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Human Immunodeficiency Virus Type 1 Reverse Transcriptase

3′-AZIDODEOXYTHYMIDINE 5′-TRIPHOSPHATE INHIBITION INDICATES TWO-STEP BINDING FOR TEMPLATE-PRIMER*

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Madhuri Jaju, William A. Beard, and Samuel H. Wilson‡

From the Sealy Center for Molecular Science, University of Texas Medical Branch, Galveston, Texas 77555-1068 and Laboratory of Biochemistry, NCI, National Institutes of Health, Bethesda, Maryland 20892

Human immunodeficiency virus type-1 (HIV-1) reverse transcriptase (RT) catalyzes DNA synthesis by an ordered sequential mechanism. After template-primer (T•P) binds to free enzyme, the deoxynucleoside triphosphate to be incorporated binds to the RT and T•P binary complex ($RT^{T•P}$). After incorporation of the bound nucleotide, catalytic cycling is limited either by a conformational change (for processive synthesis) or release of the enzyme from the extended T•P (for single-nucleotide incorporation). To explore cycling through these alternate rate-limiting steps, we determined kinetic parameters for single-nucleotide incorporation by HXB2R HIV-1 RT with chain-terminating nucleotide substrates 3′-azido-3′-deoxythymidine triphosphate (AZTTP) and dideoxythymidine triphosphate on a homopolymeric T•P system, poly(rA)-oligo$(dT)_{16}$. Inhibition of processive deoxythymidine monophosphate incorporation by these chain-terminating substrates was also examined. Because AZTTP is a substrate, its K_m should be equivalent to K_i, and since K_m for AZTTP should be influenced by the dissociation rate constant for $RT^{T•P}$, we examined the effect of altering $RT^{T•P}$ dissociation on AZTTP kinetic parameters. The dissociation rate constant was modulated by making use of different T•P substrates, viral sources of RT, and a mutant RT altered at a residue that perturbs T•P binding.

As expected from earlier work, the time course of AZTMP incorporation on poly(rA)-oligo$(dT)_{16}$ was biphasic, with a burst followed by a slower steady-state phase representing k_{cat} (0.42 min^{-1}) which was similar to the rate constant for $RT^{T•P}$ dissociation. Additionally, K_m for AZTTP (110 nM) was lower than its equilibrium dissociation constant (1200 nM). AZTTP inhibition ($K_{i,AZTTP}$) of processive dTMP incorporation and incorporation of a single nucleotide were similar. However, a simple correlation between the $RT^{T•P}$ dissociation rate constant and $K_{i,AZTTP}$ was not observed. These results indicate that a simple ordered model for single-nucleotide incorporation is inadequate and that different forms of $RT^{T•P}$ exist which can limit catalysis. The results are discussed in the context of a two-step binding reaction for T•P where the binary $RT^{T•P}$ complex undergoes an isomerization before binding of the deoxynucleotide substrate.

Human immunodeficiency virus type-1 (HIV-1),[1] the agent responsible for acquired immunodeficiency syndrome, encodes a DNA polymerase critical for viral replication. This enzyme, reverse transcriptase (RT), is an important molecular target for antiviral therapy. RT catalyzes both RNA and DNA template-dependent deoxynucleoside 5′-triphosphate (dNTP) incorporation. For example, 3′-azido-3′-deoxythymidine (AZT), which is converted to the triphosphate form (AZTTP) by cellular kinases, is able to inhibit viral replication *in vivo* and RT *in vitro* (Mitsuya *et al.*, 1990). Studies of enzyme-inhibitor interaction with an aim to facilitate drug design have contributed greatly to the understanding of the RT-catalyzed mechanism. By measuring specific steps in the multistep reaction scheme, we can undertake structure-function evaluation of mutant RTs to eventually identify important structural determinants for enzyme function and inhibition.

RT follows an ordered sequential mechanism for the DNA synthesis reaction pathway. The deoxynucleoside triphosphate to be incorporated onto the primer (3′-OH) binds to a complex of template-primer (T·P) and enzyme (Majumdar *et al.*, 1988). The crystallographic structure of HIV-1 RT has suggested that amino acid residues, whose mutation confers AZT resistance to the virus, map to a putative single-stranded template-binding site (Kohlstaedt *et al.*, 1992). In accord with this idea, the sensitivity of RT to chain-terminating inhibitors is dependent on the apparent interaction between these residues and the single-stranded template (Boyer *et al.*, 1994). Pre-steady-state kinetic characterization of AZTMP incorporation with HIV-1 RT has also been published (Reardon, 1992). Potent inhibition by AZTTP of *in vitro* reverse transcription is the result of efficient incorporation of the chain-terminating nucleoside analogue AZTMP (Reardon and Miller, 1990; Reardon, 1992). The slow catalytic rate in the presence of AZTTP was suggested to be dissociation of RT from the RT-template-terminated primer complex (Reardon and Miller, 1990; Reardon, 1992), and subsequent studies confirmed this to be the case (Kati *et al.*, 1992; Reardon, 1993).

The present report confirms and extends these earlier findings. We have examined inhibition of HIV-1 RT by AZTTP during processive dTTP incorporation, as well as during single-nucleotide incorporation of 2′,3′-dideoxythymidine triphosphate (ddTTP). During processive nucleotide incorporation, the $RT^{T·P}$ complex dissociation rate constant is not on the primary reaction pathway, whereas for single-nucleotide incorporation

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‡ To whom correspondence should be addressed.

[1] The abbreviations used are: HIV-1, human immunodeficiency virus type-1; RT, reverse transcriptase; T·P, template-primer; dNTP, deoxynucleoside 5′-triphosphate; AZT, 3′-azido-3′-deoxythymidine; AZTTP, 3′-azido-3′-deoxythymidine 5′-triphosphate; ddTTP, 2′,3′-dideoxythymidine 5′-triphosphate; dTTP, deoxythymidine 5′-triphosphate; dNMP, deoxynucleoside monophosphate; p66, 66-kDa RT polypeptide; p51, 51-kDa carboxyl terminally processed p66.

it is a required step. Incorporation of a chain-terminating nucleotide analogue effectively limits RT to a single-nucleotide incorporation and, therefore, its kinetic constants should be influenced by the dissociation rate constant for T·P, assuming that the simple ordered kinetic scheme described above is correct. To test this hypothesis, we have examined the effect of altering the stability of the $RT^{T\cdot P}$ complex on the kinetic parameters for AZTTP. The $RT^{T\cdot P}$ dissociation rate constant was modulated by using different T·P substrates or by taking advantage of altered enzyme/T·P interactions observed with RT derived from a different strain of HIV-1, NY5 (Beard and Wilson, 1993), or a site-directed mutant of HXB2R RT, L289K (Goel *et al.*, 1993). The kinetic scheme described above was not adequate to explain our results, and a modified scheme is proposed.

EXPERIMENTAL PROCEDURES

Materials—Poly(rA), oligo(dT)$_{16}$, oligo(dT)$_{10}$, oligo(dT)$_8$, ddTTP, and dTTP were from Pharmacia LKB Biotechnol. [α-^{32}P]dTTP (3000 Ci/mmol) and [γ-^{32}P]ATP (4500 Ci/mmol) were purchased from DuPont NEN and ICN Biochemicals, respectively. Modified nucleoside triphosphates, [5-methyl-^{3}H]AZTTP (14 Ci/mmol), unlabeled AZTTP, and [5-methyl-^{3}H]ddTTP (2 Ci/mmol) were from Moravek Biochemicals. T4 polynucleotide kinase was from New England Biolabs. Dupont Nensorb™ 20 nucleic acid purification cartridges were obtained from DuPont. DE81 filter discs of 25-mm diameter were purchased from Whatman.

Heterodimeric recombinant HXB2R HIV-1 RT (p66/p51) was expressed in *Escherichia coli* and purified as described previously (Becerra *et al.*, 1991). *E. coli* expressed NY5 recombinant HIV-1 p66/p51 was from Genetics Institute (Cambridge, MA). A p66 mutant where the leucine at position 289 was altered to lysine (L289K) was kindly provided by Dr. R. Goel (Goel *et al.*, 1993). Enzyme concentrations were determined from protein determinations (Bradford, 1976) that had been calibrated by amino acid analysis.

5′-[^{32}P] End-labeling of Oligonucleotides—Oligo(dT) primers were 5′ end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase at 37 °C for 1 h. Unincorporated [γ-^{32}P]ATP was removed by passing the reaction mixture through a Nensorb™ 20 column according to the directions given by the manufacturer.

Annealing of Template-Primer—Poly(rA) was hybridized to oligo(dT)$_{16}$, in a 5:1 or 2:1 nucleotide ratio of template to primer, by heating to 100 °C for 3–5 min and slowly cooling to room temperature. Substrates not used immediately were stored at −80 °C. Hybridization of poly(rA) with radiolabeled oligo(dT)$_{10}$ or oligo(dT)$_8$ was conducted with a 10:1 nucleotide ratio of template to primer. Concentration of nucleotides, prepared in 10 mM Tris-HCl, pH 7.4, was determined by their UV absorbance at 260 nm using the manufacturer's extinction coefficients.

Reverse Transcriptase Assay—DNA synthesis reactions contained 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 100 mM KCl, 1 μM poly(rA)-oligo(dT)$_{16}$ (expressed as 3′-primer termini), dNTP, and RT as indicated. Reactions were incubated at 20–25 °C and stopped by quenching with 167 mM EDTA, pH 8.0. Extended primer was bound to DE81 filters by absorbing the total reaction mixture on the filters and drying under an infrared lamp. Unincorporated nucleoside triphosphates were removed by washing the filters four times with 300 mM ammonium formate, pH 8.0, and twice with 95% ethanol. The filter discs were dried, and nucleotide incorporation was quantified by scintillation counting in RPI Biosafe II mixture. Incorporation was corrected for background radioactivity by measuring the amount of apparent incorporation in the absence of enzyme for each concentration of radioactive substrate.

Specific Activity of ^{3}H-Labeled Nucleoside 5′-Triphosphate—With DE81 filter binding, the counting efficiency of the ^{3}H-labeled triphosphate form of the nucleoside was found to be lower than that of the ^{3}H-labeled incorporated terminal monophosphate form (Altman and Lerman, 1970). Hence, incorporated ^{3}H-ddNMP bound to DE81 filters was used as a measure of specific activity. In order to incorporate all of the ^{3}H-labeled nucleoside triphosphate (500 nM), reactions were carried out with excess RT (780 nM) and T·P (1 μM) for prolonged times (30 min). Incorporation of labeled ^{3}H-ddNTP was considered to be complete when reaction mixtures were spotted on DE81 filters and washing did not remove radioactivity.

Primer Extension Assay—Short DNA oligomers, oligo(dT)$_{10}$ and oligo(dT)$_8$, did not bind to DE81 filter discs efficiently. This resulted in an underestimation of the product formed. Consequently, kinetic parameters with these oligonucleotides were determined by extension of 5′-radiolabeled primers and the products resolved on polyacrylamide gels.

Reactions were carried out as described above with 5′-^{32}P end-labeled primers and unlabeled dNTP. DNA synthesis reactions were stopped with EDTA, mixed with an equal volume of gel-loading buffer (0.03% xylene-cyanol, 0.03% bromophenol blue, and 90% formamide), boiled for 3 min, and cooled immediately on ice. Products (n+1) were separated from free primer (n) by electrophoresis on 28% polyacrylamide denaturing sequencing gels. Product formation was quantified by excising the radioactive bands and scintillation counting.

Kinetic Analysis—A simple model describing single-nucleotide incorporation is illustrated in Scheme I where E represents reverse transcriptase, T·P is the template-primer, dNTP is the deoxy- or dideoxynucleoside triphosphate, and T·P$_{+1}$ is the template-primer after a single-nucleotide incorporation.

$$E + T\cdot P \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E^{T\cdot P} + dNTP \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} E^{T\cdot P}_{dNTP} \xrightarrow{k_3} E^{T\cdot P_{+1}} \xrightarrow{k_4} E + T\cdot P_{+1}$$

SCHEME I

Pyrophosphate release from $E^{T\cdot P_{+1}}$ is assumed to be rapid since it binds weakly, K_i in the μM range (Majumdar *et al.*, 1988), relative to T·P. The kinetic expression for $K_{m,dNTP}$ is dependent on the assay and relative magnitudes of k_{-2} and k_3 (*i.e.* steady-state or rapid equilibrium assumptions). A summary of the steady-state kinetic constants for the mechanism illustrated in Scheme I is outlined in Table I (also see Reardon and Miller, 1990).

TABLE I

Summary of steady-state kinetic constants as defined by individual rate constants for the mechanism illustrated in Scheme I

Expressions derived using steady-state assumption and conservation of enzyme equation (see Reardon and Miller (1990)). T·P is saturating. The full expressions for k_{cat} and K_m, when incorporation is limited to a single nucleoside triphosphate, are $k_3k_4/(k_3 + k_4)$ and $[k_4/(k_3 + k_4)][(k_{-2} + k_3)/k_2]$, respectively. The expressions for k_{cat} and K_m when incorporation is processive are as given.

Approach	Assumptions	Nucleotide incorporation[a]			
		Single-nucleotide		Processive	
		K_m	k_{cat}	K_m	k_{cat}
Steady-state					
	$k_3 << k_4$	$(k_{-2}+k_3)/k_2$	k_3	Not applicable	
	$k_3 >> k_4$	$k_4(k_{-2}+k_3)/k_2k_3$	k_4	$(k_{-2}+k_3)/k_2$	k_3
Rapid equilibrium ($k_{-2} >> k_3$)					
	$k_3 << k_4$	K_d	k_3	Not applicable	
	$k_3 >> k_4$	K_dk_{off}/k_3	k_4	K_d	k_3

[a] Single-nucleotide incorporation refers to a situation where only a single nucleotide is incorporated per polymerase encounter with T·P. Such a situation occurs when the T·P is a heteropolymer and only the correct incoming deoxynucleoside triphosphate is present or when a homopolymeric T·P is used and a dideoxynucleoside triphosphate is incorporated. Processive incorporation refers to the situation where $E^{T\cdot P_{+1}}$ would not accumulate (*i.e.* $E^{T\cdot P_{+1}} = E^{T\cdot P}$).

When incorporation is limited to a single nucleotide, the equilibrium dissociation constant (k_{-2}/k_2) for nucleotide binding to $E^{T\cdot P}$ can be estimated from the nucleotide concentration dependence of the pre-steady-state burst amplitude. Under this condition, addition of dNTP to E, which is saturated with T·P, results in an exponential burst of nucleotide incorporation followed by a linear steady-state rate of T·P$_{+1}$ accumulation. The rate of the burst can be followed by rapid mixing and quenching techniques and is sensitive to the dissociation constant for dNTP binding. The dissociation constant for dNTP binding to HIV-1RT (Reardon, 1992, 1993; Kati *et al.*, 1992; Hsieh *et al.* , 1993) has been determined using this method. The amplitude of the burst is also sensitive to the concentration of dNTP and can be determined by extrapolation of the linear steady-state rate to the ordinate, where $P_{Intercept}$ represents the ordinate intercept and is equivalent to the amplitude of the burst (Wharton and Eisenthal, 1981).

$$[P_{intercept}] = \frac{[E]}{[1 + (k_3/k_4)\,(1 + (K_d/[dNTP]))]^2} \quad \text{(Eq. 1)}$$

Since $k_3 >> k_4$,

$$\sqrt{P/E} = \frac{[dNTP]}{[dNTP] + K} \quad \text{(Eq. 2)}$$

where $K = (k_4/k_3)K_d$.

Data were fitted to appropriate equations by nonlinear least-squares methods. Inhibition of apparent velocity (*i.e.* k_{obs}) by AZTTP was fitted to:

$$k_{obs} = \frac{[\text{dNTP}]\,k_{cat}}{[\text{dNTP}] + K_m\,(1 + ([\text{AZTTP}]/K_i))} \qquad \text{(Eq. 3)}$$

where k_{obs} and k_{cat} are $v/[RT]$ and $V_{max}/[RT]$, respectively (Segel, 1975).

Time course simulations were generated using HopKINSIM, a Macintosh version of the kinetic simulation program KINSIM (Barshop *et al.*, 1983) written by D. Wachsstock. Further details are given in the legend of Fig. 5.

T·P Dissociation Rate Constant—The dissociation rate constant (*i.e.* k_{off}) for T·P from wild type HXB2R and NY5, as well as a L289K mutant of HXB2R RT was determined as described before (Beard and Wilson, 1993). Enzyme was preincubated with poly(rA)-oligo$(dT)_{16}$ for 10 min before challenging with heparin (zero time). Heparin binds free RT, as well as RT which has dissociated from T·P. At time intervals after adding challenge, 10-μl aliquots were removed and mixed with 10 μl of dTTP/Mg^{2+} to determine the concentration of RT remaining bound to T·P. The final reaction conditions were 50 nM RT, 75 nM T·P (expressed as 3'-primer termini), 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 30 μM [α-^{32}P]dTTP, and 1 mg/ml heparin. EDTA was added at a final concentration of 167 mM. When the reaction mixture included 100 mM KCl, the heparin concentration was increased to 5 mg/ml to adequately compete with T·P for free RT. Incorporation of radioactive dTMP was determined by spotting the quenched reaction mixtures on DE81 filters as described in detail above.

RESULTS

Biphasic AZTMP Incorporation—The time course for [^{3}H]AZTMP incorporation with a poly(rA)-oligo$(dT)_{16}$ template-primer system was found to be biphasic (Fig. 1*A*). After a steady-state rate of nucleotide incorporation was achieved, the velocity was constant for more than 10 min corresponding to a k_{cat} of 0.42 min^{-1} (Table II). Since the ordinate intercept extrapolates above the origin, a rapid burst of product formation is indicated which is too rapid to be measured by manual mixing and sampling techniques. The linear portion of the time course, expressed as k_{cat}, was independent of enzyme concentration (Fig. 1*B*), whereas the amount of product formed in the burst phase was dependent upon the RT concentration (Fig. 1*C*). The steady-state velocity was approximately 40-fold lower than determined for incorporation of dTMP under the same reaction conditions (Table II). Similar observations of a burst phase followed by a much slower steady-state rate have been made for AZTMP incorporation (Reardon and Miller, 1990; Reardon, 1992).

The amplitude of the burst should be equivalent to the active enzyme concentration, as long as the rate of product release is much slower than the rate of AZTMP incorporation (*i.e.* $k_4 \ll k_3$, Scheme I). The ratio of AZTMP incorporated during the burst to the amount of enzyme (P/E) was 0.7 ± 0.2 (*ordinate intercept* in Fig. 1*B*) as determined from several independent experiments. The total enzyme concentration was determined by amino acid analysis, and this was confirmed by active-site titration with T·P (Beard and Wilson, 1993).

Steady-state AZTMP Incorporation—Steady-state kinetic parameters for AZTTP were determined from the incorporation following the burst. Incorporation increased in a hyperbolic fashion with increasing AZTTP concentration. From several independent determinations, the $K_{m,AZTTP}$ was 110 nM (Table II). The steady-state rate of incorporation of [^{3}H]AZTMP was much lower than the rate of incorporation of dTMP. However, the K_m for dTTP is significantly greater than for AZTTP, whereas the efficiency of incorporation calculated by k_{cat}/K_m was not significantly different for these two nucleotide substrates.

Equilibrium Dissociation Constant of AZTTP—The binding affinity of deoxynucleoside triphosphate for the polymerase binary complex with T·P has been determined by measuring the concentration dependence of the pre-steady-state rate of a single dNMP incorporation (Kuchta *et al.*, 1987; Patel *et al.*, 1991; Reardon, 1992, 1993; Kati *et al.*, 1992; Hsieh *et al.*, 1993). This approach has the advantage that the rate constant for the incorporation event is also determined. Since the amplitude of the burst is also sensitive to the K_d of AZTTP, we determined the AZTTP concentration dependence of the amplitude of the burst phase to obtain the binding affinity for AZTTP (see "Experimental Procedures"). The AZTTP concentration dependence of the square root of P/E is shown in Fig. 2. Fitting these data to Equation 2, the equilibrium dissociation constant for AZTTP was determined to be 1.2 μM (Table II).

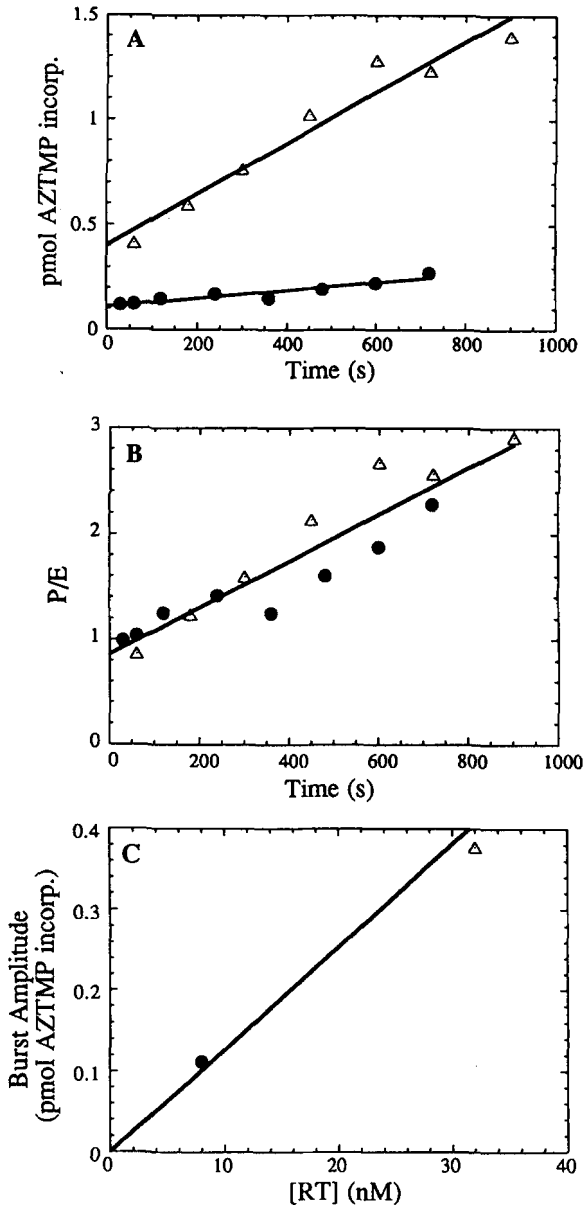

FIG. 1. **Time course of [^{3}H]AZTMP incorporation by HIV-1 RT with poly(rA)-oligo$(dT)_{16}$.** The concentration of [^{3}H]AZTTP and poly(rA)-oligo$(dT)_{16}$ was 10 and 3 μM, respectively, where the T·P concentration is expressed as concentration of 3'-primer termini. The template to primer nucleotide ratio was 2. Other reaction conditions are as described under "Experimental Procedures." *A*, incorporation of [^{3}H]AZTTP with 8 (●) or 32 nM RT (Δ). *B*, time course of AZTMP incorporation normalized to product formed/enzyme (P/E). The ordinate intercept of this plot corresponds to the amplitude of the burst phase of incorporation which extrapolated to 0.9 AZTMP incorporated/enzyme. *C*, a replot of the ordinate intercepts from *panel A* illustrates that the burst amplitude is proportional to the enzyme concentration.

TABLE II
Summary of kinetic constants for HXB2R recombinant HIV-1 reverse transcriptase and deoxynucleotide triphosphate binding

Due to the low amount of incorporation with ddTTP and AZTTP, the standard error in the fit parameters with these substrates was as much as 40%. Variation in burst amplitude and k_{cat} from independent experiments was approximately 70%.

dNTP	Primer	K_d	K_m	Burst[a]	k_{cat}	k_{cat}/K_m
		μM	nM	P/E	min^{-1}	$10^6\ M^{-1}\ min^{-1}$
dTTP	oligo(dT)$_{16}$	1.5	1500	ND[b]	18	12
ddTTP	oligo(dT)$_{16}$	2.9	30	0.5	0.12	4
AZTTP	oligo(dT)$_{16}$	1.2	110	0.7	0.42	4
	oligo(dT)$_{10}$	ND	160	ND	0.18	1
	oligo(dT)$_8$	ND	30	ND	0.18	5

[a] Product formation resulting from the rapid initial turnover of the enzyme was obtained by extrapolation of the linear phase of the time course of nucleotide incorporation to the ordinate-intercept.
[b] Not determined.

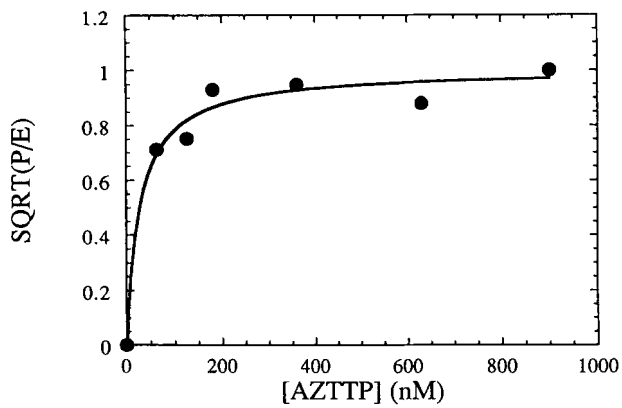

FIG. 2. **The AZTTP concentration dependence of the burst amplitude of AZTMP incorporation.** Reaction conditions were as described under "Experimental Procedures." The concentration of poly(rA)-oligo(dT)$_{16}$ and RT was 3 μM and 16 nM, respectively. The template to primer nucleotide ratio was 5. The concentration of AZTMP incorporated in the burst was determined by extrapolating the linear steady-state rate to zero time. Since the maximum incorporation observed in the burst was $P/E = 0.73$, the burst amplitude was normalized to this accumulation. The dissociation constant (K_d) for AZTTP can be estimated from the apparent dissociation constant (K, see Equation 2) as described under "Experimental Procedures."

AZTTP Inhibition—To examine the inhibition of single-nucleotide incorporation by AZTTP, the kinetics of another chain-terminating nucleotide, ddTTP, was characterized. The time course for ddTMP incorporation was similar to that with AZTMP incorporation. Following a burst of ddTMP incorporation, a constant rate of incorporation was observed, and this linear phase extrapolated to a point on the ordinate that was dependent on enzyme concentration. Like AZTMP incorporation, k_{cat}/K_m was similar to that for processive dTMP incorporation (Table II). The K_i for AZTTP, as a competing ligand for single-nucleotide incorporation using ddTTP as substrate, was 40 nM (Fig. 3*A*). In addition, for processive dTMP incorporation, the K_i for AZTTP was 10 nM (Fig. 3*B*, Table III). Thus, the Michaelis constants for AZTTP and ddTTP, and the K_i for AZTTP, are much lower than the corresponding equilibrium dissociation constant (K_d) for binding of the nucleotide to the binary $RT^{T\cdot P}$ complex (Table II). This difference will be discussed below.

Template-Primer Binary Complex Dissociation Rate Constant—The observation of a rapid incorporation of approximately one enzyme equivalent of chain-terminating nucleotide followed by a slower linear rate of incorporation indicates that a step following phosphodiester bond formation is rate determining during the linear steady-state phase. To determine if the steady-state rate is governed by the dissociation of the $RT^{T\cdot P}$ binary complex, the rate constant was measured with a RT challenge assay (Beard and Wilson, 1993). The dissociation rate constant for poly(rA)-oligo(dT)$_{16}$ as T·P with a terminal 3′-OH, under the experimental conditions of this study, was

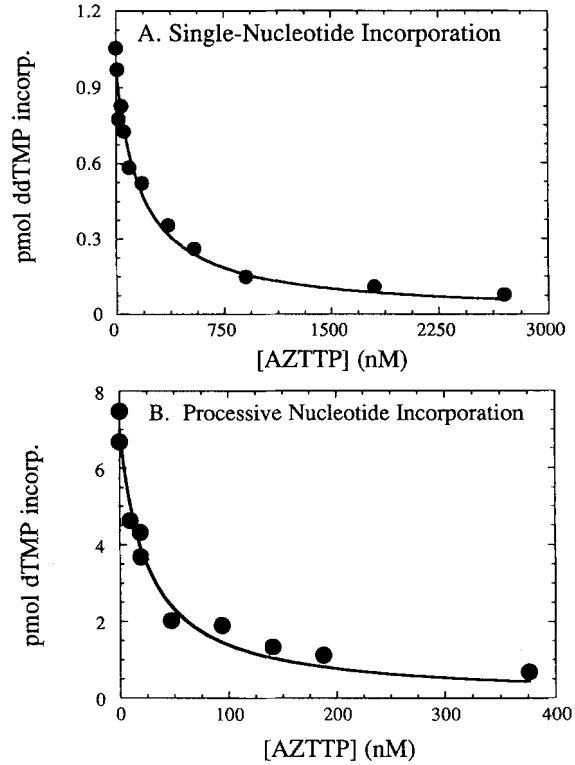

FIG. 3. **Inhibition of single-nucleotide and processive incorporation by AZTTP.** *A*, the steady-state rate of [^{3}H]ddTTP incorporation was determined from 5 min of post-burst incorporation. The concentration of ddTTP and RT were 100 and 16 nM, respectively. *B*, the steady-state rate of [^{32}P]dTTP incorporation was determined from 20 min of incorporation. The concentration of dTTP and RT was 3 μM and 2 nM, respectively. Other reaction conditions are as described under "Experimental Procedures." Inhibition data were fitted to Equation 3, and the K_i values were determined to be 40 and 10 nM for inhibition of single-nucleotide and processive-nucleotide incorporation, respectively.

TABLE III
Comparison of the $RT^{T\cdot P}$ dissociation rate constant and inhibition constant for AZTTP

RT	Strain	T·P	Relative $k_{off,app}$[a]	$K_{i,AZTTP}$[b]
				nM
p66/p51	HXB2R	poly(rA)-oligo(dT)$_{16}$	1[c]	10
		poly(rA)-oligo(dT)$_{10}$	34[c]	20
		poly(dA)-oligo(dT)$_{16}$	>>200	15,000
p66$_{L289K}$		poly(rA)-oligo(dT)$_{16}$	67	21
p66/p51	NY5	poly(rA)-oligo(dT)$_{16}$	>>200	20[d]

[a] The apparent dissociation rate constant for T·P (*i.e.* $k_{off,app}$) was determined with a challenge assay as described under "Experimental Procedures." Relative $k_{off,app}$ represents the observed increase in the apparent dissociation rate constant relative to heterodimeric HXB2R RT complexed with poly(rA)-oligo(dT)$_{16}$. The dissociation rate constants are referred to as apparent since they depend on the magnitude of the other unimolecular rate constants for a two-step binding reaction for T·P outlined in Scheme II.
[b] $K_{i,AZTTP}$ determined as described under "Experimental Procedures" for processive dTMP incorporation.
[c] Taken from Beard and Wilson (1993). The dissociation rate constant for poly(rA)-oligo(dT)$_{16}$ under the conditions of this study was 0.36 min^{-1} which is similar to k_{cat} (Table II).
[d] Taken from Kedar *et al.* (1990). The T·P was poly(rA)-oligo(dT)$_{14}$.

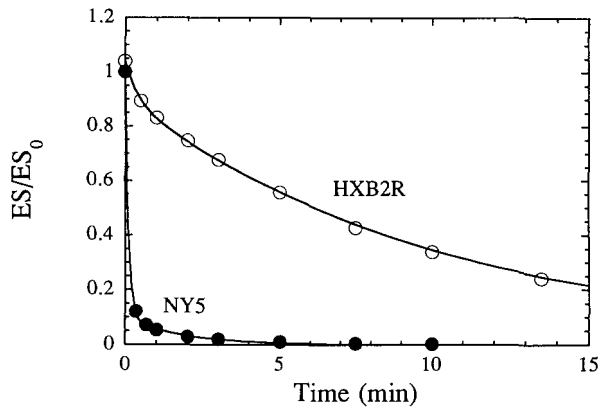

FIG. 4. **Dissociation of HXB2R and NY 5 HIV-1 RT/poly(rA)-oligo(dT)$_{16}$ complex.** Heterodimeric RT was preincubated with poly(rA)-oligo(dT)$_{16}$, and at time zero, heparin was added to bind free RT. At the indicated times, dTTP/Mg^{2+} was added to determine the amount of $RT^{T\cdot P}$ complex remaining. The reaction was allowed to proceed 10 min before stopping with EDTA. The final reaction mixture contained 50 nM RT, 75 nM poly(rA)-oligo(dT)$_{16}$, 30 μM dTTP, 1 mg/ml heparin, 50 mM Tris-Cl, pH 7.4, and 10 mM $MgCl_2$. Data were fitted to a two exponential model: $ES/ES_0 = A_1e^{-k_1t} + A_2e^{-k_2t}$, where ES/ES_0 represents the fraction of $RT^{T\cdot P}$ complex, A = amplitude, and k = apparent first-order rate constant. The fit parameters were: HXB2R RT, $A_1 = 0.22, A_2 = 0.78, k_1 = 0.43\ min^{-1}, k_2 = 0.08\ min^{-1}$; NY5 RT, $A_1 = 0.90, A_2 = 0.10, k_1 = 9.4\ min^{-1}, k_2 = 0.5\ min^{-1}$.

approximately equal to k_{cat} for AZTMP incorporation (data not shown). This not only demonstrates that T·P dissociation is the rate-determining step during steady-state single-nucleotide incorporation on a RNA template (Reardon, 1993), but indicates that the dissociation rate constant for a primer is not significantly affected by the presence of an azido group on the sugar moiety.

Kedar *et al.* (1990) found that the K_m for AZTTP was 3 μM using poly(rA)-oligo(dT)$_{14}$ as a T·P substrate, whereas Reardon and Miller (1990) found it to be 0.22 μM with a similar T·P. Scheme I predicts that the K_m for a chain-terminating deoxynucleoside triphosphate is dependent on the dissociation rate constant for T·P (*i.e.* k_4; Table I). When the dissociation rate constant ($k_4 = k_{off,T\cdot P}$) is slow relative to the rate constant for incorporation (*i.e.* $k_4 \approx k_{-1} \ll k_3$), the K_m for deoxynucleoside triphosphate is given by $k_4(k_{-2} + k_3)/k_2k_3$ and when nucleotide binding is in rapid equilibrium, $K_{m,dNTP} = (k_{-2}k_4)/(k_2k_3) = K_d(k_{off,T\cdot P}/k_3)$ (Table I). $K_{m,dNTP}$ is, therefore, expected to be lower than K_d by a factor of $(k_3/k_{off,T\cdot P})$. When $k_4 \approx k_{-1} \gg k_3$, there is not accumulation of $E^{T\cdot P+1}$ and $K_{m,dNTP} = (k_{-2} + k_3)/k_2$. If deoxynucleotide binding is in rapid equilibrium, then $K_{m,dNTP}$ is equivalent to K_d (Table I). The high K_m determined by Kedar *et al.* (1990) suggested that the dissociation rate constant for T·P from RT under their conditions was rapid. Beard and Wilson (1993) have demonstrated that the K_m for homopolymeric T·P is dependent on the source of the recombinant RT, and that the K_m for poly(rA)-oligo(dT)$_{16}$ was much greater for NY5 than for HXB2R RT with dTTP as the deoxynucleoside triphosphate. Since Kedar *et al.* (1990) used NY5 RT, the high K_m determined for AZTTP may represent the equilibrium dissociation constant for AZTTP resulting from a rapid T·P dissociation rate constant. To directly test this possibility, the dissociation rate constants for poly(rA)-oligo(dT)$_{16}$ from HXB2R and NY5 heterodimeric RT were measured under identical conditions (Fig. 4). Whereas the approximate half-life for the HXB2R $RT^{T\cdot P}$ complex was greater than 6 min, the majority (>85%) of the NY5 $RT^{T\cdot P}$ complex had decayed before the first time point (20 s) was collected. Huber *et al.* (1989) also found that a major portion of the NY5 $RT^{T\cdot P}$ complex decayed too rapidly to measure with a similar assay.

Effect of $RT^{T\cdot P}$ Dissociation Rate Constant on AZTMP Incorporation—It was shown previously that the dissociation rate constant ($k_{off,T\cdot P}$) for poly(rA)-oligo(dT)$_{10}$ was about 30-fold faster than for poly(rA)-oligo(dT)$_{16}$ from the $RT^{T\cdot P}$ binary complex (Beard and Wilson, 1993). Since this dissociation rate constant is the rate-determining step with poly(rA)-oligo(dT)$_{16}$ as T·P, we examined other T·Ps for single-nucleotide incorporation with AZTTP to determine if k_{cat} will reflect the dissociation rate constant of the T·P. We studied various circumstances where the $RT^{T\cdot P}$ dissociation rate constant was known to be increased (Table III), *e.g.* poly(rA) template annealed with oligo(dT)$_8$, oligo(dT)$_{10}$, or oligo(dT)$_{16}$ (Table II). Surprisingly, k_{cat} for AZTMP incorporation showed no significant difference with these T·Ps. This is in contrast to what is predicted from Scheme I.

For a nucleotide analogue substrate inhibitor, such as AZTTP, K_i is expected to be approximately equivalent to K_m (Scheme I, Table I). Since k_{off} for the binary complex is different with oligo(dT)$_{10}$ and oligo(dT)$_{16}$, the K_m for AZTTP with these T·Ps during a single-nucleotide incorporation should be influenced by this rate constant (k_4) and, therefore, is expected to be different. Yet, we found that the $K_{m,AZTTP}$ was not significantly different. Thus, there is no simple correlation between the dissociation rate constant for the $RT^{T\cdot P}$ binary complex and AZTTP kinetic parameters. To further evaluate the relationship between the $RT^{T\cdot P}$ dissociation rate constant and AZTTP inhibition kinetics, we examined an alternate substrate, poly(dA)-oligo(dT)$_{16}$, as T·P. The dissociation rate constant with this alternate T·P is very rapid and is unmeasurable by manual mixing methods (Table III). We found that the $K_{i,AZTTP}$ for dTMP incorporation was 15 μM, a value much higher than the $K_{i,AZTTP}$ observed with poly(rA)-oligo(dT)$_{16}$ as T·P (Table III). Thus, in this case, a faster $RT^{T\cdot P}$ dissociation rate constant qualitatively correlated with a higher $K_{i,AZTTP}$ as predicted from Scheme I. Further, the relationship between dissociation of the $RT^{T\cdot P}$ complex and AZTTP inhibition was examined with a site-directed mutant of RT where the leucine at residue 289 was replaced with lysine (*i.e.* L289K). This region has been identified as forming a portion of the primer-binding site (Sobol *et al.*, 1991; Basu *et al.*, 1992) and is important for dimerization of p66 and p51 (Goel *et al.*, 1993). The dissociation rate constant was determined to be significantly more rapid than with wild-type enzyme, whereas the K_i for AZTTP against dTMP incorporation was not affected (Table III). Again, these results indicate that kinetic parameters for AZTTP inhibition depend on factors not accounted for by Scheme I, and the magnitude of k_{off} for the $RT^{T\cdot P}$ complex does not quantitatively correlate with the magnitude of the K_i for AZTTP, as predicted by Scheme I.

DISCUSSION

Two-step Binding Reaction for Template-Primer—The kinetic model in Scheme I predicts that the K_i for AZTTP should be sensitive to the $RT^{T\cdot P}$ dissociation rate constant (k_4). This is an extremely important concept for drug design because it links RT and template-primer interactions with the apparent potency of an inhibitor that is competitive with dNTP binding. According to this idea, alterations in enzyme-nucleic acid interactions would also be expected to alter K_i for AZTTP. We found here that there are some circumstances where increases or decreases in $RT^{T\cdot P}$ dissociation rate constant do indeed correlate with increases or decreases in $K_{i,AZTTP}$. However, there are other circumstances where such a correlation clearly does not exist. Therefore, there is no uniform rule defining the relationship between $RT^{T\cdot P}$ dissociation rate constant and AZTTP kinetic parameters. Hence, the model depicted in Scheme I is not sufficient to characterize our results.

The chain-terminating deoxynucleoside 5'-triphosphates, ddTTP and AZTTP, were incorporated into a homopolymer of oligo(dT)$_{16}$ annealed to poly(rA) in a biphasic manner. After rapid incorporation of approximately one AZTMP residue/enzyme, a slower steady-state incorporation of AZTMP occurred (Fig. 1). The rapid phase represents phosphodiester bond formation or a conformational change that may limit this event, whereas the slower phase represents a subsequent step. On a heteropolymeric RNA template, this has been demonstrated to be T·P dissociation (*i.e.* k_4) from the binary complex (Reardon, 1993). Since poly(rA)-oligo(dT)$_{16}$ binds tightly to RT, $K_d < 1$ nM (Beard and Wilson, 1993), and since pyrophosphate binds only weakly, K_i in the μM range (Majumdar *et al.*, 1988), pyrophosphate would be expected to dissociate much faster than T·P. Additionally, we have determined that k_{cat} for AZTMP incorporation on a homopolymeric T·P is similar to the dissociation rate constant for the RT$^{T\cdot P}$ complex when the primer is at least 16 nucleotides in length (Tables II and III). The steady-state rate for AZTMP incorporation under low salt conditions was 0.12 min^{-1}, which is similar to RT$^{T\cdot P}$ k_{off} reported for this condition (Beard and Wilson, 1993). However, when shorter primers are employed, the steady-state rate of chain-terminating nucleotide incorporation is not similar to the rapid rate at which they dissociate (Tables II and III).

The results suggest that dissociation of $E^{T\cdot P}$ determined with the challenge assay, under some conditions, is inherently different from T·P dissociation during catalytic cycling. Thus, Scheme I is modified to reflect the existence of another $E^{T\cdot P}$ complex (designated $*E^{T\cdot P+1}$) as shown in Scheme II.

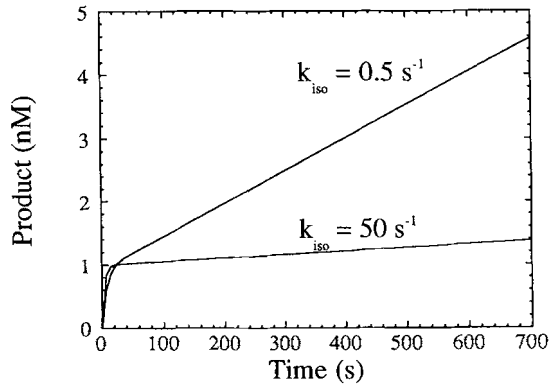

FIG. 5. **Influence of a post-binding isomerization of the RT$^{T\cdot P}$ complex on the steady-state rate for single-nucleotide incorporation.** Simulated time courses were generated from Scheme II which includes an isomerization of the RT$^{T\cdot P}$ and RT$^{T\cdot P+1}$ complexes. The RT, ddNTP, and T·P concentrations were 0.001, 10, and 1 μM, respectively. The forward isomerization rate constant (k_{iso}) was 0.5 or 50 s^{-1}, whereas, k_{on}, k_{off}, and $k_{r,iso}$ were 6×10^6 M^{-1} s^{-1} (Beard and Wilson, 1993), 5 s^{-1} (Divita *et al.*, 1993), and 0.006 s^{-1} (Table II), respectively, for both simulations. The ddNTP dissociation constant and k_3 were 1.5 μM and 0.3 s^{-1}, respectively. After a burst of product formation, the steady-state linear rate is 0.0052 and 0.0005 s^{-1} with k_{iso} of 0.5 and 50 s^{-1}, respectively. Increasing k_{iso} such that $k_{iso} > k_{off}$ results in a decrease in k_{cat} due to stabilization of $*E^{T\cdot P+1}$. This is due to the low concentration of the $E^{T\cdot P+1}$ complex under this situation that results from the low probability that $E^{T\cdot P+1}$ will dissociate to E + T·P$_{+1}$, as compared to isomerizing to $*E^{T\cdot P+1}$. More importantly, the presence of chain-terminating nucleotides would result in more potent inhibition since enzyme would get trapped as $*E^{T\cdot P}$ terminated.

$$E + \text{T·P} \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} E^{\text{T·P}} \underset{k_{r,iso}}{\overset{k_{iso}}{\rightleftarrows}} *E^{\text{T·P}} + \text{dNTP} \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} *E^{\text{T·P}}_{\text{dNTP}} \overset{k_3}{\rightarrow} *E^{\text{T·P}_{+1}} \underset{k_{iso}}{\overset{k_{r,iso}}{\rightleftarrows}} E^{\text{T·P}_{+1}} \underset{k_{on}}{\overset{k_{off}}{\rightleftarrows}} E + \text{T·P}_{+1}$$

SCHEME II

In Scheme II, T·P binding consists of two steps where k_{on} and k_{off} are the association and dissociation rate constants for T·P, and k_{iso} and $k_{r,iso}$ are forward and reverse unimolecular rate constants for a post-binding isomerization. Previous kinetic characterization of T·P binding to RT has identified such a post-binding isomerization of the $E^{T\cdot P}$ complex (Beard and Wilson, 1993; Divita *et al.*, 1993; Kruhøffer *et al.*, 1993). In contrast to Scheme I, in this model, the dissociation rate constant for RT$^{T\cdot P}$ (k_{-1} in Scheme I) does not have to be equivalent to k_{cat} (k_4 in Scheme I, Table I). The K_i for a chain-terminating nucleotide depends on the apparent dissociation rate constant during catalytic cycling (*i.e.* k_{cat}). This may or may not be equivalent to the dissociation rate constant determined with a challenge assay.

For the model depicted in Scheme II, the apparent dissociation rate constant determined by a challenge assay, or k_{cat} during single-nucleotide incorporation, will depend on the magnitude of the unimolecular rate constants. Therefore, k_{off} and $k_{r,iso}$ can be rapid, but if $k_{iso} \gg k_{off}$ and $k_{r,iso}$, the apparent rate constant describing release of T·P$_{+1}$ from $*E^{T\cdot P+1}$ (*i.e.* k_{cat}) will be slower than the rate constants for T·P$_{+1}$ dissociation in the last two steps depicted in Scheme II (*i.e.* $k_{cat} < k_{r,iso}$ or k_{off}). Fig. 5 illustrates the results from kinetic simulation demonstrating how the magnitude of k_{iso} can influence k_{cat} for single-nucleotide incorporation (*i.e.* the apparent dissociation rate constant for $*E^{T\cdot P+1}$. Note that k_{off} and $k_{r,iso}$ were not altered for these simulations. Stabilization of $*E^{T\cdot P+1}$ by increasing k_{iso} such that $k_{iso} > k_{off}$ results in a decrease in k_{cat}. This is simply due to the low concentration of the $E^{T\cdot P+1}$ complex under this situation which results from the low probability that $E^{T\cdot P+1}$ will dissociate to E + T·P$_{+1}$, as compared to isomerizing to $*E^{T\cdot P+1}$. More importantly, the presence of chain-terminating nucleotides would result in more potent inhibition since enzyme would get trapped as $*E^{T\cdot P\ \text{terminated}}$. These simulations suggest that k_{iso} is much more rapid when the T·P is poly(rA)-oligo(dT)$_{10}$ than when oligo(dT)$_{16}$ is the primer. Although the dissociation rate constant is more rapid for oligo(dT)$_{10}$ than for oligo(dT)$_{16}$ annealed to poly(rA) as determined by a challenge assay (Table III), the rapid forward isomerization would result in a slower steady-state rate than would be predicted by Scheme I and, therefore, a lower $K_{i,AZTTP}$. Additionally, the simulations suggest that the apparent dissociation rate constant is equivalent to $k_{r,iso}$ when k_{iso} is slow relative to k_{off}, such as would be the case with oligo(dT)$_{16}$. However, when k_{iso} is much greater than k_{off} (*i.e.* oligo(dT)$_{10}$), the apparent dissociation rate constant is a composite of several rate constants.

Equilibrium Binding Affinity for Chain-terminating Nucleoside Triphosphates—The burst amplitude is dependent on the concentration of the dideoxynucleotide. The apparent K_d determined by a plot of the square root of the P/E underestimates the true dissociation constant by $k_3/k_{off,app}$ (Fig. 2). In other words, the dissociation constant for dNTP is diminished by the ratio of rate constants for incorporation and T·P dissociation. This ratio of rate constants is equivalent to the processivity of RT with dTTP as the deoxynucleoside triphosphate (Beard and Wilson, 1993). Since the efficiency of incorporation (k_{cat}/K_m) of AZTMP is similar to that for dTMP (Table II), the rate of incorporation of AZTMP was estimated from that for dTMP under processive conditions. Under these conditions, incorporation is limited by the incorporation step (*i.e.* k_3). If dNTP binding is a rapid equilibrium step, the K_m determined under

processive conditions for dTTP is approximately equal to its dissociation constant. The K_d values estimated for ddTTP and AZTTP are similar to that for the K_m of dTTP determined under processive conditions, where K_m is equivalent to K_d (Table I). This result suggests that our assumptions are valid, and we note that a rapid kinetic approach also found that the dissociation constants for these deoxynucleotides were similar (Reardon, 1992).

*Influence of the T·P Dissociation Rate Constant (*i.e. *Processivity) on Inhibition by AZTTP*—Inhibition of RT by AZTTP is competitive with respect to dNTP, and AZTTP has been shown to be a substrate for HIV-1 RT leading to chain termination (Matthes *et al.*, 1987; Kedar *et al.*, 1990; Reardon and Miller, 1990). Since RT is a processive polymerase (processivity = $k_3/k_{off,app} \gg 1$ *where* $k_{off,app}$ is equivalent to k_{cat} for single-nucleotide incorporation), the low inhibition constant reflects premature termination with accumulation of $RT^{T\cdot P_{+1}}$ and/or $*RT^{T\cdot P_{+1}}$ complexes. The relevance of this mechanism of potent inhibition *in vivo* has been questioned, since in contrast to *in vitro* assays, the RT concentration is greater than the primed template concentration *in vivo* (Goody *et al.*, 1991; Müller *et al.*, 1991). Inhibition *in vitro*, therefore, is the result of premature termination and a concomitant decrease in the active enzyme concentration (*i.e.* formation of $E^{T\cdot P_{+1}}$ and $*E^{T\cdot P_{+1}}$ which are not active), whereas *in vivo* inhibition is the result of the high probability that chain termination will occur due to the great length of the genomic RNA to be transcribed. When the RT concentration is higher than the T·P concentration, chain termination does not significantly affect the active enzyme concentration.

Ma *et al.* (1992) reported that the inhibition constant, K_i, experimentally determined with homopolymeric poly(rA)-oligo(dT) is an artifact of this T·P system, which is routinely used in these measurements. In the present study, to ascertain if inhibition by AZTTP is sensitive to the character of the polymerization reaction, AZTTP inhibition of single nucleotide (ddTMP) or multiple nucleotide incorporations (dTMP) was examined on a homopolymeric template. Inhibition of dTTP incorporation by AZTTP was strong (Fig. 3*B*), and Dixon plots were linear with AZTTP concentrations giving up to 95% inhibition. In contrast, Reardon and Miller (1990) observed nonlinear Dixon plots, which they postulated may be due to a change in the dissociation rate constant for $RT^{T\cdot P}$ complex (see below). Since AZTTP is also a substrate, Scheme I predicts that K_i should be equivalent to K_m. Reardon and Miller (1990) reported that the K_i for AZTTP was lower than K_m. They suggested that the difference in K_i and K_m may reflect a change in the dissociation rate constant for T·P depending on the number of nucleotides incorporated. It has been shown that the dissociation rate constant for the binary RT-homopolymer T·P complex is dependent on the length of the primer (Beard and Wilson, 1993) and is reflected by a decrease in the termination probability with increasing number of dTMP incorporations when a gel electrophoresis assay is used (Huber *et al.*, 1989; Majumdar *et al.*, 1988; Reardon *et al.*, 1991). The K_m or K_i for AZTTP, therefore, may reflect the number of nucleotides incorporated into the primer before incorporation of AZTMP (*i.e.* primer length). We found here, however, that the K_i for AZTTP for processive and single-nucleotide incorporation was similar (Fig. 3). Additionally, the $K_{i,AZTTP}$ for processive dTMP incorporation using different length primers and a homopolymeric template (rA) is also similar (Table III).

The inhibitory potency of AZTTP as measured by the K_i, therefore, is not an artifact of the homopolymeric T·P as concluded by Ma *et al.* (1992), but is the result of the intrinsic processive nature of RT, as well as the sensitivity of K_i to the apparent dissociation rate constant for T·P (Scheme III). Whereas K_i should be equivalent to its K_m as an alternative substrate, K_m is sensitive to the pathway for incorporation. In one case, as for single-nucleotide incorporation with a processive polymerase, dissociation of RT from the terminated primer is an obligatory step and K_m underestimates K_d by $k_3/k_{off,app}$. In the other case, where processive polymerization can occur such as with a homopolymeric T·P, K_m is equivalent to K_d (Table I). This latter case could also be achieved if the dissociation rate constant for T·P from $RT^{T\cdot P}$ complex were rapid (*i.e.* a distributive polymerase). The variation in K_i for AZTTP with different T·Ps observed by Ma *et al.* (1992) could be explained if the apparent dissociation rate constant for T·P was more rapid when inhibition of single-nucleotide incorporation was examined on a heteropolymer template (large K_i) than when inhibition of incorporation was examined on runs of rA (low K_i). This is consistent with the ability of RT to bind much tighter to poly(rA)-oligo(dT) than RNA/DNA heteroduplexes (Reardon, 1992; Beard and Wilson, 1993).

The K_m reported here for AZTTP with poly(rA)-oligo$(dT)_{16}$ and HXB2R HIV-1 RT is much lower than that reported earlier with poly(rA)-oligo$(dT)_{14}$ and NY5 RT (Kedar *et al.*, 1990). The higher value obtained with NY5 RT is consistent with the more rapid $RT^{T\cdot P}$ dissociation rate constant observed with NY5 as compared to HXB2R RT (Fig. 4). Thus, the $RT^{T\cdot P}$ dissociation rate constant is dependent on both the length of the primer and the source of the RT.

In summary, this study indicates a lack of a simple correlation between the T·P dissociation rate constant and the level of inhibition observed with AZTTP that would be predicted by a simple ordered addition of substrates. The apparent dissociation rate constant during catalytic cycling can, in some instances, be different from that determined from a challenge assay suggesting that T·P binding to RT is a two-step reaction resulting in two forms of $E^{T\cdot P}$ (*i.e.* $E^{T\cdot P}$ and $*E^{T\cdot P}$).

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