 μg purified CF40.gly.NS3pro and 500 μM Ac-CTSTRR-pNA, the pH dependence for enzyme processing was determined at a constant ionic strength (I = 50 mM) using the same buffers as described in the previous section with overlapping pH ranges between pH 5.5 and 11.5. The optimum pH for proteolytic processing was again found to be pH 9.2 (Fig. 6B). By comparison the glycine-linked conjugate enzyme CF40.gly.NS3pro was significantly more catalytically active (about 4-fold) than the CF40.NS3 construct under the same conditions (Fig. 6B). The enzyme kinetics of CF40.gly.NS3pro were investigated using the same pNA substrates described above and the same reaction conditions that had been optimized for CF40.NS3pro. The results presented in Fig. 6C and Table II indicate that although the binding affinities (Kₐ) of the substrates for CF40.gly.NS3pro were similar to those for CF40.NS3pro, the catalytic rates (kₐcat) were significantly higher, leading to higher overall proteolytic efficiencies (kₐcat / Kₐ) for the covalently linked (noncleavable) form. In summary, the data in Fig. 6 (B and C) demonstrate that the glycine-linked enzyme is the more active form. We attribute this to the absence of cleavage between co-factor and protease domains in CF40.gly.NS3pro.
In Vitro Activity of Recombinant Dengue Virus NS3 Protease

**Table II**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$k_{cat}/K_m$ (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-BTSDKR-pNA (2A/2B)</td>
<td>96 ± 7</td>
<td>0.087 ± 0.001</td>
<td>903 ± 56</td>
</tr>
<tr>
<td>Ac-EVKKQR-pNA (2B/3)</td>
<td>984 ± 40</td>
<td>0.216 ± 0.002</td>
<td>220 ± 6</td>
</tr>
<tr>
<td>Ac-FAAGRR-pNA (3/4A)</td>
<td>100 ± 9</td>
<td>0.043 ± 0.001</td>
<td>425 ± 30</td>
</tr>
<tr>
<td>Ac-TTSTRR-pNA (4B/5)</td>
<td>326 ± 10</td>
<td>0.288 ± 0.001</td>
<td>883 ± 22</td>
</tr>
</tbody>
</table>

**Table III**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_i$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Ac-FAAGRR-keto-SL-CONH$_2$</td>
<td>47 ± 3</td>
</tr>
<tr>
<td>2 Ac-TTSTRR-keto-SL-CONH$_2$</td>
<td>220 ± 55</td>
</tr>
<tr>
<td>3 Ac-TTSTRR-keto-GTGN-CONH$_2$</td>
<td>368 ± 47</td>
</tr>
<tr>
<td>4 Ac-FAAGRR-CHO</td>
<td>16 ± 3</td>
</tr>
</tbody>
</table>

**Fig. 7. Dengue virus NS3pro peptide inhibitors.** Inhibitors were based on the dengue virus type 2 NS3/4A (structures 1 and 4) and NS4B/5 (structures 2 and 3) cleavage sites and synthesized as described under "Experimental Procedures."

**Synthetic Peptide Inhibitors**—Peptidic α-keto amide inhibitors have been well characterized as reversible competitive inhibitors for other serine proteases (33, 34), and most recently keto amides were shown to be potent inhibitors of the closely related HCV NS3 protease (35, 36). Fig. 7 shows several peptides that were constructed as prospective inhibitors of CF40.gly.NS3pro. Compound 1 was based on the P6-P2' residues of the 3/4A polypeptide cleavage sequence. Compound 2 contains the P6-P2' residues of the 4B/5 sequence, whereas compound 3 has P6-P4' residues of the 4B/5 sequence. Compound 1 was more potent (~5-fold) than compounds 2 and 3 (Table III). All three compounds feature an α-keto amide “trans-

**DISCUSSION**

Sequence alignment studies had previously indicated that NS3pro would be structurally similar to trypsin-like serine proteases, and this has been largely borne out by a recent crystal structure of the free enzyme (38). However, unlike trypsin, NS3pro has a marked preference for basic residues at P2 and P1 in its peptide substrates in the vicinity of the cleavage site. We considered that this unusual requirement might provide an excellent basis for design of selective inhibitors for this enzyme and, because of the essential nature of the protease to viral replication, a possible basis for design of antiviral agents. Toward this goal, the present work has examined enzymatic properties of the recombinant NS3 protease (NS3pro) from the dengue virus, defined in a preliminary way its enzymatic activity, identified and overcome difficulties with autocatalytic cleavage of the co-factor from the protease, evaluated a series of short peptide substrates derivatized for spectrophotometric detection using a para-nitroaniline chromophore, and taken the first steps toward inhibitor development.

We found that, like other flavivirus proteases, NS3pro requires a co-factor from the NS2B region for proteolytic activity. Previous modeling and deletion studies had suggested that a sequence of 40 amino acids from NS2B would likely contain the requisite co-factor domain (16) and that a central core of 14 predominantly hydrophobic residues were thought to define the essential co-factor (29). Although this is a similar situation to the hepatitis C virus NS3 protease, unlike HCV we find that (i) solutions of NS3pro and peptide substrate alone fail to lead to proteolytic activity and (ii) addition of a synthetic 13-residue peptide corresponding to the putative co-factor did not lead to proteolysis (data not shown). However, when the dengue virus NS3 protease was co-expressed with the 40-residue co-factor from the hepatitis C virus NS3 protease, unlike HCV we find that the co-factor appears to be required to thread through a narrow channel of NS3pro for structural stability and therefore probably requires simultaneous folding of enzyme and co-factor to lead to correctly bound co-factor for productive proteolysis.
Our initial work with NS3pro alone showed negligible processing of small pNA peptide substrates. When co-expressed with co-factor (CF40.NS3pro), the enzyme demonstrated some proteolytic activity (Table I) by cleaving small peptide substrates, but mass spectrometry and reversed-phase HPLC suggested that this enzyme-co-factor adduct was unstable in the presence of protease, autocatalytically cleaving itself at the CF40.NS3pro junction. This was confirmed when the kinetic properties of the enzyme improved (Table II versus Table I) for a more stable co-factor-protease construct (CF40-nonapeptide.NS3pro) corresponding to the protease fused to the 40-residue co-factor by a flexible nonapeptide linker (Gly4-Ser-Gly4). This linker is not prone to enzymatic cleavage and thus prevents enzymatic separation of co-factor from protease. These observations are in accordance with an equilibrium in the case of CF40.NS3pro between intact and cleaved adduct, and the parameters obtained for this construct are similar to those recently reported for a similar construct (18). Consequently, we propose that a noncleavable linker between co-factor and protease, described for the first time herein, is needed for optimal enzymatic activity.

By implication it would appear that NS2B functions as a molecular chaperone in assisting the folding of NS3pro to an active conformation that is presumably subtly different from that reported in the recent crystal structure of the isolated protease without co-factor (33). Other serine proteases (e.g. α-lytic protease, subtilisin) are known to require a pro-region like NS2B for productive folding that leads to protease activity. Once folding is completed, the pro-region is dispensable and does not form a component of the active enzyme. In the case of the hepatitis C virus NS3 protease, there was also a dependence on Zn²⁺ because of its structural role in stabilizing the folding of the protease. Studies here with EDTA and divalent cations confirmed our previous suggestion from homology modeling (29) that metal ions are not important for protease activity. This is supported by the crystal structures of NS3pro (38, 39), which contained no metal ions.

We have also reported the first demonstration of cleavage site preferences for NS3pro. The kinetic substrate profiles were obtained using hexapeptide substrates modified as chromogenic pNA derivatives rather than the more tedious approach using recombinant substrates. The observed cleavage efficiency and selectivity profile (Tables I and II) for the four substrates examined may provide useful clues for inhibitor development. Interestingly, the longer decapetide substrate was not cleaved as efficiently as the pNA substrates, whereas for hepatitis C virus NS3 protease the longer peptides are more effective substrates (31). The crystal structure of free NS3pro suggests that there may not be an essential requirement for amino acids in the substrate beyond P2′ on the carboxyl side of the scissile bond for substrate binding (38).

Initial attempts here to investigate the inhibition of NS3pro have revealed that this protease is not inhibited by standard serine protease inhibitors (Fig. 5) except for aprotinin, which is a potent inhibitor at submicromolar concentrations. However, this inhibitor is a large protein and probably denies substrate access to the protease active site by enveloping the enzyme. Table III shows some preliminary indications that small molecule inhibitors, based upon the peptide substrates reported herein, may be able to be developed as inhibitors of NS3pro. Although the indicated compounds are only inhibitors at micromolar concentrations, we can expect to improve upon this potency through structural optimization and with better carboxyl-terminal isosteres that permit more effective interaction with catalytic residues of this serine protease. Because of the critical and unusual requirement of dibasic residues for substrates of this flavivirus NS3 protease, there would appear to be good prospects for the design of protease inhibitors that selectively inactivate NS3pro without inactivating essential proteases for physiological function.

Acknowledgment—We thank Jodie Robinson for excellent technical assistance.

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Activity of Recombinant Dengue 2 Virus NS3 Protease in the Presence of a Truncated NS2B Co-factor, Small Peptide Substrates, and Inhibitors
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