Modulation of Hepatitis C Virus NS3 Protease and Helicase Activities through the Interaction with NS4A

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ABSTRACT: The hepatitis C virus nonstructural 3 protein (NS3) possesses a serine protease activity in the N-terminal one-third, whereas RNA-stimulated NTPase and helicase activities reside in the C-terminal portion. The serine protease activity is required for proteolytic processing at the NS3−NS4A, NS4A−NS4B, NS4B−NS5A, and NS5A−NS5B polyprotein cleavage sites. NS3 forms a complex with NS4A, a 54-residue polypeptide that was shown to act as an essential cofactor of the NS3 protease. We have expressed in *Escherichia coli* the NS3−NS4A precursor; cleavage at the junction between NS3 and NS4A occurs during expression in the bacteria cells, resulting in the formation of a soluble noncovalent complex with a sub-nanomolar dissociation constant. We have assessed the minimal ionic strength and detergent and glycerol concentrations required for maximal proteolytic activity and stability of the purified NS3−NS4A complex. Using a peptide substrate derived from the NS5A−NS5B junction, the catalytic efficiency (k_{cat}/K_m) of NS3−NS4A-associated protease under optimized conditions was 55 000 s^{-1} M^{-1}, very similar to that measured with a recombinant complex purified from eukaryotic cells. Dissociation of the NS3−NS4A complex was found to be fully reversible. No helicase activity was exhibited by the purified NS3−NS4A complex, but NS3 was fully active as a helicase upon dissociation of NS4A. On the other hand, both basal and poly(U)-induced NTPase activity and ssRNA binding activity associated with the NS3−NS4A complex were very similar to those exhibited by NS3 alone. Therefore, NS4A appears to uncouple the ATPase/ssRNA binding and RNA unwinding activities associated with NS3.

The hepatitis C virus (HCV)[1] is the main etiologic agent of both parenterally transmitted and community-acquired non-A, non-B hepatitis (*1*). It has been estimated that more than 1% of the world population is affected by the disease (*2*) and 50−80% of HCV infections become chronic, resulting in an increased risk of liver cirrhosis and of hepatocellular carcinoma (*3*). So far, no efficient therapy or vaccine is available. Therefore, there is a great need to develop HCV-specific antiviral agents to counteract this major medical problem.

HCV was identified by molecular cloning in 1989 (*4*) and was classified as a member of the family Flaviviridae (*5*). The viral genome is a 9.6 kb single-stranded positive RNA molecule that contains a single open reading frame (ORF) encoding a polyprotein of 3010−3030 amino acids (*6*−*9*). The large polyprotein precursor is proteolytically processed into at least 10 distinct products which are encoded on the viral RNA with the following order: NH2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH. C, E1, and E2 are believed to be viral structural proteins, whereas the role of p7 has not been established. The remaining viral proteins (NS2−NS5B) are believed to be nonstructural proteins, presumably components of the viral replication machinery.

Whereas the structural HCV proteins arise through the action of host signal peptidases, two viral enzymes are required for the maturation of the nonstructural region of the polyprotein (*10*−*21*). The NS2−NS3 junction is cleaved by a zinc-dependent autoproteinase composed of NS2 and the N-terminal third of the NS3 protein (*12*, *15*). The C-terminal remainder of the HCV polyprotein is further processed to give rise to mature NS3 (67 kDa), NS4A (6 kDa), NS4B (26 kDa), NS5A (56−58 kDa), and NS5B (65 kDa) proteins by the serine protease contained within the NS3 protein (*10*, *13*, *21*−*23*). The catalytic domain of the NS3 protease has been mapped to the N-terminal 180-amino acid region of NS3, containing a characteristic serine protease catalytic triad (*24*−*29*). Although the N-terminal serine protease domain of NS3 shows enzymatic activity on its own, NS4A, a 54-residue protein expressed immediately downstream of NS3 in the viral polyprotein, is a protease cofactor essential for efficient proteolytic processing. NS4A enhances NS3-dependent cleavage at all sites but is an absolute requirement for processing of the NS3−NS4A and NS4B−NS5A junctions (*18*, *24*, *30*, *31*). The existence of an NS3−NS4A stable complex was initially suggested by coimmunoprecipitation experiments (*30*, *32*−*34*) and recently confirmed by copurification of NS3 and NS4A expressed in eukaryotic cells (*35*−*37*).

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[1] Abbreviations: LDAO, lauryldimethylamine oxide; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; IPTG, isopropyl β-D-thiogalactopyranoside; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; TFA, trifluoroacetic acid; DMSO, dimethyl sulfoxide; HCV, hepatitis C virus; NS, nonstructural; FL, full-length.

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Analysis of hydropathy plots and secondary structure prediction highlight the presence of three distinct regions within NS4A. The first region encompasses residues 1−20, is highly hydrophobic, and is predicted to form a transmembrane α-helix (*38*). This domain possibly has the function of anchoring the NS3−NS4A complex onto the ER membrane. The second region, residues 21−34, is also hydrophobic and was predicted to fold in an extended *β*-strand conformation (*39*). This region has been identified as being necessary and sufficient for the binding and the stimulation of the NS3 protease (*33*, *39*−*42*). Several studies have shown that synthetic peptides encompassing this domain of NS4A are able to elicit full activation of the proteinase in vitro. The third region of NS4A, corresponding to the 20 C-terminal residues, is more hydrophilic, and its function has not yet been identified. It has been suggested that NS4A may exercise the cofactor function in one or more of the following ways: by stabilizing the active conformation of the NS3 serine proteinase; by recruiting the NS3 proteinase to the endoplasmic reticulum, where the proteolytic processing is presumed to occur; or by facilitating the folding process of the NS3 protease (*16*, *31*−*33*).

Three-dimensional structures of the NS3 protease domain both alone (*43*) and in complex with NS4A-derived peptides designed to include only the essential NS3-binding region (*44*, *45*) were recently determined by X-ray crystallography. Comparison of the different crystallographic structures suggested that NS4A activates the NS3 protease by stabilizing the enzyme active conformation. In fact, structural rearrangements upon cofactor binding ultimately affect the entire N-terminal *β*-barrel of the NS3 and result in the realignment of the protease catalytic triad in a "canonical" arrangement. Because of the large number of interactions, NS4A becomes an integral part of the serine protease fold. In agreement with the extensive interaction observed in the crystal structure, the NS3−NS4A complex has been shown to be very stable in the cytoplasm of cultured cells expressing the HCV polyprotein (*31*, *32*). This observation is in striking contrast with the relatively high equilibrium dissociation constant observed for purified NS3 (protease domain or full-length) and an NS4A-derived synthetic peptide lacking the putative transmembrane α-helix (*46*, *47*). It has been suggested that in the crystal structure NS3 and the activation domain of NS4A might assume a conformation closely related to that of the native complex (*45*). More information about the stability of the NS3−NS4A interaction in solution requires the characterization of a more physiologically relevant form of the complex, i.e., a purified full-length NS3−NS4A protein. In addition to the N-terminal protease domain, NS3 has been demonstrated to possess an RNA-stimulated nucleoside triphosphatase (NTPase) activity (*48*−*52*) and an RNA/DNA helicase activity (*49*, *53*−*58*), both associated with the 465 C-terminal amino acids of the polypeptide (*55*). The crystal structure of the minimal NTPase/helicase C-terminal domain has also been determined, both alone (*59*) and in complex with a single-stranded DNA molecule (*60*). Recently, we have demonstrated that the N-terminal protease domain has little if any effect on the ATPase/RNA helicase activity of NS3 (*47*). However, the influence that the binding of NS4A to NS3 N-terminal protease domain could exert on these enzymatic activities remains to be explored.

In this study, we present the overexpression of a native form of the full-length NS3−NS4A complex in *Escherichia coli*. We describe the procedure employed to solubilize and purify it as a stable, noncovalent heteromeric complex and the characterization of its enzymatic properties. We have analyzed the serine protease activity of the NS3−NS4A complex under optimized reaction conditions and compared it with that of the full-length NS3 protein activated by an NS4A-derived synthetic peptide containing only the core protease-activating sequence. Finally, we have explored the possible effect of full-length NS4A on the ATPase, RNA helicase, and RNA binding activities of NS3 under conditions which favor NS3−NS4A stability and solubility.

MATERIALS AND METHODS

Expression and Purification of NS3 and the NS3−NS4A Complex from Bacteria. Full-length (FL) NS3 protein (amino acids 1027−1657 of the BK strain HCV polyprotein) was expressed in *E. coli* and purified as previously described (*47*). A cDNA fragment encoding the full-length NS3−NS4A polypeptide precursor (amino acids 1027−1711) was obtained by PCR and cloned between the *Nde*I and *Hin*dIII restriction sites of the pT7-7 and pET14b (Novagen) expression vectors, respectively. In the two constructs used in this study, the coding sequence for a three-lysine solubilizing tail was added at the 3′-end of the NS4A coding region. The cysteine in the P1 position of the NS4A−NS4B cleavage site (amino acid 1711) was changed to a glycine to avoid the proteolytic cleavage of the lysine tag. Furthermore, a cysteine to serine mutation was introduced by PCR at position 1454 to prevent the autolytic cleavage into the NS3 helicase domain. The resulting constructs, pT7 NS3/4A (K) and pET NS3/4A (K), were sequenced using an Applied Biosystem 373 DNA sequencer. Both complex forms, one retaining the native N-terminal sequence (NS3−NS4A) and the other containing an amino-terminal six-histidine tag (HisNS3−NS4A), were expressed in *E. coli* BL21(DE3) cells (*61*) with a protocol previously described (*47*). Briefly, we grew transformed bacterial cultures in a defined minimal medium, and after exponential growth at 37 °C to the desired optical density, induction with IPTG was carried out at 18 °C for 22 h. All subsequent operations were performed at 4 °C unless otherwise indicated. Cells were harvested and disrupted with a Microfluidizer (model 110-S) in $^1/_{10}$ of the culture volume of a buffer containing 25 mM Hepes (pH 7.6), 1 mM EDTA, 20% glycerol, 0.5 M NaCl, 0.5% Triton X-100, 3 mM DTT, 1 mM PMSF, and COMPLETE protease inhibitor cocktail (Boehringer).

Insoluble material was pelleted at 27000*g* for 30 min in a Sorvall SS34 rotor. The clarified supernatant containing about 70% of the recombinant protein was filtered through 40 mL of DEAE-Sepharose Fast Flow resin (Pharmacia) pre-equilibrated in lysis buffer containing 0.2% Triton X-100. The filtrate was then concentrated by 50% ammonium sulfate precipitation. The NS3−NS4A sample was dialyzed against lysis buffer containing 0.1 M NaCl and 0.2% Triton X-100, whereas the HisNS3−NS4A sample was dialyzed against a buffer containing 25 mM Hepes (pH 8), 20% glycerol, 0.5 M NaCl, 0.2% Triton X-100, and 10 mM *β*-mercaptoethanol (Ni-affinity loading buffer). The protein complexes were subsequently purified by fast protein liquid chromatography (FPLC; Pharmacia), as follows. The NS3−NS4A sample was

loaded onto a 20 mL HiTrap heparin−Sepharose column (Pharmacia) and eluted with a 0.1 to 1 M NaCl linear gradient in a buffer containing 25 mM Hepes (pH 7.6), 1 mM EDTA, 20% glycerol, 0.2% Triton X-100, and 3 mM DTT (buffer A). The protein peak was detected in fractions containing approximately 0.4 M NaCl which were then pooled and dialyzed against buffer A containing 0.2 M NaCl. The HisNS3−NS4A-dialyzed sample was loaded onto a 15 mL HiTrap Chelating column (Pharmacia) charged with Ni^{2+} according to the instructions of the manufacturer and equilibrated with Ni-affinity loading buffer. After a wash with 10 column volumes of the same buffer containing 5 mM imidazole and a 3 column volume step at 50 mM imidazole, HisNS3−NS4A was eluted with 3 column volumes of the same buffer containing 200 mM imidazole and dialyzed against buffer A containing 0.2 M NaCl. Both samples were then loaded onto a poly(U) Sepharose affinity column (Pharmacia). After a wash with 5 column volumes of the same buffer, both NS3−NS4A and HisNS3−NS4A were eluted in a pure form with buffer A containing 1 M NaCl. Copurification of NS3 and NS4A was controlled by Western blot analysis of the chromatographic fractions using an anti-NS3 antiserum (*21*) and a recombinant Fab anti-NS4A monoclonal antibody (a kind gift from C. Traboni). Protein stocks were quantified by amino acid analysis and stored at a concentration of 10−20 μM at −80 °C after shock-freezing in liquid nitrogen. The final yield was 10 mg/L of culture with a purity of >80%.

Gel Filtration Chromatography. Five hundred micrograms of a purified preparation of the NS3−NS4A complex was applied to a Pharmacia Superdex 200 HR 10/30 prepacked column in a buffer containing 25 mM Hepes (pH 7.6), 1 mM EDTA, 10% glycerol, 0.3 M NaCl, 3 mM DTT, and different detergents (Calbiochem) at the concentrations indicated in the Results. The flow rate was 0.3 mL/min. The elution profile was monitored by UV absorbance, and 0.6 mL fractions were collected and analyzed by Western blotting with anti-NS3 and anti-NS4A antibodies (data not shown). Blue dextran (2000 kDa), aldolase (153 kDa), bovine serum albumin (67 kDa), and ovoalbumin (43 kDa) were obtained from Pharmacia and used as molecular mass standards.

Peptides and HPLC Protease Assays. The peptide substrate NS5A−NS5B, having the sequence H-EAGDDIVPC/SMSYTWTGA-OH, was purchased from Anaspec. All the other peptides were synthesized by solid phase synthesis based on Fmoc/t-Bu chemistry, as described previously (*46*, *62*). The identities of the peptides were determined by mass spectrometry and amino acid analysis. The concentration of stock peptide aliquots, prepared in DMSO or in buffered aqueous solutions and kept at −80 °C until they were used, was determined by quantitative amino acid analysis performed on HCl-hydrolyzed samples.

If not specified differently, cleavage assays were performed in 60 μL of a buffer containing 50 mM Hepes (pH 7.5), 0.15 M NaCl, 0.1% Triton X-100, 15% glycerol, and 10 mM DTT (protease activity buffer). When indicated, a peptide spanning the central hydrophobic core (residues 21−34) of NS4A and containing an N-terminal three-lysine tag (Pep4AK, Ac-KKKGSVVIVGRIILSGR-NH_2; *46*) was used. Saturating amounts (16 μM) of Pep4AK were preincubated for 15 min at 23 °C with 2 nM enzyme. All reactions were started by addition of the substrate to the final desired concentration. Incubation times at 23 °C were adjusted to obtain <10% conversion. Reactions were stopped by addition of 40 μL of 1% TFA to the reaction mixture. Cleavage of peptide substrates was quantified by HPLC using a MERCK-Hitachi chromatograph equipped with an autosampler, as described previously (*28*, *46*). Kinetic parameters were calculated from a nonlinear least-squares fit of initial rates as a function of substrate concentration with the aid of a Kaleidagraph software, assuming Michaelis−Menten kinetics. The dissociation constant of the NS3−Pep4AK complex was calculated from a nonlinear least-squares fit to the equation (*46*)

$$V = V_o + (V_{max}[\text{Pep4AK}])/(K_d + [\text{Pep4AK}])$$

Experiments to demonstrate the reversibility of the association of NS3 and NS4A were performed by incubating the recombinant complex at a concentration of 100 nM in the protease activity buffer containing 0 or 0.15 M NaCl for 12 h at 4 °C. Each protein sample was then diluted to 2 nM, in both 0 and 0.15 M NaCl-containing buffers, and after 5 h, they were tested for protease activity in the absence or presence of saturating concentrations of Pep4AK.

Pep4A-conjugated Affigel beads (Bio-Rad) were prepared by adding 1 mg of Pep4AK synthetic peptide to 1 mL of Affigel resin, according to the instructions of the manufacturer. The matrix that was obtained was then equilibrated in protease activity buffer. Forty microliters of Pep4A-conjugated Affigel beads (Pep4AK final concentration of 50 μM) or 40 μL of unconjugated Affigel beads (negative control) was incubated with 12 nM NS3−NS4A complex in 400 μL of protease activity buffer containing 0 or 0.15 M NaCl on a rotating wheel at 4 °C. At different time points, 10 μL aliquots were withdrawn and tested in a standard protease activity reaction (60 μL) in the presence of 0.15 M NaCl. The percentage of residual activity was calculated as the ratio between the activities measured after the same time of incubation with Pep4A-conjugated and unconjugated beads.

Protease Assays with in Vitro-Translated Substrates. In vitro translation of the HCV NS5A−NS5BΔC51 (from residue 1965 to 2470) was described previously (*63*). Briefly, in vitro transcription was performed with T7 RNA polymerase (Stratagene). The transcript was translated for 1 h at 30 °C in the presence of [^{35}S]methionine (1175 Ci/mM; Dupont NEN) using an RNA-dependent rabbit reticulocyte lysate (Promega). Aliquots of purified NS3 or NS3−NS4A were added to the translated protein substrate in the absence or presence of 15 μM Pep4AK, and the mixtures were incubated for 60 min at 30 °C. Cleavage of the labeled precursor was assessed by SDS−PAGE followed by autoradiography. The efficiency of the proteolytic reaction was calculated by quantification of the radioactivity using a PhosphorImager and ImageQuant software. The percentage of cleavage was calculated as the ratio between the amount of radioactivity associated with the NS5A product and the amount of total radioactivity associated with both the substrate and the proteolytic product.

NTPase Activity Assay. NTPase activity was directly determined by monitoring [γ-^{32}P]ATP hydrolysis by thin-layer chromatography, as described previously (*47*). Protein

titration assays were carried out by incubating 3.125−50 nM enzyme for 30 min at 37 °C under standard conditions: 25 mM MOPS/NaOH (pH 7), 2.5 mM DTT, 2.5 units of RNasin (Promega), 100 μg/mL BSA, 0.1% Triton X-100, 10% glycerol, 3 mM $MgCl_2$, 1 mM ATP, and 2 μCi of [γ-^{32}P]-ATP (6000 Ci/mmol, 10 mCi/mL; Dupont NEN) with or without 0.1 mM poly(U) in a final volume of 10 μL. NaCl (0.15 M) was added to the samples where indicated. After termination with 5 mM EDTA, 0.5 μL of each reaction mixture was spotted onto polyethyleneimine (PEI) cellulose sheets and developed by ascending chromatography in 150 mM LiCl and 150 mM formic acid (pH 3.0). The cellulose sheets were dried, and the amount of released [^{32}P]phosphoric acid was quantified with a PhosphorImager using ImageQuant software.

Helicase and RNA Binding Assays. The partially double-stranded RNA substrate was obtained by annealing a 40-mer RNA oligonucleotide (template strand) with a complementary 26-mer RNA oligonucleotide (release strand) as described previously (*47*). RNA oligonucleotides were purchased from Genset. The release strand was 5′-end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase (Pharmacia) prior to the annealing reaction. The helicase activity assay was performed in a 20 μL reaction volume containing 25 mM MOPS/NaOH (pH 7), 2.5 mM DTT, 2.5 units of RNasin, 100 μg/mL BSA, 0.1% Triton X-100, 10% glycerol, 3 mM $MgCl_2$, 0.8−100 nM enzyme, and 1.25 nM ^{32}P-labeled partial duplex RNA substrate. Where specified, 0.075 M NaCl was added to the reaction mixtures. After preincubation for 15 min at 23 °C, 5 mM ATP was added to start the helicase reaction. This was carried out at 37 °C for 30 min and then stopped by adding 5 μL of termination buffer [0.1 M Tris (pH 7.5), 20 mM EDTA, 0.5% SDS, 0.1% NP40, 0.1% bromophenol blue, 0.1% xylene cyanol, and 25% glycerol]. Aliquots (8 μL) were analyzed on a native 8% polyacrylamide gel containing 0.5× Tris-borate-EDTA. Strand separation was visualized by autoradiography, and the efficiency of the helicase reaction was calculated by quantification of the radioactivity using a PhosphorImager and ImageQuant software. The percentage of unwinding was calculated as the ratio between the amount of radioactivity associated with the release strand and the amount of total radioactivity associated with both the unwound substrate and the release strand.

Gel retardation reaction mixtures (20 μL) contained 25 mM MOPS/NaOH (pH 7.0), 2.5 mM DTT, 2.5 units of RNasin, 100 μg/mL BSA, 0.1% Triton X-100, 10% glycerol, 1.56−25 nM enzyme, and 1.25 nM ^{32}P 5′-end-labeled 40-mer ssRNA oligonucleotide corresponding to the template strand. Where specified, 0.15 M NaCl was added to the reaction mixtures. After incubation for 20 min at 23 °C, 6 μL aliquots were electrophoresed on a native 6% polyacrylamide gel containing 0.25× Tris-borate-EDTA. Bands corresponding to the protein-bound and unbound probe were visualized by autoradiography, and quantification of the radioactivity was performed using a PhosphorImager and ImageQuant software. The efficiency of the binding reaction was calculated as the ratio between the amount of radioactivity associated with the protein-bound probe and the amount of total radioactivity associated with both the protein-bound and unbound probe.

RESULTS

Expression and Purification of the Full-Length NS3−NS4A Complex. We have expressed in *E. coli* and purified both the full-length (FL) NS3 protein (amino acids 1027−1657) (*47*) and the NS3−NS4A native complex (amino acids 1027−1711). The constructs used in this study are schematically represented in Figure 1A. Initial attempts to produce the NS3−NS4A wild type complex partially failed because an NS4A-dependent autolytic cleavage occurred in the NS3 helicase domain during the expression in bacteria. A prominent 47 kDa NS3 proteolytic product identified by NS3-specific antibody staining was the most abundant protein present in the soluble fraction of the NS3−NS4A bacterial extract. This protein was not produced when FL NS3 alone was expressed in *E. coli* (*47*). A similar autolytic product had been previously observed by expressing the NS3−NS4A wild type complex both in eukaryotic cells and in cell-free protein translation systems (unpublished data). Examination of the NS3 amino acid sequence revealed two potential sites at Cys1454 and Cys1456 where autolytic cleavage could generate an N-terminal 47 kDa fragment. Out of the two, the site having Cys1454 at the P1 position more closely resembled the known NS3 consensus cleavage pattern, having an acidic residue at position P6. Therefore, we mutagenized Cys1454 into a serine and created two more NS3−NS4A expression vectors, both containing this mutation and an extra three-lysine tail at the C-terminus of NS4A to increase the solubility of the complex (Figure 1A). We have expressed in *E. coli* both constructs, one containing an amino-terminal six-histidine tag to facilitate purification and the other retaining its native N-terminal sequence, using a protocol successfully devised for the production of large amounts of soluble FL NS3 protein (*47*). Both mature NS3 (67 kDa) (Figure 1B, lane 2) and NS4A (6 kDa) (Figure 1C, lane 2) polypeptides were detected in the bacterial lysate after IPTG induction. The NS4A-containing 73 kDa band that can be seen in Figure 1C probably corresponds to residual uncleaved NS3−NS4A precursor. Both NS3 and NS4A were partially recovered in a soluble form upon disruption of cells in a buffer containing 0.5 M NaCl and 0.5% Triton X-100 (Figure 1B,C, lane 4). A nonionic detergent was strictly required for solubility, whereas a high ionic strength was demonstrated to increase the stability of the noncovalent complex (see below). Most of the uncleaved NS3−NS4A precursor was recovered in the insoluble fraction under these conditions (Figure 1C, lane 5). After filtration through DEAE-Sepharose to separate nucleic acids and concentration by ammonium sulfate precipitation, HisNS3−NS4A was further purified on HiTrap Chelating Sepharose column charged with Ni^{2+} and poly(U) Sepharose (Figure 1B,C, lanes 6−9). For the NS3−NS4A complex, HiTrap heparin−Sepharose was used as the first purification step (data not shown). In both cases, NS3 and NS4A were copurified through all the purification procedure, as judged by Western blotting with anti-NS3 (not shown) and anti-NS4A (Figure 1C) antibodies, suggesting that the two proteins were stably associated into a noncovalent heterodimer. This protocol yielded about 10 mg of purified protein complex from 1 L of culture at a concentration of 1 mg/mL with a purity of >80%, as judged by SDS−PAGE (Figure 1B).

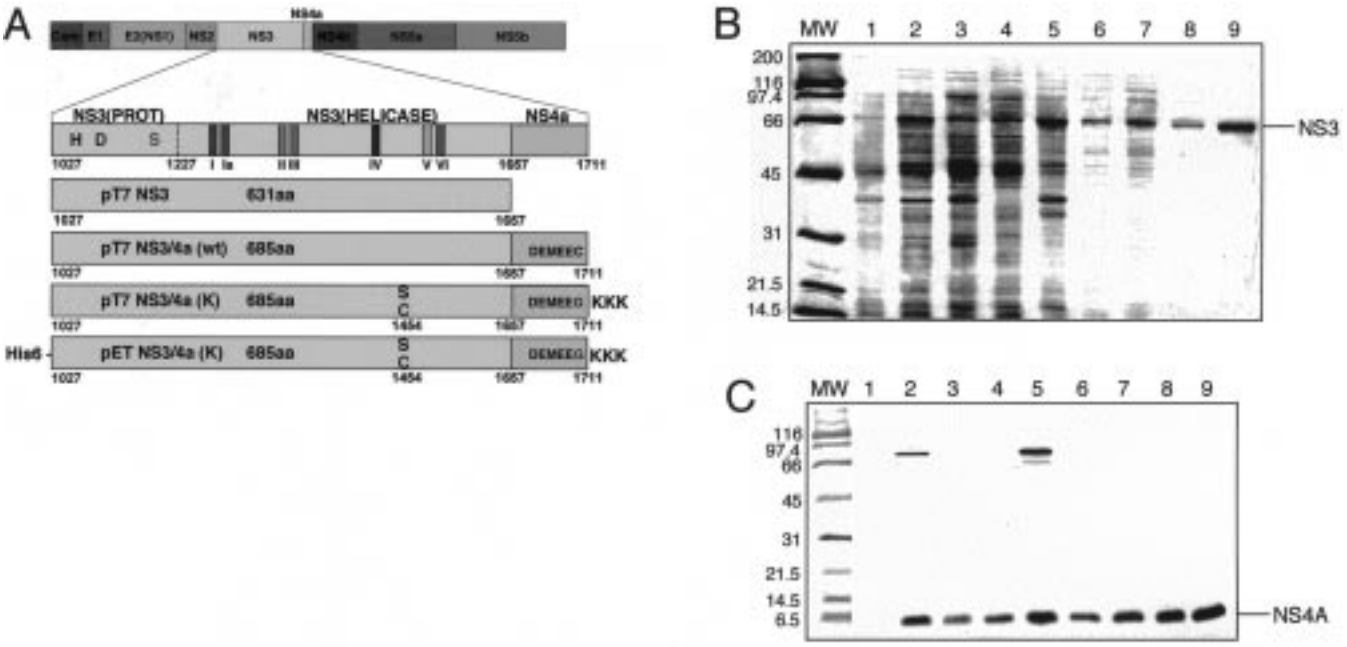

FIGURE 1: Expression and purification of FL NS3 and NS3−NS4A proteins from *E. coli*. (A) Schematic of the NS3 and NS3−NS4A constructs expressed in *E. coli.* H, D, and S make up the serine protease catalytic triad. I−VI are conserved helicase motifs. wt is wild type. His6 is a 21-residue leader peptide containing the six-histidine tag and the thrombin cleavage site of the pET-14b vector. KKK is the three-lysine tail. SDS−PAGE of HisNS3−NS4A purification steps analyzed by Coomassie blue staining (B) and by Western blot using anti-NS4A monoclonal antibodies (C): lane 1, noninduced lysate; lane 2, IPTG-induced lysate; lane 3, total extract; lane 4, soluble fraction; lane 5, insoluble fraction; lane 6, 50% ammonium sulfate cut; lane 7, Ni^{2+}-charged HiTrap Chelating input; lane 8, poly(U) Sepharose input; and lane 9, poly(U) Sepharose pool. Amount of total protein per lane: (1) 4 μg, (2) 8 μg, (3−5) 10 μg, (6) 2 μg, (7) 4 μg, (8) 0.9 μg, and (9) 1.6 μg. MW represents molecular mass markers.

Table 1: Gel Filtration Analysis in the Presence of Different Detergents

		apparent molecular mass (kDa)	
detergent	%	NS3−NS4A	NS3
none		≥2000	67
Triton	0.2	150	67
LDAO	0.2	150	67
dodecyl maltoside	0.1	150	67
octyl glucoside	0.2	≥2000	67
CHAPS	0.2	≥2000	67

State of Aggregation of NS3−NS4A. The multimerization or aggregation state of the NS3−NS4A complex purified from bacteria was determined on a Superdex 200 gel filtration column in buffers containing 0.3 M NaCl and different detergents. We found that the aggregation state of NS3−NS4A depended on the nature of the detergent included in the chromatographic buffer. Indeed, in 0.2% Triton X-100, 0.2% LDAO, or 0.1% *n*-dodecyl β-D-maltoside, more than 80% of the NS3−NS4A chromatographed as a homogeneous species in close proximity to a 153 kDa marker, whereas only a small fraction eluted in the column void volume (Table 1). The unpredicted high molecular mass of around 150 kDa could be explained either by the association of the NS3−NS4A heterodimeric form (73 kDa) with a micelle of detergent (70−80 kDa) or by the formation of a heterotetramer containing two NS3−NS4A molecules. It has to be noted that Triton X-100, LDAO, and *n*-dodecyl β-D-maltoside were used at a concentration significantly greater than their critical micelle concentration (cmc). In the presence of different detergents such as 0.2% *n*-octyl β-D-glucopyranoside or 0.2% CHAPS (Table 1), most of the NS3−NS4A complex eluted in the column void volume (≥2000 kDa) as a soluble aggregated form which remained in solution after centrifugation at 100000*g* and could be dissociated into the homogeneous heteromeric species by re-equilibration in 0.2% Triton X-100 (data not shown). The lack of solubilization by these two latter detergents could be partially due to their cmc, which is higher than the concentration used in the experiment. The observed dependence of the protein aggregation state on the nature and concentration of the detergent was a peculiarity of the NS3−NS4A complex, since FL NS3 alone was mostly recovered in a monomeric form also in the absence of detergents (Table 1). This different behavior would suggest that the inclusion in detergent micelles of an NS4A hydrophobic region potentially exposed to the solvent is strictly required for the solubilization of the complex. The dependence of NS3−NS4A stability and solubility on the ionic strength could not be assessed in gel filtration experiments due to nonspecific interactions of the complex with the chromatographic matrix at NaCl concentrations of less than 0.15 M.

Protease Activity on in Vitro-Translated Substrates. To assay the trans-cleavage activity of the purified NS3−NS4A complex on HCV polyprotein precursors, we incubated both FL NS3 and the NS3−NS4A complex at concentrations between 10 and 80 nM with the *in vitro*-translated ^{35}S-labeled precursor protein NS5A−NS5BΔC51 (*47*) (Figure 2). To assess the dependence of the NS3 protease activity on the NS4A cofactor, the experiments were performed in the presence or absence of a large excess (15 μM) of an NS4A-derived peptide, spanning the central hydrophobic core (residues 21−34) of the NS4A protein (Pep4AK) (*46*). As shown in Figure 2A, efficient processing was evident with the NS3−NS4A complex even at the lowest concentration that was tested, whereas very poor cleavage was detected with

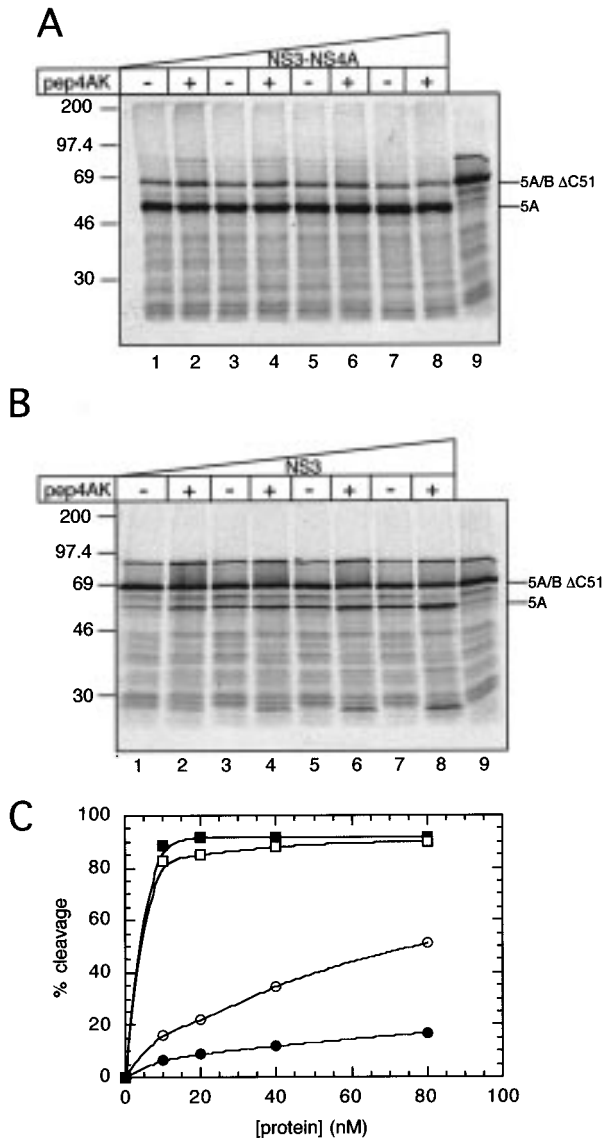

FIGURE 2: NS3−NS4A protease activity on in vitro-translated precursor substrates. The NS5A−NS5BΔC51 precursor protein was synthesized by in vitro translation of the corresponding RNA in the presence of [^{35}S]methionine as described in Materials and Methods. NS3−NS4A (A) and FL NS3 (B) stock solutions were serially diluted from 123 to 15.37 nM in 13 μL of 25 mM Hepes (pH 7.5), 1 mM EDTA, 0.2% Triton X-100, 3 mM DTT, and 20% glycerol. NaCl (1 M) was added only to samples whose migration patterns are shown in panel A. Enzyme samples were preincubated in the presence of 2 μL of H_2O (lanes 1, 3, 5, and 7; − pep4A) or 150 μM Pep4AK (lanes 2, 4, 6, and 8; + pep4A) for 10 min at 23 °C. Five microliters of NS5A−NS5BΔC51 in vitro-translated precursor was added to the protein and the mixture incubated for 1 h at 30 °C. Reactions were terminated by the addition of 40 μL of SDS sample buffer, and 15 μL aliquots were analyzed by SDS−PAGE followed by autoradiography. In lane 9 are shown control samples in the absence of NS3 or NS3−NS4A proteins. Bands corresponding to the NS5A−NS5BΔC51 substrate and to the 5A product are indicated as well as the positions of the molecular mass markers. The complementary product NS5BΔC51 was not detected in the gel system that was used because of its small size. (C) Quantitative diagram of the data presented in panels A and B. NS3−NS4A or NS3 enzyme in the presence (□ and ○) or in the absence (■ and ●) of 15 μM pep4AK.

the NS3 protein alone (Figure 2B). Furthermore, the proteolytic activity of the complex was not enhanced by the presence of the Pep4AK cofactor that, on the contrary, significantly stimulated the NS3 activity. Similar results were obtained using as a substrate the full-length NS5A−NS5B precursor (data not shown). Figure 2C shows a quantitative diagram of the data presented in panels A and B of Figure 2.

These results demonstrated that the purified NS3−NS4A complex was fully proficient in the proteolytic processing of the NS5A−NS5B protein precursor in vitro. Indeed, the intrinsic trans-cleavage activity of the complex was higher than that of the isolated NS3 protein complexed with the synthetic NS4A-derived cofactor.

Analysis of NS3−NS4A Protease Activity on Synthetic Peptide Substrates and Its Dependence on the Reaction Conditions. To devise a protease assay that was suitable for the screening of potential inhibitors, we have analyzed the efficiency of NS3−NS4A proteolytic activity on a synthetic peptide substrate under different ionic strengths and by varying the concentration of glycerol and detergent(s) (Figure 3). NS3−NS4A (2 nM) was incubated with 10 μM ($\sim K_m$; see Table 2) 18-mer peptide H-EAGDDIVPC/SMSYTWTGA-OH, derived from the NS5A−NS5B junction (*64*). The cleavage efficiency was only marginally affected by the omission of glycerol in the assay which did not have a radical effect over a wide range of concentrations (data not shown). Since pep4A-stimulated FL NS3 activity was found to be strictly dependent on high glycerol concentrations (*47*), the association of full-length NS4A apparently resulted in a more stable and glycerol-independent complex. In preliminary experiments, we have noticed that the NS3−NS4A proteolytic activity could be stimulated 10−15-fold by adding 0.3 M NaCl to the assay (data not shown). To study how ionic strength affects the functionality of the NS3−NS4A complex, we performed salt titration experiments on NS3−NS4A protease activity in the absence or presence of saturating concentrations of Pep4AK (Figure 3A). The NS4A-derived peptide had been demonstrated to stimulate about 25-fold the trans-cleavage efficiency of the isolated NS3 protein. The latter was shown to possess a very low level of basal activity in the absence of the cofactor (*47*). The cleavage efficiency of the NS3−NS4A enzyme increased 6−10-fold by increasing the salt concentration up to 0.15 M NaCl or 0.05 M ammonium sulfate (Figure 3A, left and right panels, respectively), reaching a plateau at higher ionic strengths. On the contrary, in the presence of an excess of Pep4AK, the observed proteolytic activity did not show the same dependence on the ionic strength, since it increased when the ionic strength was lowered. This finding can be explained as follows. Decreasing the ionic strength below a critical value, which is similar for both the NaCl and ammonium sulfate titration experiments ($I = 0.12-0.15$), causes the dissociation of the NS3−NS4A complex with a consequent loss of proteolytic activity. The NS3 apoenzyme that is released from the complex under these destabilizing conditions is fully activated by the excess of Pep4AK that is added to the reaction mixture. The increase in protease activity observed by reducing the ionic strength is probably caused by a parallel increase in the affinity of Pep4A-complexed NS3 for the peptide substrate (see below). Above a critical salt concentration, the NS3−NS4A complex is completely stabilized, and therefore, no free NS3 is available to be further stimulated by Pep4AK.

The effect of the nature and concentration of the detergent on the NS3−NS4A protease function is shown in Figure 3B.

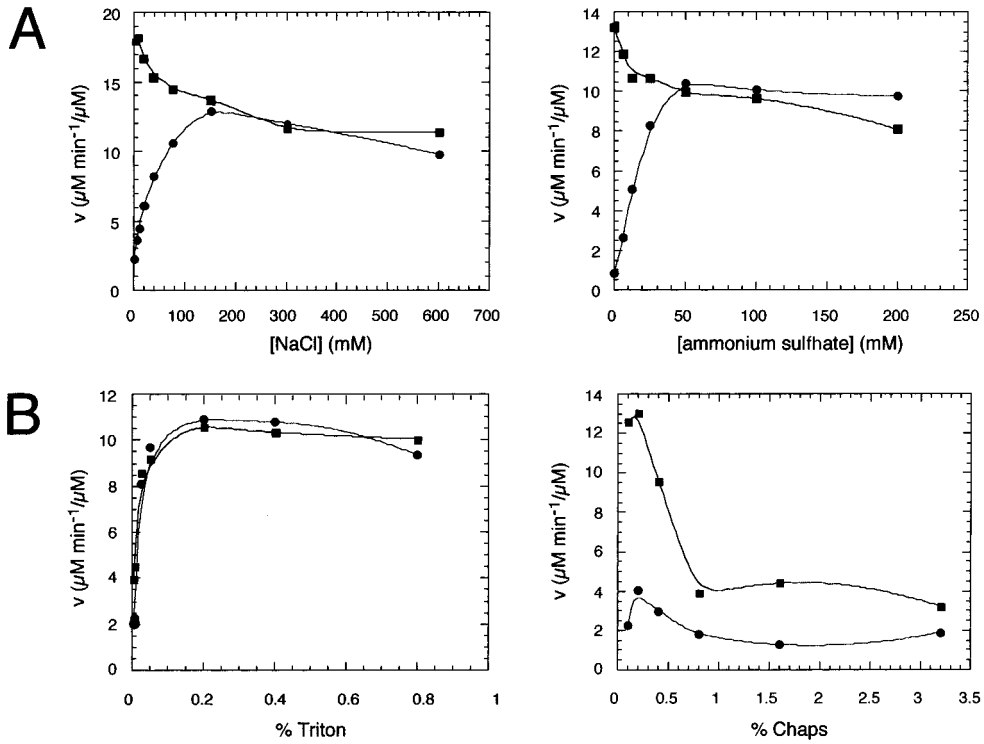

FIGURE 3: Dependence of NS3−NS4A protease activity on ionic strength and detergents. The NS3−NS4A complex (2 nM) was incubated with 10 μM NS5A−NS5B peptide substrate in the absence (●) or presence (■) of 16 μM Pep4AK in 50 mM Hepes (pH 7.5), 10 mM DTT, and 15% glycerol. Reactions were carried out at 23 °C for 10 min and stopped by addition of 1% TFA. Cleavage products were analyzed and quantified by HPLC. (A) Buffer conditions included 0.1% Triton X-100 and increasing concentrations of NaCl (left panel) or ammonium sulfate (right panel). The effect of the ionic strength was evaluated in the absence or presence of Pep4AK. (B) Conditions including 0.15 M NaCl and increasing concentrations of Triton X-100 (left panel) or CHAPS (right panel) were analyzed in the absence or presence of Pep4AK.

Table 2: Comparison between NS3−NS4A and FL NS3−pep4AK Protease Activities

	k_{cat} (min^{-1})		K_m (μM)		k_{cat}/K_m (M^{-1}, s^{-1})	
complex	0 M NaCl	0.15 M NaCl	0 M NaCl	0.15 M NaCl	0 M NaCl	0.15 M NaCl
NS3−NS4A	−	33.7	−	10	−	55780
HisNS3−NS4A	−	31.4	−	9.8	−	53467
FL NS3−pep4AK	23.4	26.7	7	41	55180	10767

Triton X-100 at concentrations close to and above its cmc (0.05%) stimulated the NS3−NS4A proteolytic activity by approximately 5-fold (Figure 3B, left panel). LDAO and *n*-dodecyl β-D-maltoside exhibited a similar effect (data not shown). Since in these experiments the curves in the presence or in the absence of Pep4AK were superimposable, the observed increase in trans-cleavage efficiency was probably not due to the stabilization of the complex by the detergent, but most likely to the prevention of NS3−NS4A aggregation. In general, nonionic detergents with a low cmc were more effective than zwitterionic detergents with a high cmc. In particular, CHAPS stimulated NS3−NS4A protease activity only 2-fold at concentrations below its cmc (0.5%) and became inhibitory at higher concentrations. Furthermore, a dissociating effect on the native complex was also observed (Figure 3B, right panel). In summary, we have assessed the minimal ionic strength and detergent and glycerol concentrations that are compatible with the maximal protease activity and stability of the NS3−NS4A complex. On this basis, reaction conditions were determined [50 mM Hepes or Tris (pH 7.5), 0.15 M NaCl, 0.1% Triton X-100, 15% glycerol, and 10 mM DTT] under which NS3−NS4A proteolytic activity reached optimized levels and was not stimulated by an excess of Pep4AK peptide. The latter, on the contrary, could greatly enhance FL NS3 trans-cleavage efficiency.

We have analyzed the time course of the NS3−NS4A-catalyzed cleavage of the NS5A−NS5B peptide at a substrate concentration of 10 μM and 2 nM enzyme under optimized conditions. No significant loss of activity was observed during incubation for up to 1 h (data not shown), thus indicating that the complex was substantially stable under the assay conditions that were used.

Kinetic Analysis of NS3−NS4A Protease Activity. We have performed a kinetic analysis of the cleavage reaction of the NS5A−NS5B peptide substrate under the optimized conditions, in the absence or presence of the Pep4AK cofactor using both NS3−NS4A (Figure 4A, left panel) and HisNS3−NS4A (not shown) purified enzymes. We obtained similar results for the two complex forms: $K_m = 10$ μM and $k_{cat} =$ 33.7 and 31.4 min^{-1}, respectively (see Table 2). These values were not significantly altered by the addition of Pep4AK (Figure 4A, left panel), thus demonstrating that the stability of the complex was independent of the substrate concentration. The absence of Pep4AK-induced protease stimulation at all substrate concentrations tested also suggested a 1:1 stoichiometry of NS3 and NS4A in both complex preparations. The trans-cleavage efficiency of both complex forms was similar under the same experimental conditions, thus indicating that the six-histidine tag at the amino-terminus of NS3 did not interfere with the correct folding or with the stability of the NS3−NS4A interaction. The turnover value

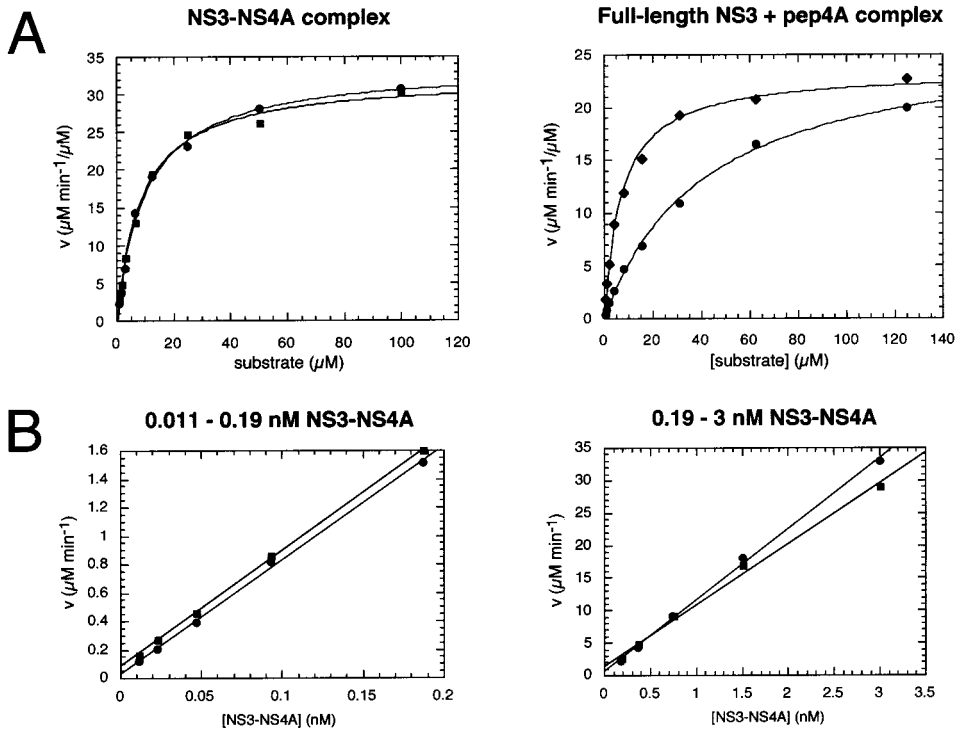

FIGURE 4: Steady state kinetic analysis of NS5A−NS5B cleavage by NS3−NS4A and FL NS3 (A) and the dependence of the initial velocity of NS3−NS4A protease activity on enzyme concentration (B). (A) NS3−NS4A (2 nM) (left panel) was incubated under optimized standard conditions [50 mM Hepes (pH 7.5), 15% glycerol, 0.15 M NaCl, 0.1% Triton X-100, and 10 mM DTT] at 23 °C for 10 min in the absence (●) or presence (■) of 16 μM Pep4AK, and increasing concentrations of NS5A−NS5B peptide substrate were added. Eight data points at substrate concentrations between 0.78 and 100 μM were used to calculate the kinetic parameters. Initial rates of cleavage were determined on samples exhibiting <10% substrate conversion. Kinetic parameters in the absence of Pep4AK (K_m = 10 μM and k_{cat} = 33.7 min^{-1}) were calculated from a nonlinear least-squares fit of the initial rates as a function of substrate concentration, assuming Michaelis−Menten kinetics. FL NS3 (2 nM) (right panel) was incubated in activity buffer containing either 0 (◆) or 0.15 M NaCl (●) and 16 μM Pep4AK. Nine data points at substrate concentrations between 0.47 and 120 μM were analyzed. Kinetic parameters (for 0 M NaCl, K_m = 7 μM and k_{cat} = 23.4 min^{-1}; for 0.15 M NaCl, K_m = 41 μM and k_{cat} = 26.7 min^{-1}) were calculated as described above. (B) NS3−NS4A (0.011−0.19 nM, 1 h assay, left panel; 0.19−3 nM, 10 min assay, right panel) was incubated under optimized standard conditions with 10 μM NS5A−NS5B substrate in the absence (●) or presence (■) of 16 μM Pep4AK at 23 °C.

associated with NS3−NS4A was similar to that observed with the Pep4AK-activated FL NS3 under both low- and high-ionic strength conditions (right panel of Figure 4A and Table 2). On the contrary, the affinity of the FL NS3−Pep4AK complex for the NS5A−NS5B peptide substrate was approximately 6-fold lower when measured in 0.15 M NaCl, thus suggesting that differences in the mode of interaction with the substrate might exist between the NS3−NS4A and FL NS3−Pep4AK enzymes.

The protease activity of NS3−NS4A was linearly dependent on the concentration of the complex between 3 nM and 11 pM (Figure 4B), thus suggesting that the dissociation constant of the NS3−NS4A complex is at least 5 orders of magnitude lower than the K_d of the FL NS3−Pep4AK complex calculated from Pep4AK titration curves under the same conditions (K_d = 0.8 μM; data not shown). Table 2 shows a comparison between the kinetic parameters obtained using the two NS3−NS4A complex forms and Pep4AK-stimulated FL NS3.

Reversibility of the NS3−NS4A Association. We have analyzed the reversibility of NS3 and NS4A association by incubating the recombinant complex at a concentration of 100 nM in the protease activity buffer containing 0 or 0.15 M NaCl for 12 h at 4 °C (Figure 5A). On the basis of the results previously discussed, we expected that during this time of incubation dissociation of NS3 and NS4A would occur in 0 M NaCl, but not in 0.15 M NaCl. We then diluted each protein sample to 2 nM, in both 0 and 0.15 M NaCl-containing activity buffers. After a further incubation for 5 h at 4 °C, we tested them for protease activity on the NS5A−NS5B peptide substrate in the absence or presence of saturating concentrations of Pep4AK. Standard NS3−NS4A protease control reactions were performed, in which a fresh protein aliquot was diluted to 2 nM and immediately tested for activity. The result of this experiment indicates that NS3−NS4A association and dissociation can be fully reversed by changing the salt concentration. In fact, both the low- and high-ionic strength samples exhibited maximal activity and no Pep4AK-mediated stimulation when subsequently incubated in 0.15 M NaCl. Conversely, only a low basal activity with a significant Pep4AK-mediated stimulation was observed when both samples were further incubated and assayed under the lower-ionic strength conditions. Compared with the control reaction, no significant decrease in NS3−NS4A cleavage efficiency was observed in both the low- and high-ionic strength protein samples, thus indicating that the enzyme was not inactivated or degraded during the long preincubation times used in this experiment.

To confirm and extend the result discussed above, we performed a different experiment to directly demonstrate that NS3−NS4A dissociation effectively occurs only under low-salt conditions. The protein complex was incubated at 4 °C with Pep4AK-conjugated beads, containing a vast excess of the NS4A-derived peptide, in 0 or 0.15 M NaCl. The amount

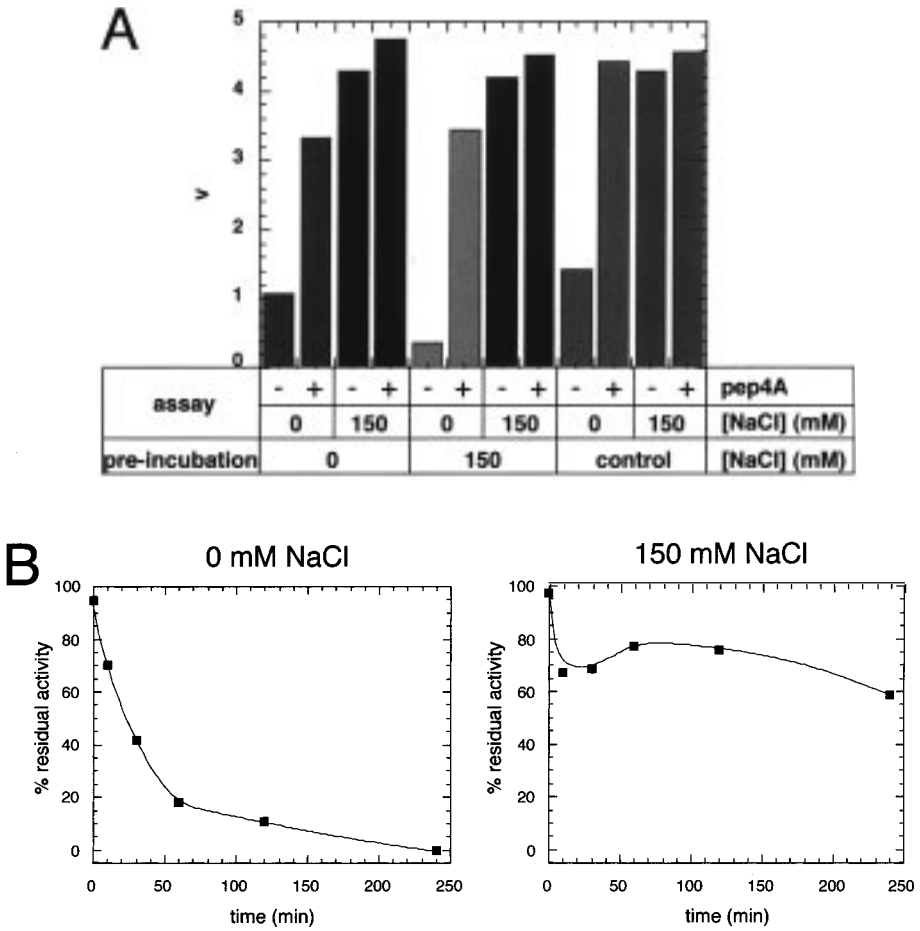

FIGURE 5: Analysis of the reversibility of NS3 and NS4A association. (A) Pure NS3−NS4A (100 nM) was incubated in protease activity buffer containing 0 or 0.15 M NaCl for 12 h at 4 °C. Both samples were diluted to 2 nM in both 0 and 0.15 M NaCl-containing activity buffers and further incubated for 5 h at 4 °C. Then 10 μM NS5A−NS5B peptide substrate was added and the protease activity tested in the absence (− Pep4A) or presence (+ Pep4A) of 16 μM Pep4AK for 10 min at 23 °C. Control reactions were performed by diluting a fresh NS3−NS4A protein aliquot to a concentration of 2 nM in both 0 and 0.15 M NaCl-containing activity buffers and in the absence or presence of 16 μM Pep4AK. Protease activity was analyzed as described above. (B) Pure NS3−NS4A complex (12 nM) was incubated at 4 °C with 40 μL of Pep4A-conjugated Affigel beads in 400 μL of both 0 and 0.15 M NaCl-containing activity buffers ([Pep4AK] = 50 μM). After increasing amounts of time, 10 μL aliquots were withdrawn and tested for proteolytic activity in a standard reaction mixture (60 μL) containing 0.15 M NaCl and 10 μM NS5A−NS5B substrate, as described above.

of NS3−NS4A in solution was estimated at different time points by a standard protease assay. In control experiments, quantitative binding of FL NS3 to the Pep4AK matrix was observed under the same buffer conditions after incubation for 4 h (data not shown). As shown in Figure 5B, a time-dependent decrease in the NS3−NS4A protease activity was observed after incubation under low-salt conditions (left panel), as an effect of NS3−NS4A dissociation and consequent depletion of free NS3 in solution upon binding to the Pep4AK matrix. This process was complete after approximately 4 h when the activity measured was reduced to background levels. The half-life of the complex could be estimated to be around 20 min under these experimental conditions. On the contrary, no significant decrease in the protease activity was observed after incubation under the higher-salt conditions (right panel), indicating that no exchange of free NS3 occurred due to the higher stability of the complex.

Western blot analysis using anti-NS3 and anti-NS4A antibodies confirmed that, after 4 h of incubation of NS3−NS4A with pep4AK-conjugated beads under low-salt conditions, free NS3 was quantitatively recovered in the matrix-bound fraction whereas NS3−NS4A was quantitatively recovered in the supernatant upon incubation in 0.15 M NaCl (data not shown).

Effect of the Interaction with NS4A on NS3 Helicase, ATPase, and ssRNA Binding Activities. To ascertain whether the heteromeric NS3−NS4A complex possesses an unwinding activity comparable with that associated with the FL NS3 protein, we attempted to devise new assay conditions that are compatible with the NS3 helicase function and under which the complex was not dissociated or aggregated. We have previously determined the optimal reaction conditions for maximal helicase activity of FL NS3 (*47*) and demonstrated that increasing concentrations of monovalent cations dramatically decrease the efficiency of unwinding. At 0.15 M NaCl, the minimal salt concentration that is compatible with NS3−NS4A complex stability (Figure 3A), NS3 helicase activity already reached background levels.

Therefore, we decided to compare NS3−NS4A and NS3 RNA helicase activities under suboptimal ionic strength conditions (0.075 M NaCl), where the complex was only moderately dissociated (Figure 3A) and NS3-associated helicase activity could still be measured. In a typical unwinding assay, protein titration was performed in 25 mM MOPS (pH 7), 0.1% Triton X-100, 10% glycerol, 100 μg/

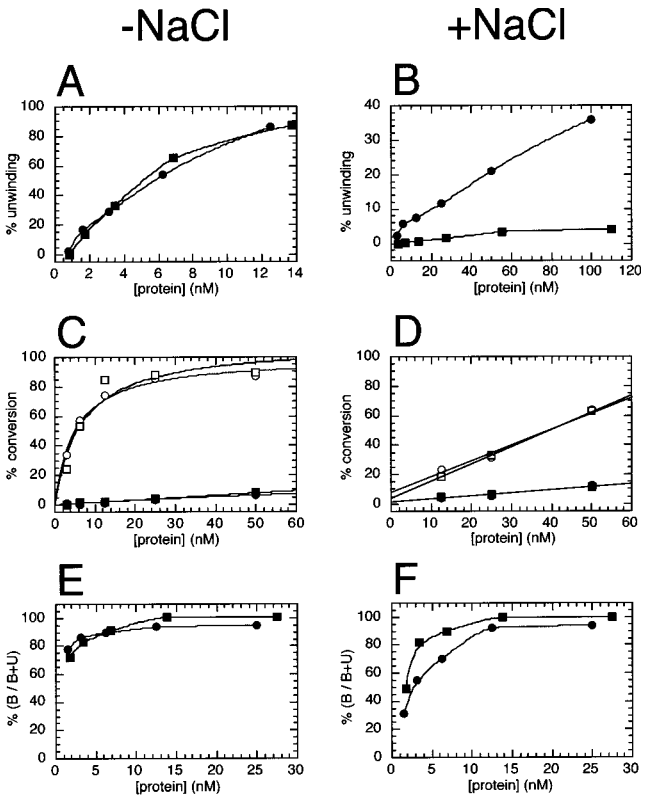

FIGURE 6: Helicase, ATPase, and RNA-binding activity of NS3−NS4A and FL NS3 proteins in the absence or presence of NaCl. NS3−NS4A (squares) and FL NS3 (circles) stock solutions were diluted in 25 mM MOPS (pH 7), 50% glycerol, 0.5 mg/mL BSA, 0.5% Triton X-100, 3 mM DTT, and 0 (A) or 0.375 M (B) NaCl, yielding a series of suitable diluted solutions. Four microliters of each of these dilutions was incubated with 1.25 nM ^{32}P-labeled partial duplex RNA substrate in a final volume of 20 μL under standard reaction conditions, as described in Materials and Methods. Six protein concentrations were analyzed in the absence of NaCl (0.86−13.75 nM NS3−NS4A and 0.78−12.5 nM FL NS3) (A) and in the presence of 0.075 M NaCl (3.44−110 nM NS3−NS4A and 3.125−100 nM FL NS3) (B). Eight-microliter aliquots were electrophoresed in a native 8% polyacrylamide gel containing 0.5× Tris-borate-EDTA. ATPase activity was analyzed by incubating increasing concentrations of NS3−NS4A and FL NS3 for 30 min at 37 °C under the helicase conditions described above and in the absence (C) or presence (D) of 0.15 M NaCl. ATP hot/cold mix (1 mM) containing 2 μCi of [γ-^{32}P]ATP was added in a final volume of 10 μL. Protein titration assays were carried out by incubating 3.125−50 nM (C) and 12.5−50 nM (D) NS3−NS4A or NS3 enzyme with (□ and ○) or without (■ and ●) 0.1 mM poly(U). Increasing concentrations of NS3−NS4A (1.7−27.5 nM) and FL NS3 (1.56−25 nM) were analyzed in RNA-binding reactions carried out in helicase reaction mixtures (20 μL) containing 1.25 nM ^{32}P 5′ end-labeled 40-mer ssRNA oligonucleotide corresponding to the template strand in the absence of $MgCl_2$ and ATP. After incubation for 20 min at 23 °C in the absence (E) or presence (F) of 0.15 M NaCl, 6 μL aliquots were electrophoresed in a native 6% polyacrylamide gel containing 0.25× Tris-borate-EDTA.

mL BSA, 3 mM $MgCl_2$, 1.25 nM partially double-stranded RNA (dsRNA) substrate, and 5 mM ATP in the absence (Figure 6A) or in the presence of 0.075 M NaCl (Figure 6B). The complex showed a helicase activity similar to that observed with NS3 alone only in the absence of NaCl, which can presumably be attributed to free NS3 that is dissociated from the NS4A cofactor (Figure 6A). The presence of the C1454S mutation in the helicase domain of this protein did not affect its unwinding efficiency. On the contrary, in the presence of 0.075 M NaCl (condition in which the complex stability was not strongly impaired), uncomplexed FL NS3 still displayed 50% of its maximal unwinding capacity, whereas only background levels could be detected with the NS3−NS4A complex up to a concentration of 110 nM (Figure 6B). This result suggests that no intrinsic helicase activity is associated with the heteromeric NS3−NS4A complex, although the NS3 protein released from the binding to NS4A fully retains the capacity of unwinding dsRNA at maximal levels under optimal conditions.

To evaluate whether the lack of duplex unwinding function exhibited by the complex could be attributed to a defect in its capacity to hydrolyze ATP, we analyzed the basal and poly(U)-induced ATPase activity of both FL NS3 and NS3−NS4A proteins under the same conditions used to assay their helicase activities and in the presence or absence of 0.15 M NaCl (panels C and D of Figure 6, respectively). A higher salt concentration could be used in these experiments since both basal and poly(U)-stimulated ATPase activities are considerably less sensitive to high ionic strength than the helicase activity. No difference in the efficiency of ATP hydrolysis was detected by comparing the titration curves of FL NS3 and NS3−NS4A at both low and high NaCl concentrations, and the degree of stimulation induced by poly(U) was also similar. These results indicate that the interaction of NS4A with NS3 does not affect the NS3 NTPase function.

Then we analyzed the RNA binding activity of NS3 and NS3−NS4A on the ^{32}P 5′-labeled 40-mer ssRNA oligonucleotide corresponding to the template strand under low- and high-salt conditions. The results of gel retardation experiments are summarized in panels E and F of Figure 6. Consistent with the moderate effect of high ionic strength on its poly(U)-stimulated ATPase activity, NS3 was able to form a stable complex with the ssRNA probe also in the presence of 0.15 M NaCl. The ssRNA-binding activity associated with the NS3−NS4A heterodimer was similar to that measured with NS3 under these conditions. Analogous results were obtained when the partial dsRNA helicase substrate was used as a probe (data not shown).

Taken together, these results suggest that the association with NS4A does not significantly influence NS3's ability to bind ssRNA and hydrolyze ATP, but does impair its RNA unwinding function. It is possible that the interaction with NS4A might uncouple NS3 ATPase and helicase activities by inducing structural changes in the NS3 C-terminal domain which result in a conformation that is less suitable for translocation on the RNA duplex, strand separation, or release of the ssRNA product. This would imply an interaction and a mutual influence of the N-terminal protease domain and the C-terminal helicase domain of NS3, modulated by the association with the NS4A cofactor.

DISCUSSION

We have devised a protocol for producing in *E. coli* milligram amounts of the full-length NS3−NS4A protein complex from the 1b HCV genotype. We have expressed two different constructs, one containing a six-histidine tag at the amino terminus of NS3 and the other retaining the native NS3 N-terminal sequence. In both versions, a C1454S mutation was introduced to prevent an NS4A-dependent NS3

autolytic cleavage within the helicase domain. We have previously produced the NS3 full-length protein in bacteria (*47*). Here we employed the same expression protocol, consisting of induction of expression at low temperatures in a defined minimal medium. We have found that both NS3−NS4A precursors were efficiently processed in prokaryotic cells at the natural NS3−NS4A cis-cleavage site, resulting in NS3 and NS4A polypeptides with the expected molecular masses. Consistent with the NS4A-mediated association of the complex with the membrane compartment, a nonionic detergent at a concentration higher than the cmc was strictly required for the extraction of NS3−NS4A in a soluble form and during further purification steps. NS3 and NS4A were copurified through all the purification procedures described in this work, leading to several milligrams of >80% pure noncovalent complex from 1 L of culture, an amount that is suitable both for enzymological studies and for the screening of potential enzyme inhibitors.

We have previously investigated the effect of a synthetic NS4A-derived core peptide on the protease activity of the isolated FL NS3 (*47*). Purified FL NS3 displayed an extremely low basal activity; the trans-cleavage activity of the full-length NS3 protein was in fact increased about 25-fold in the presence of an NS4A-derived peptide. Conversely, in this study we have shown that the NS3−NS4A purified complex possessed a very high intrinsic proteolytic activity that was not affected by the addition of saturating amounts of the peptide cofactor. The above observation led us to believe that NS3 and NS4A are present in a 1:1 stoichiometry in our complex preparations.

We have found that the dependence of NS3−NS4A protease activity on the presence of detergents correlated closely with their effect on the aggregation state of the complex, thus confirming previously published results (*37*). The highest proteolytic activity was measured with nonionic detergents such as Triton X-100, LDAO, or *n*-dodecyl β-D-maltoside at concentrations that were greater than their cmc, a condition under which NS3−NS4A was in the form of a nonaggregated heteromeric complex. The observed dependence of the protein aggregation state on the nature and concentration of the detergent was a peculiarity of the NS3−NS4A complex, since FL NS3 alone was found in a monomeric form even in the absence of detergents. NS4A contains an N-terminal hydrophobic region which is thought to be responsible for anchoring the NS3−NS4A complex to the cell membrane. It is therefore conceivable that the inclusion in a detergent micelle of the NS4A N-terminal hydrophobic portion potentially exposed to the solvent could be crucial for the solubilization of the complex and thus for its optimal proteolytic activity. Indeed, the complex eluted in gel filtration experiments with an apparent molecular mass of approximately 150 kDa, consistent with the association of an NS3−NS4A molecule with a micelle of detergent.

Notably, we have found that the dependence of NS3−NS4A protease activity on the salt concentration in the assay is readily explained by the effect of the ionic strength on the stability of the association between the NS3 and the NS4A proteins. Decreasing the salt concentration below a critical ionic strength value ($I = 0.12-0.15$) causes the dissociation of the NS3−NS4A complex with a consequent decrease in its proteolytic activity. This interpretation differs from that given in previously published reports (*37*) in which the dependence of NS3−NS4A protease activity on salt concentration has instead been correlated with the aggregation state of the complex. Furthermore, we have demonstrated that the association of NS3 and NS4A is very tight under physiological ionic strengths and that dissociation induced by lowering the salt concentration is fully reversible. The biological significance of this observation remains obscure and relates to the question of why NS4A evolved as a separate protein despite being an integral part of the NS3 serine protease structure.

Using a peptide substrate derived from the NS5A−NS5B junction and under optimized reaction conditions, the catalytic efficiency (k_{cat}/K_m) of the NS3−NS4A complex (with or without the N-terminal His tag) was 55 000 s^{-1} M^{-1}, a value very similar to that measured with Pep4A-activated FL NS3 on the same substrate under low-ionic strength conditions. This direct comparison suggests that the N-terminal and C-terminal ends of full-length NS4A do not significantly contribute to the stimulation of FL NS3 trans-cleavage activity. Nevertheless, the affinity of the FL NS3−Pep4AK complex for the NS5A−NS5B peptide substrate was approximately 6-fold lower than that measured for the NS3−NS4A complex in 0.15 M NaCl. This finding suggests that subtle differences in the mode of interaction with the substrate might exist between the NS3−NS4A and the NS3−Pep4AK complexes.

In agreement with the tight binding suggested by the structure, our data indicate that under optimized conditions the dissociation constant of NS3 and NS4A in the native complex stands in the low picomolar range. The affinity of NS3 for full-length NS4A is therefore several orders of magnitude higher than that displayed toward the NS4A-derived synthetic peptide lacking the putative transmembrane α-helix. This significant difference could be explained by a contribution of the NS4A N-terminal region to the stabilization of the complex. NS3−NS4A has been shown to be very stable in the cytoplasm of cultured cells expressing the HCV polyprotein (*31*, *32*), and it has been suggested that, while not absolutely required for complex formation and cofactor activity, certain residues in the N-terminal region of NS4A may interact with NS3 and contribute to the stability of the NS3−NS4A complex (*33*). Although highly conserved in various HCV genotypes, the 19 C-terminal residues of NS4A are not required for complex formation and proteinase activation, suggesting that this sequence has a distinct function.

NS4A has been also suggested to act as a propeptide which might assist the folding of its cognate NS3 protease and confer on it full activation upon autocleavage of the proenzyme precursor (*32*). Although NS4A may participate in NS3 folding, our evidence argues against an obligate chaperone-like role for NS4A. Since pure NS3−NS4A can be dissociated and reassociated in solution without any loss of proteolytic activity, coexpression and self-cleavage in the metabolic assembly of the functional heterodimer seem to play only a minor role, if any.

In cultured cells, the NS3−NS4A complex has been constantly found to be associated with the membrane compartment probably via the NS4A N-terminal anchoring domain (*16*). Therefore, we have compared the properties of the complex produced in bacteria to those of an NS3−NS4A complex expressed and purified from the Sf9 cell microsomal fraction, using a recombinant baculovirus vector

(data not shown). NS3−NS4A complexes from eukaryotic and prokaryotic cells exhibited identical requirements in terms of nonionic detergents and of high ionic strength for their stability and trans-cleavage activity, which were highly comparable under the same experimental conditions.

Our previous studies (*47*) demonstrated that the ATPase and helicase activities exhibited by full-length NS3 did not differ significantly from those associated with the individual helicase domain. Despite this apparent lack of functional interdependence between the N-terminal and C-terminal domains of NS3, there is no evidence indicating that these two regions are cleaved during the virus life cycle. To provide insight toward the elucidation of the physiological role of NS3 during HCV viral infection, we have tried to ascertain whether the NS3 helicase activity has any interdependent regulation via the association of NS4A with the NS3 protease domain. Several reports (*35*−*37*) have recently demonstrated an RNA helicase activity associated with NS3−NS4A complexes purified from eukaryotic cells, measured in the absence of nonionic detergents and under low-ionic strength conditions (i.e., conditions under which the NS3−NS4A complex is most likely dissociated). In contrast with the previously published studies, our data indicate that the NS4A-mediated stabilization of the FL NS3 enzyme in the active serine protease conformation could negatively affect its capacity to unwind dsRNA. The evidence we presented in this work supports a model in which NS3 could assume two alternative, mutually exclusive conformations; activation of the serine protease enzyme would be triggered by the interaction with the NS4A cofactor, whereas dissociation of the NS3−NS4A complex might instead be required for NS3 to perform the helicase function. In addition, our results would suggest that the interaction with NS4A might uncouple NS3 ATPase and helicase activities by inducing structural changes in the NS3 C-terminal domain which result in a conformation that is less suitable for translocation on the RNA duplex, strand separation, or release of the ssRNA product.

At present, the mechanism of replication of the HCV genome remains unknown, due to the lack of an appropriate in vitro cell culture system. Nevertheless, it has been recently demonstrated that NS5B, the RNA-dependent RNA polymerase, can form protein complexes in cells also with singularly expressed NS3 or NS4A polypeptides and that the association with either protein regulates its subcellular localization (*65*). Furthermore, it was also reported that the NS3 protein is present in both the cytoplasmic and nuclear compartments and that the nuclear localization of NS3 is suppressed by NS4A interaction but stimulated by p53 interaction (*66*, *67*). Therefore, NS3 enzymatic activities could be regulated in cells via different alternative protein−protein interactions which may also affect its intracellular localization.

The elucidation of such regulatory pathways will require the development of methods for studying the HCV RNA replication machinery and more detailed insight into the role of NS3 in the control of the infected cell functions.

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