Multiple Full-length NS3 Molecules Are Required for Optimal Unwinding of Oligonucleotide DNA in Vitro*

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NS3 (nonstructural protein 3) from the hepatitis C virus is a 3' → 5' helicase classified in helicase superfamily 2. The optimally active form of this helicase remains uncertain. We have used unwinding assays in the presence of a protein trap to investigate the first cycle of unwinding by full-length NS3. When the enzyme was in excess of the substrate, NS3 (500 nM) unwound >80% of a DNA substrate containing a 15-nucleotide overhang and a 30-bp duplex (45:30-mer; 1 nM). This result indicated that the active form of NS3 that was bound to the DNA prior to initiation of the reaction was capable of processive DNA unwinding. Unwinding with varying ratios of NS3 to 45:30-mer allowed us to investigate the active form of NS3 during the first unwinding cycle. When the substrate concentration slightly exceeded that of the enzyme, little or no unwinding was observed, indicating that if a monomeric form of the protein is active, then it exhibits very low processivity. Binding of NS3 to the 45:30-mer was measured by electrophoretic mobility shift assays, resulting in $K_D = 2.7 ± 0.4$ nM. Binding to individual regions of the substrate was investigated by measuring the $K_D$ for a 15-mer oligonucleotide as well as a 30-mer duplex. NS3 bound tightly to the 15-mer ($K_D = 1.3 ± 0.2$ nM) and, surprisingly, fairly tightly to the double-stranded 30-mer ($K_D = 11.3 ± 1.3$ nM). However, NS3 was not able to rapidly unwind a blunt-end duplex. Thus, under conditions of optimal unwinding, the 45:30-mer is initially saturated with the enzyme, including the duplex region. The unwinding data are discussed in terms of a model whereby multiple molecules of NS3 bound to the single-stranded DNA portion of the substrate are required for optimal unwinding.

Helicases are enzymes that provide single-stranded nucleic acid intermediates for processes such as replication, recombination, and repair of nucleic acids (1–4). These enzymes are proposed to use the energy gained from ATP hydrolysis to separate double-stranded nucleic acids. The unwinding mechanism of a helicase may reflect the oligomeric state of the enzyme. Helicases such as *Escherichia coli* DnaB (5), bacteriophage T4 gp41 (6), and bacteriophage T7 gp4 (7) have been shown to exist as hexamers. These hexameric helicases are proposed to operate through a DNA exclusion mechanism in which the hexamer encircles one strand of a duplex and excludes the complementary strand during translocation along the DNA (3, 8–10). It has also been determined that some hexameric helicases such as DnaB may drive branch migration by acting as a molecular pump (11, 12). Most helicases are believed to translocate with a directional bias along single-stranded DNA (ssDNA). Dda helicase from bacteriophage T4 was proposed to use force to remove proteins bound to DNA based upon its ability to displace streptavidin from biotin-labeled oligonucleotides in a directionally biased manner (10, 13). The ability of Dda to displace proteins from DNA increases when multiple molecules of Dda are involved in the reaction (14).

The active form of many helicases still remains unresolved. Dda was shown to be capable of functioning as a monomer during unwinding of double-stranded DNA (dsDNA) (15). PcrA helicase from *Bacillus stearothermophilus* has been proposed to function as a monomer based on x-ray crystallographic studies (16–18). Biochemical studies of the translocation activity of PcrA on ssDNA also support PcrA function as a monomer (19). Dda and PcrA are proposed to utilize an "inchworm-like" mechanism in which a monomeric helicase has two DNA-binding sites that allow a cycle of unwinding and translocation along a nucleic acid lattice. Biochemical and biophysical evidence has been presented supporting a dimeric form of the UvrD helicase (20, 21).

NS3 (nonstructural protein 3) from the hepatitis C virus contains 3' → 5' helicase activity in the C-terminal domain as well as serine protease activity in the N-terminal domain (22). In work with the helicase domain of NS3 (NS3h), Preugschat et al. (23) mixed NS3h mutants lacking strand-separating activity in steady-state unwinding assays with wild-type NS3h and observed no dominant-negative phenotype that would be expected if NS3h requires oligomerization to function. This negative result was proposed to illustrate a monomeric form of the helicase domain of NS3 and supported an inchworm-like mechanism as suggested from the x-ray crystal structure of NS3h bound to a dU8 oligonucleotide (24). In contrast, Levin and Patel (25) reported that the helicase domain of NS3 is active as an oligomer. Oligomerization of NS3h was indicated by protein-protein cross-linking and by unwinding assays in which an ATPase-deficient mutant form of NS3h was mixed.

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¶ The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; MOPS, 4-morpholinepropanesulfonic acid; EMSAs, electrophoretic mobility shift assays; F-T15,3'-fluorescein-labeled T15; F-30-mer, 3′-fluorescein-labeled 30-mer; ATPγS, adenosine 5′-O-(thiotriphosphate); HCV, hepatitis C virus.

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with wild-type NS3h under conditions of excess enzyme, which revealed that 13.5 molecules of NS3h were required for unwinding a 33-bp substrate. Levin and Patel (25) proposed a model of unwinding that utilizes a cycle of transient protein-protein interactions that result in exchange of NS3h molecules through dissociation during translocation and unwinding. Most recently, Patel and co-workers (26) have proposed a model for unwinding by NS3h that does not require protein-protein interactions, but does rely on ‘functional cooperativity’ between helicase monomers.

We have investigated the active form of full-length NS3 during the first unwinding cycle with a series of unwinding experiments in the presence of a protein trap and varying ratios of NS3 to DNA substrate. A protein trap allows the investigation of actively bound NS3 during the first unwinding cycle by removing NS3 that is not initially bound to the DNA substrate and sequestering NS3 that dissociates from the DNA substrate. Optimal unwinding was observed only under conditions that favor binding of multiple NS3 molecules to the single-stranded region of the substrate. The results are qualitatively similar to those reported by Patel and co-workers (26) for the NS3 helicase domain.

**EXPERIMENTAL PROCEDURES**

*Materials—*

Na4EDTA, SDS, bovine serum albumin, β-mercaptoethanol, MOPS, NaCl, acrylamide, bisacrylamide, MgCl2, KOH, ATP, formamide, xylene cyanol, bromphenol blue, HEPES, urea, and glycerol were from Fisher. γ-[32P]ATP was purchased from PerkinElmer Life Sciences. Poly(U) was purchased from Amersham Biosciences. DNA oligonucleotides were from Integrated DNA Technologies. Sephadex G-25 was from Sigma. T4 polynucleotide kinase was obtained from New England Biolabs Inc. Recombinant full-length NS3 was expressed and purified as described previously (27). Concentrations of nucleic acid and polypeptides were determined using absorbance and extinction coefficients (28). To create the partial duplex unwinding substrates overhang: 3'-TTTTTTTTTTTTTTTCATCATGCAGGACAGT-^{15}/H11032 and additional thymidine residues. Each DNA oligonucleotide was purified by preparative gel electrophoresis (13). Crude oligonucleotide was separated in formamide and resolved on a 7 % urea and 20% polyacrylamide gel (Hoefter Scientific Instruments). Oligonucleotide was visualized by UV shadowing and excised from the gel. Purified oligonucleotide was electroeluted using a Schleicher & Schuell Elutrap apparatus. Purified oligonucleotides were then desalted on a Waters Sep-Pak C18 column and lyophilized using a Savant Speed-Vac. The lyophilized oligonucleotides were suspended in 10 mM HEPES (pH 7.5) and 1 mM EDTA. Oligonucleotide concentration was determined by measuring absorbance at 260 nm in 0.2 M KOH and applying calculated extinction coefficients (28). To create the partial duplex unwinding substrate, a 1:2 ratio of 45-mer to 30-mer was heated at 95 °C for 10 min and allowed to cool to room temperature. The mixture of the 45-30-mer partial duplex solution was removed on a native 20% polyacrylamide gel, electroeluted, desalted, and quantified as described above.

The purified 45-mer was 5'-radiolabeled with γ-[32P]ATP by T4 polynucleotide kinase. The labeling reaction proceeded for 1 h at 37 °C, followed by heat denaturation of the enzyme at 70 °C for 10 min. The unlabeled 30-mer (1 eq) was added to the 5'-radiolabeled 45-mer. Excess γ-[32P]ATP was removed by passage through two 1-ml Sephadex G-25 spin columns. The 5'-radiolabeled 45:30-mer was heated to 95 °C for 10 min and allowed to cool to room temperature. This stock solution of the radiolabeled 45:30-mer was added to a more concentrated stock solution of the gel-purified 45:30-mer to create the substrate for DNA unwinding experiments.

**Unwinding Assays**—The NS3 unwinding experiments in Figs. 1–4 were performed using a KinTek rapid chemical quench-flow instrument maintained at 37 °C with a circulating water bath. All concentrations listed are after mixing. NS3 unwinding buffer consisted of 50 mM K’MOPS (pH 7.0), 50 μM EDTA, 0.1 mg/ml bovine serum albumin, and 10 mM NaCl. For excess enzyme experiments, 45:30-mer DNA (1 nM) was incubated with NS3 (500 nM) prior to initiating the unwinding reaction with 5 mM ATP and 10 mM MgCl2. For experiments performed with a protein trap, 5–75 μM poly(U) was incubated with ATP and MgCl2 unless otherwise stated. After rapid mixing of the NS3/45:30-mer solution with the ATP/MgCl2/poly(U) solution, the reaction was quenched with 200 mM EDTA and 0.7% SDS. To prevent re-anneling of the ssDNA products, a DNA trapping strand was added to the vial in which the quenched solution was collected. The DNA trapping strand was a 30-mer that was complementary to the 30-mer in the 45:30-mer substrate and was added in 30-fold excess of the substrate. A 25-μl aliquot of the quenched reaction mixture was added to 5 μl of loading buffer (30% glycerol, 0.1% bromphenol blue, and 0.1% xylene cyanol) and electrophoresed on a 20% polyacrylamide gel. The amount of free 45-mer and 45:30-mer was quantified using an Amersham Biosciences PhosphorImager and ImageQuant software (13, 27, 29, 30). Unwinding experiments with 25 or 100 nM 45:30-mer and NS3 were performed as described above except that 3 μM DNA trapping strand was incubated with the ATP/MgCl2/poly(U) solution to prevent re-formation of the substrate and the reaction. The addition of the 30-mer DNA trapping strand to the reaction will affect the observed rate for unwinding during multiple cycle conditions in the absence of a protein trap by serving as a binding site for NS3, but it should not affect the first cycle of unwinding by the initially bound NS3 without or with a protein trap (15). Unwinding data were fit to Scheme 1 using the program Scientist (MicroMath Research) (27). This method of data analysis utilizes differential equations to describe each of the species and provides the best fit to the data in accordance with the model. Scheme 1 describes the steps involved in product formation that account for the "lag" during the rapid phase of strand separation as well as the slow conversion of nonproductively bound NS3 to productive NS3 (31).

**Electrophoretic Mobility Shift Assays (EMSAs)**—EMSAs were used to monitor binding between NS3 and various components of the 45:30-mer DNA unwinding substrate. EMSAs were prepared by mixing the F-30-mer with a complementary 30-mer or 45:F-30-mer in fluorescence polarization buffer for 3 min at 37 °C. Fluorescence polarization with Fluorescein-labeled DNA—Fluorescence polarization was used to monitor the interaction of NS3 with the DNA substrate. Fluorescence polarization buffer consisted of 50 mM K’MOPS (pH 7.0), 50 μM EDTA, 0.1 mg/ml bovine serum albumin, and 10 mM NaCl. 3’-Fluorescein-labeled T50-G30-mer and 3’-Fluorescein-labeled 30-mer (F-30-mer) were purchased from Integrated DNA Technologies and purified by preparative gel electrophoresis. The F-30-mer was identical to the 30-mer in the 45:30-mer DNA substrate. Duplexes were prepared by mixing the F-30-mer with a complementary 30-mer or 45-mer. The blunt-end F-30:30-mer and 45:F-30-mer were purified by nondenaturing preparative gel electrophoresis as described above. Varying concentrations of NS3 were incubated with 0.1 mM F-T15, F-30:30-mer, or 45:F-30-mer in fluorescence polarization buffer for 3 min at 37 °C in a final volume of 1 ml/sample. Fluorescence polarization was monitored on a Beacon fluorescence polarization instrument (Invitrogen). Fluorescence polarization buffer was used as the blank. The change in millipolarization units was determined using a sample of 0.1 mM F-T15. The baseline fluorescence was set to zero point. After reading each sample, 10 mM MgCl2 and 1 mM ATP/Si (Sigma) were added to each sample. Following a 3-min incubation at 37 °C, the fluorescence polarization of each sample was again obtained. Fluorescence polarization data were fit to a hyperbola to obtain Kd values using KaleidaGraph (Synergy Software).

**Fluorescence Titration Assays measuring Intrinsic Protein Fluorescence**—NS3 was titrated with poly(U), and the change in protein fluorescence was measured using an SLM-Aminco luminescence spec.
Optimal DNA Unwinding by NS3 Requires Excess Enzyme

RESULTS

Unwinding under Excess Enzyme Conditions—The ability of full-length NS3 to unwind partial duplex DNA was monitored under excess enzyme conditions. NS3 (500 nM) was incubated with 45:30-mer partial duplex DNA (1 nM) prior to initiating unwinding with 5 mM ATP and 10 mM MgCl₂ at 37 °C. Under these conditions of low DNA concentration, re-anneling of ssDNA products to re-form substrate during the reaction is very slow and does not affect the observed rates (27, 29). To prevent re-anneling of ssDNA products after the reaction had been quenched with 200 mM EDTA and 0.7% SDS, a 30-fold excess of 30-mer DNA trapping strand complementary to the 30-mer in the 45:30-mer substrate was placed in the receiving vial for the KinTek rapid chemical quench-flow apparatus. The formation of ssDNA products was observed by 5′-radiolabeling the 45-mer of the substrate. Free 45-mer unwinding product was separated from the 45:30-mer unwinding substrate on a 20% polyacrylamide gel and visualized using a PhosphorImager (Fig. 1A). Under these conditions, product formation consisted of a fast phase of rapid unwinding, followed by a second slower phase (Fig. 1). The majority of ssDNA is formed during the fast phase, which is characterized by a lag in the initial portion of the unwinding curve. The lag is due to the fact that ssDNA product is not observed until the helicase unwinds most of the duplex. More than one step is required to do this, so a lag appears in the early portion of the time course for DNA unwinding (33). The unwinding data were fit to Scheme 1 using the program Scientist (27, 31). The steps in Scheme 1 are the minimal number required to fit the unwinding data for the 30-bp substrate. Two species for DNA unwinding are shown, Eₓ₁ and Eₓ₂, which correspond to productively bound enzyme and a kinetic intermediate, respectively. The intermediate is believed to represent a partially unwound substrate that occurs along the pathway to fully unwound DNA. The lag phase is observed after the lag phase, which is accounted for by the conversion of a nonproductive Eₓ complex (EₓNP) to a productive complex (Eₓ, Eₓ₁, Eₓ₂). All rates and amplitudes for each phase are listed in Table I.

To investigate NS3 unwinding under single-cycle conditions, a protein trap, poly(U), was introduced into the reaction to sequester unbound NS3. NS3 (500 nM) and the 45:30-mer (1 nM) were incubated, and the unwinding reaction was initiated with 5 mM ATP, 10 mM MgCl₂, and 75 μM poly(U) protein trap. In the presence of the poly(U) protein trap, NS3 unwound the 45:30-mer with a similar rate and amplitude in the lag phase as observed in the absence of the protein trap (Fig. 1 and Table I). This result indicates that the form of NS3 that is initially bound prior to the addition of ATP is the processive species that can separate >80% of the 30-bp substrate prior to dissociation from the DNA. The unwinding data in the presence of the protein trap, like the data collected in the absence of the protein trap, could be best fit by a lag phase, followed by rapid product formation, followed by a slower phase. All data obtained from unwinding in the presence of the protein trap were fit to Scheme 1. The efficiency of the poly(U) protein trap was investigated by incubating 75 μM poly(U) with 1 nM 45:30-mer and then initiating the reaction by adding 500 nM NS3, 5 mM ATP, and 10 mM MgCl₂. Very little product formed under these conditions, indicating that the protein trap was effective (Fig. 1).

Unwinding with Varying Ratios of NS3 to 45:30-mer—In the presence of excess enzyme, the active form of the enzyme cannot be determined. However, under pre-steady-state conditions, in which the substrate is in excess of the enzyme, then the amplitude of the burst phase can provide information about the active species of the enzyme. Dda helicase was recently shown to be capable of functioning as a monomer when unwinding short oligonucleotides under pre-steady-state conditions (15). The first cycle of unwinding by NS3 was monitored at varying ratios of NS3 to 45:30-mer to investigate the active form of NS3. The amount of 45:30-mer DNA was increased to 100 nM to conduct experiments in the presence of excess substrate. To prevent re-anneling of the ssDNA products during the reaction, 3 μM 30-mer DNA trapping strand was included in the reaction. The presence of the DNA trapping strand does not affect the first cycle of the reaction with or without the protein.
Unwinding of 100 nM 45:30-mer by NS3 was measured by gel electrophoresis (Fig. 2A). Initially, 100 nM 45:30-mer was incubated with 1000 nM NS3, and the reaction was initiated by the addition of 5 mM ATP, 10 mM MgCl₂, and 3 μM 30-mer DNA trapping strand with or without 75 μM poly(U) (Fig. 2B). The data collected were fit to Scheme 1. Under single-cycle conditions with the poly(U) protein trap, less than half of the substrate was unwound as determined by the summation of the amplitudes for each phase (Fig. 2B and Table I). The low amplitude suggests that multiple NS3 molecules are involved in the unwinding complex because a 10:1 ratio of NS3 to 45:30-mer was not sufficient to provide optimal activity. In the absence of the poly(U) protein trap, 77% of the substrate was unwound (Fig. 2B and Table I). When NS3 was lowered to 100 nM and 45:30-mer was kept at 100 nM, only ~4% of the substrate was unwound in the presence of the poly(U) protein trap (Fig. 2C and Table I). Under the same conditions in the absence of the poly(U) protein trap, ~9% of the substrate was unwound after 60 s (Fig. 2C and Table I). The data in Fig. 2 reveal that a large excess of NS3 to 45:30-mer was required for optimal unwinding activity.

To more thoroughly investigate the active form of NS3, the single-cycle experiments with the poly(U) protein trap were performed with varying ratios of NS3 to DNA (Fig. 3). 50, 100, 500, 1000, or 2000 nM NS3 was incubated with 1000 nM NS3, and the reaction was initiated by the addition of 5 mM ATP, 10 mM MgCl₂, 3 μM 30-mer DNA trapping strand, and 75 μM poly(U) (Fig. 3). Under conditions in which the NS3 concentration was below that of the substrate (50 nM NS3 and 1000 nM 45:30-mer), essentially no product was formed (Fig. 3 and Table I). As the concentration of NS3 was raised up to 2000 nM, the amplitude for the first cycle of product formation was increased to a level that was similar to that observed under optimal conditions as in Fig. 1. Thus, a ratio of 20 NS3 molecules to 1 substrate molecule is necessary for optimal unwinding.

Experiments were performed at a lower concentration of substrate so that the NS3 concentration could be in large excess over the substrate while maintaining the substrate concentration well above the Kₚ for NS3 binding to DNA. Full-length NS3 is not highly soluble under conditions in which

### Table I

Table I: Optimal DNA Unwinding by NS3 Requires Excess Enzyme

| NS3 (μM) | 45:30-mer (nM) | 75 μM poly(U) | kᵢ fast phase (s⁻¹) | Amplitude fast phase (nM) | kₛᵢ slow phase (s⁻¹) | Amplitude slow phase (nM) |
|----------|----------------|---------------|---------------------|--------------------------|---------------------|--------------------------|
| 0.5      | 1              | −             | 0.62 ± 0.01         | 0.62 ± 0.01              | 0.086 ± 0.004      | 0.26 ± 0.01              |
| 0.5      | 1              | +             | 0.62 ± 0.01         | 0.77 ± 0.01              | 0.087 ± 0.017      | 0.074 ± 0.01             |
| 0.05     | 100            | +             | 0.62 ± 0.04         | 3.9 ± 0.2                | 0.037 ± 0.004      | 5.3 ± 0.2                |
| 0.1      | 100            | +             | 1.3 ± 0.1           | 3.5 ± 0.2                |                     |                          |
| 0.5      | 100            | +             | 0.77 ± 0.03         | 16 ± 0.4                | 0.040 ± 0.006      | 10 ± 0.4                 |
| 1.0      | 100            | −             | 1.40 ± 0.05         | 46 ± 1.2                | 0.080 ± 0.006      | 31 ± 1.0                 |
| 1.0      | 100            | +             | 2.0 ± 0.1           | 25 ± 1.2                | 0.18 ± 0.10        | 19 ± 1.1                 |
| 2.0      | 100            | +             | 1.30 ± 0.03         | 52 ± 0.8                | 0.060 ± 0.005      | 27 ± 0.7                 |
| 0.01     | 25             | +             | 1.71 ± 0.05         | 1.1 ± 0.05              |                     |                          |
| 0.025    | 25             | +             | 1.41 ± 0.03         | 1.6 ± 0.2               | —                   | —                        |
| 0.125    | 25             | +             | 1.10 ± 0.08         | 4.3 ± 0.2               | —                   | —                        |
| 0.25     | 25             | +             | 1.70 ± 0.10         | 6.1 ± 0.2               | —                   | —                        |
| 0.5      | 25             | +             | 1.01 ± 0.04         | 11 ± 0.3                | 0.047 ± 0.02       | 5.1 ± 0.3                |
| 0.75     | 25             | +             | 0.90 ± 0.02         | 12 ± 0.2                | 0.084 ± 0.01       | 5.6 ± 0.2                |

**Fig. 2. Unwinding with different ratios of NS3 to 45:30-mer in the presence or absence of a protein trap.**

- **A**: Representative gel showing unwinding of 100 nM 45:30-mer by 1000 nM NS3 in the presence of a protein trap. Unwinding products were resolved on a 20% polyacrylamide gel. The blank sample (B) shows the 45:30-mer prior to unwinding, and the heated sample (H) shows the DNA trapping strand.
- **B**: Unwinding of 100 nM 45:30-mer by 1000 nM NS3 in the presence (●) or absence (○) of 75 μM poly(U) protein trap. Unwinding was initiated by rapidly mixing 100 nM 45:30-mer and 1000 nM NS3 with 5 mM ATP and 10 mM MgCl₂ in the absence or presence of 75 μM poly(U) and 3 μM 30-mer DNA trapping strand complementary to the 30-mer in the partial duplex. The unwinding reaction was performed at 37 °C, and the reaction was quenched with 200 mM EDTA and 0.7% SDS. The protein trapping efficiency of poly(U) was determined by incubating 75 μM poly(U) with 100 nM 45:30-mer prior to adding 200 nM NS3, after which 5 mM ATP and 10 mM MgCl₂ were added. C: unwinding of 100 nM 45:30-mer by 100 nM NS3 in the absence (●) or presence (○) of 75 μM poly(U) protein trap. Unwinding was performed as described for B. The unwinding data in B and C were fit to Scheme 1 using the program Scientist, and the unwinding rates and amplitudes are listed in Table I.
unwinding is optimal. High concentrations of full-length NS3 are possible at a very high concentration of NaCl (1 M), but unwinding is greatly reduced in high salt.\(^2\) The solubility/activity limitations prevented us from raising the NS3 concentration above 2 \(\mu\text{M}\); therefore, the substrate concentration was lowered to 25 nM. At this concentration, the substrate was >10-fold greater than the equilibrium dissociation constant, which is useful for interpretation of pre-steady-state experiments (see below). The substrate (25 nM 45:30-mer) was incubated with 10, 25, 125, 250, 500, and 750 nM NS3, and the reaction was initiated by the addition of 5 mM ATP, 10 mM MgCl\(_2\), 3 \(\mu\text{M} 30\)-mer DNA trap, and 75 \(\mu\text{M}\) poly(U) (Fig. 4). As shown in Fig. 3, pre-steady-state conditions in which the substrate was in excess of the enzyme (25 nM 45:30-mer and 10 nM NS3) resulted in essentially no product formation. Conditions favoring binding of multiple NS3 molecules to a single substrate (25 nM 45:30-mer and 750 nM NS3) and the reaction was initiated by the addition of 5 mM ATP, 10 mM MgCl\(_2\), 3 \(\mu\text{M} 30\)-mer DNA trap, and 75 \(\mu\text{M}\) poly(U) (Fig. 4). As shown in Fig. 3, pre-steady-state conditions in which the substrate was in excess of the enzyme (25 nM 45:30-mer and 10 nM NS3) resulted in essentially no product formation. Conditions favoring binding of multiple NS3 molecules to a single substrate (25 nM 45:30-mer and 750 nM NS3) resulted in significant unwinding. A ratio of 30 molecules of NS3 to one molecule of 45:30-mer produced an amplitude of \(\sim 70\%\) product formation, which is similar to that observed in Fig. 1. The data in Figs. 3 and 4 were fit to Scheme 1. All of the rates and amplitudes for Figs. 3 and 4 are listed in Table I. Values not listed were not defined due to insufficient product formation.

EMSA—The requirement for multiple NS3 molecules for optimal unwinding suggested that multiple molecules were bound per substrate molecule. To investigate this possibility, EMSAs were performed with the T\(_{15}\) ssDNA, 30-mer duplex (identical to the duplex portion of 45:30-mer), and 45:30-mer partial duplex (Fig. 5). Radiolabeled DNA (0.1 nM) was incubated with varying concentrations of NS3 at 37 °C and then analyzed by PAGE (Fig. 5A). The amount of T\(_{15}\), 30-mer duplex, and 45:30-mer bound was fit to a hyperbola, resulting in \(K_D = 1.3 \pm 0.2, 11.3 \pm 1.3,\) and \(2.7 \pm 0.4\) nM, respectively (Fig. 5, B and C). Binding of NS3 to the T\(_{15}\) ssDNA, 30-mer duplex, and 45:30-mer resulted in the formation of a species that did not migrate into the 4% polyacrylamide gel, but was retained in the well (Fig. 5A). This suggests that multiple NS3 molecules bind to each type of DNA investigated, leading to charge neutralization of the DNA.

Binding to the duplex region was weaker by \(\sim 10\)-fold than binding to the single-stranded regions. However, the \(K_D\) indicated that the duplex region should be completely bound under the optimal unwinding conditions in Figs. 3 and 4. Binding was investigated further using fluorescence polarization of fluorescein-labeled DNA. Equilibrium dissociation constants were measured by titrating DNA with NS3, followed by incubation for 3 min at 37 °C. The resulting \(K_D\) values were \(4.7 \pm 1.2, 15.7 \pm 2.9,\) and \(5.2 \pm 0.52\) nM for the 15-mer, 30:30-mer, and 45:30-mer, respectively. Hence, the values were similar to those observed in the gel shift assays. Binding of some helicases to DNA is modulated by the binding and hydrolysis of ATP. We added a slowly hydrolyzable ATP analog, ATP-S\(_2\), to each of the samples in Fig. 6A and measured the fluorescence polarization. Interestingly, binding was reduced for each oligonucleotide, but more so for the 30:30-mer DNA than for the

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\(^2\) A. J. Tackett and K. D. Raney, unpublished data.
15-mer and 45:30-mer DNAs. The mechanistic interpretation of this result remains to be determined, but it may support a model whereby NS3 binds to different forms of DNA as a function of ATP binding and hydrolysis, which facilitate unwinding. A recent report suggests that ATP binding and hydrolysis mediate the affinity of the NS3 helicase domain for DNA (34).

To investigate the stoichiometry of the active NS3 complex, EMSAs were performed as described above using 100 nM 45:30-mer and varying concentrations of NS3 (Fig. 7). This concentration of DNA is well above the \( K_D \) values measured for all three DNA strands (T15, 30-mer duplex, and 45:30-mer). The stoichiometric binding revealed that 90% of the DNA was shifted when the concentration of NS3 (0.5 \( \mu \)M) was in excess of the DNA by 5-fold (Fig. 7, ninth lane). This suggests that multiple NS3 molecules can bind along the 45:30-mer because much more protein is needed than expected to shift all of the DNA. The free 45:30-mer was ultimately mobility-shifted to a species that did not enter the gel (Fig. 7A). The complex between NS3 and DNA that did not enter the gel was formed under conditions in which unwinding was optimal (ratio of NS3 to DNA of \( -20:1 \)). Thus, the active species for initiation of DNA unwinding likely contains multiple NS3 molecules that bind to the single-stranded and duplex regions of the substrate. However, NS3 was not able to unwind the blunt-end 30-mer duplex rapidly.2

DISCUSSION

Understanding the mechanism for helicase activity requires knowledge of the active form of the enzyme. The active form of NS3 remains uncertain. Utilizing the C-terminal helicase domain of NS3 (NS3h), Levin and Patel (25) provided evidence for an active oligomeric form of the helicase domain; however, Preugschat et al. (23) concluded that the functional form of the helicase is a monomer. Additionally, Kim et al. (24) suggested a monomeric form of NS3h based on the x-ray crystal structure with a bound dU8 oligonucleotide. Very recently, Patel and co-workers (26) proposed a model for unwinding that invokes functional cooperativity for unwinding by the NS3 helicase domain. In this model, multiple molecules of NS3h are shown to bind to the single-stranded region of partially duplex substrates. Monomeric forms of NS3h are proposed to be non-processive, thereby requiring multiple molecules for processive DNA unwinding. We recently reported the results from DNA unwinding experiments under pre-steady-state conditions, which indicated that Dda helicase is able to unwind short oligonucleotides as a monomer (15). Here, we have applied a similar approach to the full-length form of NS3.

To investigate the active form of NS3, we first compared unwinding in the presence and absence of a protein trap to determine whether the enzyme could unwind 30 bp in a single binding event (Fig. 1). When the protein was in great excess...
over the substrate, unwinding was similar in the presence and absence of the protein trap (Fig. 1 and Table I). Product formation displayed two distinct phases as previously reported for other helicases such as bacteriophage T4 Dda, E. coli UvrD, and bacteriophage T7 gp4 (30, 33, 35, 36). The unwinding data were fit to Scheme 1 using the program Scientist (27). This model takes into account the multistep strand separation for the initial fast phase as well as the conversion of "nonproductive" to "productive" NS3 for the slower phase. The results under conditions of a large excess of enzyme over substrate illustrate that the NS3 species that is initially bound to DNA can unwind ~85% of the 45:30-mer prior to dissociation.

It is interesting to compare the activity of this particular form of NS3 with that reported by others. The fastest rate obtained from fitting the data to the two-step scheme was 2.0 ± 0.1 s−1 when the NS3 concentration was 1 μM and the substrate concentration was 100 nM (Table I). This rate corresponds to 30 bp/s at 37 °C. Others have reported unwinding of an 18-bp substrate with a rate constant of 2.2 ± 0.7 min−1, which corresponds to 0.66 bp/s (37). The form of NS3 believed to be biologically relevant contains the NS4A peptide bound to the protease domain of NS3. The report of unwinding by NS3-NS4A indicated that an 18-bp substrate was unwound at a rate of 2.5 ± 0.9 min−1, which corresponds to 0.75 bp/s. The recent report of Patel and co-workers (26) provided a rate for unwinding by NS3h of 2.2 bp/s at 22 °C, making a direct comparison with our data difficult. Thus, the full-length NS3 reported here unwinds DNA somewhat faster at 37 °C than do other forms of the enzyme reported thus far. The processivity of the full-length protein appears to be greater than that of the helicase domain. More than 80% of a 30-bp substrate was unwound by full-length NS3 under optimal conditions (Fig. 1), compared with <20% for a similar substrate in the case of NS3h (26).

Under pre-steady-state conditions, the substrate concentration is in excess of the enzyme concentration, so the product formed in the first unwinding cycle will reflect the concentration of active enzyme. Thus, varying the enzyme concentration relative to the substrate concentration should reveal the amount of NS3 that is required for optimal activity. Initially, the amount of NS3 (100 nM) was equivalent to that of 45:30-mer (100 nM), resulting in only ~4% of the substrate being unwound (Fig. 2C and Table I). This illustrates that NS3 cannot effectively unwind a 30-bp substrate at a 1:1 ratio in the presence of a protein trap. When we lowered the NS3 concentration (50 nM) below the 45:30-mer concentration (100 nM), essentially no product was observed (Fig. 3). The experiments in Fig. 2 (B and C) suggest that the most active species of NS3 is formed under conditions in which the enzyme is in large excess over DNA, allowing multiple enzyme molecules to bind per substrate molecule. This strongly suggests that monomeric NS3 is not highly processive in vitro, as suggested for NS3h (26).

The 15-mer single-stranded region of the 45:30-mer should be able to bind 1 and perhaps 2 molecules of NS3 based on the crystal structure of the enzyme (24) and the binding site size (26, 38), assuming that the full-length protein behaves similarly to the helicase domain. The unwinding data suggested that far more than 2-fold excess NS3 was needed to unwind the substrate, leading us to investigate the distribution of NS3 molecules along the 45:30-mer using EMSAs (Fig. 5). We observed that NS3 interacted with the ssDNA overhang and 30-mer duplex region of the 45:30-mer. This interaction promoted formation of a large molecular mass species that ultimately was retained in the wells of the gel (Fig. 5). Importantly, the Kd values for the single-stranded overhang (1.3 ± 0.2 nM) and 30-mer duplex portion (11.3 ± 1.3 nM) of the 45:30-mer revealed that NS3 was bound to single-stranded and duplex regions of the 45:30-mer under optimal unwinding conditions (Figs. 3 and 4). Binding assays using fluorescence polarization provided similar Kd values as the gel shift assay (Fig. 6). The absolute values for binding to ssDNA are similar to those reported by Levin and Patel (38); however, the duplex binding reported here for full-length NS3 is stronger than reported for NS3h. Binding of NS3 to DNA was reduced in the presence of ATP·γS (Fig. 6B), as was observed for NS3h (38).

Binding to the 45:30-mer was further investigated under conditions in which the DNA concentration was raised well above the Kd. Multiple species were observed, indicating that multiple molecules bound to the 45:30-mer (Fig. 7A). Based on the number of species observed on the gel and the quantity of NS3 required to saturate binding, as many as 5 molecules of
NS3 bind to the 45:30-mer under conditions in which unwinding is optimal. It is possible that NS3 melted the 45:30-mer simply in an ATP-independent manner; and therefore, all of the observed binding might have been to ssDNA rather than duplex DNA. However, we measured DNA unwinding under these conditions in the absence of ATP and observed no unwinding. Thus, NS3 binding to the ssDNA region or the ssDNA/dsDNA junction appears to be required for optimal unwinding to occur.

It is possible that some or even most of the recombinant NS3 protein is inactive. To determine the amount of NS3 that was bound to the 45:30-mer, we quantitated the bands in the lane in which approximately one-half of the DNA was bound in Fig. 7 (eighth lane). The fraction of DNA in each band correlates with the number of DNA species on the gel. As shown in Fig. 7C, the quantity of bound NS3 could be calculated by multiplying the quantity of DNA by the estimated number of NS3 molecules bound. The results of this analysis indicated that ~195 nM of a total of 250 nM NS3 was bound under these conditions. Hence, >75% of the NS3 protein was able to bind to DNA, suggesting that the majority of the protein is active. To further explore the quantity of active protein, a fluorescence titration was performed under conditions in which NS3 (100 nM) was above the equilibrium dissociation constant. Based on the binding site size of 8–11 nucleotides from the number of DNA species on the gel. As shown in Fig. 7C, the quantity of bound NS3 could be calculated by multiplying the quantity of DNA by the estimated number of NS3 molecules bound. The results of this analysis indicated that ~90% of the protein was bound at 1000 nM poly(U) (Fig. 8). If a substantial fraction of NS3 were inactive, we would observe saturation at a much lower concentration of poly(U). Therefore, the data are not consistent with a large quantity of inactive NS3. We conclude that at least 75% of the protein in our preparation is capable of binding to RNA or DNA based on the EMSA data and the fluorescence titration.

Based on the model provided by Patel and co-workers (26), binding of 2 molecules of NS3 to the ssDNA might increase the quantity of product formed. This suggests that increasing the length of the single-stranded region of the substrate might lead to enhanced unwinding by allowing more molecules of NS3 to bind. We prepared a 60:30-mer substrate and analyzed unwinding under the same conditions as described for the 45:30-mer. Similar to the 45:30-mer, high concentrations of NS3 were required for optimal unwinding of the 60:30-mer (Fig. 9). However, higher amplitudes for unwinding were observed in the case of the 60:30-mer compared with the 45:30-mer (Fig. 9B). These data are qualitatively consistent with the model previously proposed by Patel and co-workers (26), in which multiple molecules of NS3 bind to the single-stranded region of the substrate are required for optimal unwinding.

We further investigated unwinding using a substrate containing 15 nucleotides of ssDNA adjacent to 15 bp (30:15-mer). Similar amplitudes were observed with the 30:15-mer compared with the 45:30-mer, but the overall requirement for multiple helicase molecules for efficient unwinding remained (Fig. 10). For example, 25 nM NS3 incubated with 25 nM 30:15-mer produced essentially no product under single-turnover conditions. Only when the enzyme concentration was raised well above the substrate concentration was significant product formation observed. Thus, multiple molecules are required for optimal unwinding even with a short duplex. These data were fit to a single exponential that does not include a lag phase for unwinding, whereas unzipping of 30 bp can be fit with two steps. The appearance or lack of a lag phase must be interpreted with caution because a slow step prior to unwinding can greatly change the interpretation of the number of steps needed to unwind the duplex (39). A thorough description of the kinetic analysis of helicase stepping has been provided by Lucius et al. (40). Patel and co-workers (26) reported a step size of ~9 bp for NS3h, assuming that several base pairs spontaneously melt before the helicase reaches the very end of the oligonucleotide (26, 39). A recent report by Pyle and co-workers (41) suggested that full-length NS3 unwinds RNA with a large step size of ~18 bp and functions as a dimer. Our current data are insufficient to compare with the data of Patel and Pyle and co-workers regarding step size; however, the amplitude that we
Unwinding of 25 nM 30:15-mer by various NS3 concentrations. Unwinding was initiated by mixing 25 nM T30:15-mer and NS3 with 5 mM ATP, 10 mM MgCl₂, 5 µM poly(U), and 3 µM 15-mer DNA trapping strand complementary to the 15-mer in the partial duplex. The following concentrations of NS3 were used: 25 (A), 125 (●), 250 (■), and 500 (▲) nM. The reaction was quenched with 200 mM EDTA and 0.7% SDS. This experiment was performed at 25 °C rather than at 37 °C because significant melting of the 15-bp substrate occurred at the higher temperature. The data were fit to the equation for a single exponential, $A = 1 - exp(-kt)$, using KaleidaGraph. The rates and amplitudes for the fits were $0.35 \pm 0.15 \, s^{-1}$ and $8.3 \pm 0.7 \, nm$ for 125 nM NS3, $0.15 \pm 0.03 \, s^{-1}$ and $15.8 \pm 0.7 \, nm$ for 250 nM NS3, and $0.43 \pm 0.16 \, s^{-1}$ and $16.8 \pm 1.0 \, nm$ for 500 nM NS3, respectively.

Optimal DNA Unwinding by NS3 Requires Excess Enzyme

Optimal DNA unwinding by NS3 can be achieved by the presence of excess enzyme. For example, at a concentration of 250 nM NS3, the unwinding rate is $0.15 \, s^{-1}$ and the amplitude is $15.8 \, nm$. Increasing the concentration to 500 nM NS3 increases the unwinding rate to $0.43 \, s^{-1}$ and the amplitude to $16.8 \, nm$.

Substrate saturation model for optimal DNA unwinding by NS3. Multiple molecules of NS3 are shown bound to the 60:30-mer substrate. NS3 binds to the ssDNA (white) and dsDNA (gray) portions of the substrate under optimal unwinding conditions in vitro. NS3 bound to the ssDNA portion hydrolyzes ATP to serve as the motor component that pulls ssDNA through the multiprotein complex. NS3 binds relatively weakly to the dsDNA, especially in the presence of ATP, allowing the DNA to slide through the binding site. The molecules are shown bound to one another along the DNA; however, it is not known whether protein-protein interactions play a role in unwinding.

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