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## Accelerated Publications

# Inhibition of the RNase H Activity of HIV Reverse Transcriptase by Azidothymidylate†

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ABSTRACT: The effects of AZTMP and other nucleoside 5′-monophosphates on the RNA-dependent DNA polymerase and RNase H activities of a recombinant HIV reverse transcriptase have been investigated. Both activities are sensitive to inhibition by millimolar concentrations of AZTMP with $MgCl_2$ as divalent cation activator. Substitution of $Mn^{2+}$ for $Mg^{2+}$ markedly potentiates the inhibition of RNase H activity by AZTMP, reducing the $IC_{50}$ from 5 to 0.05 mM. In contrast, $Mn^{2+}$ does not alter the sensitivity of the RNA-dependent DNA polymerase activity to inhibition by AZTMP. The inhibition of RNase H activity by AZTMP can be reversed by increasing concentrations of the substrate poly(A)/poly(dT), suggesting that AZTMP may compete with the substrate for binding at the active site of RNase H. Other nucleoside 5′-monophosphates do not inhibit RNase H in the presence of $Mg^{2+}$. However, in the presence of $Mn^{2+}$, deoxy- and dideoxynucleoside 5′-monophosphates that are complementary to the DNA strand of the heteroduplex substrate are somewhat inhibitory. The RNA-dependent DNA polymerase activity is a slightly inhibited by AZTMP and ddTMP in either $Mg^{2+}$ or $Mn^{2+}$, and substitution of $Mn^{2+}$ for $Mg^{2+}$ results in inhibition by ddAMP as well. Naturally occurring ribo- or deoxyribonucleoside 5′-monophosphates are not inhibitory at concentrations up to 5 mM. Since AZTTP inhibits the RNA-dependent DNA polymerase activity of HIV reverse transcriptase at nanomolar concentrations, it is unlikely that the inhibition of this activity by AZTMP plays a significant role in the antiviral effect of AZT. However, the inhibition of the RNase H activity by AZTMP, which can reach millimolar concentrations in vivo, may account for part of the sensitivity of the virus to AZT.

The thymidine analogue 3′-azido-3′-deoxythymidine (AZT)[1] inhibits the replication of human immunodeficiency virus (HIV) in vitro (Mitsuya et al., 1985) and has been found to be effective in the treatment of acquired immunodeficiency syndrome (AIDS) (Yarchoan et al., 1986; Fischl et al., 1987). The mechanism by which AZT inhibits viral replication is thought to involve the intracellular metabolism of the nucleoside analogue to its triphosphate derivative via the action of thymidine kinase, thymidylate kinase, and nucleoside diphosphate kinase (Yarchoan et al., 1989; Mitsuya & Broder, 1987). The triphosphate derivative of AZT, AZTTP, is a potent inhibitor of the RNA-dependent DNA polymerase activity of the viral reverse transcriptase, while the DNA polymerases of the host cell are relatively insensitive to this inhibitor (Furman et al., 1986). AZTTP competes well with dTTP for binding at the active site of HIV reverse transcriptase and also functions as an alternative substrate (Furman et al., 1986; Vrang et at., 1987; Cheng et al., 1987; St. Clair et al., 1987). Incorporation of AZTMP results in chain termination and inactivation of the template/primer.

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[1] Abbreviations: HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; BSA, bovine serum albumin; AZT, AZTMP, and AZTTP, 3′-azido-3′-deoxythymidine and its monophosphate and triphosphate derivatives; ddAMP, 2′,3′-dideoxyadenosine 5′-monophosphate; ddTMP, 2′,3′-dideoxythymidine 5′-monophosphate.

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Studies on the metabolism of AZT in both uninfected and HIV-infected cells have shown that whereas the phosphorylation of AZT to the monophosphate by thymidine kinase is efficient, AZTMP is only very slowly converted to AZTDP by thymidylate kinase (Furman et al., 1986; Furman & Barry, 1988). Thus, the intracellular concentration of AZTMP is 2–3 orders of magnitude higher than that of AZTTP. In view of the fact that AZTMP accumulates to millimolar concentrations intracellularly, the present studies were undertaken to determine the effects of AZTMP as well as other nucleoside 5′-monophosphates on the RNase H and RNA-dependent DNA polymerase activities of a recombinant HIV reverse transcriptase.

## Materials and Methods

*Materials.* Ribo- and deoxynucleoside 5′-monophosphates and AZT were obtained from Sigma Chemical Co. Poly(A), poly(dT), and dTTP were purchased from Pharmacia LKB Biotechnology Inc., and $(dT)_{12-18}$ was from Midland Certified Reagents. [$^3$H]Poly(A), 300 cpm/pmol of AMP, was from Amersham, and [$^3$H]dTTP was from New England Nuclear.

*Enzyme Preparation. Escherichia coli* expressing a cloned HIV reverse transcriptase (Deibel et al., 1990), was a gift of Dr. W. Gary Tarpley of the Upjohn Co. The recombinant reverse transcriptase was purified as previously described and is a heterodimer of 66- and 51-kDa polypeptides (Tan et al., 1991). To ensure that there is no contamination with *E. coli* RNase H ($M_r$ 17 500) (Berkower et al., 1973; Kanaya and Crouch, 1983), or any other nucleases, we have further purified the reverse transcriptase ($M_r$ 120 000) by gel filtration on Superose 12 in the presence of 0.5 M KCl and 0.1% Nonidet P-40. Both RNA-dependent DNA polymerase activity and RNase H activity coeluted at 120 kDa on gel filtration, and aliquots across the peak of activity when resolved on an SDS–14% polyacrylamide gel followed by silver staining again showed only polypeptides of 66 and 51 kDa. More importantly, the ratio of polymerase activity to RNase H activity was constant across the peak (data not shown). The purified enzyme had a specific activity of approximately 12 units/pmol. One unit is defined as 1 nmol of dNMP incorporated/h under standard assay conditions.

*Preparation of 3′-Azido-3′-deoxythymidine 5′-Phosphate (AZTMP).* The synthesis of AZTMP was achieved by employing a general method for nucleoside phosphorylation first described by Yoshikawa et al. (1967). Briefly, AZT (0.88 mmol) was dissolved in triethyl phosphate (5 mL), cooled in an ice bath to 0 °C, and reacted with phosphoryl chloride (3.75 mmol). The reaction mixture was allowed to come to ambient temperature, and stirring was continued for 16 h. The reaction mixture was poured into ice water (20 mL), the pH of the solution was adjusted to 6.2 with $NaHCO_3$, the mixture was extracted with dichloromethane (3 × 10 mL), and the aqueous phase, concentrated to 5 mL, was charged on a Dowex-1 ($HCOO^-$) column, the column was washed with $H_2O$ until no more UV-absorbing material was eluted to remove any unreacted AZT or thymine (which may be formed due to the decomposition of AZT), and the product was eluted with a gradient of 0.5–4.0 M formic acid. Product-containing fractions were pooled, evaporated, and coevaporated with ethanol to dryness. The residue was dissolved in $H_2O$ (4 mL) and lyophilized to obtain 209 mg (65% yield) of chromatographically pure AZTMP: UV $\lambda_{max}$ ($H_2O$) 267 nm ($\epsilon$ 9.6 × $10^3$). The purity of the product was confirmed by thin-layer chromatography (cellulose-coated plastic plates developed in 2-propanol/$NH_4OH$/$H_2O$, 11:7:2 v/v/v; $R_f$ AZT 0.9 and AZTMP 0.7) and HPLC (partsil 10 SAX column eluted in isocratic mode by using 0.005 M $NH_4H_2PO_4$ buffer, pH 2.8, containing 10% 0.75 M $NH_4H_2PO_4$ buffer, pH 3.5, at 1.5 mL/min flow rate; retention times 3.04, 4.07, and 5.00 min for thymine, AZT, and AZTMP, respectively). Both of the chromatographic procedures showed the product to be completely pure.

*RNA-Dependent DNA Polymerase Assay.* Reverse transcriptase activity was assayed essentially as previously described (Tan et al., 1991). The standard reaction mixture contained, in a final volume of 100 μL, 40 mM Tris-HCl, pH 8.3, 2 mM $MgCl_2$, 150 mM KCl, 1 mM DTT, 2.5% glycerol, 0.025% Nonidet P-40, 0.5 mM EGTA, 8 μg of BSA, 20 μM [$^3$H]dTTP (1200 cpm/pmol), 30 μM poly(A)/oligo(dT) (3:1 in nucleotide equivalents), and 0.02–0.03 pmol of HIV reverse transcriptase. After incubation for 15 min at 37 °C, the reaction was stopped by the addition of cold 5% trichloroacetic acid containing 10 mM sodium pyrophosphate. The precipitate was collected, washed, and counted as previously described (Byrnes et al., 1976). When $MnCl_2$ was present as divalent cation, the reaction mixture was the same except that 0.6 mM $MnCl_2$ replaced 2 mM $MgCl_2$ and the enzyme concentration was increased by a factor of 10.

*RNase H Assay.* The reaction mixture contained, in a final volume of 50 μL, 40 mM Tris-HCl, pH 8.5, 16 mM $MgCl_2$ or 4 mM $MnCl_2$, 4 μg of BSA, 3% glycerol, 0.02% Nonidet P-40, 1.25 μg/mL [$^3$H]poly(A)/poly(dT) (1:1), 58 000 cpm, and 1 pmol of HIV reverse transcriptase. After incubation at 37 °C for 20 min, reactions were stopped by the addition of 150 μL of cold 10% trichloroacetic acid, and 10 μg of salmon sperm DNA was added as carrier. Following centrifugation at 25000*g* for 12 min, 75 μL of the supernatant was added to 10 mL of Biofluor scintillant and counted.

## Results

*Inhibition of the RNase H Activity of HIV Reverse Transcriptase by Nucleoside 5′-Monophosphates.* The effects of ribonuceloside 5′-monophosphates (rNMP), deoxyribonucleoside 5′-monophosphates (dNMP), dideoxyribonucleoside 5′-monophosphates, and azidothymidylate (AZTMP) on the RNase H activity of recombinant HIV reverse transcriptase with [$^3$H]poly(A)/poly(dT) as substrate are shown in Table I. In the presence of $Mg^{2+}$ as divalent cation activator, AZTMP slightly inhibited the hydrolysis of the poly(A) strand of the DNA/RNA hybrid (33% at 5 mM), whereas ribo- and deoxyribonucleoside 5′-monophosphates had little or no effect on RNase H activity. However, with $Mn^{2+}$ as divalent cation activator, RNase H became very sensitive to inhibition by AZTMP, being inhibited 91% at 2 mM. Thus the $IC_{50}$ for AZTMP decreased from approximately 5 mM in $Mg^{2+}$ (Table I) to 50 μM in $Mn^{2+}$ (Figure 1). $Mn^{2+}$ also increased the sensitivity of RNase H to inhibition by dAMP and to a lesser degree ddAMP, the deoxyribonucleotides that are capable of base pairing with the DNA strand of the poly(A)/poly(dT) hybrid. However, substitution of $Mn^{2+}$ for $Mg^{2+}$ did not affect the sensitivity of the enzyme to rNMPs, dNMPs, or ddTMP.

To determine the relative sensitivity of RNase H to AZTMP, dAMP, dTMP, ddAMP, and ddTMP, the effects of increasing concentrations of these nucleotides on RNase H activity with $Mn^{2+}$ as divalent cation activator were determined (Figure 1). RNase H activity was most sensitive to inhibition by AZTMP, being inhibited 50% at a concentration of 0.05 mM, whereas a similar degree of inhibition required approximately 20-fold higher dAMP and 60-fold higher ddAMP concentrations.

*Inhibition of RNase H by AZTMP Can Be Reversed by Higher Substrate Concentrations.* To determine the mecha-

Table I: Inhibition of the RNase H Activity of HIV Reverse Transcriptase by Nucleoside 5'-Monophosphates[a]

| nucleotide added | inhibition of RNase H activity (%) | |
|---|---|---|
| | $MgCl_2$ | $MnCl_2$ |
| AMP | 9 | 2 |
| UMP | 0 | 2 |
| GMP | 7 | 5 |
| CMP | 0 | 14 |
| dAMP | 6 | 80 |
| dTMP | 7 | 11 |
| dGMP | 8 | 8 |
| dCMP | 4 | 8 |
| AZTMP | 33 | 91 |
| ddAMP | 2 | 45 |
| ddTMP | 1 | 2 |

[a] RNase H was assayed as described under Materials and Methods. Activity in the absence of any nucleotides was 25 pmol of [$^3$H]AMP released in 15 min at 37 °C with $Mg^{2+}$ as divalent cation and 30 pmol/15 min with $Mn^{2+}$. The concentration of nucleotide was 5 mM in the presence of $Mg^{2+}$ and 2 mM in the presence of $Mn^{2+}$. All assays were performed in duplicate, and the data represent the average of at least two separate experiments.

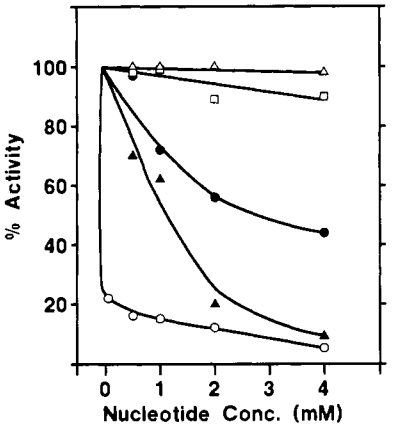


FIGURE 1: Effects of nucleoside 5'-monophosphate concentraation on RNase H activity. RNase H activity was assayed as described under Materials and Methods with $Mn^{2+}$ as divalent cation. The concentrations of ddTMP (△), dTMP (□), ddAMP (●), dAMP (▲), and AZTMP (○) were varied as indicated.

nism of inhibition of RNase H by AZTMP, we have investigated whether the inhibition can be relieved by higher substrate concentrations. The effects of increasing concentrations of AZTMP on RNase H activity at three different concentrations of [$^3$H]poly(A)/poly(dT) are shown in Figure 2. RNase H activity is significantly more sensitive to inhibition by AZTMP at low than at high substrate concentrations; 20 μM AZTMP inhibited RNase H activity 80% at 0.3 μg/mL poly(A)/poly(dT) and 60% at 0.6 μg/mL, and no inhibition was observed at 3.75 μg/mL substrate. The observation that the inhibition of RNase H activity can be reversed by increasing the concentration of the substrate suggests that AZTMP may inhibit RNase H activity by competing with the substrate for binding at the active site of the enzyme. However, more rigorous kinetic experiments under steady-state conditions are required to establish whether such is the case.

*Effects of AZTMP on the RNA-Dependent DNA Polymerase Activity of HIV Reverse Transcriptase.* The effects of nucleoside 5'-monophosphates on the DNA polymerase activity of HIV reverse transcriptase with poly(A)/oligo(dT) as template/primer is shown in Table II. With $Mg^{2+}$ as divalent cation activator, the naturally occurring ribo- and deoxyribonucleoside 5'-monophosphates had little or no effect on the DNA polymerase activity; however, AZTMP and ddTMP

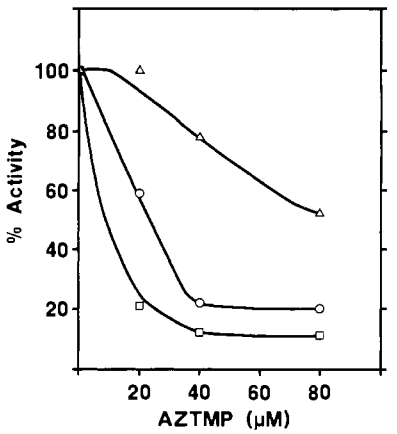


FIGURE 2: Effect of substrate concentration on the inhibition of RNase H activity by AZTMP. The assay conditions were as described under Materials and Methods with $MnCl_2$ as divalent cation. The concentration of AZTMP was varied as indicated: 0.3 μg/mL (□), 0.6 μg/mL (○), and 3.75 μg/mL (△) [$^3$H]poly(A)/poly(dT).

Table II: Inhibition of the DNA Polymerase Activity of HIV Reverse Transcriptase by Nucleoside 5'-Monophosphates[a]

| nucleotide added (5 mM) | inhibition of DNA polymerase activity (%) | |
|---|---|---|
| | $MgCl_2$ | $MnCl_2$ |
| AMP | 13 | 0 |
| UMP | 0 | 0 |
| GMP | 0 | 13 |
| CMP | 0 | 0 |
| dAMP | 8 | 4 |
| dTMP | 6 | 0 |
| dGMP | 9 | 0 |
| dCMP | 17 | 3 |
| AZTMP | 50 | 46 |
| ddAMP | 3 | 33 |
| ddTMP | 40 | 52 |

[a] RNA-dependent DNA polymerase was assayed as described under Materials and Methods. Control activity in the absence of any nucleotide was approximately 60 pmol of [$^3$H]dTMP incorporated in 15 min at 37 °C with $Mg^{2+}$ as divalent cation and 75 pmol/15 min with $Mn^{2+}$. All assays were performed in duplicate, and the data represent the average of at least two separate experiments.

were slightly inhibitory. In contrast to the results obtained with RNase H activity, the substitution of $Mn^{2+}$ for $Mg^{2+}$ did not increase the sensitivity of the DNA polymerase activity to inhibition by AZTMP and ddTMP; however, in the presence of $Mn^{2+}$ the DNA polymerase activity became somewhat sensitive to inhibition by ddAMP.

Kinetic analysis of the mechanism of inhibition of the RNA-dependent DNA polymerase activity by AZTMP revealed that the pattern is that of mixed noncompetitive inhibition in which both the maximal velocity and the Michaelis constant are affected (data not shown). This suggests that although AZTMP does not compete with dTTP for binding at the active site of the DNA polymerase activity, binding of AZTMP to the enzyme, possibly at the RNase H site, interferes with the binding of dTTP. The $K_i$ was determined to be 3 mM.

## Discussion

The present results demonstrate that the RNase H activity of HIV reverse transcriptase can be inhibited by millimolar concentrations of AZTMP when $Mg^{2+}$ is the divalent cation activator and that substitution of $Mn^{2+}$ for $Mg^{2+}$ decreases the $IC_{50}$ from approximately 5 mM to 50 μM. Inhibition of RNase H by AZTMP can be reversed by increasing concentrations of the substrate poly(A)/poly(dT), suggesting that

AZTMP may compete with the substrate for binding at the active site of RNase H. Naturally occurring ribo- and deoxyribonucleoside 5′-monophosphates have little or no effect on the RNase H activity in the presence of $Mg^{2+}$, but dAMP was somewhat inhibitory in the presence of $Mn^{2+}$, as was ddAMP.

The reason for the potentiating effect of $Mn^{2+}$ is unclear; however, a similar potentiating effect of $Mn^{2+}$ on the inhibition of the 3′ to 5′ exonuclease activity of *E. coli* DNA polymerase I by nucleoside 5′-monophosphates has also been observed (El-Deiry et al., 1984). In contrast to the inhibition of 3′ to 5′ exonuclease activities of *E. coli* DNA polymerase I and mammalian DNA polymerase $\delta$ by nucleoside 5′-monophosphates, which does not show significant base or sugar specificity (Que et al., 1978), the RNase H activity of HIV reverse transcriptase is inhibited only by 2′-deoxyribonucleoside 5′-monophosphates and preferentially those that are complementary to the DNA strand of the DNA/RNA hybrid substrate, suggesting that base-pairing between the nucleotide and the substrate is occurring. The dNMPs that are complementary to the RNA strand of the hybrid substrate, dTMP and ddTMP, are ineffective in inhibiting RNase H activity in either $Mg^{2+}$ or $Mn^{2+}$. Substitution of the 3′-hydroxyl group of dTMP by an azido group markedly increases the potency of inhibition, suggesting that the 3′-azido derivative of dAMP may be an even more potent inhibitor of RNase H than is AZTMP.

Recent studies have shown that the RNase H of HIV reverse transcriptase has both endonuclease and 3′ to 5′ exonuclease activities (Schatz et al., 1990) and that the reaction products consist of mono-, di-, and trinucleotides (Starnes & Cheng, 1989). It is not clear whether the inhibition of RNase H activity by AZTMP is due to the inhibition of the exonuclease or the endonuclease activity or both. However, the observation that RNase H can be inhibited by deoxynucleoside 5′-monophosphates and their analogues provides a structural basis for designing specific inhibitors of this enzymatic activity. In addition, since RNase H activity is essential for viral replication, it provides an important target for anti-HIV therapy.

The RNA-dependent DNA polymerase activity of HIV reverse transcriptase is also inhibited by millimolar concentrations of AZTMP with $Mg^{2+}$ as divalent cation activator, but in contrast to the RNase H activity, the sensitivity of the DNA polymerase activity to AZTMP is not potentiated by $Mn^{2+}$. The DNA polymerase activity is not inhibited by naturally occurring ribo- and deoxyribonucleoside 5′-monophosphates in either $Mg^{2+}$ or $Mn^{2+}$, but it is somewhat inhibited by ddTMP in both $Mg^{2+}$ and $Mn^{2+}$ and by ddAMP in $Mn^{2+}$.

Since AZTTP inhibits the DNA polymerase activity at nanomolar concentrations whereas AZTMP inhibits the DNA polymerase activity at millimolar concentrations, it is unlikely that inhibition of the DNA polymerase activity by the monophosphate derivative of AZT plays a significant role in the antiviral effects of this drug. However, the inhibition of RNase H activity of the enzyme by AZTMP could possibly play a role in inhibiting viral replication. Although inhibition by AZTMP requires millimolar concentrations, these levels are attainable intracellularly, due to the ability of thymidine kinase to efficiently convert AZT to the monophosphate and the low efficiency of conversion of AZTMP to the di- and triphosphate derivatives (Furman et al., 1986; Furman & Barry, 1988). Thus, it is possible that the effectiveness of AZT in vivo is the result of the inhibitory effects of both the triphosphate and the monophosphate derivatives, i.e., inhibition of the DNA polymerase by AZTTP and inhibition of the RNase H by AZTMP. Consistent with this suggestion are the recent observations that the reverse transcriptase isolated from HIV strains resistant to AZT exhibited the same degree of sensitivity to AZTTP as the corresponding enzyme obtained from AZT-sensitive strains (Larder & Kemp, 1989; Larder et al., 1989a) and that infectious viruses that carry a *pol* gene encoding AZT-resistant reverse transcriptase induced by site-directed mutagenesis showed hypersensitivity to AZT when tested in culture (Larder et al., 1987, 1989b). We are currently investigating the possibility that the sensitivity of RNase H to AZTMP is altered in reverse transcriptase isolated from AZT-resistant variants.

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## *Articles*

# Kinetic Mechanism of DNA Polymerase I (Klenow Fragment): Identification of a Second Conformational Change and Evaluation of the Internal Equilibrium Constant†,‡

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ABSTRACT: In a previously determined minimal kinetic scheme for DNA polymerization catalyzed by the Klenow fragment (KF) of *Escherichia coli* DNA polymerase I, a nonchemical step that interconverted the KF′·DNA$_{n+1}$·PP$_i$ and KF·DNA$_{n+1}$·PP$_i$ complexes was not observed in correct incorporation [Kuchta, R. D., Mizrahi, V., Benkovic, P. A., Johnson, K. A., & Benkovic, S. J. (1987) *Biochemistry 26*, 8410–8417] but was detected in misincorporation [Kuchta, R. D., Benkovic, P. A., & Benkovic, S. J. (1988) *Biochemistry 27*, 6716–6725]. In a pulse–chase experiment in this study, a burst amplitude of 100% of the enzyme concentration is observed; under pulse–quench conditions, the burst amplitude is 80%, indicative of the accumulation of the KF′·DNA·dNTP species owing to a slow step subsequent to chemical bond formation. This latter step was unequivocally identified by single-turnover pyrophosphorolysis and pyrophosphate-exchange experiments as one interconverting KF′·DNA$_{n+1}$·PP$_i$ and KF·DNA$_{n+1}$·PP$_i$. The rate constants for this step in both directions were established through the rate constants for processive synthesis and pyrophosphorolysis. Pyrophosphorolysis of a 3′-phosphorothioate DNA duplex confirmed that the large elemental effect observed previously [Mizrahi, V., Henrie, R. N., Marlier, J. F., Johnson, K. A., & Benkovic, S. J. (1985) *Biochemistry 24*, 4010–4018] in this direction but not in polymerization is due to a marked decrease in the affinity of KF for the phosphorothioate-substituted duplex and not to the chemical step. The combination of the experimentally measured equilibrium constant for the bound KF·DNA species with the collective kinetic measurements further extends previous insights into the dynamics of the polymerization process catalyzed by KF.

The Klenow fragment (KF)[1] of *Escherichia coli* DNA polymerase I contains two domains that reside on a single polypeptide chain. Unlike pol I, which has a 5′ → 3′ and a 3′ → 5′ exonuclease as well as a polymerase activity, KF has been shown to exhibit only the 3′ → 5′ exonuclease and polymerase activities (Kornberg, 1980). The polymerase activity, which resides on the larger of the two domains, has been the object of intense investigation including stereochemical (Burgers & Eckstein, 1979; Brody & Frey, 1981; Gupta & Benkovic, 1984), structural (Ollis et al., 1985; Freemont et al., 1988), and kinetic analyses (McClure & Jovin, 1975; Bambara et al., 1976; Bryant et al., 1983; Mizrahi et al., 1985, 1986a; Kuchta et al., 1987, 1988). The previously proposed kinetic pathway by which a nucleotide is correctly incorporated (Kuchta et al., 1987) involves the ordered binding of DNA then dNTP followed by a slow nonchemical event that limits the rate for a single incorporation event. Phosphodiester bond formation then occurs, which is followed by PP$_i$ release. KF then partitions between additional nucleotide incorporation or dissociation from the DNA.[2]

KF presents an ideal model (Carroll & Benkovic, 1990) with which to study the low error frequency in the synthesis of DNA owing to its relatively slow rate of polymerization compared to the bacteriophage polymerases, small size (approximately 68 000 daltons), and lack of accessory proteins. The fidelity with which pol I replicates a template strand has been estimated to be as low as $10^{-8}$ to $10^{-12}$ errors per nucleotide incorporated (Englisch et al., 1985). Recently, the kinetic mechanism for incorrect nucleotide addition to a duplex by KF has been elucidated (Kuchta et al., 1988). The mechanism

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‡ In honor of Dr. Robert Abeles on the occasion of his 65th birthday. We wish him many more.

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[1] Abbreviations: Pol I, *E. coli* DNA polymerase I; KF, Klenow fragment of pol I; KF(exo$^-$), (Asp355Ala, Glu357Ala) KF mutant devoid of any exonuclease activity; dNTP, deoxynucleoside 5′-triphosphate; PP$_i$, inorganic pyrophosphate; TEAB, triethylammonium bicarbonate; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride salt; EDTA, ethylenediaminetetraacetate sodium salt.

[2] It is gratifying to note that the principal kinetic features originally described for the polymerization of DNA catalyzed by KF also apply to T7 DNA polymerase (Patel et al., 1991).

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