Use of the Fused NS4A Peptide−NS3 Protease Domain To Study the Importance of the Helicase Domain for Protease Inhibitor Binding to Hepatitis C Virus NS3-NS4A

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ABSTRACT: The NS3 protein of hepatitis C virus is unusual because it encodes two unrelated enzymatic activities in linked protease and helicase domains. It has also been intensively studied because inhibitors targeting its protease domain have potential to significantly improve treatment options for those infected with this virus. Many enzymological studies and inhibitor discovery programs have been carried out using the isolated protease domain in complex with a peptide derived from NS4A which stimulates activity. However, some recent publications have suggested that the NS3 helicase domain may influence inhibitor binding and thus suggest work should focus on the full-length NS3-NS4A protein. Here we present the characterization of a single-chain protease in which the NS4A peptide activator is linked to the N-terminus of the NS3 protease domain. This protein behaves well in solution, and its protease activity is very similar to that of full-length NS3-NS4A. We find that this fusion protein, as well as the noncovalent complex of the NS4A peptide with NS3, gives similar K_i values, spanning 3 orders of magnitude, for a set of 25 structurally diverse inhibitors. We also show that simultaneous mutation of three residues on the surface of the helicase domain which has been hypothesized to interact with the protease does not significantly affect enzymatic activity or inhibitor binding. Thus, the protease domain with the NS4A peptide, in a covalent or noncovalent complex, is a good model for the protease activity of native NS3-NS4A.

More than 170 million people worldwide are infected with hepatitis C virus (HCV).[1] Chronic infection can lead to cirrhosis and hepatocellular carcinoma and is the most frequent underlying reason for liver transplants in the United States (*1*). The heterodimeric NS3-NS4A protein contains a serine protease domain and cleaves four of the five nonstructural protein junctions in the HCV polyprotein (*2*). NS3 is comprised of the 180-amino acid N-terminal protease domain and a 420-amino acid helicase domain. The N-terminus of the 54-amino acid NS4A serves as a membrane anchor (*3*), while the central hydrophobic portion binds to the NS3 protease domain as one strand of a β-sheet and thus is an integral part of the NS3 protease structure. Due to its essential role in viral replication, the NS3 protease has been a focus of drug discovery efforts (*4*). Several protease inhibitors have entered clinical trials, the first of which (now discontinued) was BILN 2061 (*5*), which produced more than a 3 log reduction in viral load after treatment for 2 days (*6*). This class of compounds continues to show promise for eventual use in anti-HCV therapy (*7, 8*), and NS3 remains the subject of active research.

The isolated NS3 protease domain has enzymatic activity, but this activity is stimulated significantly by addition of synthetic NS4A peptides, corresponding to the central hydrophobic portion of approximately 14 amino acids (*9*). Crystal structures of the NS3 protease domain determined in the presence (*10, 11*) and absence (*12*) of the NS4A peptide demonstrate that the peptide rigidifies sections of the protein and in particular alters the conformation of the active site catalytic triad, providing a rationale for its effect on activity. The NS4A peptide has been reported to bind weakly to the NS3 protease domain, with a slow on-rate and a low micromolar dissociation constant (K_d) achieved only in the presence of high concentrations of glycerol (*13*). In contrast, full-length NS4A coexpressed with NS3 has been reported to bind very tightly after autocleavage, with no evidence of dissociation even after dilution to subnanomolar concentrations, though binding is salt-dependent and full-length NS4A does dissociate at low salt concentrations (*14*).

Crystal structures of NS3 protease bound to the NS4A peptide show that the C-terminus of this peptide is close in space to the N-terminus of the NS3 protease, and therefore, several laboratories have constructed proteins in which the NS4A central peptide is covalently fused to the N-terminus of NS3 through a short linker (*15*−*19*). However, enzymatic and biophysical properties have been described for only one of these fusions (*18*), and little followup work making use of any of these fusion proteases has appeared. Howe et al. also constructed a full-length NS3 protein with a covalent NS4A fusion (*20*), which made it possible for this group to determine the only reported crystal structure of full-length

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[1] Abbreviations: FL-protease, NS3-NS4A heterodimer; HCV, hepatitis C virus; NS3 and NS4A, nonstructural proteins of HCV; NS4A peptide, central portion of NS4A which binds to NS3; NS3pr-NS4A$_{pept}$, noncovalent complex of the NS3 protease domain and the NS4A peptide; sc-protease, fusion of the NS4A peptide to the N-terminus of the NS3 protease domain.

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NS3 protein (*21*). One important observation was that the protease domain and NS4A peptide in this structure align very well with published structures of the protease domain of NS3 in complex with the added NS4A central peptide. In the full-length structure, the helicase domain of NS3 lies close to the protease active site with the C-terminal carboxylic acid bound in the active site, in what is expected to be a biologically relevant conformation, since this is the site of NS3-NS4A autocleavage (*21*) (Figure 1A).

Most studies of NS3 protease and its inhibitors have been carried out with one of two forms of the protein, either the 180-amino acid protease domain with a high concentration of the NS4A central peptide or the coexpressed full-length NS3-NS4A protein (FL-protease). The FL-protease is the more biologically relevant form, but it is poorly expressed and has low solubility. Because of this, many inhibitor evaluations and almost all structural studies have been performed using the NS3 protease domain-NS4A peptide combination (NS3pr-NS4A$_{pept}$). However, we and others (*22*) have found that inhibitor binding structure−activity relationships (SARs) developed with the two forms are not always the same, which raises some concern about performing NMR or crystallography studies with the protease domain to optimize inhibitors of the FL-protease. There are multiple possible explanations for the divergence in SAR with the two forms. It has recently been noted, on the basis of observation of the C-terminal residues bound in the active site of the full-length NS3 crystal structure (*23*), or modeling of inhibitors into this structure (*24*), that several residues of the helicase domain may interact with inhibitors and thus could contribute to the difference in binding for the two forms.

There are other important differences between assays carried out with the protease domain and NS4A peptide combination and FL-protease which should be taken into account. Because of the slow equilibration between the weakly bound NS4A central peptide and NS3 protease (*13*), the conformation of the inhibitor binding site is potentially different. In addition, also because of differences in protein behavior, standard buffers for assays carried out with the two forms are usually different, and this may also affect inhibitor binding. To improve our understanding of the importance of these different factors to inhibitor binding, we have carried out inhibitor binding assays with both the NS3-NS4A peptide complex and a "single-chain" covalent protease domain-NS4A peptide complex (sc-protease) similar to those reported by other groups and compared results to those obtained with the FL-protease. In addition, to directly test the hypothesis that helicase domain residues interact with protease inhibitors and contribute to binding in the FL-protease, we have mutated three of these residues (Figure 1b) to alanine and evaluated the effect of this change on inhibitor binding. Our results suggest that, in fact, the helicase domain does not play a critical role in inhibitor binding. The objective of this work was to show that protease domain-NS4A peptide complexes, either noncovalent or linked forms, can be used for structure-guided inhibitor optimization of FL-protease inhibitors. While we focus here on 25 selected compounds, the conclusions from our work are expected to apply to all peptidomimetic NS3 protease inhibitors.

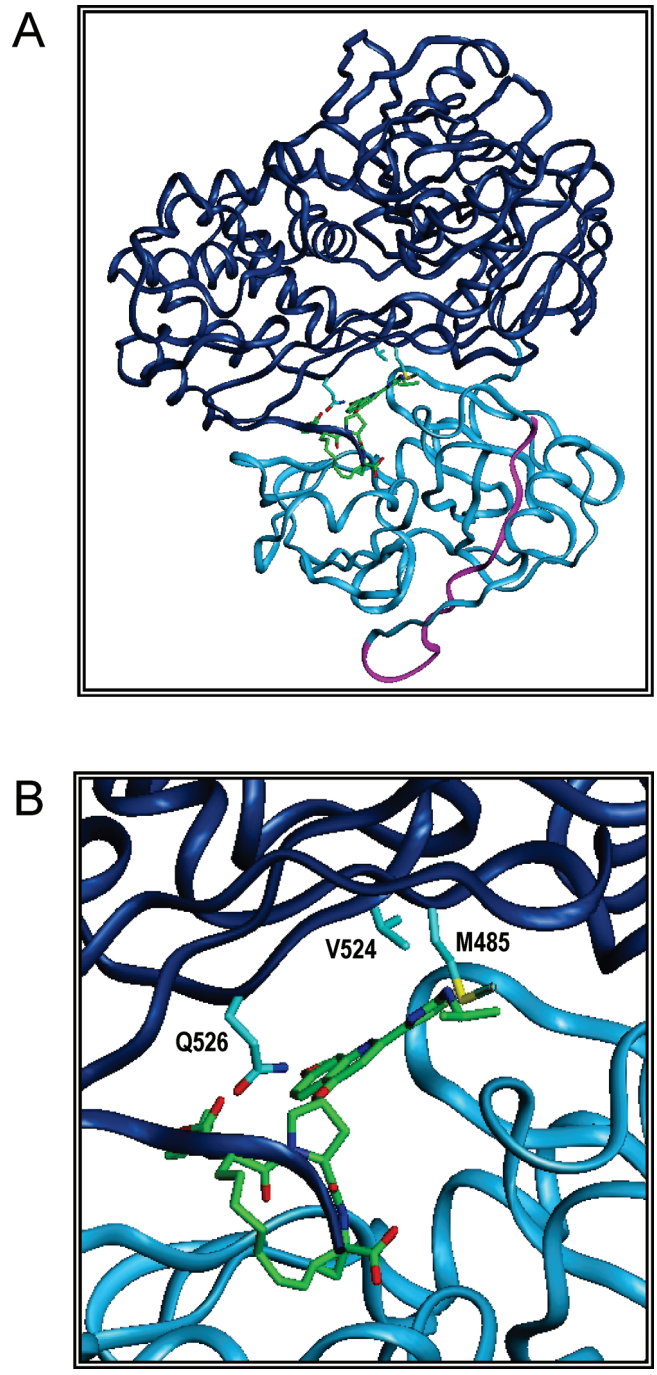


FIGURE 1: Superposition of BILN 2061 and the single-chain NS3-NS4A protein [Protein Data Bank (PDB) entry 1CU1]. (A) The helicase domain is colored dark blue, the protease domain light blue, the tethered NS4A central peptide pink, and the inhibitor green. (B) The active site is framed showing BILN 2061 (**1**) superimposed on the C-terminus of NS3; the apparent interface between helicase residues and the inhibitor is illustrated by the side chains of helicase residues M485, V524, and Q526. This superposition, without reorientation of C-terminal residues or readjustment of side chain orientations, is not realistic but does indicate the possible nature of the protein−inhibitor complex and mimics the complex models shown in some recent publications (*23, 24*).

MATERIALS AND METHODS

Inhibitors, NS4A Peptide, and Substrate. All inhibitors used in this study have been described previously or were prepared using procedures similar to those for previously published compounds (*25*−*29*). Potencies of previously

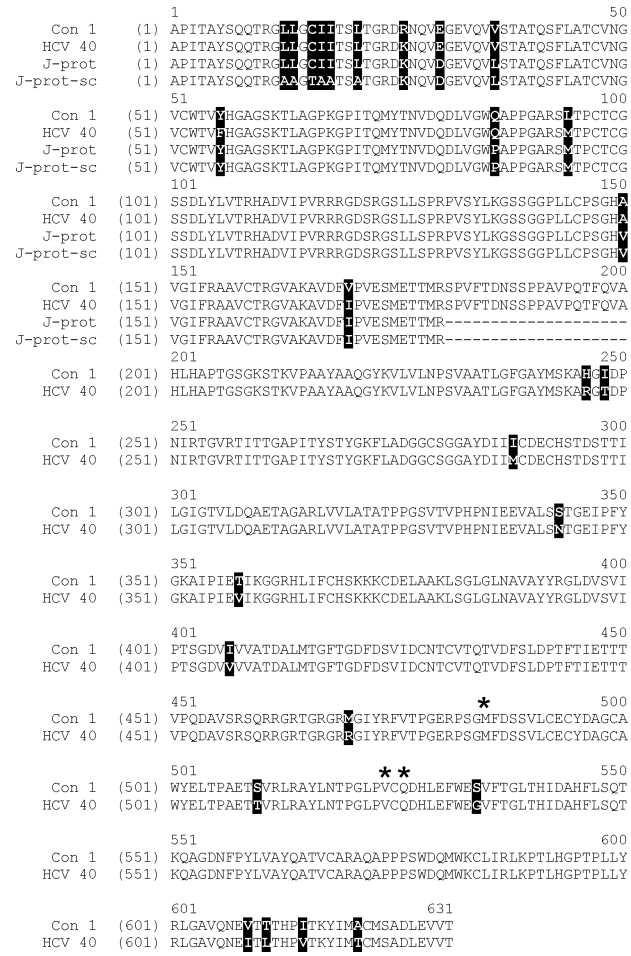


FIGURE 2: Amino acid sequence alignment of the three proteins used in this study. For comparison, the Con1 genotype 1b reference sequence is included. The NS3 portion of FL-protease corresponds to the HCV 40 sequence. The NS3 protease in NS3pr-NS4A$_{pept}$ corresponds to J-prot (J strain protease domain). The NS3 protease sequence for sc-protease corresponds to J-prot-sc; it differs from the J strain sequence only in α-helix 0. Residues which differ in at least one of the sequences are highlighted in black. The three residues in the helicase domain which were mutated to alanine are denoted with asterisks.

reported compounds have been reported in different formats of the NS3 protease assay. Values reported in this work are newly derived average values for the specific assay methods described below.

The NS4A-derived peptide KKGSVVIVGRIILSGRK was prepared using standard solid-phase peptide synthesis methodology (*30*). N-Terminal and C-terminal lysines were added to enhance solubility. The internally quenched depsipeptide fluorogenic substrate anthranilyl-DDIVPAbu[C(O)-O]AMY(3-NO_2)TW-OH, designed on the basis of the NS5A-NS5B cleavage site, was synthesized according to the previously described method (*31, 32*).

NS3 Protease and Full-Length Protein. NS3 protease with the J strain sequence (*33*) (Figure 2) was obtained from the BK strain protease by site-directed mutagenesis. The expression in *Escherichia coli* and purification of the NS3 protease domain were performed as previously described (*34*). FL-protease (strain 40; see Figure 2) was produced either in *E. coli* or using the recombinant baculovirus system and incorporates an N-terminal six-His sequence to simplify purification (*34, 35*). We have previously shown that this short tag has no effect on protease activity (*35*). The M485A, V524A, and Q526A mutations were introduced into the NS3-NS4A coding region using the QuikChange II site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene). Expression in *E. coli* and purification were performed as described for the wild-type protein.

Cloning of sc-Protease. J strain NS3 protease domain (540 nucleotides from amino acid 1 to 180) was amplified by PCR (KOD from Novagen) with forward primer 5′-CGCCGCCATATGGCGCCCATCACGGCCTACTCC-3′ and reverse primer 5′-CGCCGCCTCGAGTCACTATTACTTTTTCTTTTTCGAGGCCCGCATAGTAGTTTCCATAGACTC-3′, which also encodes a C-terminal lysine tail (ASKKKK) and three stop codons. The primers included restriction sites (underlined) for cloning into the pET 29b bacterial expression vector (Novagen). PCR products were purified by agarose gel electrophoresis with the QIAquick gel extraction kit (Qiagen) and ligated with the Rapid DNA ligation kit (Roche). The NS4A fragment was added by PCR with a primer encoding the NS4A/linker sequence MAKGSVVIVGRINLSGD, so that the next amino acid is Thr-4 of NS3. Amino acids substitutions (L13A, L14A, C16T, I17A, I18A, and L21A) were introduced into the NS4A/NS3 protease coding region using the QuikChange II site-directed mutagenesis kit (Stratagene). DNA sequencing was performed using the Big Dye-Terminator Cycle Sequencing Kit (Applied Biosystems). Except for the engineered mutations described above, the J strain protease domain is very similar in sequence to the HCV 40 genotype 1b strain full-length enzyme. The few conservative substitutions are not close to the active site (Figure 2).

Expression and Purification of sc-Protease. The protein was expressed in *E. coli* BL21(DE3) (Novagen). Cells were grown at 37 °C in CircleGrow medium (QBiogene) supplemented with 30 μg/mL kanamycin. At an OD_{600} between 0.7 and 0.9, zinc acetate was added to a final concentration of 50 μM and the culture was cooled on ice for 30 min. Protein expression was induced with 1 mM isopropyl β-D-thiogalactoside overnight at 18 °C. Cells were harvested by centrifugation, and the cell paste was frozen at −80 °C. The cell paste was resuspended in 5 mL of lysis buffer [25 mM $NaPO_4$ (pH 7.5), 0.5% CHAPS, 10% glycerol (w/v), 100 mM NaCl, and 2 mM TCEP] per gram of cells. The suspension obtained was processed in a Dounce homogenizer and Microfluidizer. The extract was clarified by a 35 min centrifugation at 29000*g* and 4 °C. The supernatant was filtered with 5, 0.8, and 0.45 μm filters. Then the filtrate was applied to a HiTrap SP-HP (GE Healthcare) column equilibrated with 50 mM $NaPO_4$ (pH 7.5), 5% glycerol (w/v), 0.1% CHAPS, and 2 mM TCEP. After a wash with the same buffer containing 100 mM NaCl, the protein was eluted with a linear gradient from 100 to 600 mM NaCl. The enzyme-enriched fractions were pooled and diluted 1:2 prior to loading onto a HiTrap Heparin column (GE Healthcare) equilibrated with the buffer described above. After a wash with the same buffer containing 100 mM NaCl, the protein was eluted with 350 mM NaCl in the same buffer. Finally, pooled fractions were concentrated with an Amicon Ultra-15 10000 moleculat weight cutoff (MWCO) (Millipore), filtered, and loaded onto a Superdex 75 column (GE Healthcare) equilibrated with 50 mM $NaPO_4$ (pH 6.0), 5% glycerol (w/v), 0.1% CHAPS, 2 mM TCEP, and 300 mM NaCl. The ^{15}N-labeled protein

used for NMR studies was expressed in Spectra 9-N medium (Spectra Stable Isotopes). Expression and purification protocols were the same as those described above.

Mass Spectrometry on sc-Protease. All mass spectra were recorded using a Voyager DE PRO MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a pulsed nitrogen laser operated at 337 nm. All data were acquired in the positive mode. Prior to MALDI-TOF MS analysis, the intact protein and the peptide mixtures were desalted using C18 ZipTips (Millipore, Billerica, MA) according to the manufacturer's instructions. Protein data were collected in the linear mode using a saturated solution of sinapinic acid (Sigma) in 50% acetonitrile and 0.1% TFA as the matrix. External calibration was performed with horse heart myoglobin (Sigma). For enzymatic proteolysis, 2 μg of sc-protease was resuspended in 50 mM ammonium bicarbonate (pH 8.0). Sequencing-grade trypsin (Promega) was added to the protein solution at an enzyme-to-substrate ratio of 1:50 (w/w), and digestion was allowed to proceed at 37 °C for 16 h. Peptide data were collected in the reflector mode using a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.1% TFA as the matrix. External calibration was performed with angiotensin and ACTH (Sigma). In all cases, sample and matrix were mixed 1:1 (v/v) prior to being spotted on the MALDI target plate.

Analytical Ultracentrifugation of sc-Protease. A sedimentation velocity experiment was performed at 20 °C using an XL-A analytical ultracentrifuge (Beckman Coulter) with an An-60 Ti rotor, using 1.2 cm double sector charcoal-filled Epon centerpieces and quartz windows. Sc-protease was exchanged into a buffer composed of 25 mM $NaPO_4$ (pH 6.0), 150 mM NaCl, and 1 mM TCEP using NAP-5 desalting columns (Amersham Pharmacia Biotech). The sample buffer density was 1.0081 g/cm^3 (by densitometer, Anton-Paar), and the viscosity was 1.0251×10^{-2} P [estimated using Sednterp (*36*)]. Vbar for the protein at 20 °C was estimated to be 0.7302 using Sednterp (*36*). The protein was diluted to give 0.9, 0.45, and 0.225 AU at 280 nm (corresponding approximately to 40, 20, and 10 μM, respectively). All three samples were centrifuged at 50000 rpm (20 °C) until sedimentation boundaries completely disappeared. Data were fitted to the Lamm equation using Sedfit, to obtain the continuous sedimentation coefficient distribution, $c(s)$ (*37*).

NMR on sc-Protease. NMR experiments were conducted using a 350 μM [^{15}N]sc-protease sample in 25 mM sodium phosphate (pH 6.0), 150 mM NaCl, and 1 mM DTT in a 95% H_2O/5% D_2O mixture. NMR data were acquired on a Bruker 600 MHz Avance III spectrometer equipped with a 5 mm triple-resonance cryoprobe at 37 °C. Two-dimensional $^1H-^{15}N$ HSQC and TROSY spectra were acquired using standard pulse sequences. The data were processed and analyzed using Topspin (Bruker BioSpin).

Kinetics and Assays for Different Protease Forms. Kinetic parameters for the sc-protease, the NS3 protease-$NS4A_{peptide}$ complex, and the FL-protease (wild-type and M485A/V524A/Q526A mutant) were determined using the depsipeptide fluorogenic substrate anthranilyl-DDIVPAbu[C(O)-O]AMY(3-NO_2)TW-OH. The cleavage reaction was continuously monitored at 23 °C on a BMG POLARstar Galaxy fluorometer (excitation and emission filters of 320 and 405 nm, respectively) in the presence of 0.5−12.5 μM substrate. Assays were carried out in either 50 mM Tris-HCl (pH 8.0), 0.25 M sodium citrate, 0.01% *n*-dodecyl β-D-maltoside, 1 mM TCEP, and a final DMSO content of 5% (citrate buffer) or 50 mM Tris-HCl (pH 7.5), 30% glycerol (w/v), 1 mg/mL BSA, 1 mM TCEP, and a final DMSO content of 5% (glycerol buffer). Protein concentrations were 1 nM for wild-type FL-protease and sc-protease, 5 nM for the triple mutant FL-protease, and 5 nM for the protease domain preincubated for 15 min with $NS4A_{peptide}$ (5 μM) to allow formation of the complex. Kinetic parameters (K_m and k_{cat}) were determined by nonlinear regression analysis of initial rates as a function of substrate concentration using GraphPad Prism (version 4.02, GraphPad Software Inc., San Diego, CA).

FIGURE 3: Schematic representation of the single-chain NS3 protease (sc-protease) construct. The sequence of the NS4A portion is given in Materials and Methods, and the sequence of the NS3 protease portion is given in Figure 2 (J-prot-sc).

The effect of $NS4A_{peptide}$ on the activity of the sc-protease, the NS3 protease domain, and the FL-protease was determined in the presence of 10 μM substrate and the different protease forms at 5 nM with or without 10 μM $NS4A_{peptide}$ in either citrate buffer or glycerol buffer. The cleavage reaction was monitored as described above.

To evaluate protease inhibition, 5 μM substrate and various concentrations of inhibitor were incubated in citrate buffer with each form of the protein (from 0.5 to 5 nM) for 30−45 min at 23 °C. For the NS3 protease-$NS4A_{peptide}$ complex, a 1000-fold molar excess of $NS4A_{peptide}$ was added. The reaction was terminated by the addition of 1 M MES (pH 5.8). The fluorescence of the N-terminal product anthranilyl-DDIVPAbu was monitored on the BMG PolarStar Galaxy instrument as described above. Calculated percent inhibition values at each inhibitor concentration were then used to determine the median effective concentration (IC_{50}) by the NLIN procedure of SAS (SAS Institute Inc., Cary, NC). K_i values were calculated from IC_{50} values using the equation $IC_{50} = K_i(1 + [S]/K_m)$, assuming $K_i \gg [E]$ (*38*).

RESULTS

Design and Expression of the NS4A-NS3 Protease Fusion (sc-Protease). Our goal was to obtain in high yield an sc-protease with well-defined structure, good solubility in simple aqueous buffers, and good enzymatic activity. Our construct was designed using several literature precedents. The starting point was the genotype 1b J strain protease domain and corresponding NS4A peptide (*33*). The rigidity and correct conformation of the linkage between the NS4A central peptide and NS3 protease sequences are important, since nonoptimal sequences yield a protein in which the NS4A peptide is not well incorporated into the protease structure; consequently, the protein is prone to aggregation (*15, 19*). The NS4A peptide sequence was linked to the N-terminus of the protease via the two-amino acid linker (Gly-Asp) inserted between the last residue of the NS4A peptide and amino acid 4 of NS3 (Figure 3). This linker sequence was shown to give a high yield of active and soluble protein after expression in *E. coli* (*19*).

The solubility of the NS3 protease can be improved by mutagenesis of hydrophobic residues on the surface distal

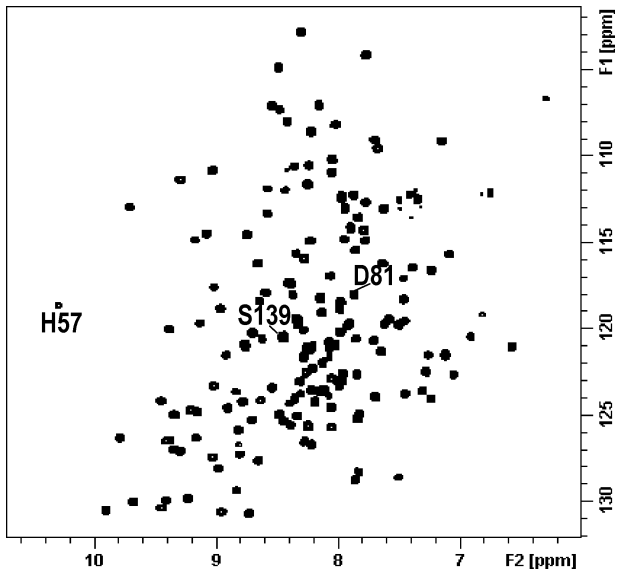


FIGURE 4: Two-dimensional $^{1}H-^{15}N$ TROSY NMR spectrum of ^{15}N-labeled sc-protease. The uniform line width of peaks and good chemical shift dispersion indicate the sc-protease is a well-behaved (nonaggregating) and folded protein. The catalytic triad residues (unpublished experiments) are labeled.

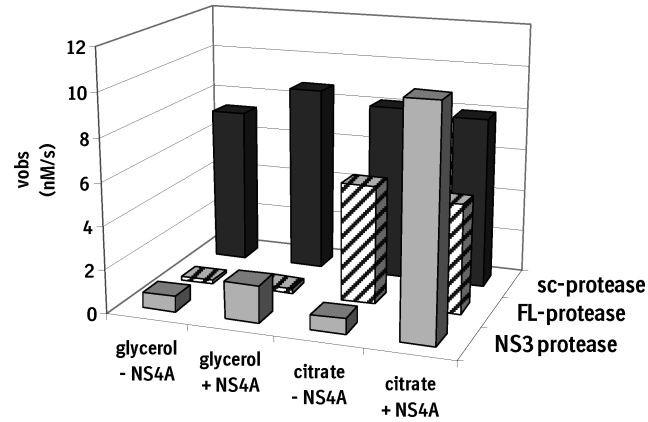


FIGURE 5: Activity of different protease forms. The velocity of the enzymatic reaction for each form of NS3 protease was measured without or with 10 μM NS4A peptide in 50 mM Tris-HCl (pH 7.5), 30% glycerol (w/v), 1 mg/mL BSA, and 1 mM TCEP (glycerol buffer) or 50 mM Tris-HCl (pH 8.0), 0.25 M sodium citrate, 0.01% *n*-dodecyl β-D-maltoside, and 1 mM TCEP (citrate buffer). Values shown are averages of three independent experiments, with coefficients of variation ranging from 10 to 20%, except for the two values for FL-protease in glycerol buffer, which had coefficients of variation of 20−30%.

to the active site (*19*). We mutated five residues on α-helix 0 (Leu-13, Leu-14, Ile-17, Ile-18, and Leu-21) to alanine. We also mutated the exposed Cys-16 to Thr because this residue might be prone to oxidation during long incubations (*19*). Finally, a polylysine tail (ASKKKK) was appended to the C-terminus to further improve solubility. The construct did not contain an affinity tag, which we found to be unnecessary given the expression level of the protein. The sc-protease was expressed in *E. coli* and purified using cation exchange, heparin, and size exclusion columns to yield approximately 20 mg of protein per liter of medium. Purity was >95% as judged by SDS−PAGE (Figure 1 of the Supporting Information). Unlike the unengineered protease domain, it could be exchanged into buffers containing no detergent or glycerol, for example, the buffer used for the NMR experiments described below, and concentrated to at least 12 mg/mL.

Biophysical Characterization of the sc-Protease. Mass spectrometry indicated that the purified protein contained a single polypeptide, with a mass of 20721 Da, in good agreement with the theoretical mass for the coding sequence minus the initial methionine (20713 Da). Tryptic digestion followed by peptide mapping confirmed that the N-terminal methionine is processed.

Sedimentation velocity experiments showed that the protein consisted of a single species with no indication of oligomerization or aggregation (Figure 2 of the Supporting Information). The calculated mass of 21000 ± 200 Da agreed well with that obtained by other techniques. The frictional ratio of 1.3 is consistent with a globular protein, suggesting that the protein is well-folded and that the fused NS4A peptide is incorporated into the NS3 fold as expected.

The $^{1}H-^{15}N$ TROSY NMR spectrum of ^{15}N-labeled sc-protease (Figure 4) showed good chemical shift dispersion as well as uniform and relative narrow line widths indicative of a folded and well-behaved (nonaggregating) protein. This result is consistent with previously reported NMR data on various NS3 constructs which showed that the addition of NS4A (*39*), either as a separate peptide or as part of a single-chain construct (*40*), has a significant effect on the dynamics and structure of the NS3 protease domain in solution, resulting in narrower line widths in the NMR spectra and in the ordering and/or rigidification of the catalytic triad residues as detected by the large upfield shift of the His-57 NH resonance at 10.4 ppm, among others (*40*).

Enzymatic Activity of the sc-Protease. In our previous publications with the NS3 protease in complex with the NS4A peptide, we performed assays in the 30% glycerol buffer described in Materials and Methods, whereas for work with FL-protease, we used a buffer containing 250 mM sodium citrate, also described in Materials and Methods (*34*). Figure 5 illustrates the activity of each form of NS3 protease in both of these buffers, in the presence or absence of excess (but not necessarily saturating) NS4A peptide. As expected, FL-protease has significant activity only in citrate buffer, and this activity is not stimulated by the NS4A peptide. The NS3 protease has similar, relatively low, activity in either buffer, and as expected, this activity is stimulated by addition of NS4A peptide. The increase in activity is much greater in citrate, rather than glycerol, buffer. This was surprising, since in previous publications by ourselves or others (*22, 34, 41*) this form of the protease has been studied in high concentrations of glycerol to stabilize the NS3 protease-NS4A peptide complex. Preliminary experiments indicate that the increase in activity is due to tighter binding of the NS4A peptide which in turn is due to an increase in k_{on} (unpublished experiments). Urbani et al. showed that the K_d for the NS4A peptide decreases significantly with an increase in the concentration of NaCl, in 15% glycerol (*41*), and the activity of NS3pr-NS4A$_{pept}$ in citrate-containing buffers has not been described. Sc-protease has similar, relatively high, activity in either buffer, and this activity is not stimulated by added NS4A peptide. We attribute this robust activity to the covalent fusion of the NS4A peptide to the NS3 protease.

Since FL-protease and both protease domain-NS4A peptide complexes (covalent and noncovalent) exhibited good activity in the citrate buffer, all experiments reported below

Table 1: Kinetic Parameters of the Single-Chain NS3 Protease Domain (sc-Protease), the NS3 Protease-NS4A$_{peptide}$ Complex (NS3pr-NS4A$_{pept}$), and the NS3-NS4A Protein (FL-Protease)[a]

	sc-protease	NS3pr-NS4A$_{pept}$	FL-protease	FL-protease (M485A/V524A/Q526A)
K_m (μM)	2.9 ± 0.3	3.4 ± 0.5	2.1 ± 0.7	4.2 ± 0.1
k_{cat} (min^{-1})	150 ± 12	140 ± 13	69 ± 5	37 ± 1.1
k_{cat}/K_m ($\times 10^5$ M^{-1} s^{-1})	8.4 ± 0.5	6.9 ± 0.3	5.5 ± 1.8	1.5 ± 0.1

[a] Kinetic parameters were determined in citrate buffer using the substrate anthranilyl-DDIVPAbu[C(O)-O]AMY(3-NO$_2$)TW-OH derived from the NS5A-5B cleavage site as described in Materials and Methods. Data are averages of at least two separate determinations.

were conducted in this buffer. Comparison of the kinetic parameters for these forms of the enzyme is provided in Table 1. Consistent with the data in Figure 5, the values of both k_{cat} and K_m are very similar for all three forms. Consequently, assays with inhibitors were carried out using the same concentration of substrate (5 μM) for each. Comparisons of inhibitor potencies are then made using K_i values calculated from the IC_{50} values as described in Materials and Methods.

Comparison of Inhibitor Potencies against Different Forms of NS3 Protease. Nearly all reported NS3 protease inhibitors are peptidic compounds derived from the N-terminal cleavage products (*4*). The 25 inhibitors used in this study have the generic structure shown in Chart 1 and were chosen to span the structural diversity of inhibitors from our NS3 protease inhibitor program. Results reported for this set are considered representative of the full Boehringer Ingelheim NS3 protease inhibitor collection. The 25 compounds span a 1000-fold range in K_i against FL-protease, from 1 nM for the former clinical candidate BILN 2061 (**1**) to 2 μM for less optimal tripeptides such as inhibitor **25**. We intentionally avoided using compounds with K_i values at or close to the wall of the FL-protease assay (one-half the enzyme concentration or 0.25 nM), so that the assumption $K_i \gg [E]$ is true or approximately true. As illustrated by the examples in Chart 1, inhibitors range from tripeptides (macrocyclic or linear) to modified hexapeptides (**17**) and contain different substituents at residues important for activity. Specifically, substitutions at the C-4 position of the proline range from hydroxyl (**19**) to 2-(4-thiazolyl)quinoline-4-oxy (**1**), and the C-terminus is either a free carboxylic acid, an acyl sulfonamide (**3** and **19**), or another group such as aza urea (**17**). A complete list of structures with respective K_i values obtained with all enzyme forms is provided as Table 1 of the Supporting Information; the subset of inhibitors shown in Chart 1 was also retested in a separate experiment with the results listed in Table 2 (vide infra).

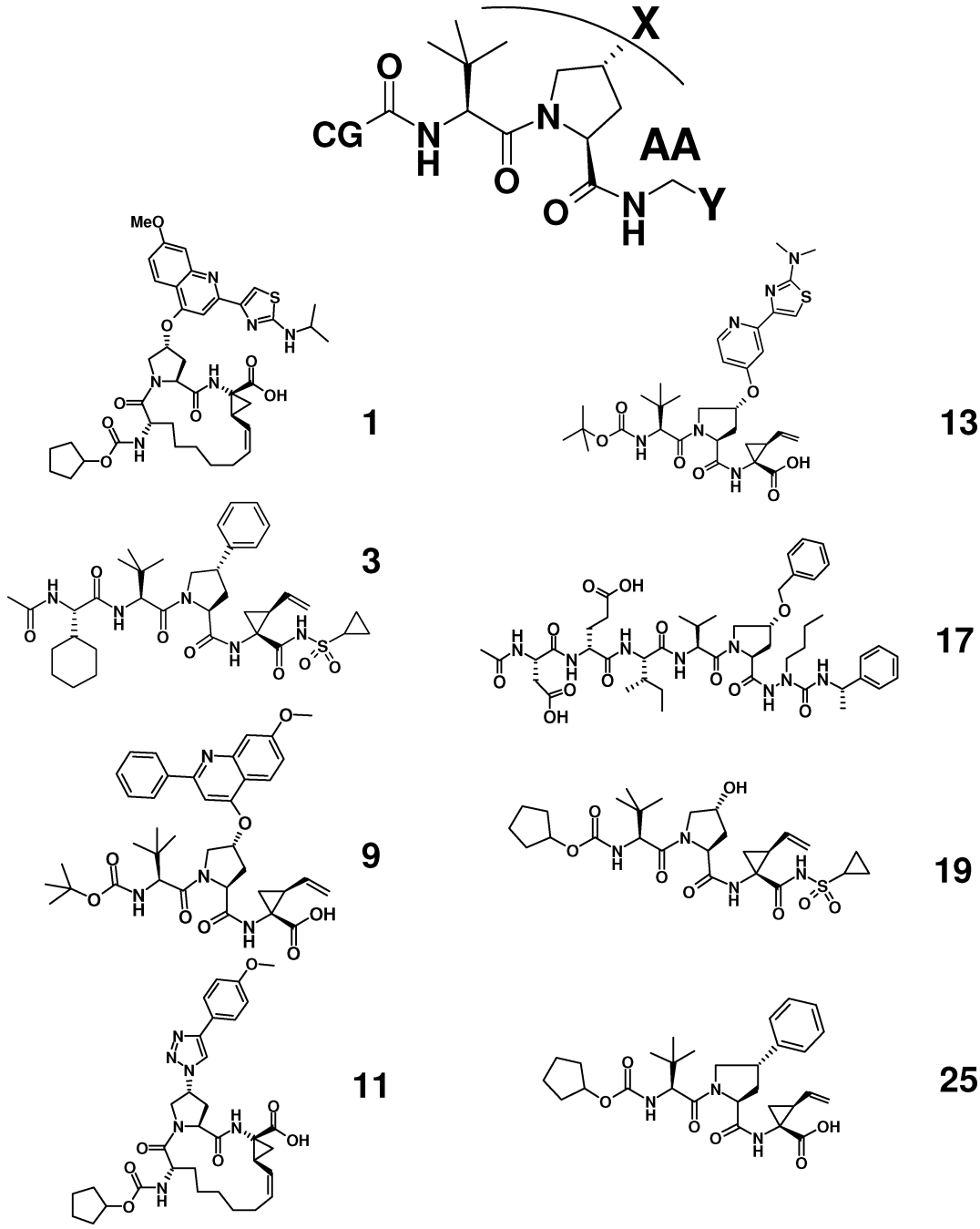
The set of compounds was tested with the three forms of NS3 protease, in citrate buffer. Results for each of the protease domain constructs are compared to those of FL-protease in Figure 6. In assays with the NS3 protease domain in complex with the NS4A peptide (Figure 6A), K_i values are on average 3.0 ± 1.9-fold higher than for the FL-protease. A slope of 0.94 and an r^2 value of 0.95 were obtained from the data in this log−log plot. The analogous comparison for sc-protease (Figure 6B) provided a very similar correlation (slope of 0.94 and r^2 value of 0.96). On average, K_i values are 3.5 ± 2.1-fold higher than against FL-protease. It is possible that the strong overall correlation between the results for sc-protease and those for FL-protease might conceal a greater deviation or more variability for certain inhibitor subclasses. However, a closer examination of the results suggests that this is not the case. Markers for each inhibitor in panels A and B of Figure 6 indicate the nature of the proline substituent (small, medium, or large proline substituent) and of the C-terminus (acid, acyl sulfonamide, or other groups). For each subclass of inhibitors, results with either form of the protease domain with the NS4A peptide are in good agreement with those for FL-protease. Similar observations can be made for other subclasses, e.g., macrocyclic versus linear inhibitors (see the full set of results and structures in Table 1 of the Supporting Information). Inhibitors were also tested against the two forms of the protease domain in glycerol buffer. Results were similar to those in Figure 6 and Table 1 of the Supporting Information, but the correlation between FL-protease and the NS3 protease domain in complex with NS4A peptide was better in citrate buffer (unpublished experiments). Thus, the assay buffer makes a modest, but not critical, contribution to inhibitor structure−activity relationships.

Effect of Helicase Domain Mutations on Inhibitor Activity. As described in the introductory section and illustrated in Figure 1, a superposition of BILN 2061 into the active site of the full-length NS3 protein crystal structure suggests that the large proline substituent of this inhibitor binds in the proximity of the helicase and might interact with residues in this domain. Similar observations were recently made by modeling BILN 2061 into the active site of this structure after deletion of the six C-terminal residues of NS3 (*24*). However, our finding that activity against the covalent and noncovalent forms of the protease domain-NS4A peptide complex closely parallels that against FL-protease, independent of the size of the substituent on the proline, suggests that the helicase domain does not directly interact with bound inhibitors such as BILN 2061. To further examine whether helicase domain residues interact with bound BILN 2061, we mutated three residues (Met-485, Val-524, and Gln-526) to alanine. These residues appear to be in the proximity of large inhibitor proline substituents in some published models (Figure 1B) (*23, 24*). FL-protease containing these three mutations was characterized in enzymatic assays. Protease (Table 1) and helicase and ATP hydrolysis (unpublished experiments) activities were not significantly affected by the triple mutation. A subset of eight inhibitors was tested against WT and triple mutant FL-proteases in parallel. Calculated K_i values were found to be 1.9 ± 0.4-fold higher for the mutant, with no systematic variation due to inhibitor structure (Table 2). Thus, we conclude that K_i values were not significantly affected by the triple mutation.

DISCUSSION

The crystal structure of a single-chain NS4A-full-length NS3 fusion protein (*21*) has stimulated a significant body of experimental work on NS3. However, in solution NS3 is unlikely to be fixed in this conformation, as was assumed in

Chart 1: General Structure and Specific Examples of Inhibitors Used in This Study[a]



[a] Structures and K_i values for the full set of 25 compounds can be found in Table 1 of the Supporting Information. K_i values for these eight compounds were also obtained in a separate set of experiments, listed in Table 2.

some recent publications (*23, 24*), especially considering that the C-terminus of NS3 must dissociate from the protease active site to allow binding of intermolecular substrates or inhibitors. This is supported by recent structures of two flavivirus protease-helicase fusion proteins. Solution small-angle X-ray scattering (SAXS) data on both the Dengue virus NS2B-NS3 protein (*42*) and the Kunjin virus NS3 protein (*43*) showed that the protease domains adopt different orientations relative to the helicase domains. For the Dengue protein, the same conformation was observed by SAXS and X-ray crystallography. Although the different conformation was suggested to be due to sequence differences from HCV NS3-NS4A (e.g., a longer linker between the two domains in the HCV protein), the relative position found for the HCV NS3 domains may simply be an artifact of crystal packing and/or construct design. In fact, it has been hypothesized that the helicase domain cannot interact with the protease domain within the replication complex, in which the N-terminus of NS4A and amphipathic α-helix 0 of NS3 are expected to be associated with cellular membranes (*44*).

Both the protease and helicase activities of HCV NS3 are essential for viral RNA replication, but why are two seemingly unrelated activities covalently linked? Does this provide some advantage to the virus? It has been reported that the protease domain stimulates helicase activity (*45*−*48*). This may be an electrostatic effect on RNA binding (*45*) or

Table 2: K_i Values for WT and M485A/V524A/Q526A Triple Mutant FL-Proteases[a]

inhibitor	K_i(WT) (nM)	K_i(mutant) (nM)
1	0.62 ± 0.12	1.6 ± 0.2
3	1.4 ± 0.2	2.8 ± 0.3
9	9.5 ± 1.8	15 ± 3
11	22 ± 5	55 ± 5
13	41 ± 2	78 ± 9
17	71 ± 6	110 ± 10
19	250 ± 40	410 ± 40
25	2000 ± 100	3600 ± 200

[a] K_i values were determined using the substrate anthranilyl-DDIVPAbu[C(O)-O]AMY(3-NO_2)TW-OH, as described in Materials and Methods. K_i values were calculated from IC_{50} values as described in the text. Values are averages of three separate determinations. Inhibitors were tested in parallel against both enzymes, in experiments separate from those performed with the full set of 25 inhibitors.

an effect on NS3 oligomerization (*47*). However, even simple N-terminal sequences, such as a His tag, can stimulate unwinding activity (*46*). A different orientation of the protease domain, relative to the helicase domain, may still allow the protease domain to exert an effect on RNA unwinding. Within the cell, the linkage may be important for tethering the helicase domain, and thus bound genomic RNA, to the membrane surface at which replication occurs (*49*).

It has been more difficult to determine whether the helicase domain exerts an effect on protease activity. This has been a challenge to study experimentally, since most of the biochemical and biophysical work on the protease domain has been carried out using a noncovalent complex with the NS4A central peptide, which behaves differently from full-length NS4A in complex with NS3. The difference is in part due to the poor solubility of NS4A or the NS4A peptide and the weak interaction of NS4A with NS3 observed in many buffers (*41*). Taremi et al. showed that a sc-protease construct had behavior generally similar to that of FL-protease (*18*), but a recent publication has suggested that the helicase domain makes an important contribution to protease activity. Work by Beran and Pyle, using full-length NS4A complexed with either FL-protease or the protease domain, suggested that the helicase domain provides a moderate 5-fold stimulation of protease activity (*50*). However, the degree of complex formation was not quantified in this work, and the affinity of the NS4A protein for either the NS3 protein or protease domain has not been reported in the buffer used. On the basis of the relatively low reported values of k_{cat}, the reported activity is probably mostly due to NS3 without bound NS4A (compare values in ref *50* to those in ref *34*). Here, we have shown that either covalent or noncovalent NS4A peptide-NS3 complexes have kinetic parameters similar to those of FL-protease. All published experiments have been carried out using peptide or depsipeptide substrates. However, the natural substrate for NS3 protease is the viral polypeptide, so the helicase domain may contribute to cleavage of large protein substrates. In any case, it could be that the helicase domain simply acts as a spacer to allow correct folding of the NS4A cofactor into NS3 protease, rather than directly influencing protease activity.

The primary reason that the HCV NS3 protein has been intensively studied is that it is a validated target for antiviral drugs with great potential to cure HCV infection. Whatever contribution the helicase domain makes for protease activity

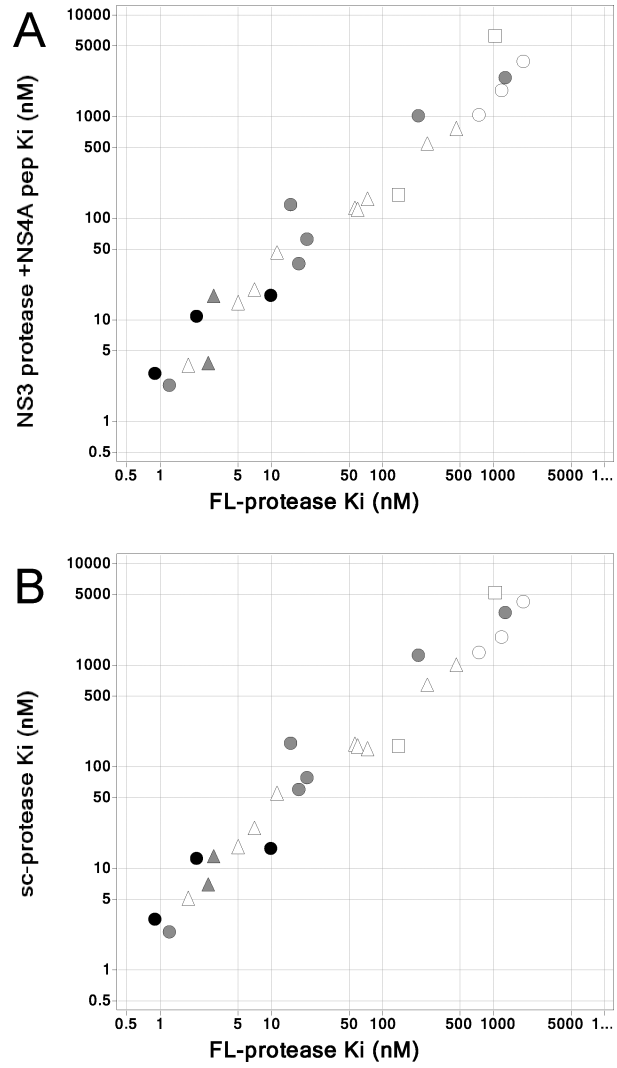


FIGURE 6: Comparison of K_i values for different NS3 protease forms of a panel of 25 inhibitors. The nature of the P1 moiety is shown as follows: carboxylic acid (●), acyl sulfonamide (▲), or others (■). Data marker shading reflects the size of the proline substituent: small in white, medium in gray, and large in black. (A) Correlation plot of inhibition of the NS3 protease-NS4A peptide complex (NS3pr-$NS4A_{pept}$) and FL-protease. (B) Correlation plot of inhibition of sc-protease and FL-protease. Values for each of the three forms of NS3 protease are averages of two or more independent experiments. K_i values were calculated from IC_{50} values as described in the text. This figure was created using Spotfire DecisionSite version 16.0.

in vivo, our comparison of several inhibitors suggests that very similar results can be obtained with the FL-protease and the sc-protease which lacks the helicase domain. This conclusion is further supported by the results we obtained with the FL-protease variant in which the three residues that appear most likely to interact with the inhibitor BILN 2061, when it is superimposed onto the substrate cleavage sequence in the active site, were truncated to alanine. Dahl et al. recently reported similar results for mutation of one of these residues (Gln-526), though they did observe a 10-fold decrease in the level of binding for BILN 2061 on mutation of the neighboring residue His-528 to serine (but not when it was mutated to alanine) (*23*). The structural rationale for this result is unclear on the basis of the model of Liverton et al. (*24*), in which the side chain of His-528 was proposed

to be hydrogen-bonded to the carbamate oxygen of BILN 2061. In any case, the sum of available evidence described above suggests that there must be an alternative explanation to a direct interaction of this inhibitor with the helicase domain.

Virtually all structural studies of inhibitor-NS3 protease interactions have been performed using the isolated protease domain because FL-protease is difficult to obtain and poorly behaved. Thus, it is particularly important to ensure that conclusions derived from NMR or crystallographic analyses of inhibitor-protease domain complexes are fully representative of those which would be obtained using the more physiologically relevant FL-protease, if such studies were possible. Our results suggest that sc-protease is indeed a good model for the study of many classes of NS3 protease inhibitors. The tight NS3-NS4A interaction due to the covalent linkage of these moieties should facilitate additional biophysical and structural studies, in a wide range of buffers, which have been difficult or impossible with other forms of the protein. It is likely that the direct interaction of the NS3 helicase domain with inhibitors, suggested by some recent publications, is not significant. Definite confirmation of this will only be obtained with the structure of an inhibitor bound to full-length NS3. Unfortunately, it has now been almost 10 years since the full-length structure was reported (*21*), and no such structure has been disclosed.

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SUPPORTING INFORMATION AVAILABLE

Table with structures of all 25 inhibitors used in this study, as well as average K_i values against each form of the protease and additional figures for protein characterization, including a Coomassie-stained gel for each form of the protease and analytical ultracentrifugation sedimentation velocity analysis of the sc-protease. This material is available free of charge via the Internet at http://pubs.acs.org.

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