# Kinetic Analysis of Template·Primer Interactions with Recombinant Forms of HIV-1 Reverse Transcriptase†

William A. Beard‡,§ and Samuel H. Wilson*,‡,§

*Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, and Sealy Center for Molecular Science, University of Texas Medical Branch, Galveston, Texas 77555-0851*

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ABSTRACT: The reverse transcriptase (RT) from the human immunodeficiency virus (HIV) exists predominantly as a heterodimer (p66/p51), but can also form a homodimer of p66 subunits (p66/p66). RT binds to template·primer (T/P) tightly to form the first complex in the reaction sequence poised to conduct DNA synthesis upon the addition of dNTP and $Mg^{2+}$. We have made use of this property to kinetically analyze poly(rA)–$(dT)_n$ interactions with recombinant homo- and heterodimeric HIV-1 RT derived from HXB2R proviral DNA. A T/P challenge assay was used to quantitatively follow RT–T/P complex formation. The homo- and heterodimeric forms of RT bound to poly(rA)–$(dT)_{16}$ in a kinetically similar fashion. There was no more than a 2-fold difference in $k_{cat}$ or for any T/P parameter examined: $K_m$, $K_d$, $k_{on}$, $k_{off}$ determined from a binary complex or from a complex incorporating dTMP, processivity, and stoichiometry of binding. In contrast, it was found that the T/P $K_m$ with heterodimeric RT derived from the NY5 strain was significantly greater than that determined for HXB2R enzyme, indicating that a kinetic diversity exists between RT derived from different viral strains. Since HXB2R RT binds to poly(rA)–$(dT)_{16}$ tightly, $K_d < 1$ nM, active-site titrations are facilitated. At saturation, one T/P binds per two polypeptides, suggesting that RT binds substrate productively as a dimer and that if monomers are present they must rapidly form dimers in the presence of T/P. In contrast, when the template to primer nucleotide ratio was diminished, the apparent number of T/P binding sites increased to 2 per dimer. The $K_d$ for poly(rA)–$(dT)_n$ was dependent on the length of the primer, with short primers binding with a lower affinity primarily due to a more rapid dissociation rate constant. The dissociation reaction could often be better fitted to a double-exponential decay, suggesting that multiple conformations of the RT–T/P complex exist.

Human immunodeficiency virus type 1 (HIV-1)[1] is generally believed to be the etiologic agent for acquired immunodeficiency syndrome, AIDS. The reverse transcriptase (RT) of this retrovirus is responsible for converting the single-stranded (+) viral RNA genome into double-stranded proviral DNA prior to its integration into the host genomic DNA. The HIV-1 RT has therefore been the target for antiviral drug design (Mitsuya et al., 1990). The cloning of the RT in biologically active form has made large quantities of enzyme available for biophysical and kinetic studies. Low-resolution crystal structures (Arnold et al., 1992; Kohlstaedt et al., 1992) and pre-steady-state kinetic studies (Kati et al., 1992; Reardon, 1992) have recently been published. Such detailed structural and kinetic information is a prerequisite for rational drug design of more specific and potent inhibitors.

HIV-1 RT is a multifunctional enzyme with three recognized enzymatic activities: RNA- and DNA-dependent polymerase activity as well as ribonuclease H (RNase H) activity. In vivo, the heterodimeric form (p66/p51) of RT is believed to be the result of carboxy-terminal proteolytic cleavage of one subunit of the homodimer (p66/p66) (Chandra et al., 1986). Steady-state kinetic studies indicated that DNA synthesis proceeds by an ordered mechanism in which template·primer (T/P) binds to free heterodimeric RT to form the first complex in the reaction pathway (Majumdar et al., 1988). Differences in kinetic behavior between the homo- and heterodimeric forms of RT may give insight into how structural differences between these forms may translate functionally. Much of the enzymology, especially site-directed mutagenesis with HIV RT, has been done with the homodimeric form. It is especially important, therefore, that the two forms be shown to be kinetically equivalent before the homodimeric form can be considered a model for the heterodimeric viral RT. Comparison of the T/P interactions between these forms is especially critical since potent inhibition of polymerase activity by 2′,3′-dideoxynucleotides, such as AZT, is due to incorporation of these analogues into the growing primer strand, resulting in chain termination and accumulation of the RT–T/P complex (Reardon & Miller, 1990; Reardon, 1992). Modeling of an RNA/DNA template·primer into its putative binding site has suggested that enzyme residues which confer AZT resistance may interact with the template strand (Kohlstaedt et al., 1992).

We have examined T/P interactions with p66/p51 and p66/p66 forms of the RT by using a challenge assay to measure

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* To whom correspondence should be addressed at the Sealy Center for Molecular Science, University of Texas Medical Branch.

‡ National Cancer Institute, National Institutes of Health.

§ Sealy Center for Molecular Science, University of Texas Medical Branch.

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[1] Abbreviations: HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; RNase H, ribonuclease H; nt, nucleotide; p66/p66, homodimer of identical 66-kDa RT polypeptides; p66/p51, heterodimer of 66-kDa and a 51-kDa C-terminally processed p66; dNTP, deoxynucleoside 5′-triphosphate; T/P, template·primer; $(T/P)_{nt}$, template to primer nucleotide ratio; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; AZT, 3′-azido-2′,3′-dideoxythymidine.

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the affinity and stoichiometry of binding to poly(rA)–oligo(dT). The use of this homopolymeric substrate also facilitates quantitative measurement of the dissociation and association rate constants for T/P binding to RT by taking advantage of the processive nature of this polymerase. In addition to the kinetic comparison of these recombinant RT forms, this information will also allow us to determine the role of particular amino acid side chains in binding of ligands and catalysis by gauging the effects determined with altered RTs through site-directed mutagenesis or by "natural" viral strain variation.

## MATERIALS AND METHODS

*Materials.* Recombinant HIV-1 RT, p66/p66 and p66/p51, was purified from *Escherichia coli* transformed with a plasmid containing the precise coding region for HXB2R HIV-1 RT (Becerra et al., 1991). *E. coli* expressed NY5 recombinant HIV-1 p66/p51 was from Genetics Institute, Cambridge, MA. Enzyme concentration was determined from protein determinations (Bradford, 1976) which had been calibrated by amino acid analysis.

Poly(rA), $(dT)_n$, and dTTP were from Pharmacia. [$\alpha$-$^{32}$P]-dTTP (3000 Ci/mmol) was from New England Nuclear, and sodium heparin (170 USP units/mg) was from United States Biochemical Corp.

*Template·Primer Annealing.* Concentrations of poly(rA) and primers were estimated from the manufacturer's extinction coefficients. Lyophilized oligonucleotides were dissolved in 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA. Template·primers were mixed at the appropriate nucleotide ratios and heated to approximately 100 °C for 3 min and cooled slowly (3–4 h) to room temperature. Annealed T/Ps were stored at −20 °C.

*Reverse Transcriptase Assays.* Enzyme activities were determined using a standard reaction mixture (50 $\mu$L) containing 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 30 $\mu$M [$\alpha$-$^{32}$P]dTTP, and the indicated concentration of poly(rA)–$(dT)_{16}$ (expressed as 3′-hydroxyl primer termini). Reactions were initiated by addition of enzyme (expressed as concentration of dimer), incubated at 25 °C for 10 min, and stopped by the addition of 20 $\mu$L of 0.5 M EDTA, pH 8. Quenched reaction mixtures were spotted onto Whatman DE-81 filter disks and dried. Unincorporated [$\alpha$-$^{32}$P]dTTP was removed by four washes of 0.3 M ammonium formate, pH 8, followed by two washes of 95% ethanol and one wash of acetone. The dried filters were counted in 5 mL of RPI Bio-Safe II.

*Challenge Polymerase Assay.* A single processive cycle of deoxynucleotide incorporation by RT was measured by adding a polymerase trapping agent, heparin, with [$\alpha$-$^{32}$P]dTTP/$Mg^{2+}$ to a mixture of enzyme preincubated with poly(rA)–$(dT)_{16}$. Heparin binds free enzyme as well as enzyme which dissociates from T/P during the course of the reaction. Time courses were followed by adding 110 $\mu$L of $MgCl_2$, heparin, and [$\alpha$-$^{32}$P]dTTP to 110 $\mu$L of RT which had been preincubated with T/P for 10 min. At time intervals, 20 $\mu$L aliquots were removed from the mixture and quenched with 10 $\mu$L of 0.5 M EDTA, pH 8. Incorporation of radioactive dTMP was determined by spotting the quenched reaction mixtures on DE-81 filters as described in detail above. Final concentrations were 15 nM RT, 75 nM T/P, 30 $\mu$M dTTP, 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, and 1 mg/mL heparin.

To determine the concentration of RT bound to poly(rA)–$(dT)_{16}$, a 40 $\mu$L solution of 10 nM enzyme, expressed as the concentration of dimer, was preincubated with varying concentrations of poly(rA)–$(dT)_{16}$. After 10 min, the reaction was initiated with 10 $\mu$L of 5 mg/mL heparin and 150 $\mu$M [$\alpha$-$^{32}$P]dTTP. After an additional 10 min, the reaction was stopped by the addition of 20 $\mu$L of 0.5 M EDTA, and the amount of incorporation was determined as above. When 100 mM NaCl was included in the reaction mixture, the final heparin concentration had to be increased to 5 mg/mL to adequately compete with T/P for free RT.

Table I: Steady-State Kinetic Parameters for dTMP Incorporation on Poly(rA)–$(dT)_{16}$ with Different Forms of Recombinant HIV-1 RT[a]

| strain | form | $K_m$ (nM) | $k_{cat}$[b] ($s^{-1}$) | $k_{cat}/K_m$ ($\times 10^8$ $M^{-1}$ $s^{-1}$) |
|---|---|---|---|---|
| HXB2R | p66/p66 | 0.9 ± 0.2 | 0.18 ± 0.01 | 2 |
| | p66/p51 | 1.4 ± 0.2 | 0.28 ± 0.01 | 2 |
| NY5 | p66/p51 | 390 ± 10 | 0.38 ± 0.01 | 0.01 |

[a] Assays were performed as described under Materials and Methods. The template/primer nucleotide ratio, $(T/P)_{nt}$, was 10. [b] $k_{cat} = V_{max}/[RT]$.

*Dissociation Rate Constant.* To measure the dissociation rate constant of T/P from RT (i.e., $k_{off}$), dTTP/$Mg^{2+}$ was added at time intervals after heparin addition so as to follow the breakdown of the RT–T/P complex. Enzyme was preincubated with T/P for 10 min before challenging with heparin (zero time). At time intervals after adding challenge, 10 $\mu$L aliquots were removed and mixed with 10 $\mu$L of dTTP/$Mg^{2+}$ to determine the concentration of RT remaining bound to T/P as above. The final reaction conditions were 50 nM RT, 40–75 nM T/P (expressed as primer 3′ termini), 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 30 $\mu$M [$\alpha$-$^{32}$P]dTTP, and 1 mg/mL heparin. EDTA was added to stop the reaction as above.

*Association Rate Constant.* The binding of poly(rA)–$(dT)_{16}$ to homo- and heterodimer RT was measured by following the appearance of the RT–T/P complex. RT was mixed with T/P, and at time intervals after mixing, 10 $\mu$L aliquots were removed to determine the concentration of RT–T/P complex as described before. The final reaction conditions were identical to those in determining the dissociation rate constant. Additional details are given in the table and figure legends.

*Data Analysis.* Data were fitted to appropriate equations by nonlinear least-squares methods. The association rate constant for RT binding to poly(rA)–$(dT)_{16}$ was estimated with HopKINSIM, a Macintosh version of the kinetic simulation program KINSIM (Barshop et al., 1983) rewritten by Danny Wachsstock.

## RESULTS

*Steady-State Parameters.* The $K_m$ for poly(rA)–$(dT)_{16}$ is the same for both the homo- and heterodimeric forms of recombinant HXB2R RT, whereas $k_{cat}$ is slightly greater for the heterodimer (Table I). The $K_m$s are significantly lower than reported earlier for immunoaffinity-purified RT produced in H9 cells infected with HIV-1 strain B (di Marzo Veronese et al., 1986; Cheng et al., 1987; Majumdar et al., 1988). Recombinant heterodimeric RT derived from NY5 proviral RNA has been expressed in *E. coli* and purified (Huber et al., 1989). This recombinant RT has been reported to be kinetically similar to the enzyme purified from virus particles (Bebenek et al., 1989; Majumdar et al., 1989; Kedar et al., 1990). Reexamination of these steady-state parameters under the conditions used in this study indicated that the $K_m$ for

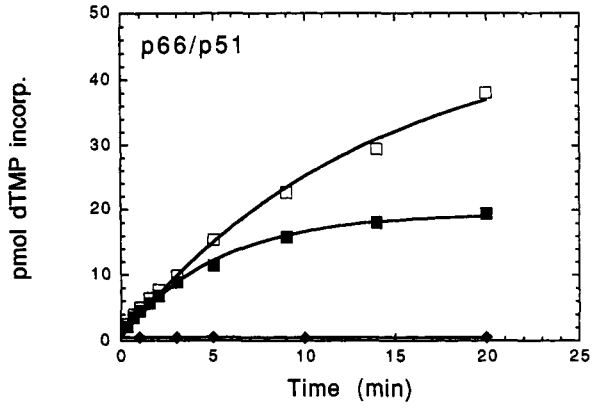


FIGURE 1: Effect of challenge on time course for dTMP incorporation with HXB2R HIV-1 RT. RT was preincubated with poly(rA)–$(dT)_{16}$ [template/primer nucleotide ratio of 10, $(T/P)_{nt}$ = 10] for 10 min. Time course initiated with $Mg^{2+}$/dTTP (□) or with $Mg^{2+}$/dTTP and heparin (■). Time course when heparin is included in the preincubation (♦). The final reaction mixture contained 15 nM heterodimeric HXB2R RT, 75 nM poly(rA)–$(dT)_{16}$ (expressed as primer 3′-termini), 30 μM dTTP, ±1 mg/mL heparin, 50 mM Tris-HCl (pH 7.4), and 10 mM $MgCl_2$. Data were fitted to a straight line (preincubation with heparin) or a single rising exponential correcting for the residual incorporation observed when heparin is included in the preincubation (challenged or unchallenged time courses). The amount of background incorporation with the heparin preincubation was equivalent to 0.0003 pmol of dTMP incorporated/min. The unchallenged and challenged time courses were described by the first-order rate constants 0.074 and 0.193 $min^{-1}$, respectively.

poly(rA)–$(dT)_{16}$ is significantly higher for NY5 than HXB2R RT, whereas $k_{cat}$s are similar (Table I). The consequence is a significantly lower apparent $k_{cat}/K_m$ for NY5 RT relative to HXB2R. It should be noted that the studies described here were done at 25 °C, whereas the earlier studies were performed at 37 °C. Increasing the assay temperature to 37 °C increased $k_{cat}$ for homodimeric HXB2R RT to 1.4 $s^{-1}$, similar to that reported for the viral enzyme (Majumdar et al., 1988), with only a 2-fold increase in $K_m$ (data not shown).

*Challenged Time Courses for Template·Primer Binding to HXB2R RT.* Equilibrium binding of RT and poly(rA)–$(dT)_{16}$ was examined with a challenge assay that has been used previously with *E. coli* Pol I (Bryant et al., 1983) and recombinant NY5 and LAV HIV-1 RTs (Huber et al., 1989; Reardon et al., 1991). RT preincubated with T/P results in RT–T/P complex formation. When heparin, a trapping agent that competes with T/P for free RT, and $Mg^{2+}$/[α-$^{32}$P]dTTP are added to this preincubation mixture, the RT–T/P complex binds dTTP and undergoes processive dTMP incorporation (Figure 1). Heparin binds free RT as well as enzyme dissociating from the T/P so that dTMP incorporation is limited to a single processive cycle. The initial rate of dTMP incorporation is the same in the absence or presence of trapping agent. The effectiveness of the challenge is demonstrated by including the trapping agent in the preincubation phase of the reaction. Under this condition, no incorporation of dTMP is observed. In the absence of challenger, the time course of dTMP incorporation is linear for approximately 10 min, but the rate of incorporation decreases at longer time periods. This decrease in velocity can be described by a simple exponential and probably represents enzyme terminating polymerization as a result of interference by a downstream primer. This is supported by the observations that the rate constant describing the decay in activity ($k_{termination}$) is independent of enzyme concentration, increases with decreasing gap size between primers, and is not observed when a heteropolymeric DNA template·primer with a long template region is used in a polymerization assay (data not shown). In some instances, denatured calf thymus DNA was used as a challenger. It, however, did not compete for free RT as effectively as heparin, as evidenced by the small amount of incorporation when enzyme was preincubated with calf thymus DNA [see Reardon et al. (1991)]. When time courses were corrected for "background" incorporation, they were the same as observed with heparin.

Table II: Comparison of Challenged Time Courses for Homo- and Heterodimeric Forms of Recombinant HXB2R HIV-1 RT[a]

| RT | $k_{obs}$[b] ($min^{-1}$) | $k_{off}^{pol}$[c] ($min^{-1}$) | $k_{cat}$[d] ($s^{-1}$) | $(N/E)$[e] |
|---|---|---|---|---|
| p66/p66 | 0.21 ± 0.02 | 0.12 | 0.20 | 100 |
| p66/p51 | 0.19 ± 0.01 | 0.12 | 0.20 | 100 |

[a] Assays were as described in Figure 1. [b] First-order rate constant describing the single-exponential decay of activity of a challenged time course. [c] T/P dissociation rate constant during catalytic cycling which reflects dissociation from the ternary enzyme complex with dTTP and T/P. This dissociation rate constant is calculated from the relationship $k_{off}^{pol} = k_{obs} - k_{termination}$, where $k_{termination}$ is determined from a first-order fit of an unchallenged time course. [d] $k_{cat} = v_i/[RT]$, where $v_i$ is the initial velocity determined from the velocity at $t = 0$ from the single-exponential fit of a challenged time course. [e] $(N/E) = k_{cat}/k_{off}^{pol}$.

As observed with *E. coli* Pol I (Bryant et al., 1983), the time course for processive polymerization can be described by a model where nucleotide incorporation results from competition between incorporation and dissociation of the extended primer from the RT–T/P complex (Figure 1). This model predicts that the time course should follow the relationship

$$N/E = (k_{cat}/k_{off}^{pol})(1 - e^{-k_{off}^{pol}t}) \quad (1)$$

where $N/E$ is the number of dTMP nucleotides incorporated per dimer of RT, $k_{cat}$ is the steady-state rate constant observed in the absence of trapping agent, and $k_{off}^{pol}$ is the dissociation rate constant for the RT–T/P complex during processive polymerization. However, as described above, there is an additional pathway resulting in termination. In this case, the time course would be predicted to follow

$$(N/E)_{obs} = (k_{cat}/k_{obs})(1 - e^{-k_{obs}t}) \quad (2)$$

where $k_{obs}$ is equivalent to $k_{termination} + k_{off}^{pol}$. After a sufficiently long time period (i.e., $t \gg 1/k_{obs}$), $(N/E)_{obs}$ represents the average number of nucleotides incorporated during a single processive cycle and is equivalent to the ratio of the competing pathways (i.e., incorporating vs nonincorporating), $k_{cat}/(k_{termination} + k_{off}^{pol})$. From eq 1, the "intrinsic" processivity is equivalent to $k_{cat}/k_{off}^{pol}$, where the nonincorporating pathway is now only the dissociation of T/P ($k_{off}^{pol}$) during catalytic cycling. These parameters for the challenged time courses with homo- and heterodimeric RT are tabulated in Table II. The "intrinsic" processivity ($N/E$) and the dissociation rate constant for T/P dissociation during processive incorporation ($k_{off}^{pol}$) were similar for the two forms of RT examined.

*Active-Site Titrations.* Incubation of RT with increasing concentrations of poly(rA)–$(dT)_{16}$ results in an apparent increase in the total incorporation of dTMP during a processive cycle of incorporation. When the T/P concentration exceeds the RT dimer concentration, the incorporation measured after a 10 min incubation in the presence of heparin does not increase further (Figure 2). Since the total incorporation is proportional to the concentration of RT–T/P complex formed in the preincubation phase of the reaction, the binding isotherm is characteristic of a tight binding ligand ($K_d \ll$ [RT]). An isotherm where $K_d$ = 1 nM is also illustrated in Figure 2. Tight T/P binding ($K_d$ < 1 nM) was observed for both forms of the RT which probably bind poly(rA)–$(dT)_{16}$ with similar

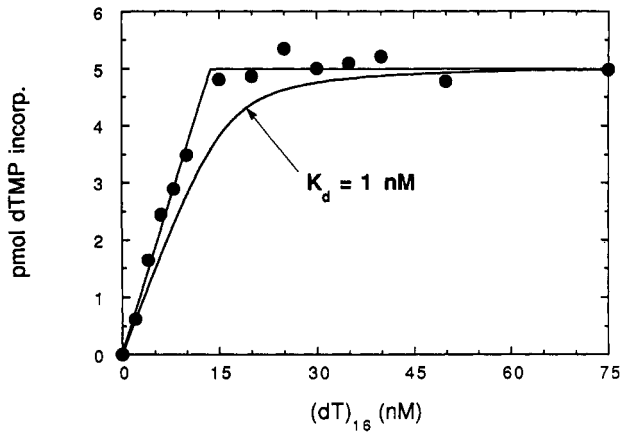


FIGURE 2: Active-site titration of homodimeric HIV-1 RT with template·primer. RT (8 nM) was preincubated for 10 min with poly(rA)–$(dT)_{16}$ at the indicated concentrations of 3′-primer termini, $(T/P)_{nt}$ = 2. The reaction was started with the addition of $Mg^{2+}$/dTTP and heparin and stopped after 10 min. Final reaction conditions were as described in Figure 1. Data were fitted to the quadratic binding equation to determine the template·primer binding site concentration assuming an infinitely small $K_d$. A theoretical binding isotherm where $K_d$ = 1 nM is also illustrated. The number of T/P binding sites per homodimer was 1.7, and the number of nucleotides incorporated per binding site ($N/nE$) was 9, where $n$ = (fit number of binding sites)/[$E$] and $E$ represents the concentration of dimer.

Table III: Stoichiometry of Binding of Poly(rA)–$(dT)_{16}$ to Homo- and Heterodimeric Forms of Recombinant HXB2R HIV-1 RT[a]

| RT | $(T/P)_{nt}$[b] | $n$[c] | 100 mM NaCl | $(N/nE)$[d] |
|---|---|---|---|---|
| p66/p66 | 2 | 1.8 ± 0.3 (3)[e] | – | 11 ± 4 |
| | 5 | 0.7 ± 0.2 (2) | – | 32 ± 4 |
| | 10 | 0.9 ± 0.2 (3) | + | 14 ± 3 |
| p66/p51 | 2 | 2.4 ± 0.4 (2) | – | 11 ± 1 |
| | 5 | 1.1 ± 0.1 (2) | + | 13 ± 2 |
| | 10 | 1.0 ± 0.1 (2) | – | 27 ± 1 |

[a] Reactions were as described under Materials and Methods. [b] The template/primer nucleotide ratio. [c] Number of T/P binding sites per RT dimer. [d] Number of nucleotides incorporated per binding site. [e] Number of independent determinations. Values represent the weighted average and the standard error.

affinities since $K_m$ for both forms with this T/P ≈ 1 nM (Table I).

Although binding of poly(rA)–$(dT)_{16}$ was too tight to get a reliable measure of the equilibrium dissociation constant, the tight binding facilitated active-site titrations. The number of T/P binding sites can be determined from the inflection point in the binding isotherm (Figure 2). When the template to primer nucleotide ratio, $(T/P)_{nt}$, was greater than 2, only a single T/P binding site per RT dimer (i.e., $n$ = 1) was determined (Table III). Addition of NaCl also did not significantly weaken binding or change the observed number of binding sites. When the $(T/P)_{nt}$ was lowered to 2 (i.e., the average single-stranded RNA template was 16 nts in length per primer), a second T/P binding site was measured for both homo- and heterodimeric RT (Figure 2 and Table III). This apparent second binding site for T/P was not involved in polymerization since $k_{cat}$ for RT on these substrates was unaffected (data not shown).

Reardon et al. (1991) have demonstrated by steady-state kinetic analysis that T/P binding is affected by the length of the primer. Short primers annealed to poly(rA), such as $p(dT)_{10}$, bound to RT much weaker than primers greater than 14 nts. To ascertain if the equilibrium dissociation constant for poly(rA)–$p(dT)_{10}$ is similar for the homo- and heterodimeric RTs, enzyme was preincubated with increasing

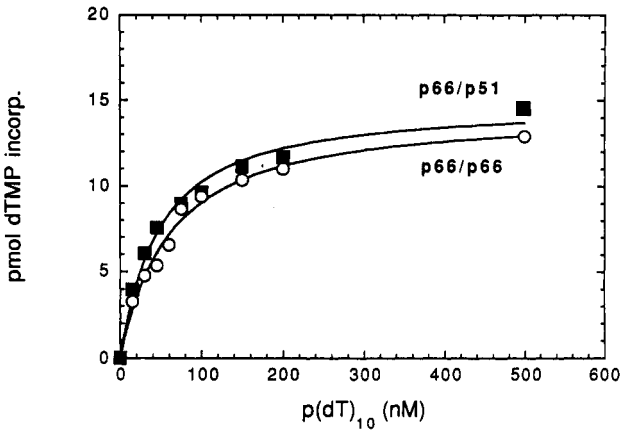


FIGURE 3: Dissociation constant for poly(rA)–$p(dT)_{10}$ and homo- and heterodimeric forms of HXB2R HIV-1 RT. RT (10 nM) was preincubated for 10 min with poly(rA)–$p(dT)_{10}$ at the indicated concentrations of 3′-primer termini, $(T/P)_{nt}$ = 10. The reaction was started with the addition of $Mg^{2+}$/dTTP and heparin and stopped after 10 min. Final reaction conditions were as described in Figure 1. Data were fitted to the quadratic binding equation. The best fit (solid line) of the data gave an apparent $K_d$ of 55 ± 7 and 40 ± 6 nM for homo- (○) and heterodimeric (■) RT, respectively.

concentrations of poly(rA)–$p(dT)_{10}$ and challenged to determine the concentration of RT–T/P complex formed during this preincubation phase. The results from these titrations are illustrated in Figure 3. The binding of poly(rA)–$p(dT)_{10}$ to RT is clearly much weaker than that observed for poly(rA)–$(dT)_{16}$ (Figure 2) and similar for both forms of RT (p66/p66, $K_d$ = 55 ± 7 nM; p66/p51, $K_d$ = 40 ± 6 nM).

*Dissociation of the RT/Poly(rA)–$(dT)_n$ Complex.* Since association rate constants are in many instances diffusion-controlled, the dissociation constant (i.e., $K_d$) is often primarily dependent on the rate constant for the breakdown of the complex (i.e., $k_{off}$). Examination of the dissociation rate constant for the RT–T/P complex is therefore a sensitive measure of the nature of the interactions occurring between RT and T/P. The breakdown of the RT–T/P complex can be followed by challenging the complex with heparin, to trap RT dissociating from T/P, and assaying for the concentration of complex remaining after increasing periods of challenge. If binding of RT to T/P is a simple equilibrium (RT + T/P ↔ RT–T/P), a monophasic exponential decrease in incorporation is expected, representing the dissociation rate constant ($k_{off}$). The time courses for the dissociation of p66/p51–T/P complexes are shown in Figure 4. The decay of these complexes is dependent on the length of the primer. The template with the shorter primer [i.e., $p(dT)_{10}$] dissociated much more rapidly than both $(dT)_{16}$ (±5′-phosphate) and $p(dT)_{20}$. In addition, the presence of a 5′-phosphate on $(dT)_{16}$ stabilized the complex further. Homodimeric RT exhibited the same dissociation rate constants with these T/Ps (Table IV).

The decay of the RT–T/P complex in the presence of a trapping agent has been reported to be biphasic with both homopolymeric (Huber et al., 1989) and heteropolymeric T/Ps (DeStefano et al., 1991). In these studies, there was a rapid decay of a population of this complex, which was too fast to measure, followed by a much slower decay. When RT was preincubated with poly(rA)–$p(dT)_{12-18}$, the decay of the RT–T/P complex was clearly biphasic, indicating that the heterogeneity of the primers resulted in complexes of differing stability (data not shown). During the course of this study, poly(rA)–$(dT)_{16}$ was observed to dissociate from T/P complexes with both homo- and heterodimeric RT in a monophasic

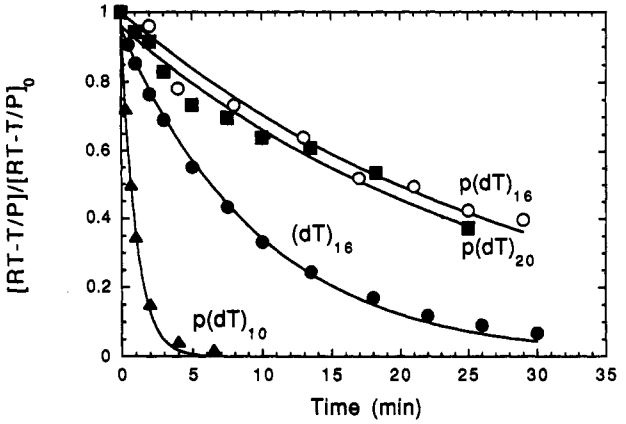


FIGURE 4: Dissociation of heterodimeric HIV-1 RT–template·primer complex. Heterodimeric RT was preincubated with poly(rA)–(dT)$_n$, (T/P)$_{nt}$ = 10, and at $t$ = 0, heparin was added. At the indicated times, $Mg^{2+}$/dTTP was added to determine the amount of RT–T/P complex remaining. The reaction was allowed to proceed for 10 min before being stopped with EDTA. The final reaction mixture was similar to that in Figure 1 except that the RT concentration was 50 nM and the poly(rA)–p(dT)$_{10,16,or20}$ (▲, ○, and ■, respectively) and (dT)$_{16}$ (●) concentrations (expressed as 3′-termini) were 62.5 (5′-PO$_4$) or 37.5 nM. Data were fitted to a single-exponential model ($I_t = Ae^{-kt}$) where $I_t$ = picomoles of dTMP incorporated at time $t$, $A$ = amplitude, and $k$ = apparent first-order rate constant. Best-fit parameters are tabulated in Table IV.

Table IV: Association and Dissociation Rate Constants for Homo- and Heterodimeric Forms of HXB2R HIV-1 RT Complexed with Template·Primer[a]

| | $k_{off}$ (min$^{-1}$) | | $k_{on}$ (×10$^6$ M$^{-1}$ s$^{-1}$)[b] | |
|---|---|---|---|---|
| template·primer | p66/p66 | p66/p51 | p66/p66 | p66/p51 |
| poly(rA)–p(dT)$_{10}$ | 1.23 ± 0.10 | 1.01 ± 0.04 | 0.4 | 0.4 |
| poly(rA)–p(dT)$_{16}$ | 0.038 ± 0.001 | 0.035 ± 0.003 | >0.6 | >0.6 |
| poly(rA)–p(dT)$_{20}$ | 0.060 ± 0.002 | 0.037 ± 0.003 | >1.0 | >0.6 |
| poly(rA)–(dT)$_{16}$ | 0.125 ± 0.005 | 0.103 ± 0.003 | >2.1 | >1.7 |

[a] Determined as outlined under Materials and Methods and in Figures 4 and 5. [b] Calculated assuming that $K_d = k_{off}/k_{on}$ = 55 or 40 nM for p(dT)$_{10}$ binding with homo- and heterodimer RT, respectively. Other values calculated assuming $K_d$ < 1 nM.

as well as in a biphasic manner. Greater than 98% of the primers were of the correct length as determined by polyacrylamide gel electrophoresis. When the dissociation of the RT–T/P complex was observed to be biphasic, the two phases were often not well separated. Figure 5 illustrates that a two-exponential decay is superior to a single-exponential model describing the dissociation of T/P from RT for these data. However, when a biphasic decay was observed, a monophasic dissociation could be "induced" in a parallel assay if the (T/P)$_{nt}$ was diminished to 2 (i.e., average length of the single-stranded RNA template is 16 nts) and where [T/P] < [RT] (Table V). Again, both homo- and heterodimeric RTs responded similarly to this change in (T/P)$_{nt}$.

*Rate of Association of RT and Poly(rA)–(dT)$_{16}$.* RT–T/P complex formation can be followed by adding [$\alpha$-$^{32}$P]-dTTP/heparin at time intervals after mixing RT and T/P. As RT binds T/P, forming RT–T/P complex, there will be less free RT available to bind heparin, and therefore an increase in incorporation with time of incubation is expected. When 100 nM RT is rapidly mixed with a solution of 50 nM poly(rA)–(dT)$_{16}$ (expressed as primer 3′-termini), there is a rapid formation of RT–T/P complex as represented by near-maximum incorporation at the earliest sampling time (Figure 6). The association rate constant ($k_{on}$) was estimated by modeling a simple bimolecular reaction to these conditions.

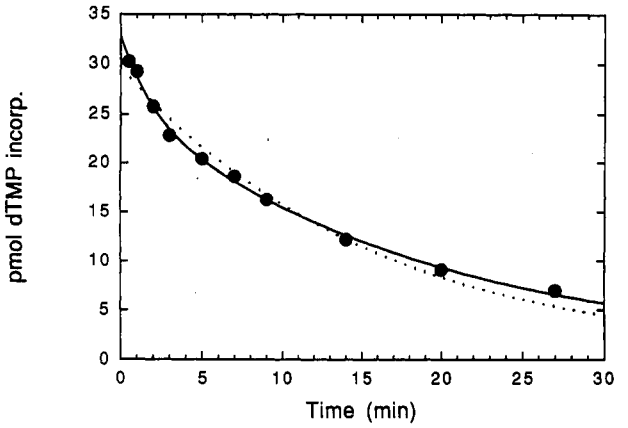


FIGURE 5: Biphasic nature of the dissociation of p66/p66 RT–template·primer complex. The final reaction mixture was similar to that in Figure 4 except that the T/P concentration was 75 nM. Data were fitted to a single-exponential model ($I_t = A_1e^{-k_1t}$, dotted line) or a two-exponential model ($I_t = A_1e^{-k_1t} + A_2e^{-k_2t}$, solid line) where $I_t$ = picomoles of dTMP incorporated at time $t$, $A$ = amplitude, and $k$ = apparent first-order rate constant. Best-fit parameters are tabulated in Table IV.

Table V: Effect of Single-Stranded RNA on the Dissociation Rate Constants Measured for Poly(rA)–(dT)$_{16}$ with Recombinant Forms of HIV-1 RT[a]

| form | (T/P)$_{nt}$ | $k_1$ (min$^{-1}$) | $k_2$ (min$^{-1}$) | $A_1/A_T$[b] | $k_{on}$[c] (×10$^6$ M$^{-1}$ s$^{-1}$) |
|---|---|---|---|---|---|
| p66/p66 | 2 | 0.109 ± 0.003 | ND[d] | 1.00 | >1.8 |
| | 10 | 0.513 ± 0.179 | 0.050 ± 0.004 | 0.23 | |
| p66/p51 | 2 | 0.115 ± 0.004 | ND | 1.00 | >1.9 |
| | 10 | 0.500 ± 0.081 | 0.130 ± 0.024 | 0.56 | |

[a] Determined as outlined under Materials and Methods and Figures 4 and 5. The enzyme and T/P concentrations were 100 and 75 nM, respectively. [b] Proportion of the amplitude in the first-exponential phase. [c] Calculated assuming that $K_d = k_{off}/k_{on}$ < 1 nM. [d] Not detected.

The dissociation rate constant was fixed at 0.002 s$^{-1}$ as determined earlier (Table IV). In addition, it was assumed that only the dimeric form of RT binds T/P. Therefore, the stock concentration of RT dimer was calculated from the respective binding constants for dimerization of p66 and p51 (Becerra et al., 1991). It was also assumed that the dimer would not dissociate upon dilution into monomers over the course of the reaction (3 min). The association rate constant was then estimated by adjusting this parameter until a good visual fit to the experimental data was obtained. The number of nucleotides incorporated after a binding event, $(N/E)_{obs}$, was determined from the maximum observed incorporation per estimated RT–T/P concentration. The best-fit for $k_{on}$ for homodimeric RT was 3 × 10$^6$ M$^{-1}$ s$^{-1}$ (Figure 6, solid line). Other estimates for $k_{on}$ are also included in Figure 6 to illustrate the sensitivity of the predicted time courses to changes in this parameter. Decreasing or increasing $k_{off}$ by a factor of 2 does not alter the quality of the fit. Varying the RT dimer concentration by similar factors changes the best-fit for $k_{on}$ by no more than a factor of 2. From three independent determinations, $k_{on}$ was estimated to be (2 ± 1) × 10$^6$ and (5 ± 3) × 10$^6$ M$^{-1}$ s$^{-1}$ for p66/p66 and p66/p51 binding to poly(rA)–(dT)$_{16}$, respectively. When the T/P concentration was increased, an additional slow exponential increase in incorporation was observed, probably reflecting the dimerization of monomers. However, the rate constant for this slow phase was dependent on T/P concentration, indicating that this explanation is oversimplified.

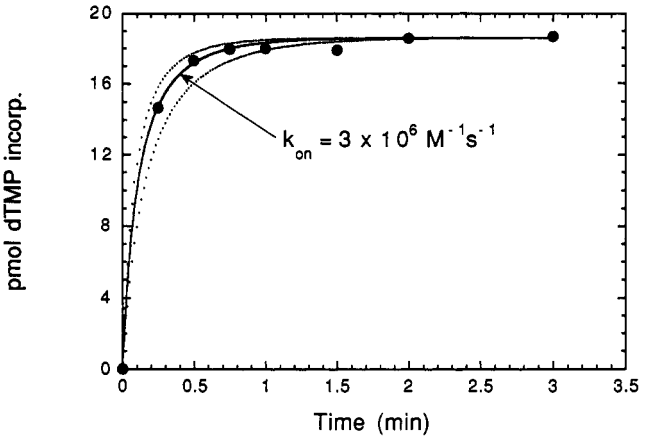


FIGURE 6: Association of homodimeric RT and poly(rA)–$(dT)_{16}$. RT (p66/p66) was rapidly added to a mixture with poly(rA)–$(dT)_{16}$, and at time intervals after mixing, aliquots were removed and added to a solution of heparin and $Mg^{2+}$/dTTP to determine the concentration of RT–T/P complex. After an additional 10 min, the reaction was stopped with EDTA, and dTMP incorporation was determined as described under Materials and Methods. The association rate constant ($k_{on}$) was estimated by simulating a simple bimolecular reaction where $k_{off}$ was fixed at 0.002 $s^{-1}$ as determined before (Table IV). The best visual fit (solid line) of the data resulted in a $k_{on}$ for poly(rA)–$(dT)_{16}$ binding to p66/p66 of 3 × $10^6$ $M^{-1}$ $s^{-1}$ and a $(N/E)_{obs}$ of 52. Theoretical time courses predicted for association rate constants of 2 × $10^6$ and 4 × $10^6$ $M^{-1}$ $s^{-1}$ are included to illustrate the sensitivity of the time courses to changes in $k_{on}$ (dotted lines). Additional details are given under Materials and Methods.

## DISCUSSION

*Polymerase Challenge Assay.* We have measured a number of parameters describing quantitatively poly(rA)–$(dT)_n$ interactions with homo- and heterodimeric HXB2R HIV-1 RT. A T/P challenge assay was used to determine RT–T/P complex formation (Figures 1 and 2). Free RT is effectively quenched in the presence of the challenger (Figure 1), whereas RT that has productively bound T/P can undergo a single processive cycle of incorporation before dissociating from the extended primer and becoming trapped by the heparin challenge. The challenge assay has several advantages. First, the use of homopolymeric templates requires only a single dNTP, thus eliminating any effects of competing nucleotides (West et al., 1992), and such templates are widely available and inexpensive. These attributes have made poly(rA)–$(dT)_n$ a commonly used T/P in the past, and the continued use in assays to measure specific steps in the reaction pathway permits comparison of results from numerous laboratories employing a variety of enzymes and techniques. The challenge assay described here has been used before to measure T/P interactions with *E. coli* Pol I (Bryant et al., 1983), as well as HIV-1 RT (Huber et al., 1989; Reardon et al., 1991). It is a rapid and simple assay which takes advantage of the processive nature of these polymerases to amplify the signal for a polymerase–T/P interaction. For every T/P binding event (association and dissociation), there are numerous incorporations of radioactive dNMP that can be routinely measured. Second, the use of a homopolymeric T/P eliminates the effect that a heterogeneous T/P sequence might have on kinetic constants. It has been noted previously that the apparent dissociation rate constant for a heteropolymeric DNA/RNA hybrid–RT complex can vary nearly an order of magnitude, depending on the nucleotide sequence (Reardon, 1992). Further, the characteristic pattern of termination sites observed with HIV-1 RT using a M13 DNA template (Bebenek et al., 1989; Huber et al., 1989; Abbotts et al., 1993) or a heteropolymeric RNA sequence (Huber et al., 1989) indicates that the heteropolymeric nucleotide sequence influences the kinetic constants for RT and T/P interactions. Recently, Kohlstaedt and Steitz (1992) demonstrated that template sequence 3′ to the primer can also influence incorporation.

Patel et al. (1991) have criticized the use of homopolymers for quantitative studies with DNA polymerases due to potential stacking problems of the short primers on the template. Although cooperative binding of oligo(dT) to a poly(dA) template was observed by Olivera and Lehman (1968), stacking did not occur with a poly(rA) template. More recently, Mesner and Hockensmith (1992) have examined the stacking of oligo(dT) on poly(dA) and poly(rA) templates. Using laser UV cross-linking to probe for stacking of dT primers, they demonstrated that at temperatures normally used to measure RNA-dependent polymerization, there was no evidence for cooperative binding of oligo(dT) to poly(rA). Additionally, in the present study, RT and poly(rA)–$(dT)_{16}$ binding demonstrates a 1:1 stoichiometry in our assays (Figures 2 and Table II). If oligo(dT) molecules were stacking on the template, the titrations would grossly overestimate the RT concentration. Instead, our measurement perfectly coincided with the RT dimer concentration in the reaction mixture, as determined by gravimetric methods. This homopolymeric substrate is therefore ideal to study RT and T/P interactions, as well as to screen mutants to identify protein–nucleic acid interactions.

*Comparison of T/P Interactions with Homo- (p66/p66) and Heterodimeric (p66/p51) RT.* Much of the enzymology, especially site-directed mutagenesis, with HIV RT has been done with the homodimeric form. It is especially important that the two forms are characterized kinetically before the homodimeric form can be considered a model to do basic kinetic or structure–function studies. We found that the homo- and heterodimeric forms of HXB2R HIV-1 RT interact with poly(rA)–$(dT)_{16}$ in a kinetically similar fashion. Between these two forms of RT, there was no more than a 2-fold difference for any T/P parameter examined ($K_m$, $K_d$, $k_{on}$, $k_{off}$, $k_{off}^{pol}$, processivity, and stoichiometry of T/P binding) or in $k_{cat}$. This suggests that the C-terminus of one of the p66 subunits of the homodimer does not make significant T/P interactions, and does not have a major influence on the rate of incorporation. The homodimeric form of the RT can therefore serve as a model for heterodimeric RT interactions with T/P.

*Kinetic Diversity among HIV-1 RT Derived from Different Viral Strains.* Although HIV viral genetic diversity is well recognized, it has generally been assumed that RTs derived from HIV-1 are kinetically, as well as structurally, similar. Small structural changes, however, may be accompanied by important kinetic and functional changes. There is no clear pattern in the literature for the kinetic behavior observed for homo- and heterodimer forms of RT. When the activities of these two forms of RT were compared, it was reported that the heterodimer is more active (Lowe et al., 1988; Le Grice & Gruninger-Leitch, 1990), is less active (Anderson & Coleman, 1992), or has similar activity (Restle et al., 1990) as the homodimeric form. These discrepancies are probably not only the result of the different conditions and assays used to determine activity, but also may reflect different enzyme sources. Whereas the $k_{cat}$ for HXB2R heterodimeric RT was slightly greater than that measured with homodimer, the $K_m$s for poly(rA)–$(dT)_{16}$ were identical (Table I). The steady-state parameters under the conditions used in this study indicated that the $K_m$ for poly(rA)–$(dT)_{16}$ is significantly

higher for NY5 than HXB2R RT (Table I). The measured $K_m$ for NY5 heterodimer is similar to that reported for immunoaffinity-purified viral RT (di Marzo Veronese et al., 1986; Cheng et al., 1987; Majumdar et al., 1988). The $K_m$ for poly(rA)–$(dT)_n$ has been reported to range from 4 to 60 nM for other recombinant sources of HIV RT (Reardon & Miller, 1990; Dudding et al., 1991; Krug & Berger, 1991). Although these various studies were done under slightly different conditions, the results here demonstrate that there may be differences in kinetic interactions between substrates and RTs derived from different viral strains. Our results suggest caution in extrapolation of results from enzymes derived from different strains.

NY5 heterodimer has 26 amino acid residues which are different from the corresponding residues in HXB2R (Myers et al., 1989). Chemical modification (Mitchell & Cooperman, 1992) and UV cross-linking studies (Sobol et al., 1991; Basu et al., 1992) have suggested that side chains within a region from residues 195 to 300 of p66 make primer or T/P contacts. Within this region of primary sequence, there are three differences per subunit between HXB2R and NY5 RT with a dramatic difference being a proline or alanine occurring at position 272 with HXB2R and NY5 RT, respectively.

*T/P Binding to HXB2R RT.* Reardon et al. (1991) reported that the $K_d$ for T/P, as determined by steady-state analysis, was dependent on the length of the primer. Short oligo(dT) primers (≤14 nts), annealed to poly(rA), bound RT relatively weakly ($K_d$ = 20–30 nM), whereas oligo(dT) primers of 16 and 20 nts in length bound tightly to RT ($K_d \approx 0.1$ nM). Our findings are in accord with these earlier findings (Figures 2 and 3). Additionally, Müller et al. (1991) reported a $K_d$ of 0.35 nM for poly(rA)–$(dT)_{15}$ binding to BH10 HIV RT, whereas Huber et al. (1989) reported a $K_d$ of 3 nM for poly(rA)–$(dT)_{20}$ binding with NY5 HIV RT using a challenge assay. These affinities are much greater than those reported for $p(dT)_{15}$ or $p(dT)_{16}$ in the absence of template, $K_d$ = 300–1500 nM (Painter et al., 1991; Sobol et al., 1991; Basu et al., 1992). The presence of the template therefore increases the primer affinity for HIV RT dramatically. These $K_d$s for T/Ps are also considerably lower than those reported for $tRNA^{Lys,3}$, the natural primer for minus strand DNA synthesis, $K_d$ = 80–300 nM (Sobol et al., 1991; Andreola et al., 1992; Kohlstaedt & Steitz, 1992).

The dissociation rate constant is a sensitive parameter to changes in affinity of an enzyme for a ligand, and direct measurement can reveal multiple conformations of the ligand–enzyme complex in some instances. In contrast to Reardon et al. (1991), we find that dissociation of the RT–T/P complex is much more rapid when the primer is $p(dT)_{10}$ than when it is $p(dT)_{20}$ (Figure 4 and Table IV). Reardon et al. (1991) did not directly determine $k_{off}$, but chose to measure $k_{off}^{pol}$. Although we find that $k_{off}^{pol}$ approximates the dissociation rate constant for the T/P in a binary complex (Tables II and IV), the lack of a difference between these two primers with this assay probably is the result of rapid extension of the shorter primer leading to an observed dissociation rate constant similar to that of a primer of longer length. It should be noted, however, that $k_{off}$ and $k_{off}^{pol}$ would be expected to measure the dissociation rate constant of T/P from the binary complex and ternary complex with dTTP, respectively.

It has typically been assumed that T/P and RT are in a simple equilibrium (Reardon, 1992; Kati et al., 1992), so that knowing any two of the binding parameters will allow one to calculate the remaining one (i.e., $K_d = k_{off}/k_{on}$). Previous studies examining the dissociation rate constant of NY5 RT from homopolymeric T/P (Huber et al., 1989) or heteropolymeric RNA template primed with DNA (DeStefano et al., 1991) indicated the dissociation was biphasic with a substantial rapid phase that was too fast to measure. These results suggest that there are situations where the assumption of a simple equilibrium is not justified. Therefore, it is important to demonstrate the validity of a "simple equilibrium" assumption for T/P binding to RT. The only way to definitively demonstrate this is to compare the equilibrium dissociation constant with the ratio of the dissociation and association rate constants ($k_{off}/k_{on}$). Since equilibrium binding between RT and poly(rA)–$(dT)_{16}$ was too tight to measure reliably ($K_d$ < 1 nM), we were unable to compare the equilibrium dissociation constant with the dissociation constant calculated from the molecular rate constants ($k_{off}/k_{on} \approx 0.6$ nM). However, the biphasic character of the dissociation rate constant suggests that multiple RT–T/P conformations exist and that the equilibrium measurements sample all populations.

In contrast to the dissociation of T/P from NY5 RT (Huber et al., 1989), two well-defined exponential decays of the poly(rA)–$(dT)_{16}$/RT complex were not observed with either homo- or heterodimeric HXB2R RT. The decays, however, could often be better fitted to a double-exponential decay (Figure 5). The multiphasic nature of the dissociation precludes a simple calculation of the association rate constant ($k_{on}$) from the equilibrium $K_d$ and $k_{off}$. Yet, as the two phases decayed with similar half-lives for HXB2R RT, an estimate of $k_{on}$ can be calculated (Table IV). These values are in reasonable agreement with those determined directly (see below).

Although these multiple conformations may be of little kinetic relevance with HXB2R HIV-1 RT, the work of Huber et al. (1989) suggests that with recombinant enzyme from the NY5 strain, two distinct populations of RT exist which bind to T/P differently. These results also indicate that kinetic differences exist between these two recombinant sources of RT as noted above. Kati et al. (1992) have reported that the dissociation of heteropolymeric DNA from recombinant RT, derived from BH10 proviral DNA, is a single exponential, suggesting a single form of their preparation of RT binds T/P productively. Active-site titrations, however, indicated only 50% of their enzyme preparation was active.

It is possible that the multiple decays noted above reflect a post-T/P binding isomerization of the RT–T/P complex:

$$RT + T/P \leftrightarrow RT\text{–}T/P \leftrightarrow RT^*\text{–}T/P$$

Rapid kinetic approaches to measure initial complex formation may help answer this question. It should be pointed out that the challenge assay monitors binding of the T/P to the polymerase active site. Isomerization of the RT–T/P may reflect different binding modes to other portions of the T/P binding site (e.g., RNase H active site). Our observation of the absence of two phases when the primers are spaced close together suggests stabilization of one form under this condition (Table V).

The association rate constant was determined by assuming that only the dimeric form of RT can bind T/P. This was calculated from the $K_a$ for binding of the individual polypeptides (Becerra et al., 1991). Together with the dissociation rate constant, the association rate constant was determined from simulated time courses, $k_{on} \approx (2\text{–}5) \times 10^6\ M^{-1}\ s^{-1}$ (Figure 6). These values are significantly lower than the apparent $k_{cat}/K_m$ values determined under processive polymerizing conditions (Table I). This is due to the nature of the reaction where the T/P need only bind to RT one time in a processive cycle where multiple incorporations will occur before it will dissociate. This will artificially increase the apparent $k_{cat}$/

$K_m$ by approximately $N/E$ [$k_{on} \approx (k_{cat}/K_m)/(N/E) \approx 2.0 \times 10^6$ $M^{-1}$ $s^{-1}$; Tables I and II].

The quantitative characterization of homopolymeric poly(rA)–$(dT)_n$ and RT interaction also allows for comparison with different substrates such as heteropolymeric T/Ps. This is particularly relevant with regard to the rapid kinetic study by Reardon (1992) which used similar assay conditions to ours, as well as the same viral strain of recombinant RT. Whereas the measured association rate constants are similar, the dissociation rate constant determined with poly(rA)–$(dT)_{16}$ ($k_{off} \approx 0.002$ $s^{-1}$) is at least an order of magnitude lower than with heteropolymeric RNA/DNA T/P ($k_{off} = 0.04$ $s^{-1}$). This is consistent with the lower equilibrium dissociation constant determined with the homopolymeric T/P. Kati et al. (1992) have recently reported kinetic constants for RT binding with heteropolymeric DNA/DNA T/P. Using a DNA template with a different sequence, recombinant source of RT, and assay temperature (37 °C), the association rate constant was calculated to be $4.3 \times 10^7$ $M^{-1}$ $s^{-1}$, an order of magnitude greater than that measured by Reardon (1992).

*Stoichiometry of T/P Binding Sites.* The equilibrium dissociation constant for poly(rA)–$(dT)_{16}$ was too low to measure reliably ($K_d < 1$ nM, Figure 2). This tight binding, however, was useful in determining the number of T/P binding sites. When the single-stranded template (i.e., rA) region was $\gg$16 nts/primer, a single T/P binding site per dimer was measured (Table III). When the T/P nt ratio was diminished to 2 (i.e., the single-stranded template was on average 16 nts/primer), the number of T/P binding sites increased to 2 per dimer. This "new" T/P binding site does not represent a polymerase catalytic site since $k_{cat}$ is not affected by reducing the length of the single-stranded RNA template. The ability of RT, under some circumstances, to bind an additional template may play a role in both of the strand transfer reactions that are required for double-stranded proviral DNA synthesis (Goff, 1990). Peliska and Benkovic (1992) have recently demonstrated that strand transfer may proceed through an RNA/DNA/RNA–RT intermediate. Alternatively, RT binding to T/P may sterically exclude binding to a primer that is only 16 nts away. Crystallographic (Kohlstaedt et al., 1992) as well as neutron-scattering (Lederer et al., 1992) studies have indicated that the heterodimer is elongated with the p66 subunit having the dimensions of 110 by 30 by 45 Å (Kohlstaedt et al., 1992).

*Dimerization Constant for Subunit Association Is Influenced by the Binding of T/P.* The dissociation constant for subunit association would predict that both homo- and heterodimer would be primarily monomeric at the concentrations of enzyme used in these assays, as well as at the concentrations that are typically used for steady-state measurements (Becerra et al., 1991). Since it is generally believed that RT is active as a dimer, the subunit association constant is a critical parameter in defining the activity of a preparation of RT. However, establishment of equilibrium after dilution of enzyme occurs slowly so that the dimer concentration, in the absence of substrates, should be calculated from the stock concentration of enzyme and not from the assay concentration. The subunit association constant for HXB2R RT has been determined by analytical ultracentrifugation (Becerra et al., 1991) and for BH10 RT by gel filtration HPLC (Restle et al., 1990). The association constants for heterodimer formation for these different recombinant forms differ by over 3 orders of magnitude. It remains to be determined if this is also due to the source of enzyme or the techniques employed. Active-site titrations with HXB2R RT indicated that in the presence of T/P, enzyme is completely dimeric (Reardon, 1992; and Table III) although only 40–50% of the total enzyme concentration would be dimeric as calculated from the stock concentrations of apoenzyme used in our studies. In contrast, active-site titrations with enzyme derived from BH10 proviral DNA, but which differed slightly from the recombinant enzyme used in the Restle study due to differences in plasmid construction, indicated that only 50% of the RT was active (Kati et al., 1992). The apparently low active-site titration in the presence of high concentrations of T/P suggests that the inactive enzyme in their preparation was not due to a population of monomeric enzyme.

In attempting to measure the association rate constant of homopolymeric T/P to RT, it was necessary to keep the concentration of T/P less than the dimeric concentration of enzyme determined from the stock concentrations. When the T/P concentration was greater than the dimer concentration of RT, an additional exponential increase of incorporation was observed probably reflecting the association of monomers. Andreola et al. (1992) have measured two apparent equilibrium binding sites for tRNA with an preparation of p66 enzyme by fluorometric titration. Their results are also consistent with a nucleic acid induced dimerization of p66 with a concomitant increase in activity.

*Concluding Remark.* The RTs from AZT-sensitive and -resistant virus strains have been shown to be similar by steady-state kinetic characterization (Lacey et al., 1992). Examination of T/P interactions with viral AZT-resistant RT mutants is suggested by the observation that these mutations map to the putative template binding site of RT (Kohlstaedt et al., 1992). The results reported here can now be used to compare HIV-1 RTs from other sources, as well as RTs altered by site-directed mutagenesis to eventually determine structural and functional relationships.

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