Hepatitis C Virus NS3 Helicase Forms Oligomeric Structures That Exhibit Optimal DNA Unwinding Activity in Vitro

HCV NS3 helicase exhibits activity toward DNA and RNA substrates. The DNA helicase activity of NS3 has been proposed to be optimal when multiple NS3 molecules are bound to the same substrate molecule. NS3 catalyzes little or no measurable DNA unwinding under single cycle conditions in which the concentration of substrate exceeds the concentration of enzyme by 5-fold. However, when NS3 (100 nm) is equimolar with the substrate, a small burst amplitude of ~8 nm is observed. The burst amplitude increases as the enzyme concentration increases, consistent with the idea that multiple molecules are needed for optimal unwinding. Protein-protein interactions may facilitate optimal activity, so the oligomeric properties of the enzyme were investigated. Chemical cross-linking indicates that full-length NS3 forms higher order oligomers much more readily than the NS3 helicase domain. Dynamic light scattering indicates that full-length NS3 exists as an oligomer, whereas NS3 helicase domain exists in a monomeric form in solution. Size exclusion chromatography also indicates that full-length NS3 behaves as an oligomer in solution, whereas the NS3 helicase domain behaves as a monomer. When NS3 was passed through a small pore filter capable of removing protein aggregates, greater than 95% of the protein and the DNA unwinding activity was removed from solution. In contrast, only ~10% of NS3 helicase domain and ~20% of the associated DNA unwinding activity was removed from solution after passage through the small pore filter. The results indicate that the optimally active form of full-length NS3 is part of an oligomeric species in vitro.

Helicases are molecular motors that catalyze unwinding of double-stranded DNA or RNA by converting chemical energy from ATP hydrolysis into mechanical energy for nucleic acid strand separation. Helicases are required for virtually all cellular processes involving nucleic acids, including replication, transcription, translation, repair, and recombination (1–4). Several diseases characterized by premature aging and increased incidence of cancer, including Bloom and Werner syndromes, have been linked to mutations in helicase genes (5).

The quaternary structure of the active forms of helicases varies considerably, with some requiring dimer or oligomer formation for strand separation activity and others functioning efficiently as monomers. Bacteriophage T4 gp41 helicase, for example, forms a hexameric structure that sequesters single-stranded DNA by encircling it (6). Numerous other helicases function as hexamers (7). Bacteriophage T4 Dda helicase is active as a monomer (8) but unwinds DNA substrates more efficiently under conditions that allow binding of multiple helicase molecules per substrate molecule (9). Escherichia coli helicase Tral, also called helicase 1, can function as a robust, highly processive helicase as a monomer (10). In order to understand the mechanism by which helicases unwind duplex DNA, the quaternary structure of the active species must be known.

More than 170 million people worldwide are infected with the hepatitis C virus (HCV), predominantly through blood-borne routes, such as contaminated blood/blood product transfusion and shared needle injection (11). The great majority (85%) of patients develop chronic HCV infection characterized by subclinical but persistent and progressive inflammation and fibrosis of the liver, which can ultimately result in liver cirrhosis, hepatic failure, or hepatocellular carcinoma. HCV infection has become the primary reason for liver transplants among adults in Western countries (12). Moreover, current therapy is inadequate in treating all patients afflicted with chronic HCV, and an urgent medical need exists for an effective anti-HCV agent (13). HCV contains a positive single-stranded RNA genome that encodes a polyprotein of about 3010 amino acids that is processed into structural (capsid, E1, E2, and p7) and nonstructural (NS2, NS3, NS4a, NS4b, NS5a, and NS5b) proteins by cellular and virus-encoded proteases (14). The NS3 protein is a 631-amino acid residue bifunctional enzyme with a serine protease localized to the amino-terminal 180 residues...
and an NTPase–RNA helicase located in the carboxyl-terminal 451 residues (15) and is an attractive target for development of an antiviral agent for HCV (16). NS3 helicase activity was identified; the associated protein was purified more than 10 years ago (17), and the crystal structures of NS3 helicase domain (NS3h) and full-length NS3 have been reported (18–20). However, the kinetic and chemical mechanisms for NS3h or full-length NS3 remain an active area of investigation.

The full-length form of HCV NS3 is a bifunctional enzyme consisting of protease and helicase domains. Both protease and helicase activities are required for viral replication. NS3 has a 3′- to 5′ directional bias, unwinds both RNA and DNA, and binds preferentially to U-rich or T-rich nucleic acid substrates (21–23). Some studies have focused on the activity of NS3h regarding the oligomeric state of full-length NS3 by using different approaches. Tackett et al. (26) have reported that binding of multiple NS3 molecules to the same substrate molecule is required for optimal unwinding of a 30-bp DNA duplex. Serebrov and Pyle (27) have reported that, based on comparison of concentration-dependent processivity and binding data, the active form of NS3 helicase is a dimer with an 18-bp kinetic step size. Dumont et al. (28) have reported that NS3 is active as a monomer with an 11-bp kinetic step size in optical trap unwinding experiments. Regarding the helicase domain, Levin et al. (40) have reported a dominant negative effect of an ATPase-deficient mutant on NS3h helicase unwinding, suggesting that multiple molecules bound to the same substrate can influence NS3h activity. The results for NS3h have been interpreted in terms of functional cooperativity, which does not rely on strong protein–protein interactions. In the functional cooperativity model, NS3h is active as a monomer, but the presence of multiple molecules increases processivity. A similar model has been put forth for Dda helicase (9, 30–32).

Frick et al. (33) compared the DNA and RNA unwinding activities of NS3 and NS3h from HCV genotype 1a, leading to the conclusion that the full-length protein unwinds RNA better than the helicase domain alone. Hence, the presence of the protease domain clearly influences the interaction of NS3 with nucleic acid. The protease domain has also been reported to facilitate dimerization of NS3 (34). In this work, we have investigated the quaternary form of NS3 by using chemical cross-linking and biophysical approaches as well as DNA unwinding measurements under pre-steady state conditions.

**EXPERIMENTAL PROCEDURES**

**Materials**—A high pressure liquid chromatography column (Bio–Sil SEC 250–5 column) and gel filtration molecular weight marker standard (molecular weight range 1,350–670,000) were purchased from Bio-Rad. HEPES, β-mercaptoethanol, SDS, MOPS, Tris, NaCl, Na$_2$EDTA, BSA, acrylamide, bisacrylamide, MgCl$_2$, KOH, formamide, xylene cyanol, bromphenol blue, urea, and glycerol were purchased from Fisher. BSA$^\alpha_2$ was purchased from Pierce. The Silver Stain Plus kit and 4–15% Tris–Cl Ready Gels were purchased from Bio-Rad. Sephadex G-25, PK/LDH, NADH, ATP, and PEP were from Sigma. Polyuridine was purchased from Amersham Biosciences. DNA oligonucleotides were from Integrated DNA Technologies and purified by preparative gel electrophoresis. [$\gamma$-$^32$P]ATP was purchased from PerkinElmer Life Sciences. T4 polynucleotide kinase was obtained from New England Biolabs. Recombinant full-length NS3 was derived from the HCV Con 1b replicon consensus sequence and was purified as described (26). NS3h was also derived from the HCV Con 1b replicon consensus sequence and was purified as described (35).

**Steady State DNA Unwinding**—NS3 (100 nm) was prepared in 25 mM MOPS (pH 7.0), 10 mM NaCl, 0.1 mM EDTA (pH 8.0), 2 mM β-mercaptoethanol, and 0.1 mg/ml BSA. DNA substrate (15 nt/30 bp, the overhang strand radiolabeled with $^32$P) was added to 100 nm, and the mixture was incubated at 37 °C for 5 min. The unwinding reaction was initiated by the addition of 5 mM ATP, 10 mM MgCl$_2$ (unless otherwise specified), and a 30-fold excess of DNA trap to bind the displaced strand and prevent renatenable to the radiolabeled product. After 1–10 min, a 10-μl aliquot of the reaction mixture was transferred to a centrifuge tube containing 200 mM EDTA, 0.7% SDS, 0.1% bromphenol blue, 0.1% xylene cyanol, and 6% glycerol. The double- and single-stranded DNA were resolved via native 20% polyacrylamide gel. The radiolabeled substrate and product were detected using a PhosphorImager (Amersham Biosciences). Quantitation was performed with ImageQuant software (GE Healthcare), and the ratio of single- to double-stranded DNA was plotted as a function of time. Data were fit to a linear function using Kaleidagraph (Synergy Software, Reading, PA).

**Single Turnover and Pre-steady State DNA Unwinding**—NS3 (500 nm) and DNA substrate (2 mM 15 nt/30 bp, the longer strand radiolabeled with $^32$P) were prepared in 25 mM MOPS (pH 7.0), 10 mM NaCl, 0.1 mM EDTA (pH 8.0), 2 mM β-mercaptoethanol, and 0.1 mg/ml BSA. Reaction components were incubated at 37 °C using a circulating water bath. The unwinding reaction was initiated by the rapid addition of 5 mM ATP, 10 mM MgCl$_2$ (unless otherwise specified), 30-fold excess DNA trap, and 10-fold excess polyuridine protein trap using an RQF-3 Rapid Quench Flow instrument (KinTek Corp., Austin, TX). The reaction was quenched after 0.1–10 s by adding 200 mM EDTA and 0.7% SDS. Bromphenol blue (0.1%), 0.1% xylene cyanol, and 6% glycerol were added to each, and the double- and single-stranded DNA were resolved via native 20% polyacrylamide gel. The radiolabeled substrate and product were detected using a PhosphorImager (Amersham Biosciences). Quantitation was performed with ImageQuant software (Amersham Biosciences), and the ratio of single- to double-stranded DNA was plotted as a function of time. Data were fit to a two-step mechanism for DNA unwinding as described (26).

**Chemical Cross-linking**—Chemical cross-linking was carried out by incubating NS3/NS3h (final concentration is 2 μM) in a buffer containing 50 mM MOPS K$^+$ (pH 7.0), 10 mM NaCl at 37 °C. After 30 min, B$^\alpha_2$ (2 mM in H$_2$O) was added to a final concentration of 500 μM. Aliquots of the reaction mixture were removed at various time points, quenched by adding 1 M glycine (pH 8.0) and protein sample buffer (62.5 mM Tris–Cl (pH 6.8), 0.45% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.004% bromphenol blue), incubated at 100 °C for 5 min, analyzed by SDS-PAGE (4–15% gradient gel), and visualized by silver stain-
ing. Another chemical cross-linking was carried out by incubating NS3 (final concentration is 2 μM) in the same buffer as above along with poly(dT) (varying concentrations) at 37 °C. After 30 min, BS3 (2 mM in H2O) was added to a final concentration of 500 μM. After 20 min, the reaction was quenched. The reaction was analyzed by SDS-PAGE (4–15% gradient), and protein was visualized by silver staining.

**Dynamic Light Scattering**—The hydrodynamic radii of wild type NS3 and NS3h were measured by dynamic light scattering using a DynaPro MS/X dynamic light scattering instrument from Protein Solutions, Inc. NS3 was used at a concentration of 0.5 mg/ml, whereas NS3h was used at 1.0 mg/ml. Additionally, 50 μl of each sample was filtered through a 0.22-μm filter. The quartz cell was regulated at 20 °C. Measurements were made at a fixed angle of 90° using an incident laser beam of wavelength 830 nm. Twenty measurements were made with an acquisition time of 10 s for each measurement. The data were analyzed using the graphical size analysis software, Dynamics, provided with the instrument.

**High Pressure Gel Filtration Chromatography**—Gel filtration was carried out at room temperature using a Bio-Sil SEC 250-5 column (300 × 7.8 mm). Elution was monitored by measuring absorbance at 280 nm. The flow rate was 1 ml/min. 20 μl of molecular weight standard mixtures were injected into the column with running buffer containing 100 mM K2HPO4, KH2PO4 (pH 7.0), and 10 mM or 150 mM NaCl. NS3 or NS3h was incubated for 5 min in running buffer before being injected onto the column.

**Protein Filtration through Small Pore Filters**—Whatman Anotop disposable syringe filters (ThermoFisher) were used to remove oligomeric protein from solution for NS3 and NS3h. An aliquot of protein (100 μl) was allowed to thaw on ice and then passed through the filter (0.1 or 0.02 μm). Equal volumes of filtered or unfiltered protein were then assayed for protein concentration by using a modified Bradford assay ( Pierce).

**ATPase Assays**—Identical volumes of filtered or unfiltered NS3 or NS3h were examined for ATPase activity by using the phosphoenolpyruvate kinase/lactate dehydrogenase-coupled assay (36). The specific activity for ATP hydrolysis was calculated, assuming that the entire enzyme concentration passed through the filter. NS3 or NS3h was incubated with 5 mM ATP in 25 mM MOPS (pH 7.0), 10 mM MgCl2, 10 mM NaCl, 0.1 mg/ml BSA, 4 mM phosphoenolpyruvate, 10 units/ml pyruvate kinase, 15 units/ml lactate dehydrogenase, and 0.9 mM NADH. ATP hydrolysis rates were determined by measuring the conversion of NADH to NAD+ at 380 nm in the presence of 100 μM polyuridine. Hydrolysis rates were calculated using an extinction coefficient of 1,210 M−1 cm−1 for NADH.

**RESULTS**

**Kinetic Simulations of DNA Unwinding under Pre-steady State Conditions**—We have previously shown that the Dda and TraI helicases can unwind DNA substrates as monomeric enzymes (8, 10). We used a functional approach in which the active form of these two enzymes was determined by using an active site titration. DNA unwinding experiments were performed under pre-steady state conditions in which the substrate concentration exceeded the enzyme concentration. Under pre-steady state conditions, the quantity of product in the first cycle of DNA unwinding is reflective of the quantity of active enzyme and is referred to as the burst amplitude of the reaction. For Dda and TraI, the burst amplitude was similar to the concentration of enzyme, indicating that each helicase was capable of unwinding DNA in a monomeric form. Dda is only capable of unwinding short duplexes (low processivity), whereas TraI appears to be capable of unwinding much longer duplexes (high processivity).

A kinetic model for a two-step process for unwinding a 30-bp DNA substrate is shown in Fig. 1A. The model was designed to simulate DNA unwinding by NS3 helicase. Only two kinetic steps are required for unwinding a 30-bp substrate, because NS3 has a very large kinetic step size of ~18 bp (27); however, smaller, physical steps occur during the slow, rate-limiting kinetic step (28, 37). The kinetic scheme allows the enzyme to dissociate from the substrate according to rate constant ket, or unwind the substrate to form a partially unwound intermediate, ES*. The enzyme substrate intermediate can dissociate or undergo a second unwinding step to produce product. For the kinetic simulation, ket was set at 2.0 s−1, which was the fastest rate constant that was measured for DNA unwinding in a previous report (26). The concentration of enzyme substrate complex (ES) was set at 50 nM, based on preincubation of 50 nM enzyme with excess concentration of substrate (250 nM). The dissociation rate constant was initially set at 0 s−1, which simulates an infinitely processive helicase. If NS3 is assumed to function as a monomer, then the kinetic simulation shows clearly that 50 nM NS3 should produce 50 nM ssDNA product under these conditions (Fig. 1B, filled squares). As the dissociation constant increases from 0 to 8 s−1, the quantity of product declines. Hence, a helicase can produce less than the expected burst amplitude due to low processivity.

NS3 can unwind most of the 30-bp substrate (15 nt/30 bp) under single turnover conditions in the presence of a large excess of enzyme concentration over substrate concentration (Fig. 1C) (26). The fact that most of the substrate (~80%) was converted to product in the presence of excess enzyme indicates that NS3 can be relatively processive, but it says nothing about the form of the protein that is responsible for unwinding the substrate, because the enzyme is in excess of the substrate. When NS3 (50 nM) was incubated with excess substrate (250 nM), no product was observed (Fig. 1D, filled circles). If monomeric NS3 exhibited the level of DNA unwinding activity suggested by the data in Fig. 1C, then 40 nM ssDNA product should be observed, because ~80% of the bound enzyme would be expected to unwind the DNA. However, little or no product is actually observed under these conditions. If the protein trap is removed, then some product is observed over time (open circles in Fig. 1D), indicating that NS3 is somewhat active under these conditions. We conclude that a monomeric form of NS3 is not responsible for rapid, processive DNA unwinding as observed in Fig. 1C.

**NS3-catalyzed DNA Unwinding Is Fast and Relatively Processive When Enzyme Concentration Is in Excess of Substrate Concentration**—It is possible that the lack of a highly active monomeric form of NS3 is due to less than optimal reaction conditions. For example, binding of NS3 to DNA is sensitive to
the concentration of salt in the reaction mixture. The conditions for conducting DNA unwinding may influence the rate and amplitude of product formation when the concentration of NS3 is in excess of the substrate. We examined several different concentrations of ATP and MgCl₂. NS3 (500 nM) was incubated with the 15-nt/30-bp substrate (2 nM), and the reaction was initiated upon the addition of ATP. Similar rates and amplitudes for product formation were observed regardless of whether the concentration of ATP was equal to, greater than, or less than the concentration of MgCl₂ (supplemental Fig. 1). The addition of varying concentrations of detergent reduced the rate slightly but did not change the amplitude for unwinding (supplemental Table 1). Under all conditions studied, the fraction of substrate converted to product and the rate of unwinding were similar. Hence, the species of NS3 responsible for unwinding the 30 base pairs of DNA under conditions of excess enzyme is a relatively fast and processive form of the enzyme under a variety of conditions.

**NS3-catalyzed DNA Unwinding Exhibits a Non-zero Intercept When Substrate Is in Excess of Enzyme**—We have already shown that no burst amplitude is observed when the substrate concentration exceeds the enzyme concentration by 5-fold (Fig. 1C). If NS3 forms an active species, such as a dimer, then it might be possible to observe a burst amplitude for DNA unwinding when the enzyme concentration is equal to or in slight excess of the substrate concentration. The dissociation constant for the 15-nt/30-bp DNA substrate was previously reported to be 2.7 nM when measured by gel shift assay and 5.2 nM when measured by fluorescence polarization (26). We conducted DNA unwinding experiments at 100 nM DNA and 100 nM NS3 in order that enzyme and substrate would be well above the dissociation constant. NS3 was incubated with the 15-nt/30-bp DNA substrate followed by initiation of unwinding by mixing with ATP and MgCl₂. Varying the concentration of ATP (Fig. 2A) and MgCl₂ (Fig. 2B) and the addition of varying concentrations of detergent (Tween 20) had little effect on the steady state rate of unwinding. The observed rates for product formation ranged from 2.0 to 4.2 nM/min. These data all exhibit a non-zero intercept at the y-axis, indicating a small burst amplitude, which ranged from 8 to 16 nM. The magnitude of the burst amplitude is much lower than expected for a monomeric

![Figure 1](https://example.com/figure1.png)

**FIGURE 1.** Kinetic simulation for pre-steady state DNA unwinding by NS3. A, kinetic scheme describing DNA unwinding by NS3. Two steps are needed to unwind a 30-bp DNA substrate. NS3 can dissociate from substrate at each step. A trapping strand is included in the simulation to prevent enzyme from rebinding to substrate. B, kinetic simulation for pre-steady state unwinding of 250 nM 15-nt/30-bp partial duplex DNA by 50 nM NS3 in the presence of a protein trap (75 μM nt polyuridine). Shown are results from kinetic simulations in which the unwinding rate constant, $k_u$, was held constant at 2.0 s⁻¹, whereas the dissociation rate constant, $k_d$, was set at 0 s⁻¹ ( ), 0.4 s⁻¹ ( ), 1.0 s⁻¹ ( ), 2.0 s⁻¹ ( ), 4.0 s⁻¹ ( ), and 8.0 s⁻¹ ( ). C, single turnover conditions were used to measure DNA unwinding of a substrate containing 15 nt of ssDNA overhang and 30 bp (15 nt/30 bp). NS3 (500 nM) was incubated with the DNA substrate, and the unwinding reaction was initiated upon rapid mixing with ATP (5 mM) and MgCl₂ (10 mM). A protein trap (polyuridine, 75 μM nt polyuridine) was introduced along with the ATP. Reactions were quenched with EDTA (200 mM), and products were examined by native gel electrophoresis. The data were fit to a two-step mechanism for unwinding using the program Scientist, resulting in an observed unwinding rate ($k_u$) of 1.03 ± 0.1 s⁻¹ and an amplitude of 0.8 ± 0.1 nM. D, DNA unwinding was conducted under the same conditions as in the kinetic simulation. NS3 (50 nM) was incubated with a 15-nt/30-bp DNA substrate (250 nM), followed by initiation of the unwinding reaction by the addition of ATP (5 mM) and MgCl₂ (10 mM). Product formation was measured in the presence ( ) or absence ( ) of a protein trap (75 μM nt polyuridine).
or dimeric enzyme. If a monomer or dimer is the only active species and is the primary form of NS3 in solution, then a much higher burst amplitude would be observed based on the rate and processivity measured under conditions of excess enzyme (Fig. 1C). One possible explanation for the low burst amplitude is that the most active form of NS3 in vitro is an oligomer, thereby assuring that multiple molecules of NS3 are bound to each DNA substrate. This conclusion does not exclude the possibility that the monomeric or dimeric forms are completely inactive.

“Burst Kinetics” Are Observed When NS3 Is Similar to the Substrate Concentration—To further examine the apparent burst amplitudes indicated in Fig. 2, rapid mixing experiments were performed with 100 nM substrate and increasing concentrations of NS3. The results indicate that rapid unwinding does occur under these conditions, resulting in a low but measurable quantity of ssDNA. NS3 (100 nM) unwound the DNA (100 nM) with a burst amplitude of ~7.5 nM (Fig. 3 and Table 1), consistent with the results from Fig. 2. Increasing NS3 concentration up to 400 nM resulted in a linear increase in the burst amplitude (Fig. 3B). The linear increase in amplitude as a function of NS3...
concentration indicates that the enzyme species that unwinds the substrate has similar activity over the concentration range being examined (50–400 nM). It might be expected that an oligomeric form of NS3 would exhibit a sigmoidal increase in activity with increasing enzyme concentration if an oligomer is the optimally active species. However, it is possible that some oligomerization occurs at NS3 concentrations lower than 50 nM. We turned to biochemical and biophysical approaches to investigate the oligomeric nature of NS3.

The question arises as to whether the DNA unwinding activity observed in vitro relates directly to RNA unwinding activity. Several quantitative, ensemble approaches have been reported for studying RNA unwinding in which the NS3 concentration greatly exceeds the substrate concentration (27, 38, 39). Our results with NS3 indicate that little or no RNA unwinding occurs when the enzyme concentration is similar to that of the substrate concentration, although fast and relatively processive unwinding occurs when the enzyme concentration greatly exceeds the substrate concentration (supplemental Fig. 3). This trend is the same as that observed for NS3-catalyzed DNA unwinding; therefore, it is reasonable at this time to conclude that an oligomeric form of NS3 is responsible for optimal RNA unwinding under these conditions.

Chemical Cross-linking of NS3 Reveals Oligomeric Structures—Chemical cross-linking has been used previously to evaluate the ability of NS3h to form oligomeric structures, and higher order species were observed (40). To directly compare NS3 cross-linking with that of NS3h, both proteins were treated with the homo-bifunctional cross-linker BS3 under identical conditions. NS3h can be cross-linked, which is consistent with previous observations (40), although dimers were primarily observed under conditions used here (Fig. 4A). NS3 forms larger cross-linked species and in greater quantity than NS3h under identical conditions (Fig. 4B). During longer incubation times, most of the NS3 cross-linked structures do not migrate into the gel. This indicates that NS3 may form oligomeric structures in solution much more readily than NS3h.

Dynamic Light Scattering Supports an Oligomeric Form of NS3 but a Monomeric Form of NS3h—Dynamic light scattering is an alternative way to determine size distributions of particles in solution. Fig. 5, A and B, represent the size distribution histograms of NS3h and NS3, respectively, obtained in buffer conditions that mimic those used in the DNA unwinding assay (10 mM NaCl, 50 mM MOPS-K+ at pH 7.0). NS3h exhibits a monomodal, monodisperse profile with a hydrodynamic radius of ∼3.9 nm, as measured by dynamic light scattering. NS3 is shown to exist as a monomodal, polydisperse protein with a hydrodynamic radius of ∼29.4 nm (Fig. 5B). These results indicate that NS3 exists as an oligomeric species made up of 7–10 molecules under these conditions.

Size Exclusion Chromatography Indicates That NS3 Exists as an Oligomer in Solution—Several attempts to examine the oligomeric form of NS3 by sedimentation equilibrium and sedimentation velocity methods failed due to precipitation of NS3. We turned to size exclusion chromatography to further analyze the oligomeric form of NS3. Full-length NS3 eluted from the column near the void volume, which corresponds to a molecular size equal to or greater than ∼700 kDa (Fig. 6A). In contrast, NS3h elutes from the column at the expected position for a monomeric protein of 50 kDa. Interestingly, the same quantity of each protein was injected on the column, but the peak for NS3h was much larger than that for NS3. This result suggests that some NS3 may not elute from the column. NS3 and NS3h were then incubated with higher salt concentration (150 mM NaCl), resulting in similar retention times for each protein as observed under lower salt conditions, but the quantity of NS3 eluting from the column was increased when compared with the peak from NS3h (Fig. 6B). Similar results were observed.

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**TABLE 1**

Kinetic parameters for DNA unwinding under presteady state conditions

DNA (100 nM, 15-nt/30-bp partial duplex) was incubated with NS3 (50–400 nM), followed by rapid mixing with ATP and Mg2+ at 37 °C. Data were fit to a two-step unwinding mechanism to obtain the observed forward rate constant for each step, k_u, and the amplitude for the reaction (26).

| NS3 | k_u (s^-1) | Amplitude |
|-----|------------|-----------|
| 50  | 2.7 ± 0.5  | 7.8 ± 0.5 |
| 100 | 1.1 ± 0.3  | 14 ± 1    |
| 200 | 1.0 ± 1.0  | 23 ± 2    |
| 300 | 1.0 ± 0.1  | 40 ± 1    |
| 400 | 1.1 ± 0.1  | 48 ± 3    |

**FIGURE 4.** Time dependence of NS3 cross-linking using BS3 indicates oligomerization. A, silver stain of a SDS-PAGE (4–15% gradient) gel showing time dependence of cross-linking of NS3h. The experiment was carried out by adding 500 μM BS3 to a solution containing 2 μM NS3h in 50 mM MOPS-K+ (pH 7.0) and 10 mM NaCl, which had incubated at 37 °C for 30 min. Aliquots of the reaction mixture were removed at various times and quenched by adding 1 M glycine (pH 8.0). B, silver staining of a SDS-PAGE (4–15% gradient) gel showing time dependence of cross-linking of NS3 under the same condition as above for NS3h.
when NS3 was examined in the presence of an oligonucleotide, indicating that the oligomeric structure was similar in the presence and absence of ssDNA (supplemental Fig. 2). We conclude that NS3 can exist as an oligomeric species in solution and that higher salt concentrations somewhat reduce protein-protein interactions.

Passage of NS3 through Small Pore Filters Removes the Majority of the Protein and the Enzyme Activity—Small pore filters can remove large, oligomeric protein complexes from solution. To determine whether the enzymatic activity of NS3 was associated with the oligomeric structures, NS3 and NS3h were passed through 0.1- and 0.02-μm filters (low protein binding Anotop syringe filter; Whatman) followed by measurements of protein concentration and enzyme activity. A modified Bradford assay using Coomassie Brilliant Blue 250 was used to measure protein concentration. When comparing filtered NS3 versus unfiltered NS3, greater than 90% of NS3 was removed upon passage through the 0.02-μm filter, whereas lesser amounts were removed from the 0.1-μm filters (Fig. 7A). The ATPase activity of NS3 was measured in the presence of 100 μM polyuridine. The reduction in activity correlated strongly with the reduction in protein concentration (Fig. 7B). It is possible that NS3 adheres to the membrane filter, so NS3h was also examined. Only ~25% of protein was removed when NS3h was passed through the 0.02-μm filter, and the reduction in NS3h ATPase activity correlated with the reduction in protein concentration (Fig. 7, A and B).

The DNA unwinding activity of filtered versus unfiltered enzyme was also examined. No product formation was observed when examining NS3-catalyzed DNA unwinding of filtered protein (Fig. 7C), indicating that the activity resides in the filtered protein. In contrast, most of the DNA unwinding activity was recovered in the case of filtered NS3h when compared with unfiltered NS3h (Fig. 7D). These results support the conclusion that NS3 exists in an oligomeric form under these conditions and that its enzymatic activity resides within this oligomeric form.
Helicases coordinate ATP hydrolysis with movement along nucleic acid and concomitant unwinding of DNA (3). A mechanism for coupling ATP hydrolysis to molecular motion has been described for PcrA helicase (from SF1) and is supported by extensive x-ray crystallography (41) and biochemical results (42). ATP binding and hydrolysis is proposed to drive PcrA through multiple conformational changes that allow the enzyme to move in an “inch-worm” fashion along ssDNA. A related mechanism has been described for NS3h, based on x-ray crystallographic analysis (24). These inchworm mechanisms do not require a helicase to oligomerize in order to function. Some helicases clearly oligomerize into hexameric structures to exert their function (7, 43). However, evidence suggests that for some SF1 helicases, optimal DNA unwinding activity occurs in the presence of a dimeric form of the enzyme. In the case of UvrD and Rep helicases, optimal DNA unwinding in vitro occurs when these enzymes form dimeric (44) or oligomeric structures (45), although UvrD monomers readily translocate on ssDNA (46). It is possible that some factor that stimulates DNA unwinding in vivo is simply not present in vitro, which may contribute to the need for oligomerization in vitro.

Previous work from other laboratories has indicated that NS3 exhibits intramolecular protein-protein interactions. A yeast two-hybrid assay and results from gel filtration experiments by Khu et al. (34) indicate that dimerization may be important for helicase activity. Analysis of biochemical activity has also led to the conclusion that a dimeric form of NS3 is active (27). We employed biochemical and biophysical methods to investigate the oligomeric state of the NS3 helicase. The unwinding results are revealing when the amplitudes for product formation are considered under differing reaction conditions. When the enzyme is in great excess over substrate (500 nM NS3, 2 nM DNA substrate), most of the substrate is quickly converted to product, indicating a relatively processive form of the NS3 (Fig. 1C). However, when the DNA substrate is in great excess of the enzyme (50 nM NS3, 250 nM DNA substrate), no measurable product is formed under single turnover conditions (Fig. 1D).

These data strongly indicate that the monomeric form of NS3 is not responsible for the fast and relatively processive unwinding observed in Fig. 1C. A recent report indicates that the activity of NS3h can be stimulated substantially by inclusion of a single-stranded binding protein (SSB) in the reaction mixture and that NS3–4A unwinding activity is also stimulated but to a lesser extent (47). We tested E. coli SSB to determine whether the burst amplitude of NS3 is enhanced under pre-steady state conditions. The amplitude for product formation was increased by less than 2-fold, but the rate constant for DNA unwinding was increased by ∼7-fold (supplemental Fig. 4). These results do not change our conclusion that NS3 functions optimally as an oligomer, but the role played by multiple molecules of NS3 during DNA
unwinding can be compensated in part by the presence of SSB. The mechanism proposed by Rajagopal and Patel is that SSB serves to stabilize the helicase at the unwinding junction and prevent its dissociation. Such a mechanism could also apply to an oligomeric form of NS3.

A kinetic model termed functional cooperativity was previously described for NS3h in which multiple enzyme molecules acting on the same substrate molecule are required for optimal DNA unwinding (29). The full-length NS3 and the helicase domain (NS3h) share the property whereby fast and processive DNA unwinding is observed only when the enzyme concentration greatly exceeds the substrate concentration. The DNA unwinding results in Figs. 1–3 can be explained by the functional cooperativity model, by a model in which NS3 exists in an oligomeric form, or by a combination of these two phenomena.

The biophysical data reported here support an oligomeric form of NS3 as the species that is responsible for optimal DNA unwinding. Dynamic light scattering reveals that NS3h exists as a monomeric species, whereas NS3 forms oligomeric species (Fig. 5), consistent with the kinetics data. The size exclusion chromatography experiments show that oligomeric species of NS3 form readily (Fig. 6). Small pore filters that are able to remove large oligomeric proteins were used to investigate whether the active form of NS3 was found within the oligomeric structures. Filtration of NS3 and NS3h through the small pore filters shows that the active form of NS3 is retained by the 0.02-μm filter, whereas the majority of protein and enzyme activity of NS3h readily passes through the filter (Fig. 7). The fact that NS3 behaves quite differently than NS3h indicates that the NS3 protease domain may modulate the protein-protein interactions. One report provides evidence that the minimal region required for this interaction maps to a specific subdomain of 174 amino acids in the N terminus of the helicase region (34).

The overall conclusion from this work is that NS3 oligomerizes in solution at concentrations that are typically utilized inensemble biochemical experiments and that the oligomer contains the optimally active form of NS3. Oligomerization of NS3 may be due to nonspecific protein–protein interactions that occur due to the absence of other proteins that are known to interact with NS3. NS3 and HCV polymerase (NS5B) have been shown to interact and influence each other’s activity in vitro (48–52). If the oligomeric form of NS3 is heterogeneous, then some quantity of protein might be inaccessible to the DNA substrate. For example, an oligomeric structure made of 7–10 molecules of NS3 might contain some molecules that are properly oriented for DNA unwinding activity, whereas other molecules might be buried within the nonstructured oligomer. We are currently investigating the nature of the oligomer, including its dependence on solution conditions and the presence of other HCV proteins. Recent studies indicate that NS3 concentration greatly exceeds the concentration of HCV RNA in the cell (53). Therefore, it is possible that each strand of HCV RNA is acted on by many molecules of NS3.

Based on the kinetic simulation and experimental results in Fig. 1, it is clear that NS3 monomers have much lower activity than the optimal form of NS3 in vitro. The fact that the unexpectedly low burst amplitude is observed under pre-steady state conditions can be explained if the monomer is far less processive than the optimally active form or if the monomer has a much lower rate of unwinding. A small degree of DNA unwinding is observed in the presence of excess DNA substrate and in the absence of a protein trap (Fig. 1D), indicating that some DNA unwinding activity is retained under conditions that disfavor binding of multiple molecules to the same substrate molecule. However, the low activity may be due to a monomeric form of NS3 or a low amount of oligomeric NS3. Further work is required to discern between these possibilities.

The protease domain clearly has a major role in defining the quaternary structure of NS3. Recent work indicates that this role extends to the enzymatic activities of NS3. A report from Frick et al. (33) indicates that NS3 unwinds RNA better than NS3h. We have previously reported results from single-turnover studies that showed NS3h (500 nM) was capable of unwinding a 30-bp DNA substrate (2 nM) poorly, yielding only ~10% product (35), which is consistent with the extensive studies reported earlier by Levin et al. (40). In contrast to NS3h, full-length NS3 exhibits much higher processivity, unwinding ~80% of the 30-bp substrate under identical conditions. We previously reported that under the conditions employed here, NS3 is capable of binding to the ssDNA and double-stranded DNA portions of the 15-nt/30-bp substrate. The ability of NS3 to “coat” the entire DNA substrate may play an important role in the processive DNA unwinding. NS3h does not bind tightly to the duplex region of the substrate under conditions reported here,5 which may help to explain the low activity of NS3h relative to NS3.

In conclusion, under conditions in which NS3 concentration exceeds the substrate concentration, NS3 unwinds DNA in a fast and relatively processive manner. However, when DNA substrate greatly exceeds the enzyme concentration, NS3 unwinding activity is greatly reduced. The reason for this difference appears to be directly related to fact that NS3 exists as an oligomer in solution under conditions studied here and that the substrate must be bound by multiple protein units of the oligomer for optimal unwinding to occur.

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