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Stepwise Mechanism of HIV Reverse Transcriptase: Primer Function of Phosphorothioate Oligodeoxynucleotide†

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ABSTRACT: Primer recognition by purified HIV reverse transcriptase has been investigated. Earlier we found that the reaction pathway for DNA synthesis is ordered, with template·primer and free enzyme combining to form the first complex in the reaction sequence (Majumdar et al., 1988). We now find that $d(C)_{28}$ is a linear competitive inhibitor of DNA synthesis against poly[r(A)]·oligo[d(T)] as template·primer, indicating that $d(C)_{28}$ and the template·primer combine with the same form of the enzyme in the reaction scheme, i.e., the free enzyme. The phosphorothioate oligodeoxynucleotide $Sd(C)_{28}$ also is a linear competitive inhibitor against template·primer. However, the K_i for inhibition (~2.8 nM) is ~200-fold lower than the K_i for inhibition by $d(C)_{28}$. Since the inhibition is linear competitive, the dissociation constant is equal to the K_i for inhibition. Filter binding assays confirmed high-affinity binding between $Sd(C)_{28}$ and the enzyme and yielded a K_D similar to the K_i for inhibition. Substrate kinetic studies of DNA synthesis using $Sd(C)_{28}$ as primer, and poly[r(I)] as template, revealed that the K_m for $Sd(C)_{28}$ is 24 nM. The K_m for this primer is, therefore, 8-fold higher than the K_D for enzyme–primer binding (2.8 nM). These results enable calculation of real time rate values for the enzyme–primer association ($k_{on} = 5.7 \times 10^8$ M^{-1} s^{-1}) and dissociation ($k_{off} = 1.6$ s^{-1}).

The HIV reverse transcriptase is responsible for replication of the HIV viral genome. Shortly after retrovirus infection of a cell, the enzyme catalyzes both RNA-directed DNA synthesis and DNA-directed DNA synthesis in the cytoplasm. In the process, the enzyme is capable of recognizing its natural primer, Lys-tRNA subspecies 3, and of the template switching steps necessary for replication of the complete viral genome (Gilboa et al., 1979). The enzymatic mechanism of these events and of the DNA synthesis reaction itself is the subject of the current investigation.

As a step toward understanding the mechanism of HIV reverse transcriptase, we recently applied steady-state kinetic and processivity analysis to obtain a kinetic scheme for the overall DNA synthetic reaction (Majumdar et al., 1988). Our data suggested that the free enzyme interacts with the primer substrate in the initial phase of the DNA synthesis reaction pathway. Here, we extended these studies by developing a primer analogue with exceptionally high affinity for the free enzyme. We then made use of this primer to determine both a thermodynamic dissociation constant for the enzyme–primer complex and a kinetic constant (K_m) for DNA synthesis. A mathematical treatment of these constants then enabled calculation of on and off rates for the interaction between free enzyme and primer. The results are discussed in the context of a kinetic model for reverse transcriptase activity and of the use of the high-affinity primer, a phosphorothioate oligodeoxynucleotide, as a potential antiviral agent (Matsukura et al., 1987).

Experimental Procedures

Materials

[^{3}H]dTTP and [^{3}H]dCTP were from New England Nuclear. [γ-^{32}P]ATP was from Amersham. The chain length of each polynucleotide was calculated from the sedimentation coefficient or from gel electrophoretic analysis. Normal and sulfur-substituted oligodeoxynucleotides were synthesized chemically and were characterized by NMR spectroscopy, as reported earlier (Stec et al., 1984; Stein et al., 1988). Synthesis was conducted with a mixture of phosphorothioate diastereoisomers. Hence, each phosphorothioate oligomer is a mixture of stereoisomers. $r(A)_{810}$,[1] where 810 is the average chain length, and $r(I)_{127}$, where 127 is the average chain length, were

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[1] Abbreviations: TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane; Pol I, *Escherichia coli* DNA polymerase I; poly[r(A)], polyriboadenylate; poly[r(C)], polyribocytidylate; poly[r(I)], polyriboinosinate; oligo[d(T)], oligomer of deoxythymidylate; $nd(C)_{28}$ or $d(C)_{28}$, 28-residue-long normal oligodeoxycytidylate; $Sd(C)_{28}$, 28-residue oligodeoxycytidylate with sulfur substituted at a nonbridge oxygen of each phosphate atom; $3'\text{-}S_9\text{-}O_{18}\text{-}5'\text{-}d(C)_{28}$, $3'\text{-}O_9\text{-}S_9\text{-}O_9\text{-}5'\text{-}d(C)_{28}$, or $3'\text{-}O_{18}\text{-}S_9\text{-}5'\text{-}d(C)_{28}$, partially sulfur substituted oligodeoxynucleotides where sulfur substitutions are tandemly connected (blocks) and the subscript represents the number of substitutions relative to the 3′ or 5′ ends. Polynucleotide subscripts indicate precise chain length unless otherwise noted. The kinetic nomenclature and constants are according to Cleland (1963a,b) and Fersht (1985).

obtained from Pharmacia; hereafter, they will be referred to simply as "$r(A)_{810}$" and "$r(I)_{127}$". Pol I large fragment and polynucleotide kinase were from New England Biolabs. To prepare the template·primer complexes, $r(A)_{810}$ and $d(T)_{14}$ or $r(I)_{127}$ and S or ndC_{28}, the template and primer were mixed in a weight ratio of 5:1 in 10 mM KCl. The solution was heated in boiling water for 3 min and then allowed to cool to room temperature spontaneously. The solution was chilled to 0–4 °C before addition to other reaction mixture components, also at 0–4 °C. Alternatively, the solution of annealed template·primer could be stored at −20 °C. Sources of other materials have been described (Detera et al., 1981). Nitrocellulose membrane filters (BA 85, 0.45 μM, 25-mm circles) were from Schleicher and Schuell.

HIV-1 reverse transcriptase, highly purified by an immunoaffinity procedure (Cheng et al., 1987), was a gift from M. G. Sarngadharan. The enzyme preparation was found to be free of exonuclease with $d(T)_{14}$ as substrate. Recombinant HIV-1 reverse transcriptase used in the filter binding study was generously supplied by Genetics Institute (Boston, MA). This enzyme and the natural enzyme purified from virions were found to be indistinguishable in reaction properties.[2] DNA polymerase α was purified from calf thymus (Albert et al., 1982). DNA polymerase γ, purified from pig liver by using DEAE-Sephadex, phosphocellulose, and heparin-agarose chromatography, was a generous gift of D. Mosbaugh. Human DNA polymerase β was purified as described by Abbotts et al. (1988).

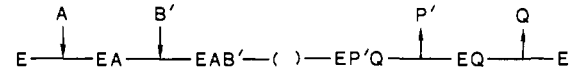
Methods

Reverse Transcriptase Assay. Standard reaction mixtures in a final volume of 25 μL containing 50 mM Tris-HCl, pH 8.2, 100 mM KCl, 6 mM $MgCl_2$, 10 mM dithiothreitol, 0.2–1 nM enzyme plus template·primer, and dNTP were incubated for 30 min at 36–37 °C. Unless indicated otherwise, [^{3}H]-dTTP or [^{3}H]dCTP was 32 μM, $r(A)_{810}$ or $r(I)_{127}$ was 10 μM (nucleotide), and $d(T)_{14}$ or $d(C)_{28}$ was 2 μM (nucleotide). The reactions were terminated by addition of 10% TCA containing 50 mM $NaPP_i$. [^{3}H]DNA products were collected on nitrocellulose filters and washed with 5% TCA containing 50 mM $NaPP_i$. Radioactivity was measured as described (Schrier & Wilson, 1975); recovery of DNA products was >90% as measured by DE-81 paper chromatography. The specific activity of [^{3}H]dNTP was 3000–7500 dpm/pmol. Linearity of dNMP incorporation with time of incubation was observed for various concentrations of dNTP and the two different types of template·primer systems. The rate of dNMP incorporation was proportional to the amount of reverse transcriptase with each condition shown. Data points shown for the determination of kinetic constants are the average from two or more determinations. The velocity of the polymerization reaction is expressed as pmol of dNMP incorporated/(min·mL of reaction mixture).

Kinetic constants first were evaluated graphically by plotting reciprocals of substrate concentrations (A) and the velocity of reaction (v) according to the relationship $v = V_{max}A/K_m + [A]$. Slopes (K_m/V_{max}) and intercepts ($1/V_{max}$) so obtained were plotted against the reciprocal of the other substrate. Finally, constants were obtained by least-squares fits of data to the eq 1–3 below, using the interactive curve-fitting program MLAB described by Knott and Schrager (1972). Experiments for Hill plot analysis of phosphorothioate oligodeoxynucleotide inhibition were as described. Data were plotted according to the equation $\log (v_0 - v/v) = n \log [I] + \log (1/K_i)$, where

[2] S. H. Wilson, unpublished observation.

Scheme I



[I] is inhibitor concentration, n represents slope of the line and molecular order of the inhibition, K_i is the dissociation constant, and v_0 and v are velocity of reaction in the absence and presence of inhibitor.

Data Analysis. We have shown, by substrate kinetics and product inhibition analyses, that the polymerization reaction catalyzed by HIV reverse transcriptase can be depicted as shown in Scheme I (Majumdar et al., 1988), where the free enzyme (E) combines with the substrates template·primer (A) and nucleoside 5′-triphosphate (B) in an ordered fashion. The general equation representing this mechanism for a DNA polymerase where A and Q are identical is

$$v = V_{max}[A][B']/[K_iAK_mB' + K_mA[B'] + K_mB'[A] \times (1 + K_mA/K_iQ) + [A][B'](1 + K_mA/K_iQ)] \quad (1)$$

At a relatively high dissociation constant (K_iQ) of the enzyme–product complex (EQ), the K_mA/K_iQ term is negligible and can be ignored. A similar situation arises if the free enzyme does not bind to product Q. Under these conditions, eq 1 can be rewritten as

$$v = V_{max}[A][B']/(K_iAK_mB' + K_mA[B'] + K_mB'[A] + [A][B']) \quad (2)$$

In the case of the $r(I)_{127}$·$Sd(C)_{28}$ template·primer system used here, the substrate A is different from the product Q, since the product is a polymer of d(C) rather than Sd(C); as shown under Results, binding to the product, a d(C) oligomer, is much weaker than binding to the primer, a Sd(C) oligomer. Therefore, the K_iQ term is ignored and eq 2 applies.

A modification of the scheme above separating the initiation of synthesis from subsequent processive synthesis also has been considered for the HIV reverse transcriptase (Majumdar et al., 1988) and is described by

$$v = V_{max}[A][B']/(K_mAK_mB' + K_mA[B] + K_mB'[A] + [A][B]) \quad (3)$$

The kinetic constants obtained by fitting the data to eq 1–3 are compared in Table I.

Assay of DNA Polymerases. The reactions with α, β, and γ DNA polymerases were carried out in a 25-μL reaction mixture for 30 min at 37 °C containing 50 mM Tris-HCl, pH 8.2, 50 mM KCl, 6 mM $MgCl_2$, 1 mM DTT 1.3 μg of activated calf thymus DNA, and 32 μM of each of dATP, dCTP, dTTP, and dGTP. [^{3}H]dATP (3000–4000 dpm/pmol) was used as tracer. The incorporated, TCA-precipitable radioactivity was measured as described above.

Membrane Filter Binding Assay for Reverse Transcriptase. We have routinely used Schleicher and Schuell 25-mm, 0.45-μm nitrocellulose membrane filters (lot nos. 903217 and 909817). The filters were soaked in wash buffer containing 50 mM Tris-HCl, pH 8.2, 6 mM $MgCl_2$, 50 mM KCl, 5 mM 2-mercaptoethanol, 5% dimethyl sulfoxide, and 2.5 μg/mL BSA. The binding reactions were carried out at 37 °C for 30 min in a total volume of 100 μL of reverse transcriptase assay mixture without the template·primer or dNTP. Fifteen picomoles of enzymes protein was used per binding mixture along with the indicated amounts of end-labeled [^{32}P]$Sd(C)_{28}$. Each reaction was diluted with 1.0 mL of buffer at 0 °C and then transferred immediately to pretreated filter disks; the tube was washed three times with ~1.5 mL of wash buffer without BSA. The filter was then washed three times, 5–6 mL each, with wash buffer without BSA and air-dried for 10 min

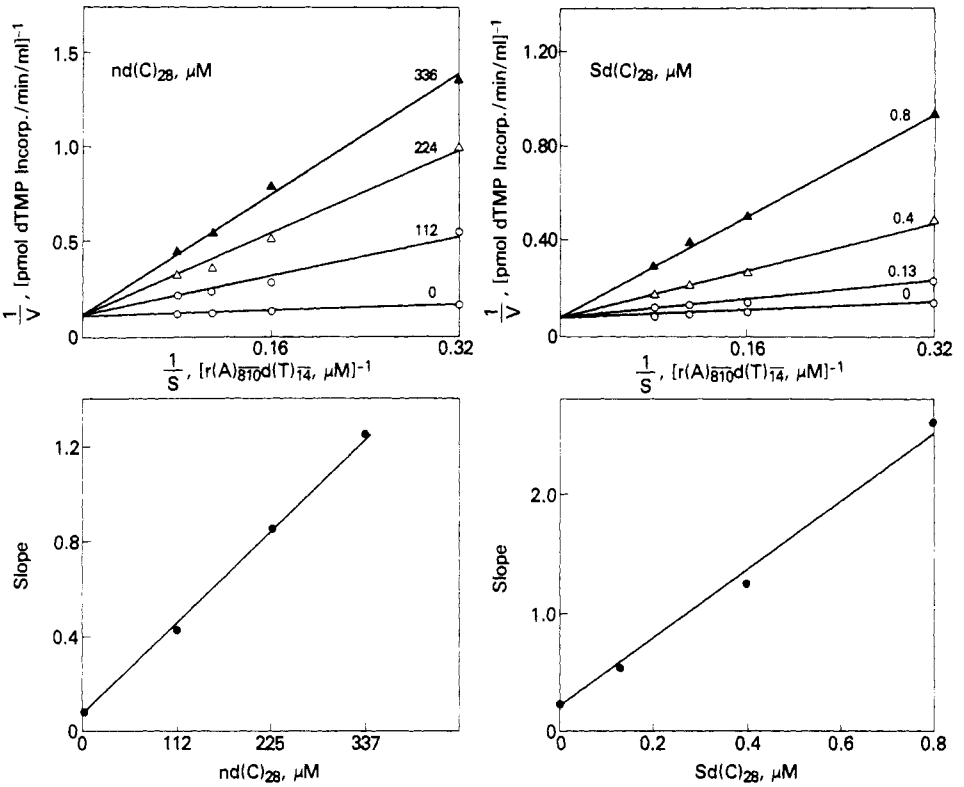


FIGURE 1: Substrate kinetics and inhibition of HIV reverse transcriptase by 28-residue-long normal and phosphorothioate oligonucleotides. The dTTP concentration was 32 μM with varying concentrations of $r(A)_{810}\cdot d(T)_{14}$. The fixed variable oligomer concentrations are indicated. Replots of primary plots are shown in the lower panels.

(complete drying not necessary). The filter-bound radioactivity was determined as above. The background radioactivity was measured in reaction mixtures without enzyme, and the background value was subtracted. The value for background was 2–4% of total input radioactivity and varied slightly with lot of filters. Bound ligand was subtracted from total ligand in each reaction to obtain the concentration of free ligand. Data were plotted according to the equation $[\text{bound}]^{-1} = (K_D/n)[\text{free}]^{-1} + n^{-1}$, where n and K_D represent independent binding sites and intrinsic binding constant, respectively.

End Labeling of Oligodeoxynucleotides. Synthetic oligodeoxynucleotide containing a 5′-OH group was 5′-end-labeled by polynucleotide kinase reaction in a final volume of 40 μL containing 5 pmol of 5′-OH, 30–35 μCi of [γ-^{32}P]ATP, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 1 mM spermidine, 1 mM EDTA, and 40 units of polynucleotide kinase. The reactions were incubated at 37 °C for 1 h. The mixture was extracted with phenol/chloroform and precipitated with 95% alcohol. The labeled oligonucleotide [(4.4–5.4) × 10^7 cpm/nmol] was dissolved in 100 μL of TE buffer (50 mM Tris-HCl, pH 7.5, 10 mM KCl, 0.005 mM EDTA). The solution was diluted 8–10 times in 10 mM KCl before addition to the reaction mixture.

Results

Substrate kinetic studies of DNA synthesis by purified HIV reverse transcriptase had suggested an ordered mechanism, with free enzyme and template·primer combining to form the first complex in the reaction pathway (see Methods). It was found also that a heterologous oligodeoxynucleotide (i.e., potential primer) is a competitive inhibitor against the template·primer, whereas heterologous polynucleotide template either failed to inhibit or showed uncompetitive inhibition against the template·primer (Majumdar et al., 1988). This suggested the importance of interaction between free enzyme and primer in the template·primer recognition process. In the current study of primer–enzyme interaction, we found that a phosphorothioate analogue of an oligodeoxynucleotide primer is a much stronger inhibitor of HIV reverse transcriptase than the oxygen-containing homologue. Analysis of inhibition by the phosphorothioate oligomer, $Sd(C)_{28}$, was conducted. DNA synthesis was measured with the template·primer system $r(A)_{810}\cdot d(T)_{14}$ with dTTP as the nucleotide substrate. Hill plot analysis of the inhibition by $Sd(C)_{28}$ revealed a simple linear pattern, and the slope of the line was ~1; this is consistent with molecular order of inhibition of 1 and with limitation of just one rate-limiting step in the steady state for overall DNA synthesis.

Kinetic Analysis of Inhibition by Phosphorothioate Oligodeoxynucleotides. To further characterize the phosphorothioate inhibition and compare K_i values, substrate kinetic studies were conducted. Fixed concentrations of either normal $d(C)_{28}$ or the phosphorothioate oligonucleotide $Sd(C)_{28}$ were used with $r(A)_{810}\cdot d(T)_{14}$ as the variable substrate; the poly[r(A)]·oligo[d(T)] replication system was used with the idea that the oligodeoxynucleotide inhibitors would not hybridize with the template or primer. In Figure 1, it is shown that double-reciprocal plots at fixed "inhibitor" concentrations are linear in the cases of both $d(C)_{28}$ and $Sd(C)_{28}$. The plots form converging patterns intersecting at the ordinate. The replot of slopes obtained with each oligodeoxynucleotide was linear also (Figure 1, lower panels). These results indicate that both oligodeoxynucleotides block enzymatic activity through competitive inhibition with the template·primer for binding to the free enzyme. The K_i value of the normal oligomer was ~200-fold higher than that of $Sd(C)_{28}$ (Table I), suggesting that the sulfur substitution strongly increases the affinity of the enzyme for the oligomer. The linear slope replot with $Sd(C)_{28}$ is consistent with the linear Hill plot described above.

The effect of phosphorothioate oligodeoxynucleotide chain length on the inhibition was examined. Using a 14-residue-

Table I: Comparison of Inhibition of HIV Reverse Transcriptase by $d(C)_{28}$ and Phosphorothioate Analogues

inhibition	K_i nucleotide ($\times 10^{-6}$ M)	K_i molecular ($\times 10^{-9}$ M)	pattern of inhibition
$d(C)_{28}$	16	560	linear competitive
$Sd(C)_{28}$	0.08	2.8	linear competitive
$5'\text{-}S_9\text{-}O_{18}\text{-}3'\text{-}d(C)_{28}$	0.70	25	linear competitive
$5'\text{-}O_9\text{-}S_9\text{-}O_9\text{-}3'\text{-}d(C)_{28}$	0.78	28	linear competitive
$5'\text{-}O_{18}\text{-}S_9\text{-}3'\text{-}d(C)_{28}$	1.46	52	linear competitive
$Sd(C)_{14}$	0.50	18	linear competitive

long oligomer, $Sd(C)_{14}$, we found that the inhibition pattern was linear competitive as with $Sd(C)_{28}$; however, the K_i value was five times higher (Table I). Thus, the decrease in chain length appears to be associated with a decrease in affinity between the enzyme and the phosphorothioate oligodeoxynucleotide. To examine the question of whether the position of the sulfur substitution in an oligodeoxynucleotide chain has an effect on the inhibition, we synthesized three 28-residue oligodeoxycytidylates in which a block of 9 phosphorothioate residues was placed either at the 5′ end, in the middle, or at the 3′ end of the chain. Results with these "block" phosphorothioates indicate that inhibition with each is linear competitive against $r(A)_{810}\cdot d(T)_{14}$, but inhibition constants were 12–25 times higher than with $Sd(C)_{28}$ (Table I); there was only a 2-fold difference in the K_i values obtained with the three "block" polymers. We conclude that the position of the 9-residue block of sulfur substitution along the 28-residue chain has no significant effect on the inhibition.

Filter Binding Assay for Interaction of $Sd(C)_{28}$ with Reverse Transcriptase. Because the K_i value for inhibition with $Sd(C)_{28}$ is exceptionally low compared with that for $d(C)_{28}$, we sought to confirm enzyme–$Sd(C)_{28}$ interaction by a thermodynamic binding assay. The nitrocellulose membrane filter binding technique has been applied to examine DNA binding of various transcription regulatory proteins, notably *lac* repressor and phage λ repressor binding to operator DNA (Winter et al., 1981; Johnsson et al., 1980), aminoacyl tRNA synthetase binding to tRNA (Yuras & Berg, 1967), and, more recently, *trp* repressor binding to wild-type and mutated operator sites (Klig & Yanofsky, 1988). Although the technique does not permit studies under true equilibrium binding conditions, dissociation constants (K_D) determined by this technique, in some instances, approach those determined by true equilibrium binding techniques. Hence, we applied the filter binding assay to obtain a K_D value for the HIV reverse transcriptase–$Sd(C)_{28}$ interaction. It was expected that if the free enzyme binds $Sd(C)_{28}$, the K_D might be similar to the K_i obtained from kinetic studies. A single-stranded DNA, such as $Sd(C)_{28}$, binds to a nitrocellulose filter in the absence of protein; however, we reasoned that such a short oligodeoxynucleotide might bind weakly enough to be removed by washing with buffer. Indeed, extensive washing of the filter reduced binding in the absence of protein to a negligible level, i.e., ~1.5% of input ^{32}P-labeled $Sd(C)_{28}$. In a preliminary experiment, 1–16 nM (molecular) $Sd(C)_{28}$ was used to determine a concentration range at which binding is predominantly saturable, rather than unsaturable due to nonspecific binding. A biphasic binding curve was observed when reciprocal plots were constructed for $[bound]^{-1}$ vs $[free]^{-1}$ $Sd(C)_{28}$. At concentrations of $Sd(C)_{28}$ higher than 10 nM, the data points were indicative of unsaturable binding. Unsaturable binding also was observed when a DNA polymerase that is not inhibited by $Sd(C)_{28}$, DNA polymerase β, was substituted

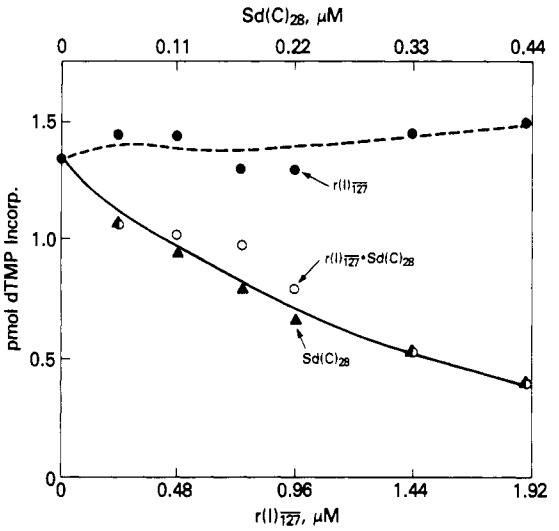


FIGURE 2: Effects of annealed and unannealed template and primer on HIV reverse transcriptase activity with heterologous template·primer $[r(A)_{810}\cdot d(T)_{14}]$. Experiments were as described under Experimental Procedures using 12 μM (nucleotide) template·primer and 32 μM dTTP. $r(I)_{127}$ and $Sd(C)_{28}$ were annealed in a 5:1 ratio.

for the HIV reverse transcriptase. With the reverse transcriptase, however, saturable binding could be observed when data points were obtained at lower oligomer concentrations; concentrations below 4 nM yielded simple linear double-reciprocal plots intersecting the ordinate, indicative of saturable binding. The dissociation constants determined from several such experiments were 3.7–4.8 nM (0.09–0.13 μM nucleotide). These K_D values are close to the K_i value determined by kinetic experiments (Table I) for the linear competitive inhibition by $Sd(C)_{28}$. The filter binding results are taken only as corroborative evidence for the K_D value obtained from the study of kinetics of inhibition.

Comparison of Inhibition of HIV Reverse Transcriptase by Annealed and Unannealed Primer or Template. The results have shown that $Sd(C)_{28}$, which is a potential primer for DNA synthesis, blocks the reverse transcriptase by linear competitive inhibition against a heterologus template·primer. Next, we examined whether unannealed $Sd(C)_{28}$ and annealed $r(I)_{127}\cdot Sd(C)_{28}$ could inhibit the enzyme similarly. This experiment was conducted to determine if the presence of template in hybridized form would alter the inhibition by $Sd(C)_{28}$. The complex $r(I)_{127}\cdot Sd(C)_{28}$ was used to study inhibition of the polymerase activity at 12 μM $r(A)_{810}\cdot d(T)_{14}$. The results (Figure 2) indicate that unannealed $r(I)_{127}$ does not inhibit, whereas the annealed template·primer inhibits strongly. This inhibition by $r(I)_{127}\cdot Sd(C)_{28}$ was very similar to that observed with $Sd(C)_{28}$ alone. As expected from the proposed HIV reverse transcriptase reaction scheme in which a dNTP does not interact with the free enzyme, dCTP did not inhibit the rate of dTMP incorporation (not shown).

Hill plot analysis of the inhibition with $r(I)_{127}\cdot Sd(C)_{28}$ revealed a linear pattern with a slope of ~1; the plot was similar to that for inhibition by $Sd(C)_{28}$ alone. The data points with the two highest levels of $r(I)_{127}\cdot Sd(C)_{28}$ did not fall on the line, however, suggesting that there is a secondary inhibitory effect at higher concentration of $r(I)_{127}\cdot Sd(C)_{28}$. It should be noted that the potential template poly[r(C)] inhibits uncompetitively in the $r(A)_{810}\cdot d(T)_{14}$ reaction system (Majumdar et al., 1988), and the secondary inhibition observed here with $r(I)_{127}\cdot Sd(C)_{28}$ could be due to this type of inhibition with higher concentrations of the template. Taken together, we conclude from these results that, with the homopolymer systems used, the template·primer recognition step by the HIV reverse tran-

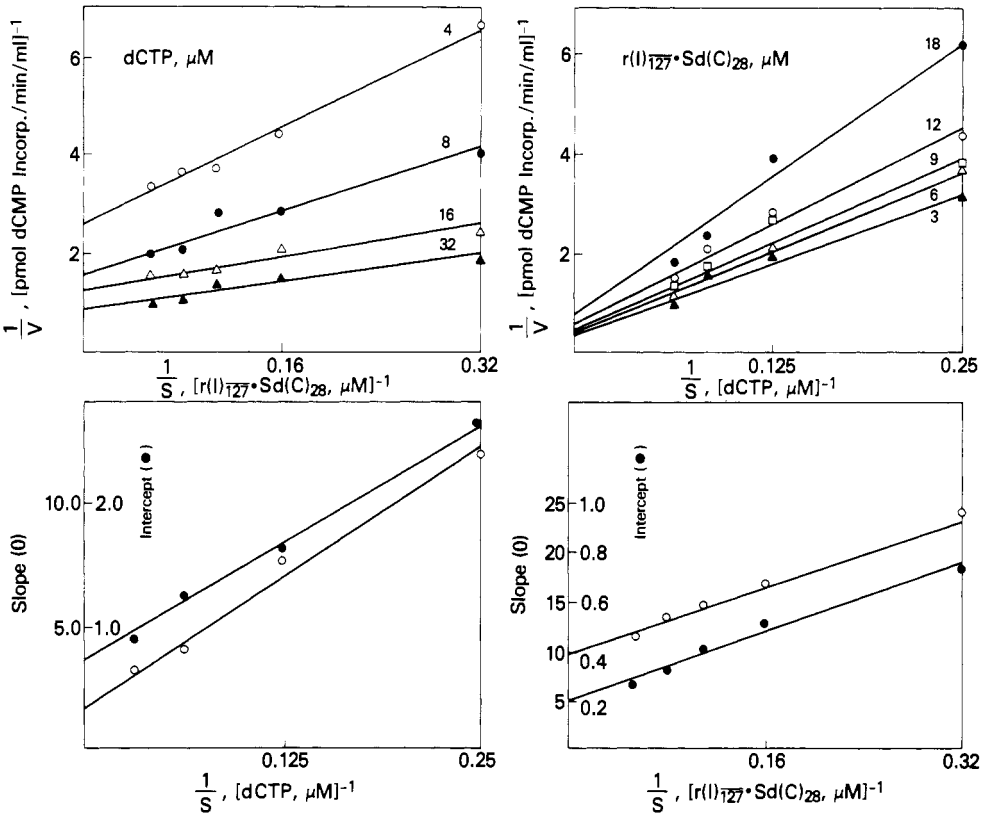


FIGURE 3: Substrate initial velocity patterns of DNA synthesis with $Sd(C)_{28}$ as primer. The concentrations of the fixed variable substrate are indicated. Double-reciprocal plots are shown in the upper panels, and replots of slopes and intercepts are shown in the lower panels. The reaction conditions were as described under Experimental Procedures.

scriptase involves formation of an initial enzyme–primer complex. The template clearly directs association of the nucleotide substrate, but the free enzyme recognizes the primer rather than the template.

Comparison of DNA Synthesis with Normal and Phosphorothioate Oligonucleotides as Primer. Since, the binding constant for the $Sd(C)_{28}$–enzyme interaction is known, it was of interest to determine if $Sd(C)_{28}$ can serve as primer for DNA synthesis. For comparison, $Sd(C)_{28}$ or $d(C)_{28}$ was tested for their ability to support dCMP incorporation with $r(I)_{127}$ a template. $r(I)_{127}$•$Sd(C)_{28}$ supported a 12 times faster rate of dCMP incorporation than $r(I)_{127}$ and the normal oligomer. No dCMP incorporation was observed in the absence of $d(C)_{28}$ or $Sd(C)_{28}$. The routine template•primer system, $r(A)_{810}$•$d(T)_{14}$, at the same template to primer ratio and concentration, supported a rate of nucleotide incorporation about 4-fold lower than that of $r(I)_{127}$•$Sd(C)_{28}$. Since the steady-state polymerization rate in the $r(A)_{810}$•$d(T)_{14}$ system is ~3 nucleotides s^{-1}, the rate in the $r(I)_{127}$•$Sd(C)_{28}$ system is approximately 12 nucleotides s^{-1}.

Substrate Kinetics. The ability of HIV reverse transcriptase to synthesize with the $r(I)_{127}$•$Sd(C)_{28}$ template•primer system provided an opportunity for study of kinetic constants of the enzyme with a replication system where the K_D for enzyme–primer binding is known. Thus, it is possible with this system to evaluate whether the rate of overall DNA synthesis is controlled by the first step, i.e., formation of the enzyme–primer complex. Reciprocal plots of results from substrate kinetic experiments yielded a series of linear converging lines with either template•primer or nucleotide as variable substrate (Figure 3). The replots of slopes and intercepts in each case were linear, and no substrate inhibition was evident. The overall kinetic patterns were similar to those obtained earlier with the poly[r(A)]•oligo[d(T)] template•primer system

Table II: Apparent Kinetic Constants for dCMP Incorporation by HIV Reverse Transcriptase with $r(I)_{127}$•$Sd(C)_{28}$ as Template•Primer

kinetic parmater[a]	eq 1	eq 2	eq 3
K_mA (template•primer)	5.8 (0.62)[b]	4.0 (0.41)	3.0 (0.21)
K_mA (primer)	0.97	0.67	0.50
K_mB'	18.3 (1.1)	15.7 (1.0)	19.2 (2.6)
K_mB			12.2 (0.83)

[a] K_mA = μM nucleotide; K_mB, K_mB' = μM dCTP; K_mA (primer) is $^1/_6K_mA$ (template•primer); 0.67 μM and 0.50 μM are equal to 23.8 nM and 17.8 nM molecular, respectively. [b] Values in parenthesis are standard error.

(Majumdar et al., 1988). Kinetic constants were obtained by using eq 1–3 (Table II). Values for constants with each equation are similar.

The values for primer K_m, 17.8 or 23.8 nM, are of particular interest. The values are 6.4- or 8.5-fold higher than the value of K_D for the enzyme–primer interaction. Based upon this, a simplified scheme of the overall reaction can be used to calculate values for k_{on} and k_{off} for the formation of the enzyme–primer complex:

$$\text{E + primer} \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} \text{E–primer} \xrightarrow{k_{cat}} \text{E + product}$$

such that $K_i = K_D = k_{-1}/k_1$ and $K_m = K_D + k_{cat}/k_1$. K_m is taken as 23.8 nM, k_{cat} is 12 s^{-1}, and K_D is 2.8 nM. Rate values are therefore calculated as

$$\text{E + primer} \underset{1.6\ s^{-1}}{\overset{5.7 \times 10^8\ M^{-1}\ s^{-1}}{\rightleftarrows}} \text{E–primer} \xrightarrow{12\ s^{-1}} \text{E + product}$$

This indicates that for the $r(I)_{127}$•$Sd(C)_{28}$ system k_{cat} is ~8-fold greater than k_{-1} and, of course, that the K_m value is dominated by the k_{cat}/k_1 term. The association rate of 5.7×10^8 M^{-1} s^{-1} is similar to values reported for several tRNA/synthetase

interactions (Pingoud et al., 1975) and is about the same as the theoretical diffusion-controlled rate of association [see, e.g., Fersht (1985)]; the value also is similar to values reported recently for the interaction of *Escherichia coli* RNA polymerase and various TAC promoters (Mulligan et al., 1985) and λ promoter (Hawley et al., 1985).

Discussion

Previous studies of polymerization by HIV reverse transcriptase suggested that the enzyme mechanism is order, with template·primer adding to the enzyme before the first nucleoside 5′-triphosphate. In the present study, we extended this idea by showing that the enzyme binds a model primer as tightly as it binds a complex of the same primer annealed to template, and conversely, free template is not bound. Hence, our interpretation is that the initial step in the template·primer recognition process is primer binding to the free enzyme.

A phosphorothioate oligodeoxynucleotide, $Sd(C)_{28}$, was found to bind to the enzyme with exceptionally high affinity and to competitively inhibit synthesis directed by a heterologous template·primer. The K_i value for inhibition by $Sd(C)_{28}$ was ~200-fold lower than the K_i value with an oxygen-containing oligomer of the same base and chain length. Exceptionally tight binding of $Sd(C)_{28}$ to the enzyme could be due to the ability of the phosphorothioate groups to form hydrophobic interactions with the enzyme. This is reasonable because the van der Waals attractive potential for sulfur is 17-fold greater than that for oxygen, and since van der Waals energies are additive, summation of energies over the surface of the entire primer binding grove could contribute a large difference to the free energy of binding.

The observation of a relatively high steady-state polymerization rate (k_{cat}) with the template·primer system containing a phosphorothioate primer was somewhat surprising. This is because tighter enzyme–substrate binding is usually associated with a diminution in k_{cat}. The explanation for this paradoxical result may be related to enzyme processivity. Thus, most of the dNMP incorporation and termination events are probably occurring during processive synthesis and, hence, are distal to the phosphorothioate primer molecule. Nevertheless, study of reverse transcriptase processivity with the $r(I)_{127}$·$Sd(C)_{28}$ template·primer system should be interesting.

The substrate kinetic studies with $Sd(C)_{28}$ as primer indicated that the K_m for primer is greater than the K_D for enzyme–primer binding. This observation indicates that the K_m value (i.e., the dissociation constant for all enzyme complexes) is dominated by dissociation of a complex other than the initial enzyme–primer complex and permits the calculation of the rate for enzyme–primer interaction using the Briggs–Haldane kinetics relationship $K_m = K_D + k_{cat}/k_{on}$ and $k_{off} = K_D k_{on}$. The values, respectively, are K_m = 23.8 nM, K_D = 2.8 nM, k_{cat} = 12 s^{-1}, $k_{on} = 5.7 \times 10^8$ M^{-1} s^{-1}, and k_{off} = 1.6 s^{-1}. The assumption in this calculation is that enzyme intermediates in the reaction scheme subsequent to the initial enzyme–primer complex do not accumulate. This assumption appears reasonable. Accumulation of such intermediates would lower the K_m value by reducing the K_D term in the equation above. Since this term is relatively low in the present case, the effect of intermediates could only be minimal. A diagnostic feature of strict Briggs–Haldane kinetics, where intermediates do not accumulate, is that k_{cat}/K_m approaches k_{on} (Fersht, 1985); this, indeed, is the case in the present experiment with the phosphorothioate primer. Finally, it also is reasonable to assume that k_{on} and k_{off} are not themselves influenced by dNTP because the filter binding derived K_D and the K_i (equivalent to K_D in this case) determined from kinetic experiments in the presence of dNTPs were similar.

In studies of *E. coli* Pol I, McClure and Jovin (1975) reported a K_D for the polymerase–poly[d(A-T)] interaction of 5 nM, and in recent studies of Pol I large fragment, Kuchta et al., (1987) reported a K_D value of 5 nM for the interaction with a short heteropolymer oligonucleotide template·primer. Thus, these values are similar to the K_D value found here. The latter workers also reported k_{off} and k_{on} values of 0.06 s^{-1} and 1×10^7 M^{-1} s^{-1}, respectively; this value for k_{on} is about 50-fold lower than the value derived here for the HIV reverse transcriptase–primer interaction. The explanation for this difference is not clear. k_{on} values in the range of 5×10^8 M^{-1} s^{-1} have now been found for a variety of nucleic acid enzymes, and the value is about the same as the theoretical diffusion-controlled rate of association. A lower value for Pol I large fragment–template·primer interaction could reflect the presence of an intermediate step(s) in the enzyme–template·primer recognition process for that enzyme.

Earlier, in studies with the routine reverse transcriptase template·primer system, $r(A)_{810}$·$d(T)_{14}$, a primer K_m of 150 nM molecular had been observed (Majumdar et al., 1988). The k_{cat} for this replication system is ~3 s^{-1}. It is reasonable to assume that the k_{on} value is about the same as the value found here for the $r(I)_{127}$·$Sd(C)_{28}$ system, i.e., 5.7×10^8 M^{-1} s^{-1}. Thus, we can calculate the k_{cat}/k_{on} term as 5 nM, K_D as 145 nM, and k_{off} as 87 s^{-1}. Therefore, the primer K_m in this routine system is dominated by the dissociation of the initial enzyme–substrate complex, as in "usual" Michaelis–Menten kinetics. In other words, dissociation of complexes occurring after the initial enzyme–primer complex is much slower than dissociation of the initial complex.

The concept of an interaction between a reverse transcriptase and a primer is well-known for avian reverse transcriptase and its natural primer, Trp-tRNA (Panet et al., 1975; Haseltine et al., 1977; Sawyer & Hanafusa, 1979; Peters & Hu, 1980). This enzyme is associated with Trp-tRNA in the virion, and in addition, the purified enzyme forms a tight complex with Trp-tRNA, even in the absence of AMV (template) RNA (Panet & Berliner, 1978; Haseltine et al., 1977). Likewise, MuLV reverse transcriptase in the virion is associated with its natural primer tRNA, Pro-tRNA. This association is found in mutant virions lacking template RNA, and hence, it appears that the association is enzyme directed, at least in part, rather than completely template directed (Levin & Seidman, 1979, 1981). With purified MuLV reverse transcriptase, however, no specific in vitro complex could be isolated with Pro-tRNA (Haseltine et al., 1977; Panet & Berliner, 1978).

Finally, various phosphorothioate oligonucleotides are being evaluated as potential antiviral agents for the acquired immunodeficiency syndrome (Yarchoan & Broder, 1987; Matsukura et al., 1987). Antiviral effects of phosphorothioate oligodeoxynucleotides have been found and can be placed in two general categories: oligonucleotide sequence dependent and sequence independent (Matsukura et al., 1987). A proposed mechanism for sequence-dependent antiviral activity involves "antisense" hybridization to viral mRNA, whereas the mechanism of the sequence-independent antiviral activity has been obscure. On the basis of the findings described here, a plausible mechanism for the sequence independent activity could be reverse transcriptase–phosphorothioate oligonucleotide interaction.

With the idea in mind of phosphorothioate oligonucleotides as antiviral agents, we evaluated the effect of $Sd(C)_{28}$ on the activity of various DNA polymerases. The results (Table III) show that cellular α- and γ-polymerases, as well MuLV reverse

Table III: Effect of $Sd(C)_{28}$ on Activities of DNA Polymerases

enzyme	0.67 μM (nucleotide) $Sd(C)_{28}$	pmol of dTMP incorporated
HIV reverse transcriptase	−	1.5
	+	0.2
MuLV reverse transcriptase	−	29.0
	+	1.5
α-polymerase	−	2.7
	+	0.8
β-polymerase	−	6.6
	+	6.9
γ-polymerase	−	0.7
	+	0.1
DNA polymerase I (large fragment)	−	11.4
	+	11.4
DNA polymerase I	−	10.1
	+	9.2

transcriptase, are sensitive to the phosphorothioate oligomer. The K_i values are about the same as that observed for HIV reverse transcriptase (not shown). In contrast, DNA polymerase β and Pol I are not inhibited by the agent. These results indicate that two of the normal cellular DNA polymerases are sensitive to phosphorothioates in vitro. Therefore, the phosphorothioate oligonucleotides are, on this basis, potentially toxic for the host cell, although such toxicity does not appear to be strong (Matsukura et al., 1987).

Registry No. $Sd(C)_{28}$, 115401-96-2; $d(C)_{28}$, 25609-92-1; dCMP, 1032-65-1; reverse transcriptase, 9068-38-6.

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