NS3 helicase actively separates RNA strands and senses sequence barriers ahead of the opening fork

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RNA helicases regulate virtually all RNA-dependent cellular processes. Although much is known about helicase structures, very little is known about how they deal with barriers in RNA and the factors that affect their processivity. The hepatitis C virus encodes NS3, an RNA helicase that is essential for viral RNA replication. We have used optical tweezers to determine at the single-molecule level how the local stability of the RNA substrate affects the enzyme rate of strand separation, whether separation occurs by an active or a passive mechanism, and whether processivity is affected. We show that sequence barriers in RNA modulate NS3 activity. NS3 processivity depends on barriers ahead of the opening fork. Our results rule out a model where NS3 passively waits for the thermal fraying of double-stranded RNA. Instead, we find that NS3 destabilizes the duplex before separating the strands. Failure to do so before a strong barrier leads to helicase dissociation and limits the processivity of the enzyme.

Barrier Dependence of NS3 Pausing and Stepping. To follow the unwinding of these substrates by NS3, we flowed NS3 and ATP together in buffer U into the fluidic chamber at 22 ± 1°C (5 mM NS3 and 1 mM ATP unless otherwise noted). We have shown previously that the helicase activity we monitored using this assay most likely originates from NS3 monomer (4). Independent single-molecule experiments, we also did single-cycle unwinding experiments in bulk and showed that the NS3 monomer does have helicase activity, and its processivity is consistent with single-molecule observations [supporting information (SI) Fig. 7]. Next, using the constant force feedback mode of the instrument, RNA-AG was held at 7.0 ± 0.1 pN, while changes in its end-to-end distance were simultaneously monitored (4). At this force, no spontaneous opening of RNA-AG was observed in the absence of either NS3 or ATP. Therefore, the extension change of RNA-AG seen when both NS3 and ATP are present must be due to helicase-catalyzed hairpin unwinding and can be converted into the number of RNA base pairs unwound at the given force as a function of time (4, 15, 16).

Strikingly, NS3 unwinding is affected by the same sequence-dependent barriers in the RNA-AG hairpin seen in force unwinding (Fig. 2). For most unwinding events (>80%), NS3 proceeds very rapidly in the A-U region of the hairpin with a

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mean step size of 11 ± 2 bp and then undergoes a long pause at the boundary (±2 bp) between A-U and G-C regions. Subsequent unwinding of the G-C region by NS3 has a mean step size of 9 ± 2 bp but is characterized by a slow stepping velocity (defined by the slope of the step; see Materials and Methods) and long pauses. Most unwinding trajectories were followed by a sharp decrease in the extension of the molecule due to dissociation of the helicase and rezipping of the hairpin. In contrast, when G-C pairs are placed in front of A-U pairs (RNA-GA), unwinding events are >50-fold less frequent than RNA-AG when the molecule is held at 7 pN. Frequent unwinding is observed when the molecule is under higher tension to help the enzyme enter and unwind the G-C region. As shown in Fig. 2B at 17 pN, the slow unwinding of the G-C region is followed by a sharp increase in the extension of the RNA to a fully unfolded state, because under these forces the A-U region of RNA-GA is already mechanically unstable.

Clearly, the pause duration and the stepping velocity of NS3 are both affected by the barriers in RNA, regardless of their location in the hairpin. More importantly, the fact that NS3 undergoes a long pause even before stepping into the G-C region (see arrows in Fig. 2A) indicates that the enzyme pauses in front of still intact, stable base pairs. Barrier effects on the dynamics of NS3 are quantitatively summarized in Table 1. Briefly, the mean pause duration in front of the G-C segment is 10-fold longer than that before the A-U segment, whereas the corresponding mean stepping velocity is 3-fold slower. Previous results indicate that force affects neither pause duration nor stepping velocity, which is also confirmed in this study for RNA-AG unwinding (SI Table 2). We have shown previously that the pause is part of the helicase enzymatic cycle and that exit from the pause is not due to binding of multiple helicase molecules (4). The fact that barriers affect pause duration more than stepping velocity indicates that pausing and stepping are associated with different biochemical events during NS3 unwinding. Previously, single-molecule unfolding experiments have been used to determine the strength and location of the various mechanical barriers in RNA (11). The above results show that these barriers indeed affect the activity of NS3 in vitro and therefore are likely to be relevant to the in vivo functionality of RNA-based motors.
Table 1. Dependence of NS3 unwinding kinetics on duplex RNA sequence

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Pause duration, s</th>
<th>Stepping velocity, bp/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% A-U</td>
<td>0.20 ± 0.03</td>
<td>62 ± 26</td>
</tr>
<tr>
<td>52% G-C</td>
<td>0.60 ± 0.06</td>
<td>51 ± 26</td>
</tr>
<tr>
<td>100% G-C</td>
<td>2.0 ± 0.3</td>
<td>22 ± 17</td>
</tr>
</tbody>
</table>

Data from RNA-AG were analyzed for pause duration and stepping velocity on 100% A-U and 100% G-C. Data from RNA3 (4) were analyzed for pause duration and stepping velocity on 52% G-C. The RNA3 substrate was characterized previously and has the following sequence: GGGAGACAUUGGUAGACAGACUACUGUACACAUCCAGAUCUCCCCCATGGG-\text{H}18528. The dashed line is calculated based on Eq. (1) with Eq. (5) and SI Table 3.

Analysis of Stepping Velocity Indicates Active Unwinding. While steps coincide with base pair opening by NS3, the barrier-dependent pause durations suggest that during a pause, NS3 either passively awaits the spontaneous fraying of the duplex to move or actively interacts with the duplex for a duration that depends on the stability of the latter. Analysis of the stepping velocity at various barriercs furnishes a way to establish this point. In the first case, unwinding is a passive process; the enzyme establishes no destabilizing interactions with the RNA and only moves forward opportunistically, upon spontaneous thermal fraying of the duplex (5, 7). Because the kinetics of thermal fraying is much faster than that of helicase movement (17, 18), the opening and closing of RNA base pairs can be assumed to be in rapid equilibrium when compared with the rate constant of helicase forward movement (see SI Text). In these circumstances, it is the free energy of base pair opening rather than the activation energy that determines how fast the helicase unwinds. As we derive (see SI Text), the mean stepping velocity of a passive helicase can be written as a function of the rate of helicase translocation (19, 20) on ssRNA and the thermodynamic stability of base pairs in front of the helicase (see SI Text): \[ \ln v = \ln s + \ln k_3 - \ln \left[ \frac{1 + c \exp \left( \frac{-\Delta G_{\text{open}}}{RT} \right)}{1 + c} \right], \] where \( v \) is the mean stepping velocity, \( s \) is the step size of the helicase, \( k_3 \) is the rate constant of helicase translocation on ssRNA and is assumed to be sequence-independent, and \( c \) gives the contribution of external force to base pair free energy, \( c = \exp\left(-F \Delta s/(k_B T)\right) \), where \( F \) is the force on the substrate and \( \Delta s \) is the end-to-end extension change in the substrate upon opening of a base pair at force \( F \). Importantlly, by definition, a passive helicase does not change the energetic of base pairs and, thus, \( \Delta G_{\text{open}} \) is simply the standard free energy to open a base pair at zero force and can be estimated from nearest-neighbor stability data (21).

To determine the extent to which NS3 relies on thermal fraying, we plotted the stepping velocity expected from this model for a purely passive helicase as a function of \( \Delta G_{\text{open}} \) (Fig. 3 A and SI Fig. 8) and compared it with the stepping velocity of NS3. As shown by the dashed line in Fig. 3 A, the stepping velocity of a purely passive helicase decreases with increasing free energy of base pair opening. Interestingly, the stepping velocity of NS3 (blue symbols) is less sensitive to the presence of barriers than what is expected for a passive helicase. In particular, for the opening of 4 bp, equivalent to one substep of NS3 observed previously (4), the mean stepping velocity of NS3 on G-C base pairs is 14,000-fold faster than a passive helicase, indicating that NS3 actively reduces the free energy of base pair opening.

We also performed calculations for substeps of other sizes (SI Fig. 8). For a 1-bp substep (22), the stepping rate of NS3 is still 2- to 3-fold faster than a passive helicase on substrates with 50% or more GC content (Fig. 3C). Thus, rather than passively awaiting the thermal fraying of the RNA, NS3 interacts with the duplex and actively speeds up RNA unwinding.

To lower the free energy of base pair opening, the helicase can either destabilize the dsRNA or stabilize the separated single strands upon unwinding. To distinguish between these two possibilities, we investigated how the durations of the pauses preceding the NS3 steps depend on the barrier ahead. We found that pause durations are much shorter than those expected for a passive helicase (Fig. 3C), a result consistent again with a scenario in which NS3 does not passively wait for the thermal fraying of base pairs before stepping but instead actively destabilizes the RNA duplex during the pause. This conclusion is also supported by calculations done for 1-, 2-, 3-, and 5-bp openings (data not shown). The minimum amounts of free energy reduction in base pair opening introduced by the active enzyme, calculated from Fig. 3 A, are 1.8, 6.7, and 10.1 RT for 4 bp made up of 100% A-U, 52% G-C, and 100% G-C, respectively (SI Text and SI Table 3).

Barrier Dependence of NS3 Processivity and Residence Time. Next, to understand the interaction of NS3 with the RNA substrate, we investigated how the barriers present in RNA influence the

Fig. 3. NS3 moves on RNA by destabilizing duplex RNA. (A) The mean stepping velocity of NS3 (blue symbols) depends much less on \( \Delta G_{\text{open}} \) than a passive helicase (dashed line), where the stepping velocity for 52% G-C was taken from a previous study (4). The free energy is in units of RT, where \( R \) is the gas constant and \( T = 295 \text{ K} \). The \( y \)-intercept of the dashed line is set identical to the \( y \)-intercept of a straight line that fits NS3 stepping velocity. This straight line (not shown) gives a slope of \( -0.09 \) for 4 bp being unwound each time and defines an upper bound of 106 s\(^{-1}\) for NS3 stepping velocity on dsRNA. (B) Dependence of helicase mean stepping velocity on the free energy of base pair opening. The dashed line is calculated for a passive helicase with a substep size of 1 bp. (C) Dependence of helicase mean pause duration on the free energy of base pair opening. The dashed line is calculated based on Eq. 5 (SI Text) at 7 pN for a purely passive helicase. The \( y \)-intercept given by a straight line (not shown) that fits NS3 data defines a lower bound of 0.02 s for NS3 pause duration on dsRNA. The \( y \)-intercept for the dashed line is chosen to be the same as that of the straight line for this comparison. All of the error bars represent 68% confidence interval.

not the case for NS3; rather, the residence time of NS3 on RNA helicase off-rate on ssRNA and independent of the position and on the substrate before detachment) should be the inverse of the mean residence time (i.e., the average time that the helicase stays or an active helicase. In particular, for a passive helicase that and dissociate. This conclusion would be valid for either a passive competition between on-pathway unwinding and off-pathway probability of detachment while pausing than while stepping. NS3 may have a higher encountering a barrier? Because a strong barrier induces the chance of the enzyme to move off of the main helicase pathway detachment can explain these observations (23). Strong barriers on the residence time of NS3 on RNA suggests an active interaction between NS3 and the barrier, which for stronger barriers leads to the accelerated detachment of the helicase (24). Indeed, the rate coefficient of NS3 dissociation upon unwinding G-C sequences is 0.5 ± 0.2 s⁻¹ at 7 pN, which is 2-fold faster than that on A-U sequences at the same force. These results are inconsistent with a purely passive helicase (24) but reveal a motor that actively destabilizes and separates the RNA strands. The picture that emerges then is one in which the enzyme must interact with the dsRNA to destabilize the duplex, and failure to do so speeds up the enzyme detachment from the substrate.

**Tunable Response of NS3 to Sequence Barriers.** The model described above predicts that the probability of enzyme dissociation should depend on the physical size of the barrier (i.e., the number of G-C pairs), because its size will determine the stability of the barrier and the tendency of the duplex to rezip. To test this model, we designed two more RNA hairpin substrates (Fig. 5A). Instead of having all A-U pairs followed by G-C pairs, substrate RNA-6GC has 22 A-U pairs followed first by 6 G-C pairs, then 5 A-U pairs, and then 27 G-C pairs; while substrate RNA-3GC has 22 A-U pairs followed first by 3 G-C pairs, then 5 A-U pairs, and then 30 G-C pairs. We first characterized the mechanical unfolding properties of these substrates. As shown in SI Fig. 10A, RNA-6GC unfolds in three transitions by mechanical force; the unfolding of the first 22 A-U pairs occurs at 11.5 ± 0.1 pN; the next 6 G-C pairs followed by 5 A-U pairs unfold at 20.5 ± 0.2 pN; and the next 27 G-C pairs unfold at 25.8 ± 0.3 pN. RNA-3GC also unfolds in three transitions by mechanical force (SI Fig. 10B), but the second transition corresponding to 3 G-C pairs and 5 A-U pairs occurs at 17.0 ± 0.1 pN.

The unwinding of RNA-6GC and -3GC by NS3 is shown in Fig. 5 B and C, respectively. Most unwinding events on RNA-6GC show the effect of the 6 G-C pairs spanning positions 23–28 with NS3 responding either by detachment or a long pause followed by rapid unwinding upon encountering the barrier. In contrast, the most unwinding events on RNA-3GC do not show any significant effect of the first 3 G-C pairs on NS3 unwinding and processivity but undergo long pauses or detachment when NS3 faces the second G-C barrier in the substrate. These observations are quantitatively summarized in Fig. 5D by using a cumulative processivity plot, which describes the fraction of unwinding events at each duplex length taking place before detachment. The preferential detachment of NS3 on RNA-6GC over -3GC shows that NS3 processivity depends on the strength of the barrier. Most interestingly, although RNA-6GC and -3GC are identical in sequence up to base pair 25 (indicated by the dashed line in Fig. 5D), the fraction of NS3 molecules that dissociate begins to differ at base pair 20 and at base pair 25 already differs by 40% between these two substrates. These results reveal that the probability of NS3 detachment depends on the sequence ahead of the opening fork as far as 6 bp. RNA thermodynamic stability is well described by nearest-neighbor interactions (21). The fact that NS3 detachment is affected by RNA 6 bp away from the opening fork indicates that NS3 interacts with the duplex RNA at least 6 bp ahead of the fork and that this interaction, in turn, affects the stability of the enzyme–substrate complex.

**Discussion**

To rationalize the above observations, we propose a model for the movement of NS3 as it unwinds a RNA duplex. In this model, NS3 makes at least two points of contact with the substrate; one at the fork, possibly tracking the ssRNA during
active unwinding, and the other ahead of the fork, contacting the duplex RNA to (i) actively destabilize it during the pause preceding the step and (ii) establish new thermodynamically more favorable interactions with the RNA (see Fig. 6 for a free energy diagram and a cartoon model of these interactions). The processivity of the enzyme depends on the formation of a stable contact with the RNA ahead. Such a contact may involve partial melting of the duplex and, therefore, its duration should be barrier-dependent. It is easier for the enzyme to form a stable contact on A-U than on G-C sequences because A-U duplexes are thermodynamically less stable. Depending on the strength of the barrier (affected variously by its sequence, its length, etc.), the interaction with duplex RNA ahead of the fork leads to either stabilization of the NS3–RNA complex competent for unwinding or the accelerated detachment of NS3 from the substrate. We term this phenomenon “barrier sensing.” In the latter case, NS3 dissociates from the RNA even before the duplex is unwound. A very important prediction of this model is that once NS3 succeeds in making a stable contact with the RNA ahead, the speed of subsequent strand separation as defined by the stepping velocity should be less sensitive to the strength of the barriers, because the free energy of base pair opening has already been lowered by the helicase at the end of a pause. Indeed, the stepping velocity of NS3 on A-U versus G-C only differs by 3-fold, in contrast to the 10-fold difference in the mean pause duration before stepping (Table 1), and NS3 stepping velocity is much faster than that of a passive helicase (Fig. 3A).

Collectively, our data strongly favor NS3 being an active helicase instead of a purely passive Brownian ratchet. Lohman and coworkers (26, 27) have conducted elegant pre-steady-state bulk experiments to test whether a helicase uses a passive or an active mechanism of unwinding. In these experiments, it was shown that Rep or UvrD helicase can bypass a non-DNA linker and also with the sensitivity of NS3 helicase activity to the structure of the duplex from bulk studies (25).

Mechanical force has been used to study folding and unfolding of single RNA molecules (11, 13) (SI Fig. 11). However, it remained unclear how relevant the information deduced from these studies is to biochemical processes involving RNA helicases and other motors, such as ribosomes, that must unwind dsRNA regions during their translocation. The present study validates the results of single RNA molecule unfolding experiments by revealing an important correspondence between mechanical force and the enzymatic action of the NS3 helicase: duplex sequences that require a higher mechanical force to
unfold and constitute barriers in mechanical unfolding experiments also cause the helicase to pause longer and to move slower.

Numerous nucleic acid translocases were shown to move at rates limited by the thermal fraying of nucleic acid base pairs (7, 28). In contrast to these enzymes, NS3 moves on nucleic acids faster than the rate imposed by the thermal fraying of base pairs. We propose that this faster, catalyzed movement is facilitated by a decrease of the stability of the RNA duplex brought about by contacts between the helicase and duplex RNA ahead of the opening fork. The sensitivity of NS3 helicase to sequence barriers in RNA should be relevant to a broad range of motors using RNA as substrate, such as ribosomes that translate through secondary structures in mRNAs (29) and whose dynamics (pauses, rate of translocation, and frame-shifting) may depend on the sequence barriers encountered therein.

Materials and Methods

RNA Substrates and NS3 Protein. The RNA molecules used throughout this study were all made from in vitro transcription using T7 RNA polymerase (Ambion) as described (4) with modifications. The detailed procedures are reported in SI Text. Full-length NS3 from HCV genotype 1a was overexpressed in M15(pRep4) (Qiagen) and purified by using the protocol described in ref. 30. NS3 concentration was measured by using DTT, pH 6.5). Bulk unwinding assays under single-turnover conditions in 20 mM sodium phosphate buffer with 6 M guanidinium chloride, pH 6.5.

Data Analysis. NS3 steps and pauses were analyzed as described previously by using a custom-written MATLAB program (4). To examine whether a detachment of the helicase is within a step or within a pause, the shortest pause was required to be longer than 70 ms. To calculate the distribution of NS3 mean residence time on a substrate, a random sampling analysis of NS3 residence time was performed. For unwinding traces collected for a given RNA substrate, 50 traces were randomly chosen each time to compute the mean residence time. This random selection process was repeated 1,000 times to obtain the distribution of the resulting mean residence time. The errors for step size and stepping velocity were standard deviations. The errors for pause duration and residence time were standard errors of the mean. The errors for fraction of unwinding were standard deviations obtained from bootstrap analysis for the same data sets sampled 1,000 times.

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Optical Tweezers Experiments. We used a counterpropagating dual-beam optical tweezers instrument (12) to manipulate individual RNA molecules. Unless otherwise noted, the force-extension measurements of the RNA hairpin were done at 22 ± 1°C in standard buffer (10 mM Tris·Cl/100 mM NaCl, pH 7.0), and the helicase unwinding experiments were done as described (SI Text) at 22 ± 1°C in buffer U (20 mM MOPS/30 mM NaCl/0.9% vol/vol glycerol/0.75 mM MgCl2/0.1% Tween 20/2 mM DTT, pH 6.5).