Unwinding of Unnatural Substrates by a DNA Helicase†

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ABSTRACT: Helicases separate double-stranded DNA into single-stranded DNA intermediates that are required during replication and recombination. These enzymes are believed to transduce free energy available from ATPase activity to unwind the duplex and translocate along the nucleic acid lattice. The nature of enzyme−substrate interactions between helicases and duplex DNA substrates has not been well-defined. Most helicases require a single-stranded DNA overhang adjacent to duplex DNA in order to initiate unwinding. The strand containing the overhang is referred to as the loading strand whereas the complementary strand is referred to as the displaced strand. We have investigated the interactions between a DNA helicase and the DNA substrate by replacing the displaced strand with a nucleic acid mimic, peptide nucleic acid (PNA). PNA is capable of forming duplex structures with DNA according to Watson−Crick base pairing rules, but contains a *N*-(2-aminoethyl)glycine backbone in place of the deoxyribose phosphates. The PNA−DNA hybrids had higher melting temperatures than their DNA−DNA counterparts. Dda helicase, from bacteriophage T4, was able to unwind the DNA−PNA substrates at similar rates as DNA−DNA substrates. The results indicate that the rate-limiting step for unwinding is relatively insensitive to the chemical nature of the displaced strand and the thermal stability of oligonucleotide substrates.

Helicases are enzymes that unwind double-stranded (ds)[1] nucleic acids in processes such as replication and recombination where single-stranded (ss) nucleic acids are required as intermediates (*1*−*3*). The energy needed to perform this function is provided by ATP hydrolysis, although the precise relationship between unwinding and ATP hydrolysis is not known. Binding to ssDNA stimulates ATP hydrolysis for most helicases, whereas dsDNA usually does not provide the same level of activation. In fact, most helicases require a ssDNA "tail" adjacent to a dsDNA region in order to initiate unwinding of the duplex.

The ssDNA may serve as a loading strand and starting point for helicases to unwind dsDNA by translocating with a strong directional bias on ssDNA. Evidence that translocation on ssDNA occurs with a strong directional bias has been provided by a number of laboratories (*4*−*7*). Most recently, the ability of helicases to displace streptavidin from only one end of biotin-labeled oligonucleotides has been interpreted in terms of a directional bias in translocation (*8*). Translocation on ssDNA with a directional bias is generally associated with an inchworm mechanism for unwinding of dsDNA. In an inchworm mechanism, two binding sites for DNA are proposed which can be provided by a monomeric or multimeric enzyme. One of the sites remains in contact with the DNA while the second site "inches" forward along the lattice to bind to a new section of DNA as a result of ATP binding and hydrolysis (*9*). An inchworm model has been proposed for the PcrA helicase based on crystal structures of the enzyme with a ss/ds DNA junction in the presence of ADP and an ATP analogue (*10*). In the model for PcrA, a monomeric enzyme is proposed to contain binding sites for dsDNA and ssDNA.

Others have suggested that specific interactions between a helicase and the dsDNA region of a substrate are not necessary for unwinding to occur (*11*). It is possible that by translocating with a directional bias, a helicase can sequester ssDNA that forms naturally at a ss/dsDNA junction due to thermal fluctuations (*12*). Such a mechanism has been suggested for the NS3 helicase based on consideration of the cocrystal structure of the enzyme bound to ssDNA (*13*).

An alternative mechanism has been proposed in which the role of the ssDNA tail differs from that suggested for the inchworm mechanism. This mechanism is termed the "active rolling" mechanism and has been proposed for Rep and UvrD helicases from *E. coli* (*1*, *14*). Biochemical evidence for a functional, dimeric helicase has led to a model in which each subunit participates in binding and unwinding of dsDNA. The subunits alternate positions at a ss/dsDNA junction in a process driven by binding and hydrolysis of ATP.

Some helicases have been found to exist as hexamers, and these enzymes may contain elements of the proposed models for dimeric or monomeric helicases. In each of the proposed mechanisms, the nature of specific, enzyme−substrate interactions between the helicase and the DNA has not been adequately addressed. Regardless of whether a particular helicase functions according to the rolling or inchworm mechanism, or by some other mechanism, the specific chemical interactions responsible for strand separation have

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[1] Abbreviations: dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; PNA, peptide nucleic acid.

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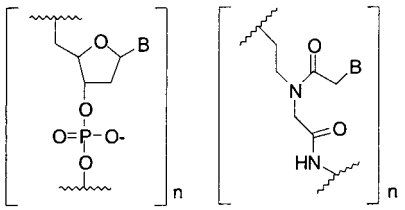

FIGURE 1: Structures of DNA and PNA backbones.

not been defined. To begin investigating this question, we have performed unwinding reactions using substrates in which the displaced strand has been replaced with an unnatural nucleic acid mimic. In this study, the displaced strand of a partially duplex substrate has been replaced with a strand of peptide nucleic acid (PNA) (Figure 1). PNA contains the normal purine and pyrimidine bases linked to a polymer made of repeating *N*-(2-aminoethyl)glycine units (*15*). Rates for unwinding of the modified substrates were measured in the presence of the bacteriophage T4 Dda helicase. Dda is a member of the helicase superfamily 1, as are Rep and PcrA (*16*). In this report, Dda was observed to unwind substrates containing the unnatural displaced strands with similar rates as the natural DNA substrates.

EXPERIMENTAL PROCEDURES

Materials. ATP (disodium salt) and Sephadex G-25 were obtained from Sigma. Hepes, Na_4EDTA, BME, BSA, $Mg(OAc)_2$, KOAc, SDS, xylene cyanole, bromophenol blue, NaCl, glycerol, and KOH were obtained from Fisher. T4 polynucleotide kinase was purchased from New England Biolabs. [γ-^{32}P]ATP was purchased from New England Nuclear. DNA oligonucleotides (Operon Technologies) were purified by preparative polyacrylamide gel electrophoresis, and stored in 10 mM Hepes (pH 7.5) and 1 mM EDTA. RNA (Dharmacon Research Inc.) was purified according to the manufacturers' protocol. PNA strands were prepared, purified, and characterized as described (*17*). Purified PNA and oligonucleotides were quantified by UV absorbance at 260 nm in 0.2 M KOH with calculated extinction coefficients. Recombinant Dda (*18*) was overexpressed and purified from *E. coli* as previously described (*5*).

Helicase Substrates. Purified oligonucleotides were 5′-radiolabeled with T4 polynucleotide kinase at 37 °C for 1 h. The kinase was inactivated by heating to 70 °C for 10 min, and unincorporated [γ-^{32}P]ATP was removed by passing the labeled oligonucleotides through two Sephadex G-25 spin columns. Helicase substrates were prepared by mixing equivalent amounts of loading strand oligonucleotide with the appropriate complementary oligonucleotide or PNA strand, followed by heating to 95 °C for 5 min, and then slow cooling. Sequences of substrates are shown in Figure 2.

Helicase Unwinding Assay. Unwinding assays were performed with a Kintek rapid chemical quench-flow instrument (Kintek, Austin, TX) maintained at 25 °C with a circulating water bath. All concentrations listed are after mixing, unless stated otherwise. The helicase reaction buffer consisted of 25 mM Hepes (pH 7.5), 0.1 mM Na_4EDTA, 2 mM BME, 0.1 mg/mL BSA, and 10 mM KOAc. Substrate (2 nM) was

DNA:DNA I, DNA:RNA

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5′-TTT TTT TTT TTT TTT CTG TCC TGC ATG ATG-3′
                      3′-GAC AGG ACG TAC TAC-5′
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DNA:PNA I

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5′-TTT TTT TTT TTT TTT CTG TCC TGC ATG ATG-3′
                   COOH-GAC AGG ACG TAC TAC-NH2
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DNA:DNA II

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5′-TTT TTT TTT TTT TTT CAT CAT GCA GGA CAG-3′
                      3′-GTA GTA CGT CCT GTC-5′
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DNA:PNA II

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5′-TTT TTT TTT TTT TTT CAT CAT GCA GGA CAG-3′
                   COOH-GTA GTA CGT CCT GTC-NH2
```

FIGURE 2: Dda unwinding substrates. All substrates contain a 5′, T_{15} overhang. Sequences I and II differ by inversion of the duplex region.

Scheme 1

$$ES_{NP} \xrightarrow{k_{NP}} ES_1 \xrightarrow{k_U} ES_2 \xrightarrow{k_U} \text{ssDNA}$$

incubated with 125 nM Dda (unless otherwise stated) for 10 min. Following the incubation, the unwinding reaction was initiated by rapidly mixing the enzyme−substrate solution with 5 mM ATP and 10 mM $Mg(OAc)_2$. The quench solution was 400 mM Na_4EDTA, pH 8.0 (before mixing). To prevent reannealing of ssDNA products after the unwinding reaction, 50 nM trapping strand (a 15mer that was complementary to the displaced 15mer of the substrate) was placed in the receiving vial. In one experiment, the 15mer trapping strand (50 nM) was introduced into the reaction mixture along with ATP and $Mg(OAc)_2$. Aliquots (25 μL) from each sample were mixed with 5 μL of nondenaturing gel loading buffer (30% glycerol, 0.1% bromophenol blue, 0.1% xylene cyanole) and analyzed by electrophoresis on a 20% native polyacrylamide gel. The fractions of ss 30mer and 30mer remaining in the partial duplex form were determined with a Molecular Dynamics Phosphorimager and ImageQuant software (*19*). Results from experiments in which Dda and substrate were incubated prior to the reaction were fit to a mechanism according to Scheme 1 using the program Scientist (Micromath). Data fitting was performed by describing differential equations for each of the species in Scheme 1, and then obtaining the best fit to the multiphase progress curves for formation of ssDNA product (*20*).

Melting Temperature Analysis. Attempts to measure melting temperatures of the 30:15mer substrates were unsuccessful, presumably due to the ssDNA region. Therefore, only the duplex portion of the substrates was used to measure the T_M values. The hybrids were prepared by heating a 1:1 mixture of oligonucleotides for 5 min at 95 °C, and then allowing the mixture to cool slowly to room temperature. Duplexes (4 μM) in 25 mM Hepes (pH 7.5), 0.1 mM Na_4EDTA, 2 mM BME, and 10 mM KOAc were heated from 25 to 95 °C at a rate of 0.7 °C/min. The change in absorbance at 260 nm was observed with a Pharmacia Biotech spectrophotometer equipped with a Peltier heating unit, and the substrate melting temperature was derived from the melting curve using Swift software (Pharmacia Amersham).

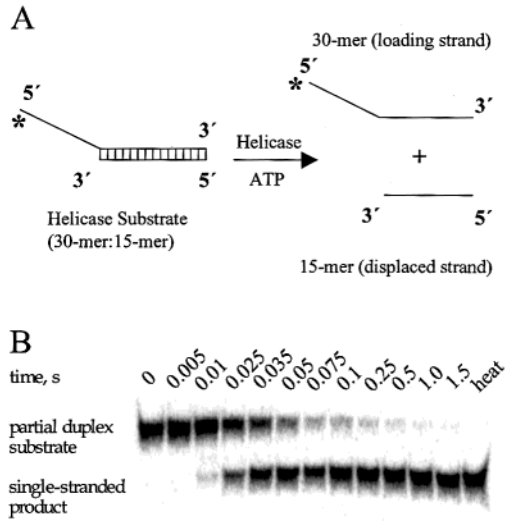

FIGURE 3: (A) Assay for measuring helicase-catalyzed unwinding of partially duplex substrates. The strand with the ss overhang is referred to as the "loading strand", while the complementary strand is referred to as the "displaced strand". (B) Separation of ssDNA product from partially duplex substrate by native polyacrylamide gel electrophoresis. Shown here are results from an experiment utilizing the 30:15mer substrate, DNA:PNA I.

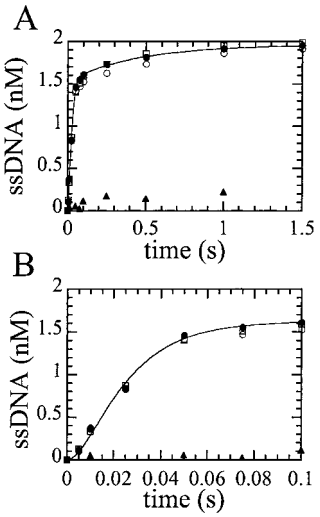

FIGURE 4: Unwinding of DNA:DNA I with Dda produced a fast, lag phase followed by a slower phase. (A) Incubation of 2 nM DNA:DNA I with 125 nM Dda (●), 250 nM Dda (○), or 500 nM Dda (□) prior to initiating the reaction with 5 mM ATP. The line represents the best fit of the data at 125 nM Dda to a model described by Scheme 1. Initiation of the reaction by mixing Dda (125 nM) with DNA:DNA I (2 nM) led to much slower unwinding (◆). (B) Expanded view of the fast, lag phase from panel A.

ATPase Assays in the Presence of PNA. Dda ATPase activity was monitored with a coupled, spectrophotometric assay as previously reported (*5*). 50 nM Dda was titrated with 50, 150, or 500 nM 15mer PNA, and the conversion of NADH to NAD^+ was followed at 380 nm with a Pharmacia Biotech spectrophotometer using Swift software (Pharmacia Amersham). As a control, the ATPase activity of Dda was measured in the presence of 100 nM 15mer DNA. ATP hydrolysis rates were determined by using an extinction coefficient of 1210 M^{-1} cm^{-1}.

RESULTS

Helicase Unwinding Assay. The goal of these experiments was to determine the sensitivity of Dda to the structure of the displaced strand during the unwinding reaction. Substrates were prepared by annealing a 30mer to a 15mer to form a partially duplex substrate (Figure 2). The strand containing the 5′-ssDNA overhang is referred to as the loading strand because it facilitates initiation of unwinding of the duplex. The sequence of the single-stranded overhang was $(dT)_{15}$ in order to reduce the likelihood of formation of secondary structures. The loading strand is believed to be the strand on which Dda translocates with a strong directional bias in the 5′-to-3′ direction (*5*, *8*). The displaced strand consisted of a 15mer DNA, RNA, or PNA.

It was necessary to establish that the observed rates were limited by strand separation rather than by association of the enzyme with substrate. Experiments were performed in which excess Dda (125 nM) was incubated with DNA:DNA I (2 nM), followed by initiation of the reaction by rapid mixing with ATP and Mg^{2+} (Figure 3A). The low concentration of substrate precluded reannealing of product strands during the rapid unwinding reaction. Reactions were quenched by addition of EDTA (400 mM). To prevent spontaneous reannealing of products after the reaction, a 15mer trapping strand that was complementary to the displaced strand was introduced after adding the quench solution. Single-stranded reaction products were separated from duplex substrates by nondenaturing polyacrylamide gel electrophoresis and quantitated using a phosphorimager (Figure 3B).

Under these conditions, rapid product formation was observed. Three concentrations of Dda were tested, each resulting in essentially identical progress curves for product formation (Figure 4A). For comparison, the unwinding reaction was initiated by mixing Dda with DNA substrate, which resulted in much slower unwinding (Figure 4A). These results suggest that the substrate was saturated with Dda during incubation prior to the reaction, and that association of enzyme with substrate did not limit product formation. The shape of the progress curve was similar to that reported for unwinding by UvrD (*21*, *22*). A distinct, fast lag phase in which ~80% of the substrate was unwound was followed by a slower phase in which the remaining 20% of substrate was converted to product. The lag phase is shown in Figure 4B, and likely results from multiple steps required for forming fully unwound DNA, indicating at least one intermediate along the reaction pathway. The presence of the lag phase and the similarity in the unwinding curves at different Dda concentrations indicate that the unwinding reaction is rate-limiting under these conditions.

The slower phase was also unchanged at varying enzyme concentration, suggesting that it did not arise from association of enzyme with substrate. In a separate experiment, the 15mer trapping strand was introduced in the reaction along with ATP and Mg^{2+}. If Dda dissociates from the substrate, the 15-mer should compete with substrate for protein and slow the observed reaction. However, no changes in the resulting rate constants and amplitudes for product formation were observed (Table 1). Therefore, the slow phase likely represents a conformational change of nonproductively bound substrate to productively bound substrate. In the cases where Dda was incubated with substrate prior to initiating the reaction, all of the substrate was converted to product at each Dda concentration. Dda is generally considered a nonpro-

Table 1: Unwinding Rates of 30:15-mer Substrates at Varying Concentrations of Dda Helicase[a]

Dda	k_U, s^{-1} (lag phase)	amplitude, nM (lag phase)	k_{NP}, s^{-1} (slow phase)	amplitude, nM (slow phase)
125 nM	78 ± 11	1.56 ± 0.12	2.2 ± 2.2	0.40 ± 0.14
125 nM[b]	87 ± 9	1.67 ± 0.09	1.7 ± 1.7	0.35 ± 0.15
250 nM	81 ± 11	1.48 ± 0.10	1.6 ± 1.5	0.48 ± 0.18

[a] The rates for single-turnover unwinding experiments were determined by fitting the data to a mechanism according to Scheme 1 using the program Scientist as discussed in the text. Four parameters were obtained from the fit. Errors reported are 95% confidence intervals. [b] Trapping strand (50 nM) was introduced into the reaction mixture with ATP.

cessive helicase (*23*, *24*); however, the substrate used in these experiments was short enough for complete unwinding in a single binding event.

The simplest model that takes into account all of the observed phases for the progress curves is shown in Scheme 1. Productively bound substrate (ES_1) is rapidly converted to an intermediate (ES_2) due to unwinding by Dda. A second unwinding step leads to product formation (ssDNA). Although only one intermediate is shown, it is possible that additional intermediates occur along the reaction pathway. Ali and Lohman (1997) measured the lag phase for unwinding by UvrD helicase as a function of varying length substrates to estimate the step-size for unwinding (*21*). Such experiments are in progess with Dda; therefore, the number of steps in Scheme 1 represents only the minimum required to account for the lag phase. Because ~20% of the substrate was unwound more slowly, the model contains a slow step in which nonproductively bound substrate (ES_{NP}) slowly converts to productively bound substrate (ES_1), to account for the slow phase in the progress curve. Dissociation of enzyme from the substrate at each step in the reaction is possible, but was not considered here because all of the DNA was unwound, indicating that little or no dissociation occurred with this short substrate over the time frame of the reaction. Results were fit to a model according to Scheme 1 to obtain rate constants and amplitudes for each phase of the progress curve. The amplitudes of the lag phase at 125, 250, and 500 nM Dda were 1.56 (±0.12), 1.48 (±0.10), and 1.48 (±0.08) nM, respectively (Table 1). The rate constants for unwinding during the lag phase, k_U in Scheme 1, were also similar at each concentration of Dda, ranging from 78 (±11) to 87 (±9) s^{-1} (Table 1). From these data, it was clear that incubation with 125 nM Dda was sufficient to saturate the substrate; therefore, these conditions were chosen for further studies using the unnatural substrates.

Unwinding of DNA:RNA and DNA:PNA Substrates. Initially, the effect of changing the sequence of the substrate was investigated by comparing unwinding of two sequences containing only DNA. The two DNA−DNA substrates, DNA:DNA I and DNA:DNA II (Figure 2), were unwound with very similar observed rate constants of 78 (±11) and 77 (±9) s^{-1}, respectively (Table 2). This result indicates that altering the sequence of the substrate has little effect on the observed unwinding rate, consistent with the sequence independence observed with most helicases. Changing the displaced strand from DNA to RNA also had little effect on the unwinding rate (Figure 5A, Table 2). The lag phase for unwinding of substrate DNA:RNA, which was identical in sequence to DNA:DNA I (Figure 2), occurred with an observed rate constant of 91 (±11) s^{-1} and amplitude of 1.67 (±0.13) nM. Thus, Dda is able to unwind DNA−RNA hybrids as efficiently as DNA−DNA hybrids.

Table 2: Unwinding Rates of 30:15-mer Substrates and Melting Temperatures of Corresponding 15:15-mer Duplexes[a,b]

substrate	k_U, s^{-1} (lag phase)	amplitude, nM (lag phase)	T_M, °C
DNA:DNA I	78 ± 11	1.56 ± 0.12	47.6
DNA:DNA II	77 ± 9	1.53 ± 0.10	47.6
DNA:RNA	91 ± 11	1.67 ± 0.13	44.2
DNA:PNA I	64 ± 5	1.64 ± 0.10	76.4

[a] A preliminary account of this study was reported in ref *35*. [b] Reactions were initiated by incubating Dda (125 nM) with substrate (2 nM) followed by rapid mixing with ATP and Mg^{2+} as described under Experimental Procedures. The rates and amplitudes for single-turnover unwinding experiments were determined as described in Table 1. Errors reported are 95% confidence intervals. The rate constants and amplitudes of the slow phase (not shown) were similar to DNA:DNA I for each substrate (Table 1).

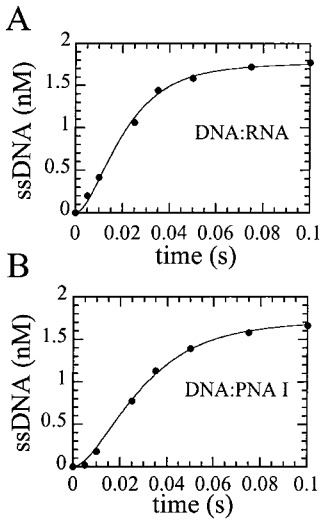

FIGURE 5: Unwinding of 2 nM DNA:RNA (A) and 2 nM DNA:PNA I (B) with 125 nM Dda. Unwinding produced a fast, lag phase followed by a slower phase as observed for the DNA:DNA substrates. The line represents the best fit of each data set to the model depicted in Scheme 1. See Table 2 for rate constants and amplitudes obtained from fitting the data.

PNA has been found to bind to DNA through normal Watson−Crick hydrogen bonds, although the melting temperatures for PNA−DNA hybrids are significantly higher than the corresponding DNA−DNA duplexes (*25*). A 15mer PNA was hybridized to a 30mer oligonucleotide to form helicase substrate DNA:PNA I (Figure 2). Unwinding of this substrate occurred with similar rate constants and amplitudes to the other substrates (Figure 5B, Table 2). The rate constant for unwinding during the lag phase was 64 (±5) s^{-1}, and the amplitude was 1.64 (±0.1) nM. To determine whether the unwinding rate of the DNA−PNA substrate was independent of sequence, a second PNA strand was annealed to a DNA sequence to prepare substrate DNA:PNA II (Figure 2). The observed rate constants for unwinding during the lag phase, 81 (±4) s^{-1}, and the amplitude of the lag phase, 1.70 (±0.05) nM, were similar to the other substrates (Table 2). Hence, replacement of the displaced DNA strand of the substrate with a strand of PNA had very little impact on unwinding by Dda under the conditions described here.

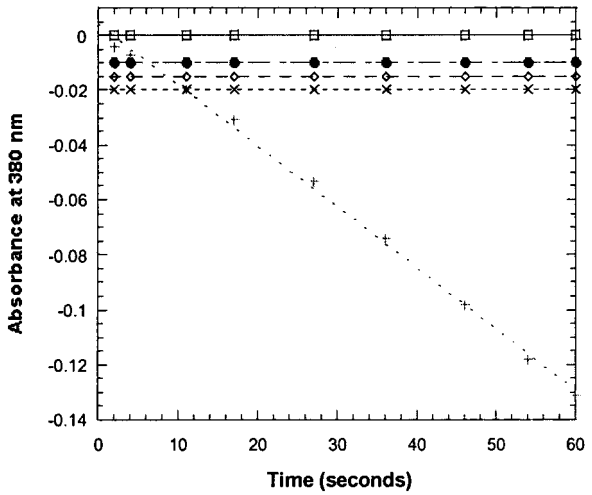

FIGURE 6: Dda ATPase assay in the presence of PNA. ATPase activity was followed by using a coupled, spectrophotometric assay that monitors the change in A_{380} as NADH is converted to NAD^+. After an initial measurement with 50 nM Dda in the absence of ssDNA (□), 15mer PNA was added in increasing concentrations: 50 nM PNA (●), 150 nM PNA (◇), 500 nM PNA (×). After addition of PNA, 100 nM 15mer DNA was added to the solution (+).

Melting Temperatures of Helicase Substrates. To compare the stability of the substrates, melting temperatures were determined. Initial attempts to measure the melting temperature of the helicase substrates were unsuccessful, presumably due to the contribution to absorbance from the ssDNA overhang. Therefore, melting temperature measurements were conducted with the duplex regions of the substrates, which provided sigmoidal melting curves. The DNA−PNA substrates melted at higher temperatures than DNA−DNA, and DNA−RNA, as expected (Table 2) (*25*). Thus, unwinding by Dda shows little sensitivity to changes in the thermal stability of oligonucleotide substrates.

PNA Does Not Stimulate the ATPase Activity of Dda. To determine whether the ability for Dda to unwind the modified substrates was due to specific interaction of the helicase with PNA, the ATPase activity of Dda was measured in the presence of DNA or PNA 15mer. A spectrophotometric assay was performed as described (*5*), and 50 nM Dda exhibited no detectable ATPase activity in the absence of DNA (Figure 6). In the presence of 100 nM 15mer DNA, a velocity of 1834 $nM \cdot s^{-1}$ was observed (Figure 6). Higher concentration of 15mer DNA provided no additional stimulation of the ATPase activity (data not shown). In contrast, no stimulation of the ATPase activity of Dda was observed in the presence of 50, 150, or 500 nM PNA 15mers (Figure 6). Thus, PNA does not interact with the helicase in the same manner as DNA, at least in regards to stimulation of the ATPase activity of the helicase.

DISCUSSION

The substrate requirements for helicases have been studied by chemically modifying the duplex in a strand-specific fashion. Chemical modification of the loading strand often has led to slower rates of unwinding for many helicases. In the case of the T7 gene 4 helicase, the enzyme was sequestered at the site of a DNA adduct on the loading strand but was unaffected by modification of the displaced strand (*26*). Site-specific DNA adducts of the antitumor drug CC-1065 inhibited Dda helicase activity only when adducts were placed on the loading strand of the substrate (*27*). Likewise, CC-1065, cyclobutane dimers, and cisplatin-GG dimers inhibited the yeast helicase, Rad 3, only when adducts were placed on the strand on which Rad 3 is proposed to bind and translocate (*28*). In each of these cases, modification of the displaced strand of DNA led to little or no effect on the rate of unwinding. These observations suggest that specific interactions between some helicases and DNA occur primarily on the loading strand of the substrate.

The hexameric helicases gp41 from bacteriophage T4 and gene 4 from bacteriophage T7 were each shown to encircle ssDNA (*8, 29*). When biotin−streptavidin blocks were placed within the duplex of a fork substrate, inhibition occurred only when the blocks were placed on the strand on which the helicase is believed to encircle (*19, 30*). However, gp41 and gene 4 prefer 5′- and 3′-ss tails ahead of the duplex for optimal unwinding, suggesting that some specific interaction with the displaced strand ahead of the duplex may enhance unwinding activity with these hexameric helicases (*31*).

In this study, the displaced strand of the substrate was replaced with a strand of RNA or PNA. The DNA−RNA substrate allows for comparison of a small change in primary structure, the 2′-OH, but a substantial change in secondary structure of the duplex. DNA−RNA duplexes adopt an A-form helix whereas DNA−DNA duplexes adopt a B-form helix. In the case of PNA, the primary structure is altered dramatically by the replacement of the deoxyribose phosphate backbone with the *N*-(2-aminoethyl)glycine backbone (Figure 1). The secondary structure of PNA−DNA duplexes is also different from DNA−DNA and contains elements of A- and B-form DNA. The base pair stacking is similar to A-form, whereas the sugar moieties are more like those in B-form DNA (*32*). The number of base pairs per helical turn is 13, as compared to 10 for B-form DNA. The thermal stability of PNA−DNA hybrids is generally higher than DNA−DNA duplexes (*25*).

Substitution of an RNA strand for the displaced strand of DNA in the substrate led to only a small change in the rate of unwinding by Dda (Table 2). This suggests that Dda shows little sensitivity to changes in the conformation of the helix. Substitution of a PNA strand for the displaced strand also led to little or no change in the rates of unwinding (Table 2). PNA provides the necessary structural and electronic features to support unwinding by Dda when occupying the position of the displaced strand. However, the chemical structure of the PNA backbone is entirely different than DNA so that any specific interactions between the helicase and the backbone of the displaced strand should be interrupted. The fact that the T_M values of the substrates varied by approximately 30 °C, yet unwinding occurred with similar rates suggests that Dda shows little sensitivity to changes in the thermal stability of these substrates.

The similar rates observed for unwinding between natural and unnatural substrates suggest that the rate-limiting step for unwinding is insensitive to the chemical nature of the displaced strand under the conditions described here. If strand separation is rate-limiting during the unwinding reaction, then it may be concluded that specific, enzyme−substrate interactions need not occur between Dda and the displaced strand. A model that is consistent with the data is that Dda unwinds

duplex DNA as a consequence of its ability to translocate on ssDNA (*8*). Translocation with a strong directional bias on the loading strand could allow the enzyme to sequester ssDNA that forms naturally due to thermal fluctuations within the duplex region (*11*, *12*). Or, the enzyme may act as a "wedge", in which the displaced strand is actively removed due to steric interactions with the translocating enzyme. In this mode, molecules in the path of the helicase such as the complementary strand are displaced due to steric interactions with the enzyme as suggested for the *E. coli* Rho transcription termination factor (*33*, *34*). Either model does not require specific enzyme−substrate interactions within the displaced strand.

These results may have bearing on applications of PNA. PNA has been used as an antisense and antigene agent in many systems. If mammalian helicases are capable of displacing PNA, then the use of PNA to target DNA as antisense agents may be limited if other helicases prove capable of overcoming challenges presented by hybrids formed with PNA.

REFERENCES

1. Lohman, T. M., and Bjornson, K. P. (1996) *Annu. Rev. Biochem. 65*, 169−214.
2. Matson, S. W., Bean, D. W., and George, J. W. (1994) *Bioessays 16*, 13−22.
3. Waksman, G., Lanka, E., and Carazo, J.-M. (2000) *Nat. Struct. Biol. 7*, 20−22.
4. Young, M. C., Schultz, D. E., Ring, D., and von Hippel, P. H. (1994) *J. Mol. Biol. 235*, 1447−1458.
5. Raney, K. D., and Benkovic, S. J. (1995) *J. Biol. Chem. 270*, 22236−22242.
6. Dillingham, M. S., Wigley, D. B., and Webb, M. R. (2000) *Biochemistry 39*, 205−212.
7. Matson, S. W., and George, J. W. (1987) *J. Biol. Chem. 262*, 2066−2076.
8. Morris, P. D., and Raney, K. D. (1999) *Biochemistry 38*, 5164−5171.
9. Hill, T., and Tsuchiya, T. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4796−4800
10. Velankar, S. S., Soultanas, P., Dillingham, M. S., Subramanya, H. S., and Wigley, D. B. (1999) *Cell 97*, 75−84.
11. Geiselmann, J., Wang, Y., Seifried, S. E., and von Hippel P. H. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 7754−7758.
12. Chen, Y. Z., Zhuang, W., and Prohofsky, E. W. (1992) *J. Biomol. Struct. Dyn. 10*, 415−427.
13. Kim, J. L., Morgenstern, K. A., Griffith, J. P., Dwyer, M. D., Thomson, J. A., Murcko, M. A., Lin, D., and Caron, P. R. (1998) *Structure 6*, 89−100.
14. Wong, I., and Lohman, T. M. (1992) *Science 256*, 350−355.
15. Nielsen, P. E. (1999) *Curr. Opin. Biotechnol. 10*, 71−75.
16. Gorbalenya, A., and Koonin, E. V. (1993) *Curr. Opin. Struct. Biol. 3*, 419−429.
17. Goodwin, T. E., Holland, R. D., Lay, J. O., and Raney, K. D. (1998) *Bioorg. Med. Chem. Lett. 8*, 2231−2234.
18. Hacker, K. J., and Alberts, B. M. (1992) *J. Biol. Chem. 267*, 20674−20681.
19. Raney, K. D., Carver, T., and Benkovic, S. J. (1996) *J. Biol. Chem. 271*, 14074−14081.
20. Picha, K. M., Ahnert, P., and Patel, S. S. (2000) *Biochemistry 39*, 6401−6409.
21. Ali, J. A., and Lohman, T. M. (1997) *Science 275*, 377−380.
22. Ali, J. A., Maluf, N. K., and Lohman, T. M. (1999) *J. Mol. Biol. 293*, 815−834.
23. Jongeneel, C. V., Formosa, T., and Alberts, B. M. (1984) *J. Biol. Chem. 259*, 12925−12932.
24. Raney, K. D., Sowers, L. D., Millar, D. P., and Benkovic, S. J. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 6644−6648.
25. Egholm, M., Buchardt, O., Christensen, L., Behrens, C., Freier, S. M., Driver, D. A., Begg, R. H., Kim, S. K., Norden, B., and Nielsen, P. E. (1993) *Nature 365*, 566−568.
26. Yong, Y., and Romano, L. J. (1996) *Chem. Res. Toxicol. 9*, 179−187.
27. Maine, I. P., Sun, D., Hurley, L. H., and Kodadek, T. (1992) *Biochemistry 31*, 3968−3975.
28. Naegeli, H., Bardwell, L., and Friedberg, E. C. (1993) *Biochemistry 32*, 613−621.
29. Egelman, E. H., Yu, X., Wild, R., Hingorani, M. M., and Patel, S. S. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 3869−3873.
30. Hacker, K. J., and Johnson, K. A. (1997) *Biochemistry 36*, 14080−14087.
31. Ahnert, P., and Patel, S. S. (1997) *J. Biol. Chem. 272*, 32267−32273.
32. Eriksson, M., and Nielsen, P. E. (1996) *Nat. Struct. Biol. 3*, 410−413.
33. Walstrom, K. M., Dozono, J. M., Robic, S., and von Hippel, P. H. (1997) *Biochemistry 36*, 7980−7992.
34. Walstrom, K. M., Dozono, J. M., and von Hippel, P. H. (1997) *Biochemistry 36*, 7993−8004.
35. Raney, K. D., Hamilton, S., and Corey, D. R. (1998) in *Peptide Nucleic Acids* (Nielsen, P. E., and Egholm, M., Eds.) pp 241−251, Horizon Scientific Press, Wymondham, England.

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