In Vitro Characterization of a Purified NS2/3 Protease Variant of Hepatitis C Virus

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SUMMARY

The cleavage of the Hepatitis C Virus polyprotein between the nonstructural proteins NS2 and NS3 is mediated by the NS2/3 protease, whereas the NS3 protease is responsible for the cleavage of the downstream proteins. Purification and in vitro characterization of the NS2/3 protease has been hampered by its hydrophobic nature. NS2/3 protease activity could only be detected in cells or in vitro translation assays with the addition of microsomal membranes or detergent. To facilitate purification of this poorly characterized protease, we truncated the N-terminal hydrophobic domain resulting in an active enzyme with improved biophysical properties. We define a minimal catalytic region of NS2/3 protease retaining autocleavage activity that spans residues 904-1206, and includes the C-terminal half of NS2 and the N-terminal NS3 protease domain. The NS2/3 (904-1206) variant was purified from Escherichia coli inclusion bodies and refolded by gel filtration chromatography. The purified inactive form of NS2/3 (904-1206) was activated by the addition of glycerol and detergent to induce autocleavage at the predicted site between Leu1026 and Ala1027. NS2/3 (904-1206) activity was dependent on zinc ions and could be inhibited by NS4A peptides, peptides that span the cleavage site or an N-terminal peptidic cleavage product. This NS2/3 variant will facilitate the development of an assay suitable for identifying inhibitors of HCV replication.

INTRODUCTION

Hepatitis C Virus (HCV)\(^1\), a member of the Flaviviridae family, is the major etiological agent of non-A, non-B hepatitis and an important cause of chronic liver disease leading to cirrhosis and hepatocellular carcinoma in humans (1, 2). The enveloped virion contains an RNA genome of positive polarity of approximately 9500 nucleotides encoding a polyprotein of about 3010 aa and is comprised of 10 viral proteins: C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B. C is
HCV NS2/3 protease

thought to be the nucleocapsid protein binding to the viral RNA genome (3), E1 and E2 are the virion glycoproteins (4) and p7 is of unknown function. The NS2-5B proteins are thought to be of nonstructural nature and are involved in replication and polyprotein processing (5). The individual proteins are processed from the polyprotein by a combination of host and viral proteases. A host signal peptidase is responsible for the cleavage of p7 from NS2 creating an N-terminus at aa 810 for NS2 (6, 7). The proteolytic release of mature NS4A, NS4B, NS5A and NS5B is mediated by a chymotrypsin-like serine protease within the N-terminus of NS3. The NS3 protease requires NS4A as cofactor and a structural zinc for efficient processing (8). Most of the HCV-encoded enzymes have been evaluated as targets for the development of new antiviral therapies, namely the NS3 protease, helicase and ATPase activities, as well as the NS5B RNA-dependent RNA-polymerase activity (9, 10, 11).

One viral enzyme that has not been extensively characterized is the NS2/3 protease. The NS2 protein extends from aa 810 to 1026 and autocleavage of the NS2/3 junction at aa 1026-1027 is mediated by a protease activity which is encoded by the NS2 region and the minimal NS3 protease domain that flank the cleavage site (aa 810-1206) (12-16). This autocleavage appears to be essential for productive replication in vivo as validated with an HCV clone devoid of the NS2/3 protease activity that fails to infect chimpanzees (17). The minimal NS2/3 protease domain for the autocleavage activity spans from amino acids 898 at the N-terminal NS2 boundary to amino acid 1207 at the C-terminal NS3 boundary (6, 12, 18, 19). Remarkably, the NS2/3 protease activity is not dependent on an active NS3 protease (12, 13) yet the NS3 protease domain cannot be substituted by another non-structural protein (19). Mutagenesis studies have shown that the residues His952 and Cys993 of NS2 are essential for the autocleavage activity.
(12, 13) but no homology between NS2/3 protease and other proteases has been identified. Gorbalenya et al. (20) have suggested that the NS2/3 protease could be a cysteine protease. However, the observation that the activity is stimulated by metal ions and inhibited by EDTA led to the suggestion that the NS2/3 protease is a metalloprotease (12, 13). Studies with classical protease inhibitors in an in vitro translation assay (15) have not resulted in a definitive classification.

Additionally, NS2/3 auto-processing is inhibited by mutations that are presumed to perturb the local conformation of the polyprotein precursor and may indicate an extreme sensitivity of the NS2/3 cleavage reaction to the correct folding of the protease (12, 13, 16). It was also demonstrated that NS2/3 activity can be detected upon co-transfection of constructs harboring defects in either the NS2 or NS3 regions, but not both, in conjunction with constructs expressing intact versions of the defective region. Furthermore, trans-cleavage activity in cells was suggested by the co-expression of an active NS2/3 protease containing a mutated cleavage site with an inactive NS2/3 protease precursor (H952A or C993A) supplying the cleavage substrate (16).

The biochemical characterization of the NS2/3 protease, as well as mechanistic and structural studies have been limited due to the lack of a pure recombinant form of the enzyme. In this study, we describe the mapping of an N-terminal truncation of the NS2/3 (904-1206) protease; its subsequent expression in E.coli, extraction from inclusion bodies, and renaturation has facilitated the biochemical characterization of this critical target for HCV intervention.
EXPERIMENTAL PROCEDURES

Materials-The detergents CHAPS and Triton X-100 were purchased from Sigma, LDAO from Calbiochem, n-dodecyl-β-D-maltoside from Anatrace Inc., NP-40 from Roche and octyl-POE from Bachem. The reducing agents DTT and TCEP were obtained from Amersham Pharmacia Biotech and Pierce respectively. Arginine hydrochloride, glycerol, Hepes, imidazole, magnesium chloride, Chelex-100 resin and BSA were all purchased from Sigma. Guanidine hydrochloride and Tris were obtained from Gibco BRL, while IPTG and urea were from Roche. Sodium chloride was purchased from Fisher, DMSO and zinc chloride from Aldrich and EDTA from Ambion. Restriction enzymes and DNase were from Amersham Pharmacia Biotech. E. coli XL-1 Blue cells were obtained from Stratagene and E. coli BL21(DE3)pLysS cells from Novagen. The following protease inhibitors were purchased from Sigma: captopril, iodoacetamide, leupeptin, N-ethylmaleimide, pepstatin, 1,10-phenanthroline, phosphoramidon, TLCK and TPCK. Aprotinin and E-64 were purchased from Roche, Pefabloc from PentaPharma AG and 1,7-phenanthroline from Aldrich.

NS2/3 (810-1206) full-length construct-The NS2/3 sequence was amplified by PCR from a HCV genotype 1b sequence obtained from cloning of the NS2-NS5B-3’non coding region by RT-PCR into the pCR®3 vector (Invitrogen) using RNA extracted from the serum of an HCV infected individual². An NcoI site at the 5’end and an amino acid sequence conferring streptavidin binding affinity, followed by a BamHI site at the 3’end were introduced by PCR. The PCR product was inserted into the vector pCR®3 using the TA Cloning® (Invitrogen). The insert was then transferred to the bacterial expression vector pET-11d (Novagen) by cutting with EcoRI followed by Klenow treatment to create blunt ends and then by a partial digestion with NcoI.
This construct was designated pET-11d-NS2/3. The DNA was transformed into *E. coli* XL-1 Blue cells, isolated and sequenced. The NS2/3 protease construct was translated *in vitro* using a rabbit reticulocyte lysate translation kit (Promega) and $^{35}$S-methionine as a label (NEN-Dupont). Translated $[^{35}\text{S}]$-labeled products were separated by SDS-PAGE (15%) and visualized with a PhosphorImager (Molecular Dynamics, Inc.). To evaluate protein expression, the DNA was transferred into *E. coli* BL21(DE3)pLysS cells followed by a 2h-induction at 37°C with 1 mM IPTG. The level of expression was verified by SDS-PAGE (15%) and immunoblot analysis using an anti-NS3 polyclonal antibody.

*NS2/3 N-terminal deletion constructs*-The N-terminal deletion constructs 815-1206, 827-1206, 855-1206, 866-1206, 904-1206 and 915-1206 were derived from the pET-11d-NS2/3 template and obtained by PCR using the appropriate synthetic oligonucleotide primers. The DNA was then transformed into *E. coli* XL-Blue cells, isolated and sequenced. Protein production and expression of the different constructs were verified as described above.

*NS2/3 (904-1206) bacterial expression constructs*-Four lysine residues followed by a hexahistidine tag were added to the N-terminus and four lysine residues were added at the C-terminus of the NS2/3 protease. Constructs were obtained using PCR and the pET-11d-NS2/3 template for the wild-type and the H952A mutant using the appropriate synthetic oligonucleotide primers. The primers also introduced a NdeI and a BamHI sites at 5’ and 3’ end respectively. These inserts were cloned into pET-11d and designated pET-11d-NS2/3 (904-1206) WT or H952A. The DNA was then transformed into *E. coli* XL-Blue cells, isolated and sequenced.
**Enzyme Expression and Production**—Full-length and N-terminal truncated variants of the NS2/3 protease were expressed in *E. coli* BL21(DE3)pLysS cells following induction with 1 mM IPTG for 2-3 h at 37°C to assess their level of expression. The highly expressed NS2/3 (904-1206) was selected for further characterization. A typical 4 L fermentation yielded approximately 20 g of wet cell paste. The cell paste may be stored at -80°C.

**Inclusion bodies extraction**—Following thawing at 23°C, the cells were homogenized in lysis buffer (5 mL/g) consisting of 100 mM Tris, pH 8.0, 0.1% Triton X-100, 5 mM EDTA, 20 mM MgCl₂, 5 mM DTT followed by a DNase treatment (20 µg/mL) for 15 min at 4°C and a centrifugation at 22,000xg for 1 h at 4°C. The resulting insoluble pellet was then washed twice by homogenization (5 mL/g) in 100 mM Tris, pH 8.0, 2% Triton X-100, 5 mM EDTA, 2 M urea, 5 mM DTT and centrifuged at 22,000xg for 30 min at 4°C. Finally, the insoluble material was washed in 100 mM Tris, pH 8.0, 5 mM EDTA, 5 mM DTT and inclusion bodies were recovered in the pellet by centrifugation at 22,000xg for 30 min at 4°C.

**Protein purification from inclusion bodies**—To solubilize the inclusion bodies, the pellet was suspended in the extraction buffer (4 mL/g) [100 mM Tris, pH 8.0, 6 M guanidine-HCl, 0.5 M NaCl] and maintained in that buffer for 1 h at 23°C. The suspension was then centrifuged at 125,000xg for 30 min at 4°C. The resulting supernatant was filtered through a 0.22-µm filter. The clarified filtrate was stored at -80°C. In order to purify the NS2/3 (904-1206), the filtrate was diluted 2-fold in 100 mM Tris, pH 8.0, 6 M guanidine-HCl, 0.5 M NaCl and applied to a Pharmacia Hi-Trap Ni⁺²-chelating column. The NS2/3 protease was typically eluted with a 50 to
500 mM imidazole linear gradient with a 250 mM imidazole peak elution. Fractions from the major peak, containing the purified enzyme, were pooled.

*Refolding of an inactive NS2/3 (904-1206)* - The purified NS2/3 (904-1206) in 6 M guanidine-HCl (typically 1-2 mg proteins/mL) was supplemented with 5 mM TCEP and 5 mM ZnCl₂. Following a 15 min incubation at 23°C, the enzyme was loaded on a Pharmacia Superose 12 gel filtration column (HR 10/30 column, 24 mL bed volume) pre-equilibrated in refolding buffer (50 mM Tris, pH 8.0, 0.5 M arginine-HCl, 1% LDAO, 5 mM TCEP). The column was run with refolding buffer at 4°C at a flow rate of 0.4 mL/min. Fractions associated with the major peak were pooled. The inactive NS2/3 (904-1206) was stored at -80°C in the refolding buffer.

*NS3 serine protease activity* - The NS3 serine protease activity of the refolded NS2/3 (904-1206) was determined by incubating serial dilutions of the refolded enzyme with 5 µM of the internally quenched fluorogenic substrate anthranilyl-DDIVPAbu[C(O)-O]AMY(3-NO₂)TW-OH in 50 mM Tris-HCl, pH 7.5, 30% (w/v) glycerol, 1 mg/mL BSA and 1 mM TCEP for 30 or 60 min at 23°C. The proteolytic activity was monitored by the fluorescence change associated with cleavage of the substrate and the appearance of the fluorescent product anthranilyl-DDIVPAbu-COOH on a BMG Galaxy 96-well plate reader (excitation: 355 nm; emission: 485 nm).

*NS2/3 protease activity* - The autocleavage reaction was initiated by diluting the NS2/3 (904-1206) in the cleavage buffer (50 mM Hepes, pH 7.0, 50% (w/v) glycerol, 0.1-1.0 % CHAPS or 0.1-1.0 % n-dodecyl-β-D-maltoside, 1 mM TCEP) to a final concentration of 0.5-0.8 µM. The enzyme concentration was determined using the Non-Interfering Protein Assay™.
HCV NS2/3 protease (GenoTechnology, Inc.). The assay mixture was then incubated for up to 24h at 23°C. For the time-course experiment at 15°C, 23°C or 37°C, the cleavage reaction was performed with 0.8 µM NS2/3 protease in 50 mM Tris, pH 8.0, 30% glycerol (w/v), 0.1% CHAPS, 1 mM TCEP for 0, 0.25, 0.5, 1, 3, 5 and 24h. The reaction was stopped by heat denaturation of the enzyme in the presence of SDS. Cleavage at the NS2/3 junction was monitored by SDS-PAGE (15%) and immunoblot analysis using either a NS3 protease polyclonal antibody or a commercially available hexahistidine-tag polyclonal antibody (Santa Cruz Biotechnology, Inc.).

NS2/3 protease inhibition—Various concentrations of classical protease inhibitors were assayed in the presence of 0.8 µM NS2/3 protease in 50 mM Hepes, pH 7.0, 50% (w/v) glycerol, 0.1% CHAPS, 1 mM TCEP. The final methanol or ethanol content never exceeded 1% (v/v). The assay mixtures were incubated for 4h at 23°C. The reactions were stopped and quantitation performed as described above. Inhibition was quantified by densitometry (UN-SCAN-IT software, Silk Scientific Inc., Orem, Utah). NS2/3 protease cleavage-site derived peptides and NS4A-derived peptides were synthesized in-house using the standard solid-phase methodology (21) or were made by Multiple Peptide Systems (San Diego, CA). Various concentrations of peptides were pre-incubated with 0.54 µM NS2/3 protease for 30 min at 23°C in 50 mM Hepes, pH 7.0, and 50% (w/v) glycerol. The autocleavage reaction was initiated by addition of n-dodecyl-β-D-maltoside to a final concentration of 0.5%. The final DMSO content never exceeded 5% (v/v). The resulting mixture was then incubated for 3h at 23°C. The reaction was stopped and quantified.
RESULTS

NS2/3 protease N-terminal truncation study-The hydrophobic nature of native NS2 (alone or linked to NS3) has limited its isolation and characterization. An N-terminal truncation study of NS2/3 was therefore undertaken to reduce hydrophobicity and identify a minimal functional domain. All of the NS2/3 protease N-terminal deletion variants were expressed by in vitro translation (Fig. 2A) and were capable of autocleavage with the exception of the NS2/3 (915-1206). Notably, the NS2/3 (904-1206) was expressed at high levels in E. coli (Fig. 2B, 2C), although it partitioned in insoluble inclusion bodies. Expression of the NS2/3 (904-1206) with soluble fusion partners, such as maltose-binding protein and thioredoxin, was unsuccessful at increasing the solubility of the protease (data not shown).

Purification and refolding of an inactive NS2/3 (904-1206)-The NS2/3 (904-1206) was therefore selected for further studies. Following its expression in E. coli, some autocleavage could be observed (Fig. 3, lane 1). However, the majority of the enzyme remained unprocessed in inclusion bodies. Consequently, the inclusion bodies were extracted, solubilized in 6 M guanidine-HCl (Fig. 3, lane 2) and purified on a Ni\(^{2+}\)-chelating column (Fig. 3, lane 3), resulting in a >90% pure NS2/3 (904-1206). The enzyme was refolded by buffer exchange on a gel filtration column with 50 mM Tris, pH 8.0, 0.5 M arginine-HCl, 1% LDAO and 5 mM TCEP. A typical chromatogram is shown in Fig. 4A (solid line). Addition of 0.5 M arginine-HCl was required to maintain NS2/3 (904-1206) solubility. Use of the zwitterionic detergent LDAO was also required for refolding. Finally, the presence of a reducing agent, either DTT or TCEP, was necessary during the refolding process to avoid aggregate formation. Greater than 95% purity, as judged by SDS-PAGE, was obtained (Fig. 4B, lane 3), and typically, 7 mg of refolded enzyme...
were purified per liter of *E. coli* cell culture. Immunoblot analyses showed that the NS2/3 (904-1206) did not auto-process upon refolding (Fig. 4B, lanes 6 and 9). The lack of autocleavage suggested that the cleavage assay conditions were either not optimal or that the enzyme was not refolded properly. In order to address the latter, the efficacy of refolding was assessed by monitoring the NS3 serine protease activity. As shown on Fig. 4A (dotted line), the NS3 serine protease activity co-eluted with the major NS2/3 protein peak. Our finding that NS2/3 (904-1206) possessed NS3 protease activity suggested proper refolding but sub-optimal reaction conditions permissive for autocleavage activity.

**Activation of the NS2/3** (904-1206)-Earlier studies with NS2/3 (810-1615) in *in vitro* translation systems suggested that detergents activate autocleavage (15). On the basis of this observation several detergents were evaluated for their ability to promote autocleavage (Fig. 5A). The detergents NP-40, Triton X-100, n-dodecyl-β-D-maltoside and CHAPS promoted autocleavage to a similar extent at concentrations varying from 0.125 to 1%. However, poor processing was observed in the presence of 0.125, 0.25 and 0.5% octyl-POE and LDAO and no processing was observed in the presence of 1% detergent.

In the absence of detergent, autocleavage was not observed even in the presence of up to 50% (w/v) glycerol (Fig. 5B, lanes 1-6). Glycerol was found to enhance the ability of 0.1% CHAPS to promote autocleavage (Fig. 5B, lanes 7-12). However, the effect of glycerol reached a plateau at concentrations higher than 30% and under these conditions, ~50% autocleavage was observed (Fig. 5B, lanes 10-12). The effect of temperature and incubation time on the NS2/3 (904-1206) activity was also examined. Autocleavage was observed at 15°C and 23°C (Fig. 6, lanes 1-14).
although more NS3 protease product was detected at earlier time points from the 23°C reaction (compare lanes 2-4 to 9-11). At both temperatures, the processing reaction appears to reach a plateau following a 3-5h incubation. Little to no product was detected from reactions performed at 37°C (lanes 15-21). All of our subsequent experiments were therefore conducted at 23°C.

Characterization of NS2/3 (904-1206)-In an attempt to verify that the protease activity was NS2/3-dependent, the activity of the wild-type enzyme was compared to the corresponding H952A mutant (Fig. 7). The absence of cleavage products from the H952A mutant, when assayed under conditions that paralleled the wild-type, confirmed that the activity was indeed NS2/3 protease dependent (Fig. 7, compare lanes 2-3 with lanes 6-7). As a control for the NS2/3 wild-type, no significant cleavage was detected upon removal of 1% n-dodecyl-β-D-maltoside from the assay buffer following a 24h incubation (Fig. 7, compare lanes 7 and 8). Furthermore, no change in the activity was observed in the presence of potent substrate-based NS3 protease inhibitors, confirming that the NS2/3 protease activity was independent of the NS3 serine protease activity (data not shown). Finally, N-terminal sequencing of the NS3 product confirmed that the cleavage occurred between the residues Leu1026 and Ala1027 (data not shown).

As a further characterization of our purified NS2/3 (904-1206), we studied its stimulation by zinc, and inhibition by EDTA as previously reported for the in vitro translated enzyme (12, 13, 15). These studies are easily biased by trace amounts of zinc present in solutions, such that addition of 1 µM zinc chloride to the cleavage buffer only resulted in a slight increase in activity (Fig. 8, compare lanes 1 and 2) and addition of 100 µM EDTA partially inhibited the enzyme
(Fig. 8, compare *lanes 1* and *3*). However, upon stringent preparation and zinc depletion of both the refolding and the cleavage buffers (with Chelex-100 resin), autocleavage was observed only upon addition of zinc to the reaction mixture and EDTA addition prevented zinc activation completely (Fig. 8, *lanes 4-6*), emphasizing the importance of zinc for NS2/3 protease activity.

The effect of classical protease inhibitors on NS2/3 (904-1206) autocleavage was evaluated and is listed in Table I. Of the inhibitors tested, the serine/cysteine protease inhibitors TLCK and TPCK, known to react with active site histidine residues, as well as the thiol-reactive agents iodoacetamide and N-ethylmaleimide, were effective inhibitors of autolysis. The metal chelators EDTA and 1,10-phenanthroline were also effective inhibitors. 1,10-phenanthroline acts via its chelating properties since 1,7-phenanthroline did not inhibit autolysis. This inhibition profile was similar to the profile of the *in vitro* translated NS2/3 (810-1615) (15), but did not provide a definitive classification of this viral protease.

NS2/3 protease cleavage-site derived peptides were also evaluated as potentially competing substrates (Table II, peptides 1 and 4), though none of them were cleaved in *trans* (data not shown). The peptide spanning residues P10-P10′ of the NS2/3 junction (peptide 1) inhibited the autolysis with an IC50 of 270 µM, whereas the peptide substrate spanning residues P6-P6′ (peptide 4) was less potent with an IC50 of 630 µM. Among the corresponding cleavage-site products, the most active was the peptide SFEGQGWRLL (IC50=90 µM, peptide 2), the N-terminal product of peptide 1.
NS4A-derived peptides were also evaluated as inhibitors of the purified NS2/3 (904-1206), based on their reported inhibition of in vitro translated NS2/3 (810-1615) (22). Peptide 6 (NS4A peptide_{genotype 1b}) inhibited the autocleavage reaction with an IC50 of 0.6 µM, whereas peptide 8 (NS4A peptide_{genotype 1a}) exhibited an IC50 of 4 µM (Table II). Peptide 7, which is distinguished from peptide 6 by one single amino acid substitution, exhibited an IC50 > 1000 µM. Remarkably, the ability of the NS4A-derived peptides to inhibit the NS2/3 protease appears to correlate with their ability to activate the NS3 serine protease (data not shown).

**DISCUSSION**

The initial characterizations of processing at the NS2-NS3 junction were based on expression of the NS2-NS3 region in cell-free translation systems or various cellular systems (12-16, 19, 22). The expression of HCV polyprotein precursors, including the NS2/3 protease, was also reported in *E. coli* (12, 23). However, no reports have thus far reconstituted auto-processing of a purified recombinant NS2/3 protease. In the present work, the production of the HCV NS2/3 (904-1206) protease in *E. coli*, its purification and initial biochemical characterization are described.

Initial expression of a NS2/3 construct in *E. coli* encompassing the entire NS2 and NS3 protease domains, NS2/3 (810-1206), resulted in low level of expression that was probably due to the hydrophobicity of the NS2 protein. A series of N-terminal truncations identified the region spanning residues 904-1206 as a functional NS2/3 protease in which the putative transmembrane domain of NS2 was deleted. When expressed in *E.coli*, high levels of NS2/3 (904-1206) were obtained as an insoluble protein and therefore required denaturation and refolding.
Various buffer conditions were investigated in the refolding of NS2/3 (904-1206) in order to keep it in solution and promote its activation after refolding. The presence of 0.5 M arginine-HCl in the refolding buffer was necessary to keep the enzyme in solution. Arginine is a polar additive known to slightly destabilize proteins in a manner comparable to low concentrations of chaotropes, and is likely to increase the solubilization of folding intermediates (24). A reducing agent was required to avoid formation of intermolecular disulfide bonds. Several detergents were evaluated. Detergents are required for refolding of hydrophobic proteins such as β-barrel membrane proteins and probably reduce aggregation during renaturation (25). LDAO was selected since it allows refolding and reconstitution of the NS3 protease activity without promoting autocleavage.

The refolded NS2/3 (904-1206) was activated by the non-ionic detergents NP-40, Triton X-100 and n-dodecyl-β-D-maltoside at concentrations above their respective CMC. CHAPS also promoted autocleavage at concentrations higher and lower than its CMC. The effect of CHAPS was enhanced by glycerol, while glycerol alone had no effect on NS2/3 activity; yet glycerol did potentiate the effect of other detergents (data not shown). The results suggest that glycerol or detergent alone is not sufficient to confer an optimal active conformation. Temperatures greater than 23°C were detrimental to the processing efficiency. Using an optimized cleavage buffer, a plateau of ~50% autocleavage was observed for the NS2/3 (904-1206) following a 5h incubation at 23°C. Further significant processing was not observed with prolonged incubation times. We cannot exclude the possibility that only a fraction of the refolded enzyme adopts an active conformation as the amount of active NS2/3 protease enzyme cannot be assessed directly by active site titration. In this respect, our estimation of the extent of autocleavage may be an
underestimate as the efficiency of processing was calculated with the assumption that all of the refolded enzyme was active. Alternatively, the plateau in processing may reflect instability of the enzyme over time since no further improvement in autocleavage was observed following a 5h-incubation.

Generally, the conditions required for NS2/3 (904-1206) activity resemble those observed for the in vitro translated NS2/3 (810-1615). Triton X-100 and NP-40 activate the autocleavage of the in vitro translated enzyme, which displays a similar temperature profile to our purified protease (15). A comparable temperature-dependent inactivation was also described for the NS3 serine protease domain (26). However, in contrast to our observation, CHAPS inhibited the in vitro translated enzyme (15). Lastly, higher processing efficiencies were reported for the in vitro translated NS2/3 (810-1615) and NS2/3 (849-1240) fused to maltose-binding protein (75% and nearly 100% respectively) (15, 22).

The following observations established the protease activity of the NS2/3 (904-1206) as NS2/3-dependent: 1) autocleavage was precisely mapped to the Leu1026-Ala1027 junction; 2) autocleavage was activated by zinc and inhibited by EDTA; 3) autocleavage was not reconstituted with the refolded H952A mutant; 4) the activity was unperturbed by a potent substrate-based NS3 protease inhibitor. The NS2/3 (904-1206) demonstrated a protease inhibitor profile consistent with the in vitro translated NS2/3 (810-1615) (15). Yet the purified enzyme did not clarify the ambiguous inhibitor profile for this unique protease. Sequence comparison with other proteases have not facilitated the NS2/3 protease classification and no consensus
motif for zinc binding is evident. Its classification as either a cysteine or a metalloprotease will require more in-depth biochemical characterization.

Inhibition of the NS2/3 processing by NS4A-derived peptides was previously reported in a cell-free translation system (22). In our study, NS4A-derived peptides inhibited the NS2/3 (904-1206) with IC50’s as low as 0.6 µM and their potency correlated with their ability to activate the NS3 serine protease (data not shown). Crystallographic studies of the NS3 protease domain complexed with the central hydrophobic domain of NS4A reveal an extensive interaction between the N-terminus of NS3 and NS4A, leading to NS3 protease conformational changes (27, 28). Consequently, inhibition by NS4A-derived peptides may be a result of an overall NS2/3 conformational change. Alternatively, interaction of NS4A-derived peptides with NS2/3 may induce local conformational changes at the cleavage site and thereby impair NS2/3 processing. Toward this end, autocleavage at the NS2/3 junction appears to be determined primarily by polyprotein folding, similarly to cleavage at the NS3/4A junction by the NS3 protease (16, 29). Inhibition by NS4A-derived peptides via a local or global NS2/3 conformational change cannot be distinguished at this point and will require further investigation.

Mixing of wild-type NS2/3 (904-1206) and the H952A mutant under optimized assay conditions, did not inhibit autocleavage (data not shown). The cleavage site derived-peptide substrates P10-P10’ and P6-P6’ were then evaluated as potentially competing substrates. In a well defined assay system using purified NS2/3 (904-1206) and an optimized cleavage buffer (containing 50% glycerol and 0.5% n-dodecyl-β-D-maltoside), the P10-P10’ and P6-P6’ peptides inhibited NS2/3 processing with IC50’s of 270 and 630 µM respectively; yet under identical assay conditions, no trans-cleavage of the peptides was observed (data not shown). The results suggest
non-productive binding of the peptide substrate at the active site. Notably, the shorter P10-P1 N-terminal cleavage product peptide was the best inhibitor with an IC50 of 90 µM, whereas the corresponding C-terminal product was devoid of inhibitory activity. Product inhibition of the NS2/3 (904-1206) was reminiscent of the NS3 protease inhibition by peptides corresponding to the N-terminal cleavage product of the three intermolecular sites processed by the NS3 enzyme (30, 31). The observation that potent competitive hexapeptide NS3 protease inhibitors, while inhibiting the NS3 serine protease, failed to inhibit the NS2/3 (904-1206) activity suggests the absence of cross-talk between the two viral protease active sites. Experiments with the in vitro translated NS2/3 (810-1615) showed no significant inhibition with 500 µM peptide substrates that correspond to the P7-P7’, P5-P5’ residues, or the N-terminal cleavage product peptides (22). This discrepancy with our results may reflect differences in the intrinsic binding affinity of these peptides for NS2/3 (810-1615) and NS2/3 (904-1206) and/or reflect differences in the assay systems used. Lastly, our results with the purified NS2/3 protease are inconsistent with the observation that certain defective NS2/3 variants partially inhibited cleavage of HCV precursor polyproteins containing a NS2/3 cleavage site (16). This difference may again be due to our use of a defined assay system.

The NS2/3 protease appears to be unique among viral proteases in that its sole role in viral maturation is its auto-inactivation. NS2/3 protease auto-processing results in the separation of the NS2 and the NS3 protease domain with the subsequent translocation of NS2 into the ER membrane (19). HCV NS2/3 protease can be viewed as a positive-stranded RNA virus accessory protease, which is defined as a protease not involved directly in the proteolytic processing of key replicative proteins. Accessory proteases fall predominantly within the papain family, are found mostly in the N-terminal region of positive-stranded RNA virus polyproteins
and are wide spread among positive-stranded RNA viruses. Though not directly involved in
genome replication, accessory proteases appear to be indispensable for virus reproduction (20,
32, 33). Remarkably, functional HCV sub-genomic RNAs replicate in the absence of the
structural proteins and NS2 in cell culture, and suggest that the NS2/3 protease activity is not
essential for RNA replication (34). However, the NS2/3 protease activity appears to be essential
for virion production in chimpanzees as no signs of HCV infection can be detected upon
inoculation with an HCV infectious clone devoid of NS2/3 protease activity (17).

NS2/3 protease also seems to share some features with proteases encoded by other positive-
stranded RNA viruses. The Rubella Virus protease appears to be the most functionally related to
the NS2/3 protease. The RV protease: (i) mediates a single cis-cleavage at its C-terminus, (ii)
has a Cys/His catalytic dyad, and (iii) requires divalent cations for its catalytic activity (35). Liu
et al. (36) recently proposed that the RV protease is a novel virus metalloprotease rather than a
papain-like cysteine protease as originally thought. It remains to be seen whether the NS2/3
protease and the RV protease define a new class of viral metalloproteases.

The availability of an active purified recombinant NS2/3 protease, obtained by refolding the N-
terminial truncated form 904-1206, will facilitate the detailed biochemical characterization of the
enzyme and the development of in vitro assays using defined components for drug discovery
purposes. Our results from inhibition studies suggest two approaches for drug design:
identification of molecules that induce a conformational change and optimization of substrate-
derived peptides.
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**FOOTNOTES**

1 The abbreviations used are: HCV, hepatitis C virus; aa, amino acid(s); CHAPS, 3-[3-cholamidopropyl]dimethyl-ammonio]-1-propane sulfonate; LDAO, lauryldiethylamine oxide; octyl-POE, n-octylpentaoxyethylene; DTT, dithiothreitol; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; BSA, bovine serum albumin; IPTG, isopropyl-β-D-thiogalactopyranoside; DMSO, dimethyl sulfoxide; TLCK, tosyl-L-lysine chloromethyl ketone; TPCK, tosyl-L-phenylalanine chloromethyl ketone; Abu, aminobutyric acid; CMC, critical micelle concentration; RV, rubella virus.

2 D. Lamarre et al., unpublished data.
FIGURE LEGENDS

FIG. 1. **Schematic representation of the HCV NS2/3 protease.** The NS2/3 protease is enlarged from the HCV polyprotein. The N- and C-terminal residues of the NS2/3 protease and the residues at the autocleavage site are indicated. The residues His952(*) and Cys 993(**), known to be essential for autocleavage, are also shown.

FIG. 2. **N-terminal truncation study.** HCV NS2/3 protease N-terminal deletion mutants spanning amino acids 810 to 1206 were cloned in the pET11d expression vector. A, NS2/3 protease constructs were translated *in vitro* using a rabbit reticulocyte lysate. Translated $[^{35}S]$-labeled products were separated by SDS-PAGE (15%) and visualized with a PhosphorImager. B, *E. coli* expression of the NS2/3 protease constructs without induction (*lanes -*) or following a 2h-induction at 37°C with 1 mM IPTG (*lanes +*) was evaluated by SDS-PAGE (15%) and C, by immunoblot analysis using an anti-NS3 polyclonal antibody. Lanes are numbered according to the first amino acid of the NS2/3 protease expressed in each transcript. The positions of the molecular mass standards are indicated as well as the NS3 protease.

FIG. 3. **Production and purification of NS2/3 (904-1206)** from inclusion bodies monitored by 15% SDS-PAGE stained with Coomassie blue. *Lane 1*: induced crude *E. coli* cell extract; *lane 2*: inclusion bodies solubilized in 6 M guanidine-HCl; *lane 3*: inclusion bodies purified on Ni$^{2+}$-chelating column. The unprocessed enzyme and the products NS2 (904-1026) and NS3 (1027-1206) are indicated.

FIG. 4. **Purification of the NS2/3 (904-1206).** A, Chromatogram obtained from the refolding and purification of the NS2/3 (904-1206) on a Superose 12 gel filtration column. Following the
addition of 5 mM TCEP and 5 mM ZnCl₂ to the purified inclusion bodies, the enzyme was refolded and eluted in 50 mM Tris, pH 8.0, 0.5 M arginine-HCl, 1% LDAO, 5 mM TCEP. The solid line represents the absorbance at 280 nm while the dotted line indicates the NS3 protease activity monitored as described in the Experimental Procedures section. B, Purification was monitored by 15% SDS-PAGE stained with Coomassie blue or by immunoblot analysis using an anti-NS3 rabbit polyclonal antibody and anti-His tag rabbit antibody. Lanes 1,4,7: purified inclusion bodies; lanes 2,5,8: load of Superose 12 gel filtration column; lanes 3,6,9: refolded enzyme. The unprocessed enzyme and the products NS2 (904-1026) and NS3 (1027-1206) are indicated.

FIG. 5. Effect of detergent and glycerol on NS2/3 protease activity. A, The autocleavage reaction was initiated by dilution of the refolded enzyme in 50 mM Hepes, pH 7.0, 50% glycerol (w/v), 1 mM TCEP containing various detergents followed by an incubation of 18h at 23°C. Each detergent was tested at the following decreasing concentrations (from left to right): 1%, 0.5%, 0.25% and 0.125%. DM, n-dodecyl-β-D-maltoside. B, The autocleavage reaction was performed in 50 mM Hepes, pH 7.0, 0-50% (w/v) glycerol, 1 mM TCEP in the absence (lanes 1-6) or in the presence of 0.1% CHAPS (lanes 7-12) for 3h at 23°C. The reaction was monitored by immunoblot using an anti-NS3 rabbit antibody. The unprocessed enzyme and the NS3 (1027-1206) product are indicated.

FIG. 6. Effect of temperature and incubation time on NS2/3 protease activity. The autocleavage reaction was performed in 50 mM Tris, pH 8.0, 30% glycerol (w/v), 0.1% CHAPS, 1 mM TCEP for 0, 0.25, 0.5, 1, 3, 5 and 24h at 15°C (lanes 1-7), 23°C (lanes 8-14) or 37°C
HCV NS2/3 protease

(lanes 15-21) and monitored by immunoblot analyses using an anti-NS3 antibody. The unprocessed enzyme and the NS3 (1027-1206) product are indicated.

FIG. 7. **Protease activity of NS2/3 H952A mutant versus NS2/3 wild-type.** The autocleavage reaction was performed in 50 mM Hepes, pH 7.0, 50% glycerol (w/v), 1% n-dodecyl-β-D-maltoside, 1 mM TCEP for 0, 2 and 24h at 23°C (lanes 1-3 for H952A; lanes 5-7 for WT) and monitored by immunoblot analyses using an anti-NS3 antibody. A 24h-incubation was also performed in the absence of detergent (lane 4 for H952A; lane 8 for WT). The unprocessed enzyme and the NS3 (1027-1206) product are indicated.

FIG. 8. **Effect of zinc and EDTA on NS2/3 protease activity.** Activity comparison of the NS2/3 protease refolded and assayed using Chelex-100 resin untreated buffers (-zinc depletion) or treated buffers (+zinc depletion). The autocleavage reaction was performed in 50 mM Hepes, pH 7.0, 50% glycerol (w/v), 0.1% CHAPS, 1 mM TCEP for 18h at 23°C. Lanes 1 and 4: assay buffer only; lanes 2 and 5: addition of 1 μM zinc chloride; lanes 3 and 6: addition of 100 μM EDTA. The reaction was monitored by immunoblot using an anti-NS3 rabbit antibody. The unprocessed enzyme and the product NS3 (1027-1206) are indicated.
| Protease Inhibitor | Maximum concentration tested<sup>a</sup> | Inhibition<sup>b</sup> |
|-------------------|-----------------------------------------|----------------------|
| **Aspartic Acid Protease Inhibitors** | | |
| Pepstatin | 0.01 mg/mL | no inhibition |
| **Serine Protease Inhibitors** | | |
| Aprotinin | 1 mg/mL | no inhibition |
| Pefabloc | 1 mg/mL | no inhibition |
| **Serine/Cysteine Protease Inhibitors** | | |
| Leupeptin | 0.1 mg/mL | no inhibition |
| TLCK | 0.5 mM | complete inhibition |
| TPCK | 0.5 mM | complete inhibition |
| **Cysteine Protease Inhibitors** | | |
| E64 | 0.2 mg/mL | no inhibition |
| Iodoacetamide | 1 mM | complete inhibition |
| N-ethylmaleimide | 0.1 mM | complete inhibition |
| **Metalloprotease Inhibitors** | | |
| Captopril | 1 mM | no inhibition |
| EDTA | 2 mM | complete inhibition |
| Phosphoramidon | 1 mg/mL | no inhibition |
| 1,10-phenanthroline | 1 mM | 80% inhibition |
| 1,7-phenanthroline | 1 mM | no inhibition |

<sup>a</sup> The final methanol or ethanol content never exceeded 1% (v/v).

<sup>b</sup> Assay was performed in the presence of 0.8 µM NS2/3 protease as described in Experimental Procedures.
### TABLE II

**Inhibition of NS2/3 Autocleavage by Peptides**

| Peptide # | Sequence                        | IC50 (µM) |
|-----------|---------------------------------|-----------|
| **NS2/3 protease cleavage site-derived peptides**<sup>c</sup> |                                   |           |
| 1         | SFEGQGWRLL-APITAYSQQT          | 270       |
| 2         | SFEGQGWRLL                    | 90        |
| 3         | APITAYSQQT                    | >1000     |
| 4         | KGWRLL-APITAY                 | 630       |
| 5         | APITAY                        | 1000      |
| **NS4A-derived peptides**<sup>d</sup> |                                   |           |
| 6         | KKGSVVIVGRIILSGRK             | 0.6       |
| 7         | KKGSVVIVGRSILSGRK             | >1000     |
| 8         | KKC[VIVGRV][LLSGK]            | 4         |

<sup>a</sup> Peptides were prepared as 20 mM stock solution in DMSO. The final DMSO content never exceeded 5% (v/v).

<sup>b</sup> Assay was performed in the presence of 0.54 µM NS2/3 protease as described in Experimental Procedures.

<sup>c</sup> The hyphen indicates the cleavage site between P1 and P1’ residues.

<sup>d</sup> Residues in boldface type indicate modifications with respect to peptide 6.
FIGURE 1

C E1 E2 P 7 NS2 NS3 4 A NS4B NS5A NS5B

810 1206

NS2/3 protease (42 kDa)

* ** autoproteolytic cleavage

Leu1026 Ala1027

NS2 (23 kDa) NS3 (19 kDa)
FIGURE 2

A. *in vitro* translated N-terminal deletion mutants

![Image of a gel showing protein bands labeled NS2/3 PR and NS3 PR.]

B. *E. coli* expression - SDS-PAGE

| IPTG | 810 | 815 | 827 | 855 | 866 | 904 | 915 |
|------|-----|-----|-----|-----|-----|-----|-----|
| -    | +   | -   | +   | -   | +   | -   | +   |

![Image of a gel showing protein bands at 71 kDa, 42, 31, 18, and 7 kDa.]

C. *E. coli* expression - immunoblot with anti-NS3

| IPTG | 810 | 815 | 827 | 855 | 866 | 904 | 915 |
|------|-----|-----|-----|-----|-----|-----|-----|
| -    | +   | -   | +   | -   | +   | -   | +   |

![Image of an immunoblot showing NS3 PR bands.]

*NS3 PR*
FIGURE 4

A. 

B. 

| SDS-PAGE | anti-NS3 | anti-His<sub>tag</sub> |
|----------|----------|-----------------------|
| 1 2 3    | 4 5 6    | 7 8 9                 |

- NS2/3 PR
- NS3 PR
- NS2
FIGURE 5

A.

| % detergent | Oct-POE | NP-40 | LDAO | Triton | DM | CHAPS |
|-------------|---------|-------|------|--------|----|-------|
|             |         |       |      |        |    |       |
| NS2/3 PR    |         |       |      |        |    |       |
| NS3 PR      |         |       |      |        |    |       |

B.

| % glycerol | 0 | 10 | 20 | 30 | 40 | 50 | 0 | 10 | 20 | 30 | 40 | 50 |
|------------|---|----|----|----|----|----|---|----|----|----|----|----|
| no detergent | 1 | 2  | 3  | 4  | 5  | 6  | 7 | 8  | 9  | 10 | 11 | 12 |
| 0.1% CHAPS |   |    |    |    |    |    |   |    |    |    |    |    |
In vitro characterization of a purified NS2/3 protease variant of hepatitis C virus
Diane Thibeault, Roger Maurice, Louise Pilote, Daniel Lamarre and Arnim Pause

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