Vinylphosphonate Internucleotide Linkages Inhibit the Activity of PcrA DNA Helicase

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ABSTRACT: During the past 5 years a great deal of structural and biochemical information has given us a detailed insight into the molecular mechanism of action of the PcrA DNA helicase and challenged previous notions about the molecular mechanism of action of helicases in general. Despite this wealth of information the mechanisms of the interaction of helicases with their DNA substrates and their unidirectional translocation along ssDNA are poorly understood. In this study, we synthesized a chemically modified DNA substrate with reduced backbone rotational flexibility and minimal steric hindrance and studied its effect on the activity of the monomeric 3′−5′ DNA helicase, PcrA. Our results show that a single modification on the backbone of the translocating strand is sufficient to inhibit the activity of PcrA helicase, suggesting that rotational flexibility of the backbone is important for efficient unwinding.

Many prokaryotic DNA-binding proteins recognize specific DNA[1] sequences through discrete DNA-binding domains within their polypeptides (*1*). Some of these proteins are dimeric and interact with specific palindromic DNA sequences, known as recognition sequences. In such cases, the 2-fold symmetry of the protein is matched by the 2-fold symmetry of the recognition sequence, and the specificity of the interaction is determined, predominantly, by the nature of the bases within the DNA and the amino acid residues that H-bond exclusively with these bases. Nonspecific DNA-binding proteins interact with the polyanionic backbone of the DNA, usually, via strategically placed positively charged residues, such as arginines and lysines. In single-stranded (ss) DNA the predominantly hydrophobic bases are much more exposed, and a ssDNA-binding protein, in addition to interacting with the DNA backbone, might also have aromatic groups (tryptophans, tyrosines, and phenylalanines) which will intercalate with the DNA bases, forming base-stacking interactions. It is therefore clear that the interactions of proteins with dsDNA can be of a different nature compared to the interactions with ssDNA and this can be seen clearly in the case of PcrA DNA helicase.

PcrA, like most helicases, is a nonspecific DNA-binding protein that binds primarily to ssDNA (*2*). This binding involves mainly a number of base stacking but also some electrostatic interactions, via aromatic and positively charged residues from conserved motifs, in a groove along the top of domains 1A and 2A (*3*). These interactions are modulated by binding and hydrolysis of ATP in the active site, between domains 1A and 2A (*3*), and as a consequence, PcrA translocates along the ssDNA by employing an inchworm-like translocation mechanism, known as "the Mexican wave" (*2*, *4*).

PcrA has also been reported to bind double-stranded (ds) DNA but with reduced affinity compared to ssDNA, as shown by DNA mobility shift assays (*5*). The structural details of the PcrA binding to a ss−dsDNA junction were revealed by the crystal structures of the enzyme complexed to a 3′-tailed synthetic oligonucleotide (*2*), and the contributions of certain amino acid residues (mainly positively charged lysines from domains 1B and 2B) to the binding and distortion of the duplex were revealed by mutagenesis, nuclease protection, and DNA footprinting studies (*6*). From these experiments it transpired that PcrA actively engages and distorts the duplex at the ss−ds junction, via an active mechanism, in response to binding and hydrolysis of ATP. The interactions of the enzyme with the ss−ds junction appear to be strengthened in the presence of ATP, implying that in this case PcrA should bind to the junction with a higher affinity than in the absence of ATP.

The interaction of PcrA with the DNA illustrates nicely the principles that were explained above. This enzyme employs a combination of electrostatic and base-stacking interactions, between aromatic residues and the bases of the nucleic acid, to bind to ssDNA whereas it interacts with dsDNA only via electrostatic interactions mediated mainly by positively charged lysines. DNA backbone flexibility may also be crucial for helicase activity as the crystal structure of PcrA bound to a dsDNA substrate with a ss 3′-tail revealed that binding of the enzyme at the ss−dsDNA junction induces substantial bending of the DNA backbone (*2*). Backbone flexibility may also be important for unidirectional translocation of this enzyme along the ssDNA via the Mexican wave mechanism. A comparison of the DNA bound to the substrate and product complexes revealed that five bases are stacked in clefts formed by aromatic residues in

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[1] Abbreviations: HCV, hepatitis C virus; PNA, peptide nucleic acid; DNA, deoxyribonucleic acid; ss, single strand; ds, double strand; ATP, adenosine triphosphate; DTT, dithiothreitol; NAD, nicotinamide adenine dinucleotide; SDS, sodium dodecyl sulfate; P_i, inorganic phosphate; TBE, Tris−borate−EDTA buffer; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance.

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the space between H597 and F192 in the product complex whereas only four bases are bound to the same region in the substrate complex (*2*). Such changes in the ssDNA-binding mode of the enzyme are likely to depend on the rotational flexibility of various bonds in the DNA backbone. We have previously reported the chemical synthesis of vinylphosphonate-linked (T*T) dimers, containing a hydrolytically stable vinylphosphonate internucleotide linkage and their subsequent use in the automated synthesis of a T-T*T-T tetranucleotide (*7*). Such modifications on the DNA are likely to have a profound effect on the rotational flexibility of the backbone. Using the same technology we synthesized long oligonucleotides containing one and four consecutive T*T linkages and used them as substrates to test the effect of these modifications on PcrA DNA helicase activity. Our results show that the presence of a single T*T dimer on the translocating strand is sufficient to inhibit substantially the helicase activity of PcrA as long as it is situated either at the ss−dsDNA junction or within the duplex. As this modification restricts rotational bond freedom in the vinylphosphonate linkage, we propose that unidirectional translocation of PcrA along ssDNA is affected by strand flexibility as manifested by rotational bond freedom between successive nucleotides. Furthermore, our data are also consistent with an "inchworm" mechanism (*8*) of translocation for this helicase and are incompatible with the "active rolling" model proposed previously for the homologous *Escherichia coli* Rep DNA helicase (*9*).

MATERIALS AND METHODS

Synthesis of Modified Oligonucleotides. The vinylphosphonate-linked 5′-DMTO-T*T-3′CEP phosphoramidite building block was synthesized according to our previously published procedure (Figure 1A and ref *7*). The long oligonucleotides were synthesized on a 40 nmol scale using standard phosphoramidite methodology (dimethoxytrityl-on), with the coupling time for the modified dinucleotide building block being increased to 180 s. The oligonucleotides were purified using ABI OPC cartridges according to the manufacturer's recommended procedure.

*Preparation of the Decamer d(GCAAAT*TTGC)$_2$ for NMR Analysis.* The decamer d(GCAAAT*TTGC)$_2$ was synthesized on a 10 μmol scale and was purified by reversed-phase HPLC (dimethoxytrityl-on). After pooling together the fractions, the solvent was concentrated in vacuo to ~10 mL (HPLC retention time: 29.8 min). Deprotection of the 5′-*O*-dimethoxytrityl group was carried out by treating with acetic acid (50%, 10 mL) and stirring for 40 min at 35 °C, after which time the mixture was extracted with ether (3 × 200 mL). After separation of the ethereal extracts, excess ether was removed from the aqueous extract on a rotary evaporator. The oligonucleotide was dialyzed by transferring the solution to dialysis tubing and placing the tube in distilled water (2 L) at 4 °C. After being stirred for 8 h, the water was replaced by fresh distilled water (2 L), and stirring was continued for a further 8 h. The water was then discarded and replaced by a high salt buffer (1 M NaCl and 100 mM NaH_2PO_4, 2 L). The salt solution was exchanged with distilled water a further two times at 8 h intervals. The oligonucleotide solution was then placed in a round-bottom flask, freeze-dried, and then transferred to an Eppendorf tube with D_2O.

NMR Sample Preparation. The salt concentration of the purified oligonucleotide was adjusted to 100 mM NaCl and 10 mM NaH_2PO_4 by addition of 0.5 M NaCl (125 μL) and NaH_2PO_4 (10 μL). NaN_3 stock (10 μL, 6 mM), EDTA stock (10 μL, 6 mM), and 3,3,3-trimethylsilylpropanoic acid (TSP) (3 μL, 1 mM) as an internal reference were added. After adjustment of the pH to 7.0 by addition of NaOD solution in D_2O, the solution was freeze-dried again and then redissolved in D_2O (700 μL).

Construction of DNA Substrates. The short oligonucleotide (6 pmol) 5′-AGCTCGAATTCGTAATCATGGTCAT-3′ was radioactively labeled at the 5′-end using [γ-^{32}P]ATP and polynucleotide kinase and subsequently annealed to a 1.2× molar excess of the appropriate longer oligonucleotide to construct substrates S1, S2, S3, S4, S5, S6, S7, S8, S9, and S10, as shown in Figure 1B. The labeled DNA substrates were purified away from unincorporated [γ-^{32}P]ATP by gel filtration through the S400 Sephadex mini-spin columns (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The labeled substrate was dissolved in 100 μL of 5 mM Tris, pH 8.2, and 1 M NaCl to give a final concentration of 0.06 pmol/μL for storage (assuming 100% recovery from the S400 mini-spin column). The high ionic strength stabilizes duplex formation and prevents duplex destabilization during storage. Subsequent addition of the labeled substrates to the reaction mixtures (for helicase or gel shift assays) resulted in 1/20 dilution of the salt. Substrates S11 and S12 were prepared as described above, but the final storage buffer contained only 5 mM Tris, pH 8.2.

Protein Purification. Overexpression and purification of PcrA were carried out as described before (*2*, *3*, *5*).

Gel Shift Assays. Gel shift assays were carried out as described elsewhere (*3*, *6*, *10*). Protein at various concentrations (0, 32.5, 65, 130, 260, 521, 1042, 2084 nM) was incubated with 6 nM radioactively labeled ssDNA (oligonucleotides S11 and S12) in a buffer containing 20 mM Tris, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, and 4 mM DTT for 20 min at room temperature in a total volume of 20 μL. After addition of 2 μL of loading buffer (0.25% bromophenol blue, 10% glycerol), the PcrA−ssDNA complexes were resolved through a 10% polyacrylamide, 5% glycerol native gel. Gels were dried and visualized using a Molecular Imager FX and associated software (BIORAD), according to the manufacturer's instructions.

ATPase Assays. The steady-state ssDNA-stimulated ATPase activity of PcrA was measured spectrophotometrically by a continuous ATP-regeneration assay, as described elsewhere (*3*, *6*, *10*). Briefly, the assay links the oxidation of NADH by lactate dehydrogenase (LDH) to the reduction of pyruvate to lactate in the process. Pyruvate is formed from phosphoenolpyruvate (PEP) by the enzyme pyruvate kinase (PK). PK converts ADP to ATP during this reaction. Thus as ATP is hydrolyzed by PcrA, it is regenerated by PK. The final oxidation of NADH results in a decrease in the absorbance at 340 nm. All reactions were carried out in 1 mL total volume in a buffer containing 20 mM Tris, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 4 mM DTT, 21 nM PcrA, 1 μM ssDNA (S9 or S10), 52 nM PcrA, and varying ATP concentrations. One micromolar S11 or S12 is equivalent to a 20× molar excess of DNA over PcrA and is saturating with respect to stimulating the ATPase rate. Rates were

calculated using the extinction coefficient ($\epsilon = 6.22 \times 10^3$ L mol^{-1} cm^{-1}) for NADH at 340 nm. All rates were expressed as turnover numbers of molecules of ATP hydrolyzed per second per molecule of PcrA. The data were plotted in a double reciprocal Lineweaver−Burk plot and fitted to a straight line. K_m and k_{cat} values for ATP hydrolysis were determined from the *X*- and *Y*-axes intercepts, respectively.

Helicase Assays. Time course helicase assays were carried out at 37 °C in a buffer containing 20 mM Tris, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 4 mM DTT, 2.5 mM ATP, 34 nM unlabeled oligonucleotide trap (in all cases the trap is the unlabeled displaced oligo), 3.4 nM radioactively labeled DNA substrate, and 21.75 nM PcrA. All reaction mixtures without ATP were preincubated at 37 °C for 5 min, and the reactions were initiated by the addition of ATP. At appropriate time intervals 20 μL aliquots were removed from the reaction mixture, and the reaction was terminated by the addition of 2 μL of stop buffer (0.4% w/v SDS, 40 mM EDTA, 8% v/v glycerol, 0.1% w/v bromophenol blue) to the aliquot which was then temporarily stored on ice. Annealed and displaced oligonucleotides were resolved by electrophoresis through a 16% polyacrylamide and 5% glycerol native gel in 0.5× TBE electrophoresis buffer at 140 V. Gels were dried and visualized using a Molecular Imager FX and associated software (Bio-Rad), according to the manufacturer's instructions. Annealed and boiled controls represent no displacement and full displacement, respectively. The annealed controls were obtained by setting up helicase reactions in the absence of ATP and incubating for the longest point of the equivalent time course. Boiled controls were simply incubated at 94 °C for 10 min prior to electrophoresis.

RESULTS

Vinylphosphonate Internucleotide Linkages Inhibit the Helicase Activity of PcrA. The incorporation of a single vinylphosphonate modification in the translocating strand at the ss−dsDNA junction (substrate S2, Figure 1B) resulted in drastic inhibition of the PcrA helicase activity, as assayed in vitro, by a simple oligonucleotide displacement assay (Figure 2A). The incorporation of four consecutive vinylphosphonate linkages at the ss−dsDNA junction (substrate S3, Figure 1B) resulted in complete inhibition of the PcrA helicase activity (Figure 2A). The position of the vinylphosphonate linkages relative to the ss−dsDNA junction was critical for the inhibitory effect. Moving these linkages six bases away from the ss−dsDNA junction into the ss 3′-tail (substrates S9 and S10) resulted in relatively normal helicase activity, while moving them for the same distance in the opposite direction, into the duplex region (substrates S6 and S7), resulted in inhibition of the helicase activity (Figure 2B,C). Interestingly, a vinylphosphonate linkage in the displaced strand of the duplex (substrate S4) had no apparent inhibitory effect (Figure 2A).

Vinylphosphonate Internucleotide Linkages Do Not Affect Significantly Other PcrA Activities. Our immediate concern was that the helicase inhibitory effect might be an indirect result of defective binding of PcrA to the modified DNA. We constructed two synthetic oligonucleotides 10 bases long, one with four consecutive vinylphosphonate linkages (substrate S11, Figure 1B) and the other unmodified (substrate S12, Figure 1B). From the crystal structure of PcrA bound to a 3′-tailed DNA we can see that the ss binding region

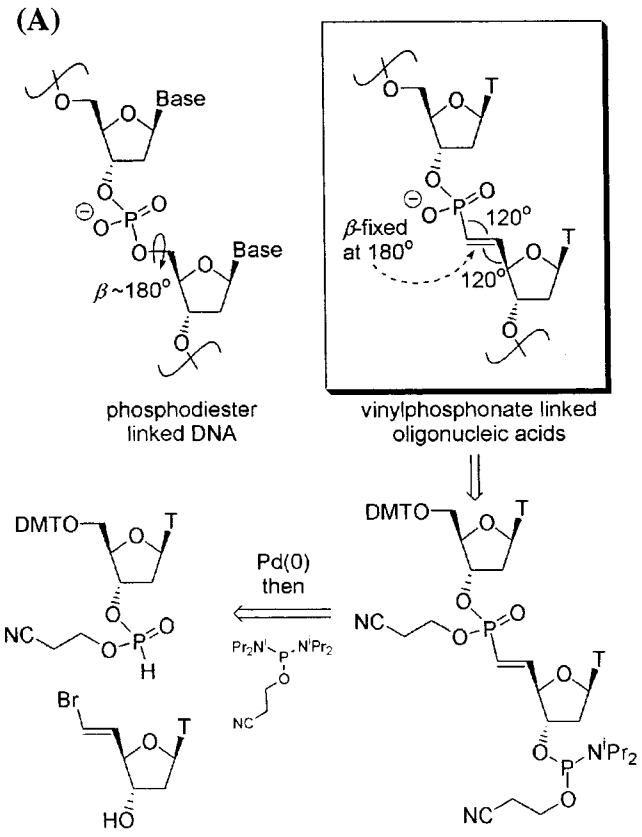

FIGURE 1: (A) Comparison of the structures of phosphodiester and vinylphosphonate-linked nucleic acids. The vinylphosphonate moiety constrains the β-torsion angle to 180° while not introducing significant steric bulk to the internucleotide linkage. The 119° P−O5′−C5′ bond angle found in B-DNA is well mimicked by the 120° P−C6′−C5′ bond angle in the modified backbone. The C6′−C5′−C4′ bond angle in the modified substrate is approximately 10° wider than the corresponding O5′−C5′−C4′ angle in the natural backbone, but the overall P−C4′ distance is only increased by 0.2 Å by the backbone replacement. (B) All of the sequences of the synthetic oligonucleotide DNA substrates used in this study. The positions of the vinylphosphonate modifications are shown by asterisks, and for the sake of clarity DNA substrates are labeled S1−S12, as indicated.

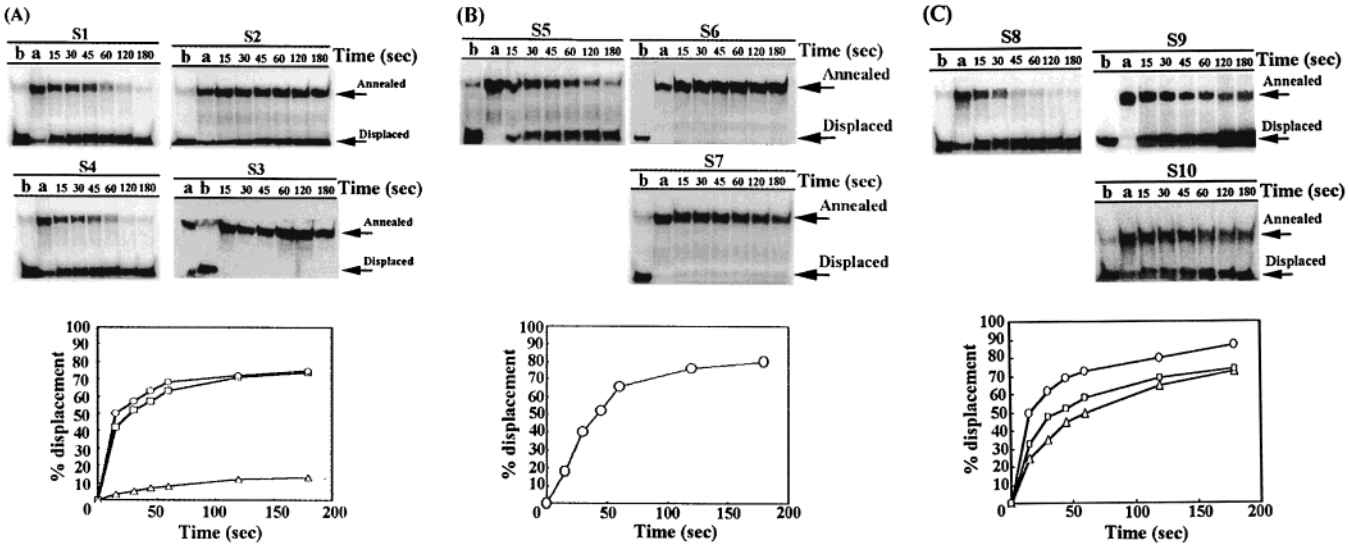

FIGURE 2: Helicase assays showing the inhibitory effect of vinylphosphonate modifications on PcrA helicase activity. Panel A: Helicase assays using S1, S2, S3, and S4 substrates, as indicated. A quantitative analysis of the results for S1 (circles), S4 (squares), and S2 (triangles) is also shown graphically. No activity could be detected for substrate S3. Panel B: Similar reactions but this time using substrates S5, S6, and S7, as indicated. The quantitative analysis of the results for S5 is shown graphically, whereas no activity was detectable for substrates S6 and S7. Panel C: Similar helicase reaction with the substrates S8, S9, and S10, as indicated. The quantitative analysis for substrates S8 (circles), S9 (squares), and S10 (triangles) is shown graphically. All reactions were carried out as described in Materials and Methods. Controls of boiled and annealed substrates are also shown in lanes labeled b and a, respectively. The annealed and displaced strands are indicated by arrows.

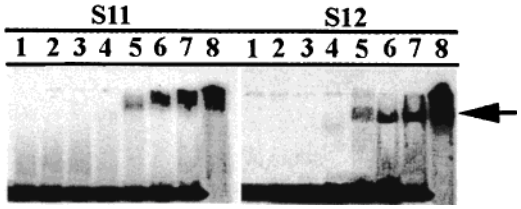

FIGURE 3: Gel shift assays showing that PcrA binds to the S11 and S12 oligonucleotides with equal apparent affinities. All binding reactions were carried out at 0 (lane 1), 32.5 (lane 2), 65 (lane 3), 130 (lane 4), 260 (lane 5), 521 (lane 6), 1042 (lane 7), and 2084 (lane 8) PcrA, using oligonucleotides S11 (modified) and S12 (unmodified), as indicated. All other experimental details were as described in Materials and Methods. The shifted bands are indicated by an arrow.

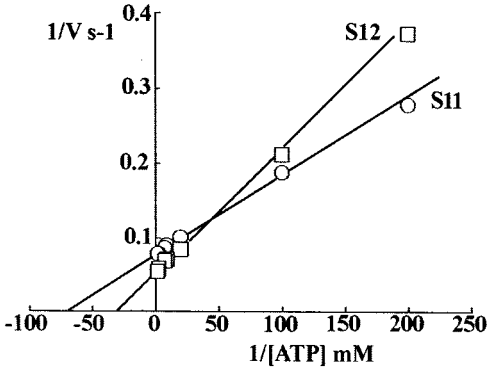

FIGURE 4: Steady-state ATPase assays using S11 and S12 oligonucleotides as cofactors. The steady-state rates for ATP hydrolysis at various ATP concentrations and in the presence of either S11 (modified) or S12 (unmodified) saturating single-stranded oligonucleotides were measured as described in Materials and Methods. A double reciprocal Lineweaver−Burk plot of the steady-state rate of ATP hydrolysis versus ATP concentration was plotted for both S11 (circles) and S12 (squares), as indicated. The k_{cat} values (expressed as turnover numbers) were determined from the *Y*-axis intercepts whereas K_m values were determined from the *X*-axis intercepts.

spans approximately 6−8 bases (*2*). Therefore, only one PcrA molecule can bind to these substrates, and upon binding PcrA will have to cope with the vinylphosphonate linkages in S11. If these modifications have affected PcrA binding to ssDNA in a nonspecific manner, we should be able to detect any binding defect by a simple gel shift assay. Our data show clearly that PcrA binds to S11 and to S12 with almost identical affinities (Figure 3). The conclusion is that the vinylphosphonate modifications do not affect general binding of PcrA to ssDNA.

Another explanation of the helicase inhibitory defect might be that, despite the fact that PcrA can bind to the modified DNA with the same affinity as the unmodified DNA, it may be unable to go through the conformational changes induced by ssDNA binding in order to activate the ATPase activity of the enzyme. In such a case, PcrA will be able to bind to the modified DNA but will be unable to hydrolyze ATP, and thus the helicase activity will be inhibited indirectly. To eliminate this argument, we proceeded to test whether the modified ssDNA can stimulate the ATPase activity of PcrA. We used a linked assay to measure the steady-state ATPase parameters of the enzymes in the presence of saturating modified (substrate S11) or unmodified (substrate S12) ssDNA. Our results show that although there is a minor reduction in the steady-state k_{cat} and K_m values for the ssDNA-stimulated ATPase activity when substrate S11 ($k_{cat} = 14\ s^{-1}$, $K_m = 15\ \mu M$) is used compared to S12 ($k_{cat} = 20\ s^{-1}$, $K_m = 40\ \mu M$) as shown in Figure 4, these differences are comparatively small and certainly not enough to explain the major inhibitory effect of helicase activity observed. Our kinetic parameters compare well with those obtained using a ss 16mer oligonucleotide ($k_{cat} = 35\ s^{-1}$, $K_m = 110\ \mu M$; ref *10*) considering that PcrA binds somewhat weaker to shorter oligonucleotides. We conclude that the modified ssDNA is approximately as effective as the unmodified ssDNA in stimulating the ATPase activity of PcrA. Col-

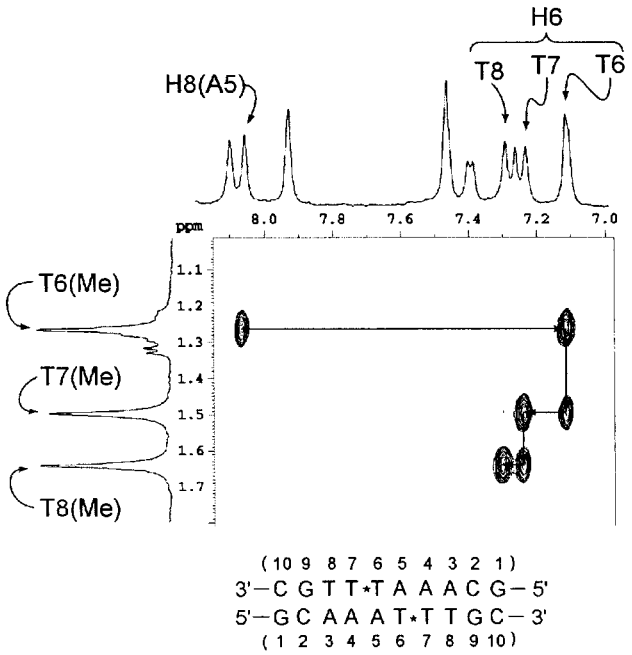

FIGURE 5: Expansion of the 2D ^{1}H-NOESY NMR spectrum of the modified decamer duplex d(GCAAAT*TTGC)$_2$ showing key NOE cross-peaks between the H8 and H6 protons of A5, T6, T7, and T8 and the thymidine methyl groups of T6, T7, and T8, thus confirming the formation of the expected double helical conformation (full data not shown).

lectively, these data suggest that the helicase inhibitory effect is a direct result of inhibition of the unidirectional translocation of the enzyme along the ssDNA lattice.

Vinylphosphonate Internucleotide Linkages Do Not Affect the Structure of the DNA Duplex. To investigate whether the vinylphosphonate modification affects the general structure of duplex DNA, we carried out a full NMR analysis of a decamer duplex d(GCAAAT*TTGC)$_2$ synthetic DNA (full data not shown). The analysis of the 2D ^{1}H-NOESY NMR spectrum of the vinylphosphonate-containing decamer duplex d(GCAAAT*TTGC)$_2$ DNA revealed that the presence of the backbone modification does not affect double helical duplex formation. An expansion of a particularly diagnostic region of the NOESY spectrum is shown in Figure 5, and key cross-peaks allowed us to carry out the sequential assignment of the H8(A5), H6(T6), H6(T7), and H6(T8) by their proximity to the three well-separated thymidine methyl groups [Me(T6), Me(T7) and Me(T8)]. These cross-peaks are characteristic of "normal" base-pair stacking in a duplex and confirm that the vinylphosphonate modification is well tolerated in these substrates without causing deviations from regular structures.

DISCUSSION

Two different translocating mechanisms have been proposed for helicase movement along the DNA. The earliest inchworm model postulated unidirectional tracking along ssDNA and simultaneous melting of the duplex immediately ahead of the translocating helicase (*8*), while the more recent active rolling model proposed DNA looping between ssDNA and dsDNA segments bound to different subunits of a dimeric (oligomeric) helicase, with binding to ssDNA and dsDNA modulated by negative cooperativity induced by ATP binding and hydrolysis (*9*). Supporting evidence for both mechanisms can be found in the literature. For example, the *E. coli* Rep helicase has been reported to be able to unwind DNA substrates, albeit less efficiently, with segments of poly(ethylene glycol) or reverse polarity DNA incorporated into the flanking ssDNA tail (*11*). These data have been interpreted in support of the active rolling model, with a dimeric Rep being able to "roll over" the physical block in front of it. However, alternative explanations of Rep targeting specifically a ss−dsDNA junction or generating considerable momentum and force that carry it through the block are also plausible. The Sgs1 DNA helicase from *Saccharomyces cerevisiae* targets specifically a variety of ss/dsDNA junctions, as shown by gel shifts and DNA footprinting, although insertions of reverse polarity segments at the junction reduced the affinity of binding and were not substrates for the helicase (*12*). However, if the reversed segments were inserted four nucleotides away from the junction, then binding was normal and the helicase activity was unaffected. Other helicases, like the gp41 and Dda helicases, can generate such considerable force during translocation that can displace streptavidin from biotin-labeled DNA with a directional bias (*13*). DnaB and T7 gene 4 replicative helicases have even been reported to translocate through covalent topoisomerase IV−DNA complexes induced by abasic sites nearby (*14*). However, physical blocks have been reported to inhibit some translocating helicases. The Rad53 helicase activity is inhibited by pyrimidine−pyrimidine dimers induced by UV irradiation (*15*). The HCV NS3 RNA helicase is also inhibited by 2′-*O*-methyl RNA (*16*). In both cases the inhibitory effects were apparent only when the physical blocks were on the translocating strand. The same blocks on the displaced strand had no apparent effect. Recent biochemical experiments have demonstrated directly that PcrA translocates unidirectionally along ssDNA in vitro, with a step size of 1 base for each ATP hydrolyzed at a speed of 50−80 s^{-1} using pre-steady-state kinetic analysis (*17*) and synthetic oligonucleotides tagged with the fluorescent base analogue 2-aminopurine (*18*).

Vinylphosphonate Linkages Inhibit Unidirectional Translocation. We constructed a unique modification designed to pose the minimum possible physical block for the translocating helicase but at the same time to restrict considerably the rotational flexibility of the DNA backbone (Figure 1A). We used modified oligonucleotides to examine the effect of this modification on the helicase activity of PcrA. A single modification on the translocating strand is sufficient to inhibit significantly the helicase activity of the enzyme while four consecutive modifications inhibit it completely. The same modifications on the displaced strand (substrate S4) have no apparent effect, consistent with previous reports for other helicases (Figure 2A and refs *15* and *16*). Here it should be made clear that the structural properties of the duplex may also be important as shown for the homologous HCV NS3 RNA helicase (*19*). Morpholino or phosphorothioate modifications on the displaced strand had no apparent effect on its helicase activity, whereas when a peptide nucleic acid (PNA) was used for the displaced strand, the rate of the helicase reaction was inhibited 25−80-fold (*19*). Such results were attributed to unique structural properties of the PNA−DNA duplexes that seem to reduce the ability of NS3 to unwind these substrates. Other helicases such as the bacteriophage T4 Dda helicase seem to be able to cope well with

PNA−DNA duplexes, as they are able to unwind these substrates apparently with similar rates to natural DNA−DNA substrates (*20*). Our vinylphopshonate linkages do not cause any drastic alterations on duplex conformation. In fact, our NMR analysis of a synthetic decamer duplex DNA carrying one vinylphosphonate linkage in the middle on each strand revealed that there is no deviation from the normal B-DNA structure (Figure 5). In addition, the fact that PcrA binds to the modified ssDNA substrate S11 (carrying four vinylphosphonate linkages) with an apparent affinity identical to the unmodified substrate S12 offers indirect evidence that the structure of the DNA backbone has not been affected significantly. Furthermore, the total insensitivity of the PcrA to the vinylphosphonate modification when the latter is placed in the displaced strand also offers additional indirect evidence for the integrity of the duplex.

The inhibitory effect is not the result of a "general DNA-binding" defect induced by the vinylphosphonate modification. PcrA binds to the modified ss oligonucleotide as efficiently as the unmodified oligonucleotide. Furthermore, the modified oligonucleotide can stimulate the ssDNA-dependent ATPase activity of the enzyme as efficiently as the unmodified oligonucleotide. This is particularly important as the previously proposed translocation cycle of PcrA along ssDNA includes an ATP-induced conformational change prior to the translocation event fueled by the energy released from ATP hydrolysis (*17*, *18*). Presumably such a conformational change sets up PcrA into a "translocation mode", and if inhibited, it will result in a stalled helicase bound to the ssDNA. Our data suggest that vinylphosphonate-modified ssDNA can still act as a "functional substrate" for DNA binding and ATP hydrolysis but does not act as an efficient translocating lattice for the translocating helicase. Although the molecular and mechanistic details of this inhibition are not clear, we propose that restrictions on the rotational flexibility imposed by the double bond in the DNA backbone form an important component of the inhibitory mechanism. A comparison of the unmodified and modified DNA substrates (Figure 1) shows that the overall geometries of the modified and unmodified DNA backbones are very similar. The P−O5′−C5′ bond angle (119° in B-DNA) is almost identical to the equivalent P−C6′−C5′ bond angle (120°) in the modified backbone. There is a small difference between the C6′−C5′−C4′ (modified substrate) and the O5′−C5′−C4′ (unmodified) bond angles, the former being 10° wider, but the overall P−C4′ distance is only increased by 0.2 Å. One might ask the question, if the structures of the modified and unmodified DNAs are similar, how is it that the helicase activity of PcrA is impaired? An important feature of the vinylphosphonate moiety is that it constrains the β-torsion angle to 180° while not introducing significant steric bulk to the internucleotide linkage. During unidirectional translocation, moving from one base to the next, a set of bonds will have to rotate in order to allow the enzyme to move on. The double bond introduced by the vinylphosphonate linkage offers a formidable energy barrier, compared to a single bond, and the enzyme cannot overcome it. Free rotational bond movement seems to be crucial for efficient translocation by PcrA.

The Translocating Mechanism of PcrA. Our data also have a direct implication regarding the molecular mechanism of unidirectional translocation by PcrA. Although both structural and biochemical information so far for PcrA suggests that the enzyme translocates unidirectionally along the ssDNA lattice by an inchworm-type of mechanism (see ref *21* for a recent review), data on the homologous Rep DNA helicase are still rather conflicting and difficult to interpret unequivocally. Rep has been reported to function as a dimer, employing an active rolling type of unidirectional translocation (*9*, *22*, *23*). This is not supported by structural evidence, since Rep crystallized as a monomer (*23*), even in the presence of ssDNA that was previously reported to induce dimerization of Rep (*24*). It should also be noted that, in the absence of DNA, Rep exists as a monomer in solution (*22*) and in the crystal structure two monomers were found to bind side by side to the same ss synthetic oligo$(dT)_{16}$, without any evidence for a functional dimer (*23*). These and other paradoxical data have led to controversy when comparing the mechanisms of action of the homologous Rep and PcrA helicases (for reviews, see refs *4*, *21*, *22*, and *25*). There is, of course, the possibility that these enzymes act by entirely different mechanisms, although for such homologous proteins with virtually identical structures this suggestion seems unlikely, albeit not impossible. It may also be that the reputed differences between Rep and PcrA are of biological rather than mechanistic importance, reflecting the possibility of serving divergent functions in vivo. Whatever the case may be with the Rep helicase, the evidence that PcrA functions as a monomer via an inchworm translocating mechanism is compelling. Our data show that a single vinylphosphonate modification on the translocating strand inhibits the helicase activity of PcrA. This is consistent with the proposed inchworm type of translocating mechanism proposed for this enzyme, rather than the active rolling mechanism proposed for Rep. If the latter were the translocating mechanism, one would expect a dimeric (or oligomeric) PcrA to be able to roll over the almost insignificant physical block provided by the vinylphosphonate double bond linkage. However, this is not the case. Interestingly, when we moved the modifications away for the ss−dsDNA junction into the 3′-tail, leaving a gap of six bases between the modifications and the junction, we observed no inhibition. Moving the modifications by the same distance in the opposite direction into the duplex restored the inhibitory effect. Presumably PcrA can bind to the six-base ss gap between the modifications and the junction, bypassing in this way the inhibitory region.

Our data suggest that free rotational bond movements of the DNA backbone are important for efficient unwinding by PcrA and might also be important for other DNA helicases in general. A wider implication may also be that rotational freedom of the DNA backbone could be of mechanistic importance in other DNA metabolizing enzymes, especially those requiring translocation along the DNA. Indeed, it is becoming apparent that the so-called "helicase motifs" are indicative of DNA translocating rather than DNA unwinding enzymes (*21*). Rotational bond freedom in the DNA backbone is likely to be of importance not only in the PcrA-type translocation mechanism but also in the active rolling and other DNA looping-type translocation mechanisms, although in these cases localized DNA backbone rotational restrictions will be dissipated by simply increasing the size of the loop during translocation. Furthermore, vinylphosphonate linkages offer us a tool for studying the significance of bond rotations in enzymatic mechanisms, like those catalyzed by DNA

topoisomerases and gyrases. We are in the process of chemically reducing the vinylphosphonate double bond into a single bond, thus relieving the rotational bond restriction. Such modification will enable us to compare directly the double versus single bonds and the significance of rotational bond freedom of the DNA backbone on the activities of nucleic acid metabolizing enzymes.

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