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Unwinding initiation by the viral RNA helicase NPH-II

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Abstract

Viral RNA helicases of the NS3/NPH-II group unwind RNA duplexes by processive, directional translocation on one of the duplex strands. The translocation is preceded by a poorly understood unwinding initiation phase. For NPH-II from vaccinia virus, unwinding initiation is rate limiting for the overall unwinding reaction. To develop a mechanistic understanding of the unwinding initiation, we studied kinetic and thermodynamic aspects of this reaction phase for NPH-II *in vitro*, using biochemical and single molecule fluorescence approaches. Our data show that NPH-II functions as a monomer and that different stages of the ATP-hydrolysis cycle dictate distinct binding preferences of NPH-II for duplex vs. single stranded RNA. We further find that the NPH-II-RNA complex does not adopt a single but at least two distinct conformations in each of the analyzed stages of ATP hydrolysis. These conformations interconvert with rate constants that depend on the stage of the ATP hydrolysis cycle. Our data establish a basic mechanistic framework for unwinding initiation by NPH-II and suggest that the various stages of the ATP hydrolysis cycle do not induce single, stage-specific conformations in the NPH-II-RNA complex, but primarily control transitions between multiple states.

Keywords

Fluorescence; FRET; Translocation; SF2; Single Molecule

INTRODUCTION

Most aspects of RNA metabolism in eukaryotes, bacteria and many viruses involve RNA helicases of the helicase superfamily 2, ubiquitous enzymes that use ATP to bind and remodel RNA and RNA protein complexes^{1, 2, 3}. SF2 helicases are further classified into distinct families, based on structural, sequence and mechanistic characteristics¹. SF2 helicases of the NS3/NPH-II family, named after NS3 from hepatitis C virus and NPH-II from vaccinia virus, are encoded by diverse viruses, where these proteins are essential for viral replication^{4, 5}. HCV NS3 functions in conjunction with the viral polymerase and several other viral proteins in complexes that replicate the HCV genome⁵. NPH-II has been implicated in transcription termination in vaccinia virus and in the export of viral RNAs out of the virion⁴.

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The biological functions of NS3/NPH-II helicases correlate with NTP-dependent unwinding activities^{6,7}, but the connections between helicase activities and physiological functions are not well understood. Nonetheless, HCV NS3 and NPH-II have become attractive model systems for the mechanistic analysis of RNA helicase activity⁸. Both, HCV NS3 and NPH-II unwind RNA and DNA duplexes, in a unidirectional, stepwise, and processive fashion^{6,8-12}. This unwinding mode resembles canonical DNA helicases¹³.

In vitro, RNA duplex unwinding by NPH-II and NS3 requires substrates with a single stranded overhang 3' to the duplex^{6,9,14,15}. The helicases bind to the overhang prior to unwinding, and are thereby oriented for subsequent translocation 3' to 5' along this "loading" strand¹⁶⁻¹⁸. These binding and orientation events collectively constitute the unwinding initiation process.

In the NS3/NPH-II helicases for which this has been tested, unwinding initiation is markedly slower than the subsequent unwinding steps^{6,10}. In NPH-II, unwinding initiation is at least two orders of magnitudes slower than subsequent strand separation steps⁶. Initiation is thus rate limiting for overall unwinding under usual reaction conditions⁶. A further important characteristic of the initiation process by NPH-II is the continuous hydrolysis of ATP before the start of the actual unwinding⁶. ATPase measurements suggest that the enzyme turns over hundreds of ATP before starting unwinding¹⁹. These findings indicate that unwinding initiation is itself a complex process.

Despite the significance of initiation for the overall unwinding reaction by NS3/NPH-II helicases, it is unclear which processes occur during this reaction phase. Here, we studied kinetic and thermodynamic aspects of this reaction phase for NPH-II *in vitro*. We examined specific stages of the ATPase cycle using non-hydrolyzable ATP analogs and a combination of biochemical and single molecule fluorescence approaches. Our data show that NPH-II functions as a monomer in which different stages of the ATP-hydrolysis cycle dictate distinct binding preferences for duplex vs. single stranded RNA. We also found that the NPH-II-RNA complex does not adopt a single but at least two distinct conformations in each of the analyzed stages of ATP hydrolysis. These conformations interconvert with rate constants that depend on the stage of the ATP hydrolysis cycle. Our data establish a basic mechanistic framework for the unwinding initiation process by NPH-II and suggest that the various stages of the ATP hydrolysis cycle do not induce single, stage-specific conformations in the NPH-II-RNA complex, but primarily control interconversion between multiple conformational states.

RESULTS

Different stages of the ATP hydrolysis cycle dictate distinct binding preferences of NPH-II for ssRNA and duplex RNA

To characterize unwinding initiation by NPH-II, we first measured RNA binding at the main stages of the ATP hydrolysis cycle. We performed density gradient centrifugation with NPH-II and radiolabeled RNA without nucleotide, with ADP, and with the non-hydrolyzable ATP analogs ADP-beryllium fluoride (ADP-BeF_x, ground state analog²⁰) and ADP-aluminum fluoride (ADP-AlF_x, transition state analog²⁰)(Fig.1). ADP-BeF_x and ADP-AlF_x have been widely employed in mechanistic and structural studies of other RNA helicases, including HCV NS3²¹⁻²⁵.

We determined how many protomers of NPH-II bound a duplex RNA with a 3' ssRNA overhang. This RNA substrate (calculated molecular weight $M_W = 21$ kD) had been previously used in unwinding studies of NPH-II^{6,16}. Without nucleotide, NPH-II ($M_W = 77$ kD) shifted the RNA to an apparent molecular weight consistent with a single protomer of

NPH-II bound to the RNA ($M_w(C) = 113 \pm 25$ kD, Fig.1A, left panel). Next, we examined binding of NPH-II to RNAs containing only the duplex or the single stranded part of the substrate (Fig.1A, middle and right panels). We observed binding of a single NPH-II protomer to the duplex, but no association to the ssRNA (Fig.1A, middle and right panels).

In the presence of the ATP ground state analog ADP-BeF_x, NPH-II shifted the RNA substrate to an apparent molecular weight consistent with binding of two protomers (Fig.1B, left panel). Binding of NPH-II to both, duplex and ssRNA was detected, but only a single protomer appeared to bind in each case (Fig.1B, middle and right panels). With ADP-AlF_x, NPH-II shifted the RNA to an apparent molecular weight most consistent with binding of a single protomer (Fig.1C). The broadening of the peak, compared to the peak without nucleotide (Fig.1A) suggests a distinct conformation of the NPH-II RNA complex. No binding to the duplex was detected, and a single protomer associated with the ssRNA (Fig. 1C). With ADP, a single protomer was bound to the RNA substrate and to the duplex, but no binding to the ssRNA was detected (Fig.1D).

Collectively, the density gradient centrifugation experiments provided three insights. First, NPH-II binds to the RNA substrate at all stages of the ATPase cycle tested here. This observation is consistent with the notion that NPH-II maintains uninterrupted substrate contact during unwinding initiation, despite the continued hydrolysis of hundreds of ATPs per substrate¹⁹. Second, NPH-II changes binding preferences between ssRNA and duplex RNA during the various stages of the ATPase cycle. This result explains the previously reported ability of NPH-II to maintain localization on the ss/duplex junction during initiation¹⁸. Third, the data revealed that NPH-II binds the RNA as a monomer, except in the ATP ground state mimicked by ADP-BeF_x, where two protomers appear to bind.

NPH-II unwinds RNA duplexes as a monomer

While instructive, the density gradient experiments left open the question whether binding of multiple protomers in the ATP ground state was required for unwinding, or merely reflected a propensity of NPH-II monomers to bind to duplex and single stranded RNA at this stage of the ATP hydrolysis cycle. To distinguish between these possibilities, we performed a functional stoichiometric titration (Fig.2). This experiment measures unwinding at substrate and enzyme concentrations that exceed the enzyme-substrate dissociation constant, and with substrate concentrations significantly higher than enzyme concentrations (Materials and Methods). The number of protomers needed for the unwinding reaction is reflected in the correlation between unwinding signal and protein/RNA ratio^{6,26,27}. To avoid complications in the data interpretation through multiple re-initiation events per substrate, we performed unwinding reactions under single cycle conditions, which preclude re-binding of the helicase to the substrate during the course of the reaction^{6,27}. Under single cycle conditions, the final extent of the reaction for a given substrate (unwinding amplitude) corresponds to the fraction of NPH-II productively bound at the reaction start⁶.

Unwinding amplitudes increased linearly with the NPH-II concentration until an enzyme substrate ratio of 1:1. No further increase was seen at higher NPH-II concentrations (Fig. 2B). This finding clearly shows that only one protomer of NPH-II per substrate is needed to reach the maximal unwinding amplitude. Oligomerization at any stage of the ATPase cycle would be reflected in a NPH-II substrate ratio greater than 1:1²⁶.

The data indicate that a single NPH-II is competent to catalyze the unwinding reaction. Binding of multiple NPH-II protomers in the ATP ground state is not required for unwinding. We therefore conclude that the binding of multiple protomers to the RNA substrate in the ATP ground state, seen by gradient centrifugation with ADP-BeF_x, reflects a functionally independent association of protomers to both single strand and duplex regions.

Observation of RNA binding and duplex unwinding by NPH-II with single molecule FRET

Having established that NPH-II unwinds as a monomer, we employed single molecule fluorescence resonance energy transfer (smFRET) to further characterize the initiation process. We used a TIR fluorescence detection setup (Materials and Methods) and fluorescently labeled RNA (19 bp duplex, 25 nt unpaired overhang 3' to the duplex), immobilized on a PEG-coated surface^{28,29} (Fig.3A). The labeling scheme allowed us to detect binding of unlabelled NPH-II to the single stranded region, conformational changes of the NPH-II -RNA complex, and unwinding events (Fig.3A).

We first recorded smFRET histograms for free RNA, and NPH-II bound to RNA with and without ATP (Fig.3B). The smFRET distribution of free RNA substrate showed a single peak at $E = 0.85$ (Fig.3B, upper panel). Addition of saturating amounts of NPH-II shifted the distribution to two peaks at $E = 0.33$ and $E = 0.15$ (Fig.3B, middle panel). This shift shows binding of NPH-II to the single stranded region and reveals two conformationally distinct NPH-II-RNA complexes.

NPH-II binding to ssRNA without nucleotide was not seen with density gradient centrifugation (Fig.1A), suggesting that NPH-II and ssRNA do not form complexes that are sufficiently persistent for detection by density gradient centrifugation. Alternatively, binding of NPH-II to ssRNA without ATP might require an adjacent duplex. Upon addition of NPH-II and ATP at concentrations where robust unwinding is seen⁶, the number of RNA complexes displaying FRET diminished over time, as expected, due to unwinding and corresponding removal of the CY3 label (data not shown). However, the smFRET distribution of the RNAs prior to unwinding was highly similar to that observed with free RNA (Fig.3B, lower panel). This finding suggests that ATP-dependent conformational changes of the NPH-II-RNA complex prior to unwinding occur at a timescale that exceeds the time resolution of the detection setup. Based on the high turnover number for ATP ($k_{\text{cat}} \sim 10^4 \text{ min}^{-1}$ ref.¹⁹), the frequency of conformational transitions of the NPH-II-RNA complex driven by ATP hydrolysis events are in fact expected to be considerably greater than the time resolution of our smFRET setup ($\tau = 0.1\text{s}$). Thus, only an average FRET value would be seen in each frame. Free RNA is also thought to sample a large number of different conformations over the time needed to acquire a single frame³⁰, and it is therefore not unexpected that both free RNA and NPH-II bound to the RNA in the presence of ATP yield similar FRET values.

To gain further insight into the characteristics of NPH-II-RNA complexes with and without ATP, we examined smFRET timetraces (Fig.3C). While timetraces of individual free RNA substrates did not reveal interpretable patterns of dynamics (Fig.3C, left panel), addition of NPH-II without ATP showed interconversion between the two FRET states seen in the corresponding smFRET distribution (Fig.3C, second panel). Addition of ATP (3.5 mM) to the NPH-II RNA complex changed the FRET values again, consistent with the corresponding FRET histogram. No interpretable patterns of dynamics of the NPH-II-RNA complex could be elucidated, except for a spike in the FRET value after several seconds, which was followed by disappearance of the signal (Fig.3C, third panel). This signature is anticipated for a strand separation event (Fig.3A). Consistent with the notion that the FRET spike reflected a strand separation event, the duration of the spike was longer at lower ATP concentrations (0.01 mM), where the strand separation process is expected to be slower (Fig. 3C, right panel). Together, the timetraces with NPH-II and ATP thus directly reflect prolonged unwinding initiation ($E \sim 0.85$) and the much faster strand separation (smFRET spike). The absence of interpretable patterns of dynamics in the NPH-II-RNA complex before the strand separation further support the notion that ATP-dependent conformational changes of the NPH-II-RNA complex occur at a timescale that exceed the time resolution of the detection setup.

At lower ATP concentrations ($[ATP] = 10 \mu M$, Fig.3C, right panel), fluctuations in smFRET values before the unwinding event appeared more pronounced than at the higher ATP concentrations (Fig.3C, right panel). Nevertheless, we could not delineate statistically significant patterns of dynamics for the NPH-II-RNA complex preceding the strand separation. We were thus not able to determine whether NPH-II shuttles back and forth on the single stranded region, as seen for the Rep, BLM and HCV-NS3 helicases^{31–33}. Extensive NPH-II dissociation from the RNA at $[ATP] < 10 \mu M$ precluded meaningful interpretations of timetraces at even lower ATP concentrations (data not shown).

Formation of NPH-II RNA complexes with non-hydrolysable ATP analogs

Since we could not delineate clear dynamic patterns of the NPH-II-RNA complex in the presence of ATP during unwinding initiation, further characterization of this reaction step required the arrest of the ATP-hydrolysis cycle at defined stages. To accomplish this arrest, we used the non-hydrolyzable ATP ground state analog ADP-BeF_x, the transition state analog ADP-AlF_x, and ADP in binding reactions of NPH-II and RNA that we monitored by smFRET (Fig.4). SmFRET distributions for the NPH-II-RNA complex with ADP-BeF_x showed three peaks at the highest experimentally accessible NPH-II concentrations (Fig.4, upper panel). Two peaks correspond to bound NPH-II, as seen without nucleotide (Fig.3B). The other peak corresponds to "free" RNA, and thus represents either RNA without NPH-II, or NPH-II bound to the duplex region, as suggested by the density gradient density centrifugation data (Fig.1B). With ADP-AlF_x, we detected two peaks, corresponding to those representing NPH-II bound to the single stranded region (Fig.4, middle panel). With ADP, only one peak was seen, corresponding to either RNA without NPH-II, or, in line with the density gradient density centrifugation data, NPH-II bound to the duplex region (Fig.4, lower panel).

Collectively, the smFRET distributions without (Fig.3B) and with nucleotides (Fig.4) provide two insights. First, NPH-II binds to single stranded RNA regions of the substrate at all of the tested stages of the ATP hydrolysis cycle, except in the ADP-bound state. The smFRET data thus confirm and extend the findings obtained by density gradient centrifugation (Fig.1). Second, complexes formed between NPH-II and single stranded RNA at the interrogated stages of the ATP hydrolysis cycle appear to exist in at least two conformationally distinct complexes. The highly similar FRET peak values of the two NPH-II bound species suggest that these two conformations are similar at the tested stages of the ATP hydrolysis cycle.

Two distinct conformations of the NPH-II RNA complex reflect a single protomer bound to RNA

To further understand the two distinct conformations, we examined whether the two smFRET peaks were caused by successive binding of multiple NPH-II protomers. Although functional titration experiments had shown that a single NPH-II protomer was fully unwinding competent (Fig.2), multiple RNA-bound NPH-II protomers in the ATP ground state were seen by density gradient centrifugation (Fig.1B). Yet, even this method did not necessarily detect all NPH-II RNA complexes, as highlighted by NPH-II binding to ssRNA regions, which was not seen by density gradient centrifugation, but evident in the smFRET data (Fig.3B). To assess a possible oligomerization of NPH-II on the RNA by smFRET, we measured smFRET distributions at increasing NPH-II concentrations without nucleotide, with ADP-BeF_x, and with ADP-AlF_x (Fig.5). The fraction of the three smFRET peaks at $E = 0.85$ (free RNA), $E = 0.33$ and $E = 0.15$ (NPH-II bound RNA) was plotted as a function of the NPH-II concentrations (Fig.5).

Without nucleotide, the fraction of the peak at $E = 0.85$ decreased with the NPH-II concentration, as expected for the binding reaction (Fig.5A). The binding constant calculated from these smFRET data was in excellent agreement with the constant obtained by non-denaturing gel shift electrophoresis (Fig.5A, Suppl. Fig.S1), indicating that the smFRET system faithfully reported quantitative aspects of NPH-II -RNA binding. Concomitant with the decrease of the peak at $E = 0.85$, the fraction of the peaks at $E = 0.33$ and $E = 0.15$ rose with the NPH-II concentration (Fig.5A). The ratio between the two peaks remained constant (Fig.5A, lower panel), indicating that neither peak arose from successive binding of multiple NPH-II protomers.

With ADP-BeF_x, a similar trend was observed (Fig.5B). Free RNA ($E = 0.85$) decreased with increasing NPH-II concentrations, and bound RNA ($E = 0.33$ and $E = 0.15$) increased (Fig.5B). The ratio between the bound peaks remained constant. The binding constant for NPH-II was about one order of magnitude higher than without nucleotide.

With ADP-AlF_x, almost no free RNA was seen, even at the lowest experimentally accessible NPH-II concentrations (Fig.5C). This observation shows that NPH-II was already bound at the lowest protein concentrations. Again, the ratio between the two peaks remained constant with increasing NPH-II concentrations (Fig.5C). Collectively, the data provided three insights. First, the two smFRET peaks reflecting bound NPH-II were not caused by successive binding of multiple protomers, and therefore correspond to distinct conformational states of a single NPH-II protomer bound to RNA. Second, the same conformational states exist at different stages of the ATP hydrolysis cycle. Third, the ratio between the states varies with the stage of the ATP hydrolysis cycle.

Interconversion between the conformations of the NPH-II RNA complex depends on the stage of the ATP hydrolysis cycle

To further understand the different ratios between the two conformational states of the NPH-II RNA complex at different stages of the ATP hydrolysis cycle, we analyzed smFRET time trajectories (Fig.6). Without nucleotide, frequent transitions were seen between the NPH-II bound states $E = 0.33$ and $E = 0.15$ and between $E = 0.33$ and $E = 0.85$ and *vice versa* (Fig. 6A, B). This pattern indicates frequent binding and dissociation events ($E = 0.33 \leftrightarrow E = 0.85$), and interconversion between the bound NPH-II states ($E = 0.33 \leftrightarrow E = 0.15$) at the experimentally accessible timescale. Time trajectories with ADP-BeF_x showed a highly similar pattern and trajectories with ADP-AlF_x showed transitions between the two lower FRET states, but only few transitions from and to the state at $E = 0.85$ (Fig.6B). This observation indicates that NPH-II binding and dissociation events occur frequently without nucleotide and with ADP-BeF_x, but much less with ADP-AlF_x, consistent with the stabilities of the respective complexes (Fig.5).

Transitions from $E = 0.15$ to $E = 0.85$ and *vice versa* were rare under all conditions (Fig.6B), suggesting that both NPH-II binding and dissociation proceeded virtually exclusively through the $E = 0.33$ state. Collectively, the data indicate a strongly preferred two step reaction path under all conditions, from $E = 0.85$ to $E = 0.33$ to $E = 0.15$, and reverse. The few transitions from $E = 0.85$ to $E = 0.15$ (and *vice versa*) may represent mostly events where the dwell time in the $E = 0.33$ state is shorter than the time resolution of the instrument, thus giving the appearance of a transition from the highest to the lowest FRET state.

To illuminate dynamic aspects of the interconversion between the FRET states, we determined kinetic parameters from the dwell times in the respective states (Fig.7). The transition from $E = 0.85$ to $E = 0.33$, which represents the binding of NPH-II to the RNA, displayed single exponential kinetics (data not shown). Transitions from the two NPH-II

bound states showed multi-exponential characteristics, indicating that each of these FRET state represents at least two kinetically discrete states. (Fig.7A). We did not detect molecular memory effects³⁴ (Suppl. Fig. S2).

Based on the two step reaction path (Fig.7B), we determined rate constants from the dwell times in the respective states (Fig.7C). Several rate constants were largely unaffected by the nucleotides, but notable nucleotide effects were seen for the dissociation of NPH-II from the RNA (k_{-1} a). ADP-BeF_x slowed NPH-II dissociation, compared to the reaction without nucleotide. ADP-AlF_x slowed dissociation to an even greater extent, consistent with results obtained by EMSA (Suppl. Fig. S3). We discuss below how slowing of the dissociation rate constant by ADP-BeF_x is reconciled with lower RNA affinity of NPH-II with this nucleotide. Significant nucleotide effects were also seen on the transition from $E = 0.33$ to $E = 0.15$ (k_2 a). The transition was slowed by ADP-BeF_x, compared to the reaction without nucleotide. This slowing explains why the FRET state at $E = 0.33$ is more populated with ADP-BeF_x (Fig.4). ADP-AlF_x accelerated the transition from $E = 0.33$ to $E = 0.15$ (k_2 a), explaining the lower population of the FRET state at $E = 0.33$ (Fig.4). Collectively, the kinetic data show that the multiple FRET states seen for the NPH-II-RNA complex interconvert with all nucleotides tested as well as without nucleotide. The nucleotides alter the dynamics of the interconversion and modulate NPH-II dissociation, but they do not induce one particular state of the NPH-II-RNA complex.

DISCUSSION

In this analysis of the unwinding initiation process by NPH-II we have shown (i) that the helicase binds and unwinds RNA duplex substrates as monomer, (ii) that different stages of the ATP hydrolysis cycle modulate binding preferences for single stranded vs. duplex RNA, and (iii) that the NPH-II-RNA complex does not adopt a single, but multiple distinct, yet interconverting conformations. The NPH-II-RNA complex traverses these conformations at all tested stages of the ATP hydrolysis cycle. However, rate constants for certain transitions depend on the particular stage of the ATP hydrolysis cycle.

NPH-II functions as a monomer

Our data show that NPH-II unwinds RNA duplexes efficiently as a single protomer (Fig.2). Notwithstanding, multiple protomers can bind independently to the substrate (Fig.1), but these protomers do not functionally interact. The ability of NPH-II to function as a monomeric RNA helicase highlights a difference to the phylogenetically related HCV NS3, which requires oligomerization to achieve optimal unwinding activity^{35,36}. Yet, HCV NS3 also functions as a monomer under certain conditions on some substrates³⁷. The data presented here for NPH-II do not rule out the possibility that multiple NPH-II protomers bind to substrates with ssRNA tails longer than those used in this study. It also remains possible that binding of multiple protomers to longer single stranded regions synergistically enhance processivity over the monomeric protein, as has been observed for HCV NS3 and the SF1 DNA helicase Dda^{36,38}. Effects of overhang lengths for unwinding by NPH-II remain to be examined.

Our data show that a NPH-II monomer is able to bind to both single and double stranded RNA (Fig.1). Although binding to double stranded RNA is not strictly required for a translocating helicase, other monomeric translocating DNA helicases, including PcrA, Rep, UvrD or Hel308, also contact duplex regions³⁹⁻⁴². These helicases use auxiliary domains, not the helicase core to bind the duplex RNA. While structural studies are required to determine how NPH-II binds to duplex and single stranded regions, one can speculate, based on analogies to other helicases, that the helicase core of NPH-II probably promotes binding

to single stranded RNA. Duplex binding may be established by C- or N-terminal domains of NPH-II.

Different stages of the ATP hydrolysis cycle modulate RNA affinities of NPH-II

A central aim of our study was to illuminate connections between substrate binding and stages of the ATPase cycle during unwinding initiation. We found that affinities for both duplex and single stranded regions are modulated by the stages of the ATPase cycle. NPH-II binds to double stranded RNA without nucleotide, with the ATP ground state analog and with ADP, but not in the presence of the transition state analog (Fig.1). Single stranded RNA is bound without nucleotide, and with ATP ground - and transition state analogs, but not with ADP (Fig.1). These findings show that NPH-II binds to RNA substrates with both single and duplex regions during the entire ATP hydrolysis cycle. This result explains how NPH-II can maintain contact to the substrate throughout many ATP turnovers during unwinding initiation. The ability of NPH-II to alternate binding between single strand and duplex region also rationalizes how the protein can remain localized at the junction throughout the initiation process¹⁸.

The analysis of NPH-II binding to a ssRNA region reveals interesting similarities to the closely related HCV NS3. Both, NPH-II and HCV NS3 bind single stranded nucleic acid tightly in the absence of nucleotide⁴³ (Fig.5A). In the ATP ground state, the affinity decreases for both proteins²⁵ (Fig.5B). Our data reveal an unanticipated facet of a seemingly straightforward result for NPH-II. While ADP-BeF_x decreases the affinity of NPH-II for RNA (Fig.5), the dissociation rate constant of NPH-II from RNA is also markedly decreased, compared to the reaction without nucleotide (Fig. 7). This decrease is not offset by corresponding changes in the association rate constant. However, ADP-BeF_x also alters the rate constant for interconversion between two conformations of the NPH-II RNA complex ($E = 0.33$, and $E = 0.15$), significantly favoring the population of the state at $E = 0.33$, from which NPH-II dissociates primarily, or even exclusively (Fig.7). Thus, dissociation events occur with a higher probability with ADP-BeF_x than without nucleotide, even though the actual dissociation rate constant is decreased.

In contrast to the perhaps non-intuitive effects of the ATP ground state analog, the transition state analog ATP-AIF_x impacts RNA binding largely as expected. It greatly stabilizes the interaction with RNA (Fig. 6C), similar to observations made with the DNA helicase Rep²². However, transitions between the two RNA-bound states of NPH-II still occur (Fig. 7B). This observation indicates that ADP-AIF_x does not completely mimic the transition state, because no conversions are expected for a "true" transition state analog⁴⁴. Notwithstanding, given that ADP-AIF_x adopts a transition state - like geometry in numerous crystal structures of helicases²⁰, the distinct characteristics of the NPH-II RNA complex with this analog render reasonable the interpretation that these characteristics correspond to features of the NPH-II RNA complex in the ATP transition state.

ADP promotes binding of NPH-II to duplex RNA, but greatly decreases the affinity of NPH-II for single stranded RNA (Figs.1,4). This decrease differs from the tight ssRNA binding induced by ADP for HCV-NS3²⁵. Thus, despite significant mechanistic similarities between NPH-II and HCV-NS3, the two proteins differ in the way how certain stages in the ATP hydrolyses cycle modulate RNA affinities.

The stages of the ATP hydrolysis cycle modulate interconversion between multiple conformations of the NPH-II RNA complex

Analysis of RNA binding by smFRET revealed unanticipated dynamics of the complex that NPH-II forms with single stranded RNA. Instead of adopting a single, defined

conformational state at each stage of the ATP hydrolysis cycle, the NPH-II RNA complex is found in at least two distinct conformations (Figs.3, 5). The multiphasic kinetics observed for the transitions between these states suggest that states with identical FRET values might consist of even more kinetically distinct states. It is not clear to which extent the structure of NPH-II changes between the states, but the two distinct FRET values indicate at least minor differences in the bound RNA. However, the states have clearly different functional properties, reflected in the probability by which NPH-II dissociates from each state.

It is remarkable that the FRET values for the different states remained the same at the different stages of the ATPase cycle examined in our study. This observation suggests that the complex formed between NPH-II and ssRNA exists in a dynamic "equilibrium" of multiple bound conformations. The different stages of the ATP hydrolysis cycle direct rates by which the conformations interconvert, but they do not appear to induce a single specific conformation.

Similar, pre-existing equilibria that are altered by ligands have been observed with several other proteins. Examples include HIV Reverse Transcriptase, which displays two binding modes^{45,46}. The "equilibrium" between these modes is altered by nucleotide or a non-nucleoside inhibitor. A further example is provided by the EF-G/ribosome interaction⁴⁷. This complex also exists in two distinct, interconverting states whose ratio changes during different stages of translation⁴⁷. Moreover, DNA polymerase I shifts rapidly between open and closed conformations in the absence of substrate, and this interconversion is changed in the presence of DNA or DNA and nucleotide⁴⁸. In each of these cases, a pre-existing "equilibrium" of conformations is altered by ligands or other functional triggers, analogous to the change seen upon addition of ATP analogs to the NPH-II RNA complex.

The direct observation of distinct, interconverting states of the NPH-II RNA complex at defined stages of the ATP hydrolysis cycle highlights inherent dynamics of NPH-II bound to the RNA. As discussed above, functional consequences of these multiple states include an intricate control of substrate affinity. It is not clear to which extent the measured rate constants for the interconversion impact the NPH-II-RNA complex under ongoing ATP hydrolysis, given that ATP turnover is significantly faster than the interconversion kinetics measured with the non-hydrolyzable ATP analogs. Further research is thus needed to discern functional implications of the inherent dynamics of the NPH-II RNA complex. It is also important to investigate whether distinct conformational states of RNA-bound complexes exist for other helicases. If so, this feature would need to be considered in the functional interpretation of structural data.

A basic, mechanistic model for unwinding initiation by NPH-II

Collectively, the data presented in this study suggest a basic model for the unwinding initiation process by NPH-II (Fig.8). Without nucleotide, NPH-II binds both, single strand and duplex regions. The complex between NPH-II and single strand RNA readily alternates between multiple conformations ($E = 0.15$, $E = 0.33$, Fig.7). ATP binding to NPH-II, as mimicked by ADP-BeF_x, slows dissociation of NPH-II from the single stranded region and changes the kinetics of the transition between the multiple conformations of the NPH-II RNA complex. However, NPH-II is also able to bind the duplex region. In the ATP transition state, mimicked by ADP-AlF_x, NPH-II no longer binds duplex RNA and binding to the single stranded region is essentially irreversible. Kinetics of the transition between the conformations of the NPH-II RNA complex change again, compared to the other stages of the ATP hydrolysis cycle. With bound ADP, i.e., after ATP hydrolysis and dissociation of the inorganic phosphate, NPH-II no longer binds the single stranded RNA, but associates with duplex RNA.

This basic model explains the ability of NPH-II to maintain contact to the RNA during unwinding initiation, despite many ATP turnovers^{6,16,18}. Binding to duplex RNA compensates for detachment from single stranded RNA and *vice versa*. The proposed initiation model does not explain why hundreds of ATP are often turned over prior to unwinding, and which specific events lead to the actual strand separation. We speculate that a certain conformation of the RNA duplex has to meet a specific conformation of the helicase, but that simultaneous occurrences of these arrangements are comparably rare.

Although we did not examine translocation by NPH-II, the proposed initiation model raises the attractive, albeit speculative possibility, that during directional duplex unwinding, NPH-II produces the forward step between the ATP transition state and the ADP-bound state, i.e., concurrent with the dissociation of inorganic phosphate. This scenario would be similar to the spring loaded translocation mechanism proposed for HCV-NS3³³.

MATERIALS & METHODS

Protein and RNA preparation

NPH-II was expressed in baculovirus infected insect cells and purified as described⁶. A bipartite RNA consisting of a 19 base pair duplex and a 24 nucleotide 3' overhang was used in the sucrose gradient sedimentation and functional stoichiometric titration assays. The sequence of the overhang-containing strand, of which the first 19 bases were annealed to a complimentary RNA, was: 5'-AGCACCGUAAAGACGCAGCCAGCAUCAAUGACAUCAGCAUCAAA. (duplex region underlined). For gradient sedimentation, the duplex RNA had the same sequence as the duplex region of this RNA, and the single-stranded substrate had the same sequence as the overhang. The substrate was radiolabeled with PNK and γ P³²-ATP. For smFRET experiments, the same bipartite substrate was used, except for two additional U's at the 3' end of the complimentary strand. The overhang-containing strand was labeled at the 3' end with Cy3. The complimentary contained a biotin modification on the 3' end and a Cy5 on the 5' end. All RNAs were purchased from Dharmacon.

Sucrose Gradient Sedimentation

Reactions (40 mM Tris-HCl, pH 8.0, 0.5 mM MgCl₂, 0.01% Nonidet P-40) containing 1 nM radiolabeled RNA, 100 nM NPH-II, and, where indicated, nucleotide - Mg²⁺ (3.5 mM) were loaded onto 4.6 mL 6-40% sucrose gradients. Where indicated, gradients also contained nucleotide-Mg²⁺ (3.5 mM). ADP-BeF_x and ADP-AlF_x were prepared as described²¹. Gradients were centrifuged in a Beckman SW-55 Ti rotor at 42,000 rpm for 15.5 hours at 4°C. 50 fractions (~90 μ L each) were collected from the top of the tube. Size standards ovalbumin (3.6S, 45 kD) aldolase (7.35S, 149 kD), and catalase (11.3S, 240 kD) were added to a gradient with the same buffer conditions and centrifuged concurrently with the reactions.

Gradient fractions were analyzed by scintillation counter to determine the amount of RNA. Size standards were monitored by SDS-PAGE. To determine the relative amount of NPH-II in each fraction, gradient fractions were probed for ATPase activity. A volume of 7 μ L of each fraction was incubated with reaction buffer (40 mM Tris-HCl pH 8.0, 0.5 mM MgCl₂ and 0.01% Nonidet P-40) and 0.01 mM radiolabeled ATP for 10 minutes. Free phosphate was separated from ATP by TLC, and the fraction ATP hydrolyzed was determined with a PhosphorImager.

Functional Stoichiometric Titration

Unwinding reactions were performed in a buffer containing 40 mM Tris-HCl (pH 8.0), 0.5 mM MgCl₂, and 0.01% Nonidet p-40. NPH-II (0.5 to 50 nM) was incubated with 20 nM RNA for 5 minutes, then ATP (3.5 mM final) and RNA scavenger (25 nt RNA, comprising the ss region of the substrate RNA, 1 μM final concentration) were added simultaneously to initiate single turnover unwinding. Aliquots were taken, the reaction was stopped in 2x Helicase Reaction Stop Buffer (50 μM EDTA, 1% SDS, 0.1 % BPB, 0.1% XC, 20% Glycerol) and samples were loaded onto a 15 % 29:1 Acryl:Bis gel containing TBE (4.5 mM Tris Base, 4.5 mM Boric Acid, 0.1 mM EDTA). The gel was run at 10 V/cm at room temperature, and subsequently dried, and exposed to a PhosphorImager screen. The fraction unwound substrate was quantified as described⁶. Unwinding amplitudes were determined by plotting the fraction unwound RNA over time and fitting the data to the integrated first order rate law, using the *Kaleidagraph* software.

Single Molecule FRET

Single molecule FRET (smFRET) measurements were performed with a custom-built TIR setup as described²⁸. Cy3 and Cy5 labeled RNA samples were immobilized in a flow-cell coated with polyethylene glycol (PEG), which prevents non-specific protein adsorption. To generate the PEG-coating, a mixture of PEG-NHS (3,000–5,000 Da) and biotinylated PEG-NHS (3,000 Da) (SHEARWATER) was covalently attached to the flow-cells that had been amino-functionalized with Vectabond (VECTOR). Biotinylated RNA was immobilized via a streptavidin (MOLECULAR PROBES) link to the biotinylated PEG surface. Buffer conditions for the reactions were identical to those in binding and unwinding reactions, except that an oxygen scavenging system consisting of glucose oxidase (SIGMA) and catalase (SIGMA) was added with 5% glucose to prevent photobleaching⁴⁹.

Fluorescent RNA molecules were excited using prism-based TIR⁴⁹ and images were collected as described²⁸. Single-molecule time traces were collected at a rate of 10 frames per second using customized software. Fluorescence and corresponding FRET values were computed as described²⁷. Dwell time analysis was performed with customized *Matlab* routines. Rate constants were determined from plots of cumulative dwell times (e.g., Fig. 7A). Rate constants listed in Fig. 7C were obtained by global fits of all plots against the kinetic scheme given in Fig. 7B, using maximum likelihood analysis performed with *Matlab*. The errors represent the deviation of the obtained rate constants from the maximum likelihood analysis, assessed by a bootstrapping algorithm on the same data.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Binding of NPH-II to RNA measured by sucrose gradient centrifugation

(A) Binding of NPH-II to three RNA substrates without nucleotide. Cartoons above the plots show the substrates; asterisks mark the radiolabel. For RNA sequences see Materials and Methods. Radiolabeled RNA in gradient fractions was quantified by scintillation counting. Plots show the fraction of total RNA (or NPH-II) in each gradient fraction (numbered at the x-axis). All experiments were repeated 3 to 5 times, and identical results were obtained. Representative data are shown. Open circles: RNA alone; filled circles: RNA in the presence of 100 nM NPH-II; crosses: free NPH-II, as quantified by the relative ATPase activity in the gradient fractions. Arrows indicate the average peak migration in the gradient of the three size standards, ovalbumin (49 kD, fraction 13), aldolase (150 kD, fraction 25), and catalase (240 kD, fraction 34). The dashed lines indicate the migration of each of the three RNAs. The apparent molecular weight of the complexes was calculated based on the sedimentation of the size standards, assuming a linear distribution of molecular weight for primarily globular protein or protein-RNA complexes. Errors represent one standard deviation from the Gaussian fit of the gradient pattern. The migration of RNA alone was slightly lower than expected based solely on molecular weight; most likely due to the higher density of free RNA and deviations from globular shape. Apparent molecular weights were for NPH-II alone: $M_w = 82.7 \pm 15.8$ kD; bipartite RNA, $M_w = 27.7 \pm 13.4$ kD; NPH-II + bipartite RNA, $M_w = 113.1 \pm 25.2$ kD; blunt-end duplex RNA alone, $M_w = 14.7 \pm 9.8$ kD; NPH-II + blunt-end duplex, $M_w = 106.0 \pm 7.5$ kD. The single-strand RNA migrated lower than the predicted molecular weight of $M_w = 8$ kD. **(B) Binding of NPH-II to RNA with ADP-BeF_x.** ADP-BeF_x was at 3.5 mM. Apparent molecular weights were for NPH-II + bipartite RNA + ADP-BeF_x, $M_w = 180.0 \pm 8.7$ kD; NPH-II + blunt-end duplex + ADP-BeF_x, $M_w = 114.7 \pm 15.1$ kD; NPH-II + single-strand RNA + ADP-BeF_x, $M_w = 106.5 \pm 6.4$ kD. **(C) Binding of NPH-II to RNA with ADP-AlF_x.** ADP-AlF_x was at 3.5 mM. Apparent molecular weights were for NPH-II + bipartite RNA + ADP-AlF_x, $M_w = 84.3 \pm 17.3$ kD. NPH-II + blunt-end duplex + ADP-AlF_x, $M_w = 22.6 \pm 12.3$ kD; NPH-II + single-strand RNA + ADP-AlF_x, $M_w = 104.9 \pm 11.4$ kD. **(D) Binding of NPH-II to RNA with ADP.** ADP was at 3.5 mM. Apparent molecular weights were for NPH-II + bipartite RNA + ADP, $M_w = 119.9 \pm 15.0$ kD; NPH-II + blunt-end duplex + ADP, $M_w = 105.7 \pm 12.7$ kD.

Figure 2. Functional stoichiometric titration of NPH-II on RNA

(A) Reaction scheme. (B) Plot of normalized unwinding amplitudes versus NPH-II concentration. Closed circles indicate the normalized reaction amplitude at each NPH-II concentration. Error bars indicate the standard deviation of multiple independent experiments. The lines with short dashes mark expected data for a single protomer of NPH-II. The line with larger dashes marks expected data for two protomers of NPH-II. The maximal reaction amplitude is reached at 16.9 ± 1.9 nM NPH-II, indicating a 1:1 ratio for $[RNA]/[NPH-II]$.

Figure 3. NPH-II binding to RNA measured by smFRET

(A) RNA construct design and principle of smFRET measurements. A 19 base pair duplex with a 24 nucleotide 3' overhang was labeled with Cy3 (green circle) and a Cy5 (red circle). The RNA was biotinylated on the 3' end of the Cy5-containing strand (grey circle) and attached via streptavidin to biotinylated PEG that was covalently linked to the glass slide. FRET is indicated by the grey arrow. NPH-II binding alters the smFRET values of free RNA. Unwinding leads to the separation of the strands, and thus results in a sharp decrease in FRET, followed by the disappearance of the Cy3 upon strand dissociation. **(B) SmFRET histograms of NPH-II binding to RNA.** Histograms were obtained by determining smFRET values for the number of molecules indicated on the right, averaged over five frames at the reaction start. In all histograms, the molecules with non-fluorescent Cy5 (FRET = 0) were subtracted. Lines indicate Gaussian fits of each FRET population, the black line marks the overall fit of the population with multiple peaks. Peak values were as follows; free RNA: $E = 0.85 \pm 0.17$; with 100 nM NPH-II: $E_1 = 0.33 \pm 0.15$ and $E_2 = 0.15 \pm 0.10$; with 100 nM NPH-II and 3.5 mM ATP $E = 0.87 \pm 0.15$. The errors mark one standard deviation of the Gaussian fit from the respective peak. **(C) Representative smFRET timetraces.** Green line: intensity of Cy3; red line: intensity of Cy5; blue line: FRET. Data were smoothed using adjacent averaging of the nearest 5 timepoints; grey lines show not averaged data. Shaded boxes mark the FRET states corresponding to the histograms in panel (B). Arrows indicate putative unwinding events.

Figure 4. NPH-II binding to RNA with ATP analogs and ADP

SmFRET histograms of NPH-II binding to RNA were obtained as described in Fig.3B (100 nM NPH-II: 100 nM, ADP-AlF_x, ADP-BeF_x, or ADP: 3.5 mM). Molecules with photobleached Cy5 ($E = 0$) were subtracted from the histograms. Lines indicate a Gaussian fit of each FRET population. SmFRET peak values were with ADP-AlF_x: $E_1 = 0.29 \pm 0.15$, $E_2 = 0.14 \pm 0.10$; with ADP-BeF_x: $E_1 = 0.34 \pm 0.17$, $E_2 = 0.19 \pm 0.12$, $E_{\text{Free}} = 0.91 \pm 0.21$, and with ADP: $E = 0.92 \pm 0.09$. Errors mark one standard deviation of the Gaussian fit of the respective peak.

Figure 5. Affinity of NPH-II to RNA without and with ATP analogs

(A) NPH-II - RNA binding without nucleotide. Upper panel: fraction of each smFRET state vs. NPH-II concentration. Fractions were calculated from areas under the respective peak in the smFRET histograms (Figs.3B, 4). Colors mark the three smFRET states, as indicated on the right. Error bars indicate the standard deviation from the fit of the smFRET peaks to the Gaussian distribution (Figs.3B, 4). Grey open circles mark the fraction free RNA measured by EMSA (Suppl. Fig.S1). Lines show the fit to the Hill equation. K_d FRET = 0.91 ± 0.12 nM, $n = 1.48 \pm 0.22$; K_d EMSA = 1.23 ± 0.59 nM, $n = 1.63 \pm 0.18$. Lower panel: ratio of the fractions at $E_1 = 0.33$ and $E_2 = 0.15$. The dotted line marks the average ratio, $R = 2.21 \pm 0.51$. **(B) NPH-II - RNA binding with ADP-BeF_x.** Data were obtained as described in panel (A). K_d FRET = 20.8 ± 7.5 nM. $R = 1.2 \pm 0.2$. **(C) NPH-II - RNA binding with ADP-AIF_x.** Data were obtained as described in panel (A). Virtually complete binding occurred even at the lowest NPH-II concentrations (K_d FRET < 0.01 nM, $R = 4.4 \pm 1.1$).

Figure 6. Interconversion between smFRET states

(A) Representative smFRET timetraces (NPH-II: 1 nM, no nucleotide). Green line: Cy3; red line: Cy5; blue line: FRET. Data were smoothed by averaging of the nearest 5 timepoints; grey lines mark unaveraged data. Intensity is indicated as arbitrary units (AU). The histogram on the right shows the distribution of smFRET states, marked by the shaded boxes. **(B) Fraction of transitions versus type of transition.** Transitions between different smFRET states were counted manually (approximately 400 for each reaction condition) and the fraction of total transitions for each transition type was determined.

Figure 7. The kinetics of interconversion between smFRET states

(A) Transitions from smFRET states corresponding to RNA-bound states. Cumulative plots of dwell times at $E = 0.15$ and $E = 0.33$ were fit to a sum of two exponentials using a maximum likelihood analysis (Materials and Methods). Error was assessed by bootstrapping. Cumulative plots of dwell times at $E = 0.85$ (free RNA) fit to single exponentials (data not shown). Dwell times at each FRET state were determined using custom Matlab software. 136 – 276 events for each reaction condition were used in the analysis. **(B). Kinetic scheme for interconversion between smFRET states.** Rate constants correspond to the transitions between each FRET state. **(C). Rate constants for the smFRET transitions.** Rate constants were calculated by globally fitting dwell time distributions to the kinetic model in panel (B) using maximum likelihood analysis. Errors were determined by bootstrapping. The amplitude of the first rate constant in bi-exponential fits is indicated as “a”. For ADP-AIF_x, k_1 and k_{-1} could not be determined by FRET, due to the high affinity of NPH-II for RNA. The value for k_{-1} is represented as a lower limit, as determined by EMSA (Suppl. Fig.S3) For ADP-BeF_x, the transition from $E_2 = 0.33$ to $E_{Free} = 0.85$ (k_{-1}) fit best to a single exponential.

Figure 8. Basic model for unwinding initiation by NPH-II

Different shapes mark the different states of NPH-II traversed during the initiation process.