The Functional Interaction of the Hepatitis C Virus Helicase Molecules Is Responsible for Unwinding Processivity*

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Although helicases participate in virtually every cellular process involving nucleic acids, the details of their mechanism including the role of interaction between the subunits remains unclear. Here we study the unwinding kinetics of the helicase from hepatitis C virus using DNA substrates with a range of tail and duplex lengths. The binding of the helicase to the substrates was characterized by electron microscopy and fluorometric titrations. Depending on the length of the ssDNA tail, one or more helicase molecules can be loaded on the DNA. Unwinding was measured under single-turnover conditions, and the results show that a monomer is active on short duplexes yet multiple molecules are needed to unwind long duplexes. Thus, increasing the ssDNA tail length increases the unwinding efficiency. The unwinding kinetics was modeled as a stepwise process performed by single or multiple helicase molecules. The model programmed in MATLAB was used for global fitting of the kinetics, yielding values for the rate of unwinding, processivity, cooperativity, step size, and occlusion site. The results indicate that a single hepatitis C virus helicase molecule unwinds DNA with a low processivity. The multiple helicase molecules present on the DNA substrate show functional cooperativity and unwind with greater efficiency, although they bind and release the substrate non-cooperatively, and the ATPase cycle of the helicase molecules is not coordinated. The functional interaction model explains the efficient unwinding by multiple helicases and is generally applicable.

Helicases are motor proteins that translocate along DNA or RNA using ATP hydrolysis. The translocation activity is required for strand separation of the duplex nucleic acids, the elimination of secondary structure in RNA, and to dissociate proteins bound to the nucleic acids (1–4). The exact mechanism of translocation and nucleic acid strand separation is not known for any helicase. However, unwinding is believed to be a stepwise process that among other things may require interaction between helicase molecules. In this paper we use single turnover unwinding kinetics experiments as well as numerical modeling to investigate the role of subunit interactions during unwinding by the helicase from hepatitis C virus.

Hepatitis C virus (HCV) contains a single stranded RNA genome that codes for a polypeptide, which is cleaved into structural and nonstructural (NS) proteins. The NS3 protein of the HCV is both a helicase and a protease. The crystal structure of NS3 shows two loosely connected domains (5). The helicase activity resides on the C-terminal domain that constitutes ~450 C-terminal amino acid residues and the protease activity on the N-terminal domain. The NS3 protease is tightly associated with its essential co-factor NS4A, which is predicted to be membrane-bound. The NS3 helicase is, therefore, tethered to the endoplasmic reticulum membrane in vivo. The protease and helicase activities appear to be independent as these domains can be expressed separately in Escherichia coli while retaining their full activity (6–9). Interestingly, NS3 helicase acts both on RNA and DNA and unwinds DNA better than RNA (10, 11). This study is conducted with the bacterially expressed helicase domain of NS3 protein (NS3h) (12). There is no consensus in the literature about the effect of the protease-NS4A on the helicase activity (10, 11, 13–16). The virus requires both helicase and protease functions, but it is not clear if the protease-NS4A activates or inhibits the helicase.

We have previously (17) characterized the helicase activity of NS3h under multiple turnover conditions using a partially duplex DNA substrate that contained a 10-nt-long ssDNA tail and a 33-bp-long duplex. Under multiple turnover conditions, the helicase rate depended on the NS3h concentration, indicating that the observed rate of unwinding is limited by the rebinding of free NS3h to DNA. We also observed that the unwinding rate decreased sharply upon the addition of small amounts of an ATPase-deficient NS3h mutant, but the amplitude of unwinding changed little. This led us to conclude that many NS3h molecules participate in the unwinding of each DNA substrate. It is not clear, however, whether a single molecule of the HCV helicase can unwind DNA and, in cases where multiple helicase molecules are required, whether they work cooperatively. This question is relevant to other helicases as well.

For the HCV helicase, most of the evidence thus far indicates that it does not form a stable oligomer in solution. Gel filtration chromatography, ultra centrifugation and x-ray crystallography show that NS3h is a monomer (17–19). Protein-protein cross-linking of NS3h occurs inefficiently, and the addition of an ATPase-deficient mutant to wild type NS3h does not inhibit its ATPase activity (17). Recently, we showed that the NS3h ATPase activity is independent of NS3h concentration when measured in the presence of a non-ionic detergent (17). Fluorometric titration experiments failed to show NS3h cooperativity.

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§ The abbreviations used are: HCV, hepatitis C virus; ds, double-stranded; ss, single-stranded; MOPS, 3[N-morpholino]propanesulfonic acid; NS3h, helicase domain of HCV non-structural protein 3; NC, nitrocellulose; nt, nucleotide(s); TLC, thin layer chromatography; NS protein, nonstructural protein; EM, electron microscopy.

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Interaction of HCV Helicase during DNA Unwinding

in ssDNA binding (20). Yet paradoxically it appears that multiple HCV helicase molecules are necessary for efficient DNA unwinding.

Classes of helicases show varying levels of intersubunit interactions. For ring helicases, the assembly into a stable cooperative hexamer ring is necessary for activity (1, 21). Whether non-ring helicases function as monomers or cooperatively as oligomers is an active area of research. Cooperative behavior in helicases can be manifested as coordination in ATPase, nucleic acid binding, or translocation activities. Such cooperativity arises from physical interaction between the helicase molecules, and it is characterized by a certain free energy of binding. However, a different type of cooperative behavior that does not require protein-protein interactions arises from the mere presence of multiple helicase molecules on the same nucleic acid substrate. The superfamily 1 helicases Rep and UvrD helicases from Escherichia coli have been proposed to function as dimers (22, 23), but multiple UvrD helicase molecules on the DNA show increased activity (23–25). RecQ and PcrA helicases have been proposed to function as monomers (26, 27), but it is not known if multiple helicase molecules have an increased activity. Dda helicase functions as a monomer during DNA unwinding (28) but appears to have optimal streptavidin displacement activity as an oligomer (29).

We have characterized the interactions of HCV helicase with DNA substrates of varying ssDNA tail lengths to determine the number of NS3h molecules bound to a helicase substrate. To determine the degree of cooperativity of NS3, a superfamily 2 helicase, we measured the unwinding of DNA substrates containing increasing lengths of the ssDNA tail. We found that multiple helicase molecules are necessary for efficient DNA unwinding. The increase in activity with increasing tail length can be explained by a functional interaction model of unwinding. In this paper we present a way to simulate and fit the helicase unwinding experimental data. We have made this model general by including features that can be applicable to all helicases and used to determine the helicase parameters such as cooperativity in helicase action, processivity of unwinding, stepping rate, and the kinetic step size.

MATERIALS AND METHODS

The helicase domain of HCV NS3 protein with a C-terminal His tag (NS3h) was expressed in E. coli carrying a plasmid pET21b-NS3HCV (12). NS3h protein was purified, stored, and quantitated as described previously (17). The reaction buffer contained 50 mM MOPS-NaOH, 5 mM MgCl2, 5 mM N-ethyldisothioctetanil, and 0.1% Tween 20, pH 7.0. Experiments were performed at 22 °C unless specified otherwise. PolyU, average length of 210 nucleotides, was purchased from Amersham Biosciences, and polyoxyetheleneorbitanmonolaurate (Tween 20), purchased from Sigma, was purified by passing through charcoal.

DNA Substrates—The sequences of the oligodeoxyribonucleotides shown in Table I were produced using a random number algorithm and checked for lack of stable secondary structure with the Oligo Analyzer (www.idtdna.com). The oligodeoxyribonucleotides were synthesized by Integrated DNA Technologies and purified by PAGE in 7 M urea, 60 °C. The concentration of DNA was determined spectrophotometrically in 8 M urea, 50 mM NaOH, 5 mM EDTA, and polyoxyetheleneorbitanmonolaurate (Tween 20), pur-

absorbance at 280 nm and fluorescence after excitation at 280 nm at an emission wavelength of 340 nm. Values of fluorescence were corrected for dilution and inner filter effect and plotted against [DNA], and the [DNA]-dependent fluorescence change was fit to Equation 1, where $F$ is the observed fluorescence, $f_L$ is the fluorescence coefficient of free NS3h, $f_{pl}$ is the fluorescence coefficient of NS3h bound to DNA, $E_t$ is total NS3h concentration, and $E_b$ is concentration of NS3h bound to DNA, and Equation 2,

$$F = f_L + f_{pl} \times E_t$$

where $D_t$ is total DNA concentration, $K_D$ is the dissociation constant, and $n$ is the number of NS3h molecules per DNA.

Helicase Assay—The kinetics of dsDNA unwinding was measured using BQF-3 Quench-Flow apparatus (KinTek Instruments, Austin, TX). NS3h (0.5 μm) was mixed with 2 μm ss/dsDNA substrate with radiolabeled strand, incubated for 10 s, and mixed with 5 mM ATP and 0.5 μm polyU for 0.1–65 s (concentrations are given for the state after the second mixing). The unwinding reaction was stopped by ejecting the mixture into a test tube containing 100 mM EDTA, 3% SDS, 15% Ficoll, and 0.1% of bromphenol blue. The intact and unwound DNA substrates were resolved on a native 10 or 13% PAGE, and their radioactivity was measured with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The fraction of unwound substrate was calculated and corrected for the presence of ssDNA at time 0 using Equation 3,

$$F = \frac{SS \times DS - SS \times DS}{DS \times (SS + DS)}$$

where $F$ is the fraction of unwound substrate, $DS$ and $SS$ are radioactivities of intact and unwound substrate bands at a given time, respectively, and $DS_a$ and $SS_a$ are the radioactivities of intact and unwound substrates at time 0, respectively.

Data Analysis—The unwinding time courses were fit to the incomplete gamma function (Equation 4),

$$F = \frac{A}{k} \int_0^x e^{-kx} - \int_0^x e^{-kx} \, dx$$

where $F$ is a fraction of unwound DNA substrate molecules, $A$ is the amplitude of unwinding, $k$ is stepping rate, and $t$ is reaction time. The number of steps, $n$, taken by the helicase to unwind the substrate was calculated as

$$n = \frac{L_{ds} - L_s}{s}$$

where $L_{ds}$ is the number of base pairs in the DNA substrate duplex, $L_s$ is the length of the shortest DNA duplex that can stay together under the experimental conditions, and $s$ is the step size. Software MATLAB with Optimization toolbox (The MathWorks, Inc., Natick, MA) was used for all calculations.

RESULTS AND DISCUSSION

Electron Microscopy (EM) of NS3h-ss/dsDNA Complexes—EM was used to determine the interactions of NS3h with the ssDNA and duplex DNA regions of the partial duplex helicase substrate. An excess of NS3h was incubated with the 66 bp-25 DNA substrate that has a 25-nt ssDNA tail and a 66-bp duplex (Table I). The complex was immobilized on a carbon grid, tungsten shadow casting, and visualized by EM. Fig. 1 shows the electron micrograph of the protein-DNA complexes that appear as a ball and a stick. The “stick” part of a complex has a thickness of 0.4 nm, which is typical for dsDNA visualized by tungsten shadow casting. The average length of the stick, 17.3 ± 2 nm, is close to the theoretical length of 66 bp B-DNA (22.4 nm). The ssDNA is not visible by the EM method used; therefore, the “ball” flanking the stick represents ssDNA covered with the NS3h protein. The EM studies show that the
complex of multiple NS3h molecules bound to ssDNA is not extended, like e.g., the complex of T7 gp4, RecA, or E. coli single-stranded DNA-binding protein with ssDNA (30–32). Instead, NS3h-ssDNA complex forms a clump regardless of the length of ssDNA (data not shown). In addition, the micrographs of NS3h-ss/H18528 dsDNA complexes show that the NS3h protein binds only to the ssDNA tail of the DNA substrate. Therefore, DNA unwinding does not initiate internally from multiple positions within the dsDNA but must proceed sequentially from the ssDNA tail end.

### Table I

| DNA substrates | Number of NS3h | Length of ssDNA |
|----------------|----------------|-----------------|
| 5'-GCTGTAACCTGTGTCACTACATGTAAGGAGGCTTGACGACATAGAGACGCAGATCAACGCAGTAACCTCAGAC | n=5 | 18bp-T5 |
| 3'-CCGACAGATGAAGTACATGAGCATCGAGATCTCC | n=10 | 18bp-T10 |
| 18bp-5 | n=25 | 18bp-T25 |
| 18bp-10 | n=50 | 18bp-T50 |
| 33bp-5 | n=75 | 18bp-T75 |

The NS3h Binding to ss/dsDNA Substrates Is Proportional to the ssDNA Tail Length—The stoichiometry and the $K_d$ of the NS3h complex with the ss/ds substrates were measured by fluorimetric titrations. The ss/dsDNA helicase substrates contained a 33-bp duplex region and ssDNA tails ranging in length from 5 to 75 nts (33 bp–5 to 33 bp–75 in Table I). The ss/dsDNA helicase substrates were made by annealing synthetic oligodeoxynucleotides. The completeness of annealing was greater than 98% as determined by native PAGE (data not shown).

As shown previously, the intrinsic fluorescence of NS3h protein decreases upon binding to ss nucleic acids (33). To measure DNA binding, the titration experiments were performed by adding increasing amounts of the ss/dsDNA substrate to a constant amount of NS3h while exciting the sample with 280-nm light and measuring the intrinsic NS3h fluorescence at 340 nm. The titration was repeated with DNA substrates of different ssDNA tail lengths. The resulting fluorescence values were corrected for sample dilution and inner filter effect. To
determine the number of NS3h proteins bound per ss/dsDNA substrate \((n)\) and the \(K_d\), the fluorimetric data were fit to Equations 1 and 2.

The number of NS3h proteins per ss/dsDNA substrate as a function of the ssDNA tail length is shown in Fig. 2A. The number of NS3h proteins bound per DNA substrate increases linearly as the ssDNA tail length increases with a slope of \(0.162 \pm 0.01\) NS3h molecules/base. This corresponds to the NS3h occlusion site of \(6.2 \pm 0.4\) bases. This value is smaller than the occlusion site obtained from similar experiments with the ssDNA substrates \((7.7 \pm 1\) bases) (20). As indicated previously (20), the observed binding behavior of NS3h protein with long ssDNA substrates is a product of binding events to multiple overlapping sites. A slightly higher binding density and a smaller occlusion site can result if NS3h has a higher affinity for binding to the ss/dsDNA junction. The \(K_d\) for the 33 bp-10 ss/dsDNA substrate with a 10-nt ssDNA tail is \(1.3 \pm 0.5\) nM (Fig. 2B). This value is consistent with \(K_d\) values previously reported for short ssDNA substrates (20). As in the case of ssDNA substrates, no cooperativity was detected in the binding of NS3h to the ss/dsDNA substrates, and equations describing a simple non-cooperative binding model fit well to the fluorimetric data (Equations 1 and 2).

The DNA Unwinding Efficiency of the NS3h Is Dependent on the Duplex as Well as the ssDNA Tail Length—The unwinding kinetics were measured using a rapid quench-flow instrument as shown in Fig. 3A. The helicase and DNA were preincubated for 10 s, and increasing the preincubation time by 3-fold did not change the unwinding kinetics (data not shown). Thus, a functional helicase-DNA complex is formed within 10 s, and lengthy preincubation times were not necessary to observe activity as reported for NS3 by Pang et al. (10). Helicase substrates of different duplex lengths from 18-bp to 40-bp and of varying ssDNA tail length, from 5 to 75 nt were studied. Two kinds of substrates with varying ssDNA tails were used. One set of substrates (18-bp and 33-bp) contained tails of a defined sequence shown in Table I and the second set (18-bp, 33-bp, 40-bp) contained \(d\)T\(_n\) tails. After pre-incubating NS3h with the DNA substrate for 10 s, unwinding was initiated by the addition of ATP and polyU RNA. PolyU was used as a trap to prevent NS3h from re-binding to the unwinding substrate once reaction was initiated. Thus, DNA unwinding was measured under single turnover conditions.

As shown in Fig. 3, B–F, the substrates with DNA length >18-bp duplex unwound with a kinetic lag, which indicates that the reaction involves a sequence of transient intermediates before the formation of the final product. The single turnover kinetics of DNA unwinding by UvrD and recBCD helicases have been described by an equation for a multistep process (stepping equation) (34, 35). The stepping equation describes the process of dsDNA unwinding as a sequence of identical steps, although the exact nature of these steps has not been determined for any helicase. The same process can be described by the incomplete gamma function (Equation 4), which is continuous with respect to \(n\), the number of steps required to unwind a particular length of duplex. This property makes the incomplete gamma a more convenient function for curve-fitting. We globally fit the unwinding kinetics of DNA with varying ssDNA tails and duplex lengths to a single kinetic step size and stepping rate (Equations 4 and 5). The resulting fit is shown as the solid lines in Fig. 3, B–F. The global fit provided a kinetic step size of 9.1 (±0.9) bp, stepping rate of 0.3 (±0.02) steps/s, and a minimal duplex length of 6.6 (±0.9) bp, which is \(L_n\), the length of the shortest DNA duplex that can stay together under the experimental conditions. Evidently, the number of NS3h molecules bound to the ssDNA tail does not affect the kinetic step size or the rate of unwinding. This indicates that the number of helicase molecules assembled on the ssDNA tail does not change the mechanism by which unwinding occurs. However, the type of helicase complex assembled on the ssDNA does affect the unwinding efficiency (the amount of DNA unwound in a single turnover).

As shown in Fig. 4, the unwinding efficiency increases as the ssDNA tail length increases, and shorter duplexes are unwound more efficiently compared with longer duplexes. The length of the ssDNA tail determines the number of NS3h molecules bound to the ss/dsDNA substrate before unwinding begins. The 18-bp duplex was unwound significantly even from a short 5-base ssDNA tail, but neither 5 nor 10 base tails were sufficient to unwind the 33-bp duplex. In the case of the 33- and 40-bp duplex substrates, the efficiency of unwinding increases gradually with the ssDNA tail length, reaching 33 and 20%, respectively, for the 75-base tail. The results also show that the efficiency of unwinding is not affected by the base composition of the ssDNA tail. The amplitudes of unwinding DNA substrates with a tail containing a defined sequence of all bases versus a \(d\)T\(_n\) tail are similar. Although fitting the unwinding data to the incomplete gamma function provided an estimate of the step size and stepping rate, these parameters do not help us to understand the dependence of the unwinding amplitude on the duplex length and the ssDNA tail length.

We have used a C-terminal His-tagged helicase domain protein for our studies that show a DNA unwinding rate of 2.7 bp/s at 22 °C (stepping rate \(\times\) step size). This rate is comparable or even faster than the reported nucleic acid unwinding rates of either the helicase domain or the full-length protease-helicase with or without the His tag (10, 11, 36). It was also shown recently that the DNA unwinding activity of the helicase domain with C-terminal or N-terminal His tag was similar (11). Therefore, it is unlikely that His tag affects the helicase activ-

Fig. 2. Stoichiometry and \(K_d\) of NS3h-helicase DNA substrates. The binding parameters of NS3h to ss/dsDNA with different ssDNA tail lengths (33 bp-5 to 75, Table I) were determined by fluorimetric titration. The S.E. are shown for each DNA. A, the number of NS3h proteins bound per DNA substrate is plotted as a function of ssDNA tail length. A straight line with a slope of 0.162 ± 0.01 NS3h/base was fit into the data. B, observed \(K_d\) values are plotted as a function of ssDNA tail length.
Fig. 3. Single-turnover kinetics of DNA unwinding by the NS3h helicase. A, the experimental setup consists of mixing the contents of three syringes with the precision electric motor (black arrow) in the following fashion. NS3h helicase was mixed with the radiolabeled ss/dsDNA unwinding substrate for 10 s followed by the mixing with ATP and polyU trap for different times (reaction time) and then mixed with the EDTA/SDS quench solution in the receiving test tube. The quenched reaction mixture was loaded on a native gel, and the DNA products were resolved and quantitated. Panels B–F show the fraction of unwound ssDNA substrate as a function of the reaction time. B shows the unwinding of the DNA substrates (Table I) 18 bp-n; ○, 18 bp-6; □, 18 bp-15; ▼, 18 bp-20; ▲, 8 bp-25; ■, 18 bp-40; □, 18 bp-65. C, 33 bp-n duplex: ○, 33 bp-6; ◻, 33 bp-10; ▼, 33 bp-25; ▲, 33 bp-50; ■, 33 bp-75. D, 18 bp-Tn duplex: ○, 18 bp-T10; ◻, 18 bp-T25; ▼, 18 bp-T50; ▲, 18 bp-T65. E, 33 bp-Tn duplex: ○, 33 bp-T10; ◻, 33 bp-T25; ▼, 33 bp-T75. F, 40 bp-Tn duplex: ○, 40 bp-T25; ◻, 40 bp-T50; ▼, 40 bp-T75. The solid lines are global fit to Equations 4 and 5 with step-size(s) of 9.1 (±0.9) bp, a stepping rate (k) steps/s of 0.3 (±0.02), and a Lss of 6.6 (±0.93) bp.

Fig. 4. Unwinding efficiency depends both on the ssDNA tail length and the duplex length. The unwinding amplitudes of 18 bp-n (○), 33 bp-n (◼), 18 bp-Tn (▲), 33 bp-n (■), 40 bp-Tn (●), 50 bp-Tn (▼), and 60 bp-Tn (▲) were obtained from fitting the unwinding kinetics to Equations 4 and 5 and plotted as a function of ssDNA tail length.

Unwinding begins from the DNA substrate fully saturated with NS3h protein (Fig. 5, bottom left). As we showed above, the number of the helicase molecules bound to the substrate is linearly proportional to the substrate's tail length (Equation 6),

\[ e_0 = \frac{L_{ss}}{m} \]  

(Eq. 6)

where \( e_0 \) is the average number of NS3h molecules bound per substrate, \( L_{ss} \) is the length of the ssDNA tail of the substrate, and \( m \) is the occlusion site. For non-integer \( e_0 \) the starting concentrations \( C_{c,1} \) and \( C_{c,-1} \) are chosen such that \( e_0 = eC_{c,1} + (e - 1)C_{c,-1} \), where \( e \) is the smallest integer greater than \( e_0 \). At time 0, the concentration of the fully saturated species \( C_{c,1} + C_{c,-1} = 1 \) or 100%, and the concentration of all other species is 0.

Upon the addition of ATP, the helicase molecules either step forward or dissociate from the DNA substrate. The stepping forward or the unidirectional translocation of the helicase results in the unwinding of the ss/dsDNA substrate. The number of successful steps that have to be taken to complete the unwinding process is proportional to the length of the DNA duplex. It is assumed that the unwinding stepping rate of each helicase molecule \( k_f \) is constant throughout the length of the duplex and independent of the number of helicase molecules bound to DNA substrate. If the duplex length of the substrate predicts a non-integer number of steps, in other words, the size of the last step is less then one, the rate of the last step is divided by the size of the last step.

The change in the species concentrations during the reaction...
Once the leading NS3h protein reaches a position end of the duplex DNA to completely separate the strands. It is reasonable to and only one NS3h molecule, presumably the leading one, has substrates that are completely unwind. It is reasonable to reveal any cooperativity in ssDNA binding, the design of the model does not rule it out. The deproteination rate of a species with more than one NS3h protein is given by Equation 8,

\[ k_{\text{doff}} = K_{\text{doff}}^{e} \] (Eq. 8)

where \( q \) is a cooperativity factor. If NS3h proteins are bound non-cooperatively and have an equal chance of falling off from the DNA substrate, \( q = 1 \). If the helicase molecules exhibit negative cooperativity, \( q > 1 \), if the helicase molecules show positive cooperativity, \( q < 1 \). If \( q = 0 \), the deproteination rate is independent of the number of the bound helicase molecules, and only one NS3h molecule, presumably the leading one, has a significant probability of falling off the substrate.

The radiometric unwinding assay detects only those DNA substrates that are completely unwound. It is reasonable to propose that the helicase complex does not have to reach the end of the duplex DNA to completely separate the strands. Once the leading NS3h protein reaches a position \( L_{a} \) bp away from the end of the duplex, the duplex strands separate spontaneously due to the wedge effect of the helicase and thermal fraying. The longest spontaneously separating stretch of the duplex, \( L_{s} \), depends on its GC content, temperature, the degree of interaction between the helicase and the separating strands, and the geometry of the helicase.

As unwinding progresses and the length of the unwound DNA strand increases, the strands can reanneal behind the unwinding complex. Because the strands remain in close proximity, we believe this process can be very fast. However, multiple helicase molecules bound to the unwound DNA can prevent reannealing of the strands behind the leading helicase molecule. The \( L_{a} \) parameter determines the minimal number of helicase molecules that are required to prevent the strands from reannealing behind the helicase complex (Fig. 6). To unwind a DNA to completion, the length of the duplex has to be shorter than the length occupied by the NS3h proteins (\( e_{f} \)) plus \( 2L_{a} \). Therefore, the minimal number of NS3h proteins \( e_{\text{min}} \) required to completely unwind a duplex DNA of length \( L_{ds} \) is given by Equation 9.

\[ e_{\text{min}} = \frac{L_{a} + 2e_{f}}{e_{f}} \] (Eq. 9)

If the unwinding processivity of a single helicase molecule is low, then the above model predicts that increasing the tail length will increase the efficiency of unwinding. A long tail flanking the duplex to be unwound provides a reservoir of helicase molecules that can replace the dissociated ones. A long tail that provides multiple helicase molecules for unwinding also prevents the DNA strands from reannealing behind the helicase complex. Thus, under single turnover conditions, a substrate with a short ssDNA tail that binds one helicase molecule will be unwound inefficiently. Such a substrate will be unwound under multiple turnover conditions, but the unwinding rate will depend on helicase concentration. On the other hand DNA substrates with long ssDNA tails can load several helicase molecules and will be unwound efficiently under both single and multiple turnover conditions. This model describes the unwinding kinetics of NS3h, but it is a general model that can also be applied to fit the unwinding kinetics of any helicase. The model can be used to determine helicase parameters such as cooperativity, processivity, stepping rate, and the kinetic step size.

**Simulation and Fitting**—The proposed model describes the observed unwinding kinetics of NS3h, and global fitting provided the precise values of the unwinding parameters. The routines for simulation and fitting were programmed in MATLAB environment. Equation 7 recorded for each unwinding.

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### Table II

**Optimized parameters of NS3h unwinding**

| Parameters          | Optimized values | Error (−) | Error (+) |
|---------------------|------------------|-----------|-----------|
| Stepping rate, \( k_{s} \) | 0.17 step/s      | 0.01      | 0.01      |
| Dissociation rate, \( k_{\text{doff}} \) | 0.16 s⁻¹         | 0.018     | 0.02      |
| Step-size, \( s \)  | 9.2 bp           | 0.5       | 0.8       |
| Minimal duplex, \( L_{a} \) | 8.4 bp          | 0.7       | 0.6       |
| Cooperativity, \( q \) | 1.01             | 0.1       | 0.17      |
| Occlusion site, \( m \) | 8.5 bp          | 0.5       | 1.1       |

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**Fig. 5. The functional interaction model of helicase unwinding**. The scheme shows all the unwinding species present in the reaction. The species are distinguished by the number of associated NS3h proteins (from 1 to \( e \)) and degree of unwinding (from 1 to s).

**Fig. 6. DNA strands can reanneal behind the helicase during unwinding**. Reannealing cannot occur if the number or NS3h proteins bound to the substrate is greater than \( e_{\text{min}} \) (Equation 9).
The substrate and is equal to the intrinsic falling-off rate, $k_{\text{off}}$. The solid lines show the degree of unwinding predicted by the model. The data are segregated according to the ssDNA tail lengths. A shows fit to substrates containing 10- or 15-nt tails: 18 bp-15 (●), 33 bp-10 (○), 18 bp-T10 (▲), 33 bp-T10 (◆), and 40 bp-T10 (□). B shows fit to substrates containing 25-nt tails: 18 bp-25 (●), 33 bp-25 (○), 18 bp-T25 (▲), 33 bp-T25 (◆), and 40 bp-T25 (□). C shows fit to substrates containing 50-nt tails, 18 bp-50 (●), 33 bp-50 (○), 18 bp-T50 (▲), 40 bp-T50 (◆), 50 bp-T50 (□), and 60 bp-T50 (▲). D shows fit to substrates containing 65- or 75-nt tails: 18 bp-65 (●), 33 bp-75 (○), 18 bp-T65 (▲), 33 bp-T75 (◆), 40 bp-T75 (□), 50 bp-T75 (▲), 60 bp-T75 (◆). The optimized parameters from the fitting are shown in Table II.

The unwinding kinetics predicted by the model with the optimized parameters shown in Table II followed the experimental data as shown in Fig. 7. The unwinding parameters obtained by optimization reveal the following properties of the NS3h helicase. Comparison of $k_f$ and $k_{\text{off}}$ show that at every step of unwinding, the NS3h helicase has an almost equal chance of falling off from the substrate or of making a step forward. Processivity of 51% per step or 93% per base was calculated from the value of $k_f/(k_f + k_{\text{off}})$. It is possible that when the leading NS3h falls off, the DNA strands close before the next NS3h molecule comes and takes its place. In this event the processivity estimated by our method will be lower than the real value. Interestingly, the value of the cooperativity factor, $q$, obtained by optimization is close to 1. As mentioned earlier, $q = 1$ indicates that all NS3h molecules bound to the DNA substrate have an equal deproteination rate or off rate. The rate is independent of the number of NS3h molecules bound to the substrate and is equal to the intrinsic falling-off rate, $k_{\text{off}}$. This indicates that NS3h molecules are bound to the ss/dsDNA substrate non-cooperatively. The size of the duplex DNA that separates without the need for the helicase to advance, $L_m = 8$ bp, which is consistent with the properties of dsDNA and suggests that melting is somewhat facilitated by the helicase wedge effect.

The unwinding step size of NS3h is estimated to be about 9 bp. A step size of 2 bp was reported for NS3h helicase based on a fluorimetric unwinding assay (19). A kinetic step size of 4 to 5 bp was reported for UvrD helicase (34), 4 bp for the recBCD enzyme (37), and 6 bp for the RNA helicase NPH II (38). As mentioned earlier, the nature of the stepping behavior remains elusive for helicases. Estimation of the number of steps (or the step size) from the appearance of the final product of the reaction relies on the assumption that all the unwinding species have identical stepping properties. Ideally, all the helicase molecules should have identical primary structure; however, multiple populations of alternatively folded proteins may still exist. If multiple populations of the helicase have different stepping rates, the unwinding kinetics will appear to have fewer steps than it actually does, making the estimation of the step size exaggerated and unreliable.

**Conclusion**—The proposed functional interaction model is the simplest model that explains why multiple HCV helicase molecules are more efficient in unwinding a long stretch of duplex DNA. In case of HCV helicase, a single NS3h molecule can unwind DNA; however, a single NS3h molecule has a low efficiency of unwinding because it has low processivity, and a single helicase molecule cannot prevent reannealing of the DNA strands behind the helicase. Multiple HCV helicase molecules bound to the helicase substrate tail increase the efficiency of unwinding, although there does not appear to be any coordination in the ATPase activity (17) or in substrate binding or dissociation. The modeling reveals the independent action of the HCV helicase molecules and explains the increased efficiency of unwinding by the availability of additional helicase molecules when one falls off from the substrate because of its low processivity. Similarly, it reveals that multiple helicase molecules occupy a longer stretch of DNA during unwinding and prevent reannealing of the DNA behind the helicase. Other helicases such as *E. coli* UvrD helicase (23, 24) show similar behavior during unwinding, and Dda helicase shows similar behavior for streptavidin displacement activity (29). In UvrD helicase both the amplitude and unwinding rate increase with increasing ssDNA tail length. Unlike HCV helicase, however, UvrD behavior could not be explained by the independent action of helicases, and there appears to be cooperativity in helicase activity (23). The functional interaction model proposed here includes a cooperativity factor, and the degree of cooperativity can be obtained by fitting the unwinding data that should reveal and the property of the particular motor protein. This model is, therefore, general and contains features that are applicable not only to helicases but also other nucleic acid unwinding systems.
acid motor proteins such as RNA polymerase, which was reported to show functional cooperativity as multiple molecules similar to helicases (39, 40).

The experiments in this paper reveal the unwinding properties of the NS3 helicase domain under in vitro conditions. The low processivity of HCV helicase monomer is likely intrinsic to its mechanism of translocation. It is not known whether processive unwinding by HCV helicase is required in vivo or its task is to separate the strands of DNA or RNA locally. If processivity is required, which is likely to be the case since the HCV helicase is believed to be involved in replicating the HCV genome, it would be interesting to know how the helicase compensates for its low processivity in vivo. Higher overall processivity can be achieved, like in vitro, by assembling multiple helicase molecules on each replication complex. A more likely solution would be to tether the helicase to its substrate to make it less likely to dissociate. This can be accomplished in a complex of helicase and HCV RNA-dependent RNA polymerase. HCV helicase is anchored to the endoplasmic reticulum membrane through a tightly associated transmembrane NS4A protein. This alone can make NS3 protein a more processive helicase. Future experiments will address these issues and parts of the unwinding mechanism not reflected in the proposed model, such as whether unwinding occurs by interaction with the duplex DNA or by force generated from unidirectional translocation.

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