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Choosing between DNA and RNA: the polymer specificity of RNA helicase NPH-II

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ABSTRACT

NPH-II is a prototypical member of the DExH/D subgroup of superfamily II helicases. It exhibits robust RNA helicase activity, and a detailed kinetic framework for unwinding has been established. However, like most SF2 helicases, there is little known about its mode of substrate recognition and its ability to differentiate between RNA and DNA substrates. Here, we employ a series of chimeric RNA–DNA substrates to explore the molecular determinants for NPH-II specificity on RNA and to determine if there are conditions under which DNA is a substrate. We show that efficient RNA helicase activity depends exclusively on ribose moieties in the loading strand and in a specific section of the 3′-overhang. However, we also document the presence of trace activity on DNA polymers, showing that DNA can be unwound under extremely permissive conditions that favor electrostatic binding. Thus, while polymer-specific SF2 helicases control substrate recognition through specific interactions with the loading strand, alternative specificities can arise under appropriate reaction conditions.

INTRODUCTION

The DExH/D proteins are a subset of helicase superfamily II, and they represent a large group of ATP-dependent remodeling proteins that are involved in virtually all known aspects of RNA metabolism (1), many forms of viral replication (2–5), and in certain DNA rearrangements (the recQ group of helicases) (6). Despite the ubiquity and importance of this protein family, there is limited understanding of their reaction mechanisms and substrate specificities. Phylogenetically, NPH-II is a prototypical member of the DExH/D family (7), and it was the first one shown to unwind RNA (8). NPH-II readily unwinds RNA substrates that contain a 3′-overhang (9), it displays a high degree of processivity, and the kinetic parameters that describe its mechanism of RNA unwinding have been described (10,11). NPH-II is both a robust motor for RNA displacement (10), and for the removal of proteins that are bound to RNA sites (12,13).

The majority of known helicases specifically unwind only one type of duplex, acting on either DNA or RNA. While a few helicases have the ability to unwind either type of polymer, such as NS3 from HCV (14) and SV40 large T antigen (15), most display strong apparent discrimination for a particular class of nucleic acid. The basis for this discrimination is unknown, and it is of great interest to understand how polymer specificity is controlled. In the case of NPH-II, unwinding of RNA has been characterized in great detail (10,11). However, there is apparent ambiguity about the activity of NPH-II on DNA substrates. Several studies indicate that NPH-II acts exclusively on RNA substrates (9,16), while one report suggests that NPH-II can unwind DNA (17).

To begin dissecting the basis of polymer specificity in SF2 proteins, and to better understand the nucleic acid specificity of NPH-II in particular, we have conducted a comparative analysis of unwinding for a family of substrates that contain varying amounts of DNA and RNA in both the top and bottom strands. Helicase unwinding of purely DNA substrates was examined, along with NPH-II activity toward hybrid duplexes (one strand of RNA and another strand of DNA) and chimeric duplexes that contain patches of DNA or RNA in the same strand. We observe that certain regions of the loading strand are of particular importance in directing the strong RNA specificity of NPH-II. In addition, we observe small amounts of DNA unwinding that may be promoted by an alternative strand displacement mechanism.

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MATERIALS AND METHODS

Protein expression and purification

NPH-II was isolated and purified as described previously (11). In brief, protein was expressed in SF21 cell suspensions (27°C), at an m.o.i. of 3, with a harvest after 72 h of infection. NPH-II was purified using a 10-His tag and both batch and column techniques, followed by a phosphocellulose column (Whatman, P-11 cell). Aliquots were stored at −80°C until needed. Preparations were evaluated for NPH-II content (by SDS–PAGE), and NPH-II concentration (Coomassie or silver stain densitometry using BSA standards) and confirmed by western blot using anti-NPH-II antibody (provided by S. Shuman) and MALDI-MS (HHMI protein core facility, Columbia University, NY). Activity was determined by ATPase and unwinding assays.

Synthesis of nucleic acid substrates

DNA and RNA oligonucleotides. DNA oligonucleotides were chemically synthesized by the HHMI Core Facility at Columbia University (New York, NY). They were purified by denaturing PAGE. RNA chimeric RNA–DNA oligonucleotides were chemically synthesized and deprotected (18) or purchased from Dharmacon, Inc. (Lafayette, CO), with subsequent deprotection and purification by denaturing PAGE. All phosphoramidites used for in-house synthesis were purchased from Glen Research. Substrates were prepared as described previously (10), and concentrations were determined by specific activity. Substrate duplexes contained a 5′-^{32}P-end label on the top strand. The substrate was stored at −80°C in a buffer of 10 mM MOPS (pH 5), 1 mM EDTA.

RNA trap molecules. Trap RNA (24 bp duplex flanked by 18 single-stranded nucleotides at the 3′ end; top strand 5′-GCC UCG CUG CCG UCG CCA GCA UAU-3′, bottom strand 5′-AUA UGC UGG CGA CGG CAG CGA GGC AGA GGA GCA GAG GGA GCA-3′) was made from synthetic RNAs (18). It was annealed and prepared as previously described (10). Concentrations were determined spectrophotometrically at A_{260} (nm) using a Hewlett Packard UV-Vis spectrophotometer (Model 8453).

NPH-II enzyme activity assays

Unwinding timecourses. Unwinding was performed using standard reaction conditions (40 mM Tris, pH 8.0, 4 mM $Mg(OAc)_2$, 15 nM NPH-II, 70 mM NaCl, 25°C) (11), unless stated otherwise. Prior to initiation of unwinding reactions, NPH-II protein (15 nM) was preincubated with duplex substrate (1–2 nM, labeled with ^{32}P at the 5′ end of the top strand) for 3 min at room temperature. Single-cycle unwinding reactions were initiated by the simultaneous addition of 3.5 mM ATP and 400 nM duplex RNA trap. Multiple cycle unwinding reactions were initiated by the addition of 3.5 mM ATP alone. Effects of the non-hydrolyzable ATP analog, adenosine 5′-(β,γ-imino)triphosphate (AMP-PNP) (Sigma Chemicals) were tested under multiple cycle conditions, at a final concentration of 5.0 mM. All reactions were conducted in volumes of 15–20 μl at 25°C. Specified timepoints were taken by adding aliquots of the reaction mix to two volumes of quench buffer (25 mM EDTA, 0.4% SDS, 0.05% BPB, 0.05% XCB, 10% glycerol). Duplex and unwound products were resolved by 15% native PAGE. Size markers for unwound species were made by heating duplex substrates at 95°C. The dried gels were visualized on a Storm PhosphorImager and reaction products were analyzed using ImageQuant software. Kinetic analysis was done using Kaleidagraph software (Abelbeck), where the fraction of unwound substrate was fitted to the integrated first-order rate law. Error analysis was performed by using at least three trials for each experiment. Reported variance in kinetic parameters was calculated with a 90% confidence limit (19).

RESULTS

DNA unwinding activity of NPH-II

To resolve apparent discrepancies in the literature regarding NPH-II specificity, and to begin exploring the basis for polymer specificity by SF2 helicases, we designed a series of experiments to evaluate DNA unwinding by NPH-II. Initial experiments were conducted with DNA substrate A (Figure 1), which has the same sequence and length as RNA substrate RNA1 (Figure 1). Under standard single-cycle conditions that have been established for unwinding of RNA (Methods), NPH-II was unable to unwind DNA A (Figure 2b, timecourse of panel A, left). In contrast, NPH-II readily unwound the analogous RNA substrate RNA1 (see Figure 3b).

To determine if apparent inactivity toward DNA could be attributable to an insufficient single-stranded region [an effect that was previously hypothesized (17)], the overhang length was doubled to 40 nt (Figure 2a, substrate B) and then tripled to 60 nt (Figure 2a, substrate C). Despite the longer overhangs, neither of these substrates was unwound by NPH-II under standard conditions (data not shown). These experiments were repeated under more permissive multiple-cycle reaction conditions and DNA unwinding was still not observed (data not shown).

To further increase permissivity of the DNA unwinding reaction, unwinding reactions were again conducted under multiple-cycle conditions, but the NaCl concentration was lowered from 70 to 11 mM. Conditions of low ionic strength are expected to promote binding by stabilizing electrostatic interactions between nucleic acid and NPH-II. Using this highly permissive salt concentration (under which NPH-II quantitatively unwinds RNA duplexes in less than 20 s), a small amount of DNA unwinding was detected (Figure 2b, panel A, right). DNA unwinding under these conditions is extremely poor in comparison to unwinding of a similar RNA substrate (data not shown). Rate constants for DNA unwinding were not computed because the reactions never achieved a significant end-point and amplitudes were extremely low (Figure 2b).

To determine if longer single-stranded overhangs would promote DNA unwinding activity under the new permissive conditions, NPH-II activity was tested with DNA substrates B and C. Substrate C, with the longest overhang (60 nt), was the least reactive, and substrate B (40 nt overhang) was unwound to a smaller extent than substrate A (Figure 2b, compare panels A, B and C, right). Therefore, despite having the same duplex length, all three substrates were unwound to differing extents by NPH-II, with an inverse relationship between unwinding and overhang length.

Because DNA unwinding by NPH-II is so inefficient and requires such radically permissive conditions (multiple cycle,

Figure 1. Substrate duplexes used in this study. Red characters signify DNA nucleotides and black characters indicate RNA. Substrates RNA1 and A are identical in sequence and length, except that all Us have been replaced by Ts in the DNA substrate. Substrates B and C are the same as A, except for the length of the overhang, which is two or three repeats, respectively, of the same 20mer sequence shown. RNA2 is a derivative of RNA1 that is shorter by 4 nt at the 5′ end of the top strand, resulting in a 30 bp duplex region and 24 nt overhang. It serves as the control duplex for substrates H and I, which have the same structure and sequence.

with 15-fold protein excess, 11 mM NaCl), it was of interest to determine whether the reaction is ATP dependent. To evaluate this possibility, the same timecourses were run with DNA substrates A–C, except that ATP was not added. Instead, time points were taken after preincubation with NPH-II (time zero is before NPH-II is added to reaction). In the absence of ATP, no unwinding was observed (data not shown). To distinguish whether the requirement for ATP was related to ATP binding, we studied DNA unwinding in the presence of the non-hydrolyzable ATP analog, adenosine 5′-adenylylimidodiphosphate (AMP-PNP). However, unwinding of the DNA substrates was not observed in the presence of AMP-PNP (data not shown). This suggests that DNA unwinding by NPH-II is an ATP-dependent process, and that ATP binding, alone, is not sufficient to promote duplex destabilization.

NPH-II reaction with DNA/RNA hybrid substrates

To further examine the nucleic acid specificity of NPH-II, we created a pair of hybrid duplex substrates that contained one RNA and one DNA strand (Figure 3a, substrate D and E). The substrates, which are analogous to RNA1 (Figure 1), are identical in secondary structure (34 bp with a 20 nt overhang) and sequence. Substrate D, which contains an RNA bottom strand and DNA top strand, was readily unwound by NPH-II (Figure 3b, substrate D). The rate constant and amplitude for unwinding are similar to that of RNA1 [Figure 3b, compare timecourses RNA1 (filled circles) and D (open circles)]. In contrast, substrate E, which contains a DNA bottom strand and an RNA top strand, was not unwound by NPH-II [Figure 3b, substrate E (squares)], as observed for the cognate DNA substrate under the same reaction conditions (Figure 2b, panel A, left). These results demonstrate that the top and bottom strands of a substrate are recognized differently by NPH-II, and are consistent with previous studies on mixed substrates (9). The results are also consistent with evidence that NPH-II tracks along the bottom strand, which contains all of the determinants for specificity (11).

Probing the basis for strand specificity with chimeric oligonucleotides

To dissect the basis for polymer specificity by NPH-II, a set of chimeric substrates was synthesized and tested for unwinding (Figure 1, substrates F–I). Having established that an all-DNA loading strand was inhibitory under single-cycle conditions, we tested the effect of a bottom strand that contains DNA throughout the duplex region and RNA in the overhang (Figure 4a; substrate F). Conversely, we examined a bottom

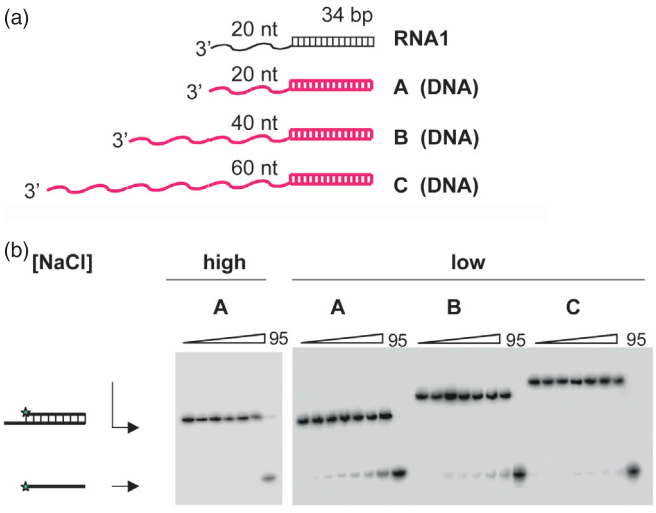

Figure 2. Unwinding of DNA substrates by NPH-II. (**a**) Substrates of 34 bp are composed of either DNA (red bold lines) or RNA (thin black lines). The duplex sequence is the same for all four substrates. The overhang for the DNA substrates is 20, 40 or 60 nt and corresponds to multiples of the 20mer sequence 3′-GUA ACU ACG ACA AUC AUG CA-5′, where U is replaced by T in DNA. (**b**) A trace of DNA unwinding is seen under 'permissive' conditions. Native gel shift assays showing NPH-II unwinding of DNA substrates A, B and C in the absence of trap RNA (multiple cycle), in a low-salt buffer of 11 mM NaCl, 40 mM Tris, pH 8.0, 4 mM MgOAc, 15 nM NPH-II, 1 nM duplex substrate and 3.5 mM ATP. Timepoints are 0, 10 s, 30 s, 45 s, 1 min, 2 min and 5 min. At the last timepoint (5 min), the maximum amount of unwinding under low-salt conditions for substrates A, B and C is 16%, 12% and 5%, respectively. The timecourse for panel A under higher-salt conditions (70 mM NaCl, left) shows no unwinding. Timepoints are 0, 0.5, 1, 2, 5 and 10 min.

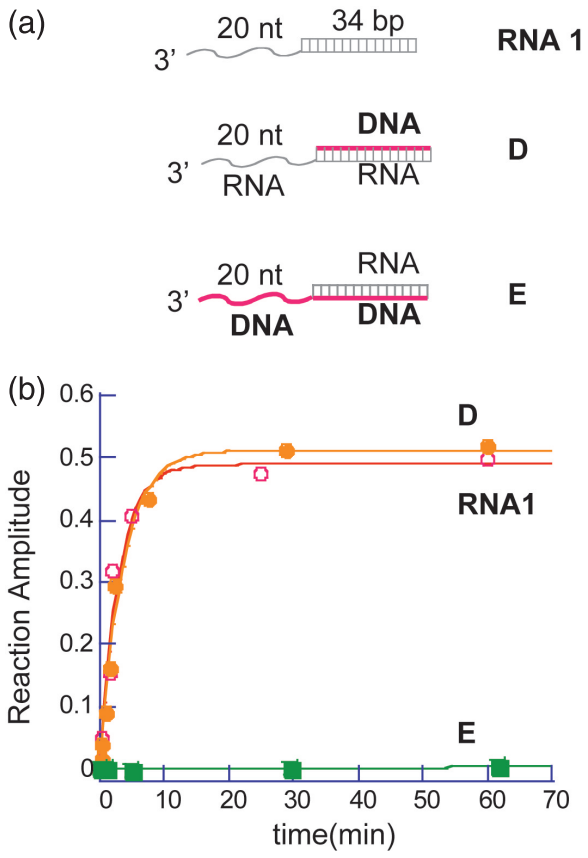

Figure 3. The role of RNA versus DNA in the substrate strands. (**a**) Schematic representation of the substrates used in this experiment, where DNA is indicated by red lines in either the top (substrate D) or bottom (substrate E) strands. U is replaced by T in the DNA strand. (**b**) Timecourses of single cycle kinetics show similar unwinding behavior of substrates with an RNA loading strand, regardless of top strand identity. Only substrates with an RNA loading strand, substrate RNA1 (filled circles, $A_{f(RNA1)} = 0.49 \pm 0.06$ and $k_{1(RNA1)} = 0.34 \pm 0.08$ min^{-1}) and substrate D (empty circles, $A_{f(D)} = 0.51 \pm 0.01$ and $k_{1(D)} = 0.28 \pm 0.11$ min^{-1}), show unwinding. No unwinding is seen for substrate E, which contains an RNA top strand and a DNA loading strand.

strand that contained DNA in the overhang and RNA in the duplex region (Figure 4a; substrate G). In addition, we synthesized substrates H and I, for which a section of DNA is placed away from, or immediately adjacent to the junction with duplex, respectively (Figure 4a).

NPH-II was unable to unwind substrate F under standard conditions, although unwinding of substrate G was significant and quantifiable (Figure 4b), which indicates that NPH-II has an RNA requirement in the duplex region of the bottom strand. Given that an entirely DNA overhang was inhibitory, we were interested in determining whether there was a portion of the overhang that contributed the most to specificity. We observed that substrate H (in which the overhang is entirely DNA except for 4 nt of RNA adjacent to the duplex junction) was unwound so readily that its behavior was indistinguishable from that of an all-RNA substrate (Figure 4b). In contrast, substrate I (containing 4 nt of DNA adjacent to the junction) was unwound to a far lesser extent. Indeed, behavior of substrates I and G were almost indistinguishable (Figure 4b).

DISCUSSION

Specificity is determined by the identity of the loading strand

These results clearly demonstrate that NPH-II requires RNA in the loading (bottom) strand in order to catalyze efficient unwinding. While this part of the study is largely consistent with previous work on hybrid duplexes (9), there are a few salient differences. In the previous study, a small amount of unwinding was observed for a hybrid substrate that contains DNA in the loading strand and RNA in the top strand (similar to substrate E). We do not observe unwinding of any substrates that contain DNA in the bottom strand under single cycle conditions. Indeed, behavior of substrate E is virtually indistinguishable to that of an all-DNA substrate.

Another distinction from previous work on hybrid substrates is that their activity had not been evaluated simultaneously with an analogous all-RNA substrate. In the present work, we compare all-RNA and hybrid substrates under identical conditions in side-by-side timecourses. In this way, it is possible to unambiguously demonstrate that specificity is dictated by the loading strand and that the polymer identity of the top strand is irrelevant for primary function of NPH-II. These findings are similar to those in which hybrid substrates were used to study polymer specificity of the NS3 helicase from hepatitis C virus. Although NS3 can unwind both RNA and DNA under the same

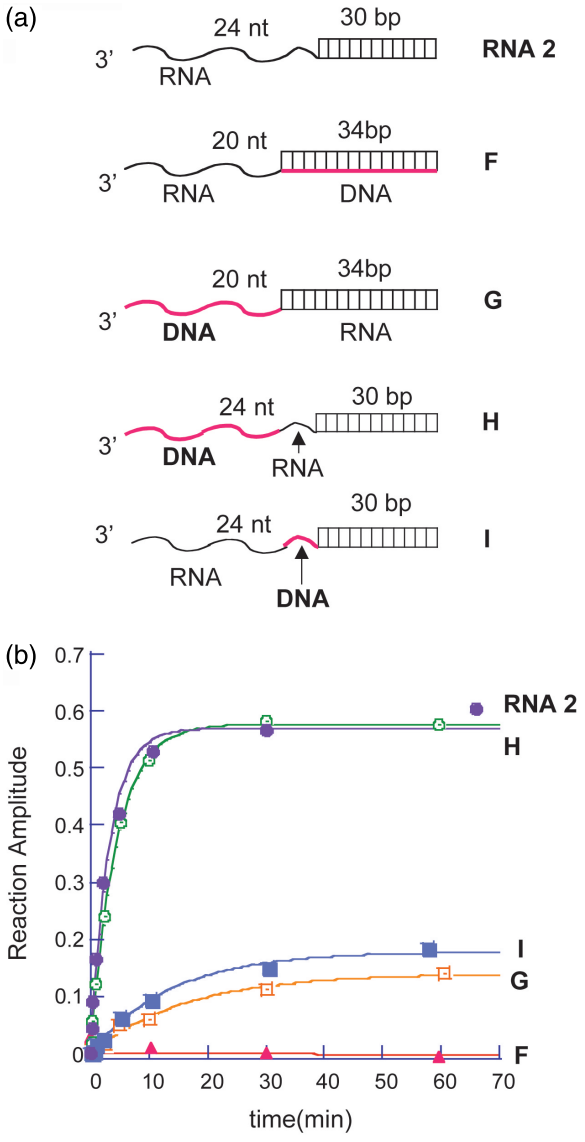

Figure 4. Probing the effects of DNA in various parts of the loading strand. (**a**) Schematic representation of substrates used in this experiment. Oligonucleotides were prepared with DNA (bold red lines) in different regions of the loading strand. (**b**) Timecourses of single-cycle kinetics for substrates RNA2 and F–I. Substrate RNA2 (filled circles) displays similar kinetics to the 34 bp duplex (RNA1), and serves here as a control for substrates H (open circles) and I (filled squares), which also have a 30 base top strand. Substrate F (filled triangles) was not unwound. Unwinding rate constants for the four substrates that could unwind are $k_{1(RNA2)} = 0.32 \pm .03$ min^{-1}, $k_{1(H)} = 0.23 \pm 0.05$ min^{-1}, $k_{1(I)} = 0.07 \pm .02$ min^{-1} and $k_{1(G)} = 0.06 \pm 0.01$ min^{-1}. Reaction amplitudes are $A_{f(RNA2)} = 0.57 \pm 0.04$, $A_{f(H)} = 0.58 \pm 0.07$, $A_{f(I)} = 0.18 \pm 0.01$, $A_{f(G)} = 0.14 \pm 0.01$.

conditions, it preferentially unwinds DNA and this specificity is dictated by the polymer identity of the loading strand (20). NPH-II and NS3 are both members of helicase superfamily 2, which suggests that members of this entire family of helicases derive their respective polymer specificities from interactions with the bottom strand. This is also consistent with the emerging view that SF2 helicases translocate by tracking along the backbone of the substrate loading strand (11).

What portion of the loading strand is recognized? A key role for the junction

Like many unidirectional helicases that initiate unwinding on 'tailed substrates' (16,21,22), NPH-II requires a long, single-stranded 3′ overhang adjacent to the duplex region. The single-stranded tail of the loading strand may provide information about polarity and may ensure that the helicase binds to the 'correct strand' before initiating unwinding (23,24). It may also provide important electrostatic determinants for productive binding of the helicase.

The minimal overhang length for efficient unwinding by NPH-II is ~19 nt, and it has been established that short overhangs of 4 nt do not support unwinding (9). Despite this requirement for a longer overhang, the present work shows that only a small portion of the overhang contributes to polymer specificity. Specifically, we demonstrate a ribose requirement for the four single-stranded nucleotides that are immediately adjacent to the duplex. This establishes that different regions of the overhang play different roles in directing the function of NPH-II, and that the residues closest to the overhang are particularly important for establishing polymer specificity.

In addition to the junction region of the overhang, there is a second region of the loading strand that exhibits a strong ribose requirement. Unwinding studies on substrates F, G and H (Figure 4b) demonstrate that RNA is required in the bottom strand portion of the duplex. The results therefore indicate that ribose sugars contribute to the initiation of unwinding (at the junction), and to the continued translocation of NPH-II along the bottom strand during the course of unwinding.

Traces of DNA unwinding by NPH-II: significance and mechanism

This work, and that of others (9,16), clearly establishes that RNA is the preferred substrate for NPH-II. However, we have demonstrated that DNA can be unwound, with very low efficiency, under an unusual set of multiple-cycle reaction conditions that promote nonspecific electrostatic interactions between proteins and nucleic acids (low salt: 11 mM NaCl). It is possible that similar conditions contributed to previous observations of DNA activity by NPH-II (17).

There are at least two mechanisms that might account for the DNA unwinding that has been observed. (i) 'Active': NPH-II catalyzes DNA unwinding via ATP-dependent translocation along the loading strand with concomitant top-strand displacement, as has been demonstrated for RNA (8,10). (ii) 'Passive': NPH-II coats the loading strand and passively displaces the top strand through a binding mechanism that does not involve active translocation of an individual helicase molecule.

Mechanism (i) is supported by the fact that DNA unwinding requires ATP. Furthermore, the failure of an ATP analog (AMP-PNP) to support unwinding suggests that ATP hydrolysis is a prerequisite for DNA unwinding. However, these results do not, by themselves, disprove alternative mechanisms for DNA unwinding. First of all, the kinetic framework for NPH-II establishes that productive binding of NPH-II can only occur in the ATP-bound state (10). Therefore, the ATP dependence of reaction does not necessarily imply an active motor function and may instead indicate that ATP (or ADP)

stimulates adoption of a specific NPH-II conformation that is conducive to binding (which would be required for Mechanism (ii), as well). The fact that AMP-PNP did not stimulate unwinding could be attributable to imperfect binding of the analog by NPH-II (no ATP analog is an exact mimic), or because ATP hydrolysis may, in fact, induce a conformational change that is necessary for productive binding (irrespective of translocation).

Mechanism (ii) is supported by several lines of evidence. NPH-II has previously been shown to bind DNA with equal affinity to RNA (9,16). Thus, the reduced unwinding of DNA cannot simply be attributed to a defect in enzyme–substrate affinity. The requirement for multiple-cycle conditions suggests that unwinding may result from filament formation along the loading strand [a RecA-like mechanism (25,26), which would displace the top strand in a manner similar to that of single-strand binding protein (SSB) (27)]. This is supported by the observation that NPH-II unwinds DNA more effectively in the presence of SSB (17). Finally, a passive binding mechanism is supported by the fact that DNA unwinding is inhibited by longer single-stranded overhangs, which 'soak up' NPH-II molecules and leave fewer protein units to aggregate along the loading strand, thereby inhibiting top-strand displacement.

Although it is inefficient, the residual (and somewhat forced) activity of NPH-II on DNA raises an important possibility. Depending on conditions, a single helicase may be able to utilize multiple mechanisms for duplex unwinding, particularly on different types of polymers. Helicase enzymes are nucleic acid binding proteins, and simple binding behavior always has the potential to dominate over other types of activities. Dual mechanistic capabilities by helicases may be advantageous and one could imagine that, at times, this serves a biological function. The existence of multiple pathways also highlights the sensitivity of reaction mechanism to experimental design.

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