Pre-Steady-State Kinetic Characterization of Wild Type and 3′-Azido-3′-deoxythymidine (AZT) Resistant Human Immunodeficiency Virus Type 1 Reverse Transcriptase: Implication of RNA Directed DNA Polymerization in the Mechanism of AZT Resistance†

Stephen G. Kerr‡ and Karen S. Anderson*

Department of Pharmacology, 333 Cedar Street, Yale University School of Medicine, New Haven, Connecticut 06520-8066

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ABSTRACT: There is lack of a correlation between biochemical studies and the observed clinical resistance of AIDS patients on long term AZT therapy. Mutant HIV-1 reverse transcriptase in the viral isolates from these patients shows a 100-fold decrease in sensitivity whereas little or no difference is observed in kinetic parameters *in vitro* using steady-state kinetic analysis. A detailed pre-steady-state kinetic analysis of wild type and the clinically important AZT resistant mutant (D67N, K70R, T215Y, K219Q) HIV-1 reverse transcriptase was conducted to understand the mechanistic basis of drug resistance. In contrast to steady-state techniques, a pre-steady-state kinetic analysis allows for the direct observation of catalytic events occurring at the active site of the enzyme, including subtle conformational changes enabling a greater degree of mechanistic detail. In this investigation the rate of incorporation of dTMP and AZTMP by wild type and mutant HIV-1 RT was determined using an RNA and the corresponding DNA template. The present study has shown a 1.5-fold decrease in the rate constant for polymerization (k_{pol}) and a 2.5-fold decrease in the equilibrium dissociation constant (K_d) for AZTTP for the mutant reverse transcriptase as compared to the wild type, for RNA dependent DNA replication. These values translate into a 4-fold decrease in selectivity (k_{pol}/K_d) for AZTMP incorporation by mutant reverse transcriptase as compared to wild type for RNA dependent DNA replication. No such decrease in selectivity was detected for DNA dependent replication. These results suggest that the basis of AZT resistance is related to RNA dependent replication rather than DNA dependent replication.

The treatment of acquired immunodeficiency syndrome (AIDS) patients with nucleoside analogs has been the method of choice to slow the progression of the disease. Nucleoside analogs are targeted against reverse transcriptase (RT), the retroviral polymerase of the human immunodeficiency virus type 1 (Rey et al., 1984; Hoffman et al., 1985), the causative agent of AIDS (Barre-Sinoussi et al., 1983; Popovic et al., 1984). The thymidine analog 3′-azido-3′-deoxythymidine (AZT, Zidovudine, Retrovir) was the first drug approved for the treatment of AIDS (Mitsuya et al., 1985). In the cell, AZT is converted to its triphosphate form by cellular kinases (Furman et al., 1986) where it competes with thymidine triphosphate for uptake by RT for incorporation into DNA (St. Clair et al., 1987). Once incorporated into DNA it brings about chain termination due to its lack of a 3′-hydroxy group (Furman et al., 1986; St. Clair et al., 1987; Goody et al., 1991). Although AZT is a potent inhibitor of HIV-1, its long term usage results in a virus that is resistant to the drug, thus limiting the therapeutic potential (Larder et al., 1989; Richman, 1993). The basis of this resistance has been mapped to mutations in the viral *pol* gene that encodes for RT (Larder and Kemp, 1989). These AZT resistant mutants of RT possess five amino acid substitutions including M41L, D67N, K70R, T215Y or F, and K219Q (Larder and Kemp, 1989; Larder et al., 1991; Kellam et al., 1992; Larder, 1994). Viruses having the quadruple mutant of RT (D67N, K70R, T215Y or F, and K219Q) have been shown to be more than 100-fold less sensitive to AZT than wild type virus, in cell culture studies (Kellam et al., 1992). Steady-state kinetic studies using the wild type and the quadruple mutant form of HIV-1 RT (homodimeric p66/p66) showed little difference in kinetic parameters for the mutant RT as compared to the wild type enzyme (Lacey et al., 1992). A more recent study with the above quadruple mutant RT (heterodimeric p66/p51, D67N, K70R, T215Y, and K219Q) and AZT triphosphate (Carroll et al., 1994) determined a ratio of incorporation of AZT monophosphate by the resistant RT to be 0.77 times that of wild type RT. An examination of a single processive cycle of RNA dependent DNA polymerization has observed differences in wild type and AZT resistant mutant which may impart advantages for viral replication (Caliendo et al., 1996). It should be noted that these results with recombinant RT were determined using

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* Author to whom correspondence should be addressed. Telephone: (203)-785-4526. Fax: (203)-785-7670. email: karen.anderson@yale.edu.

‡ Present address: Massachusetts College of Pharmacy & Allied Health Sciences, 179 Longwood Ave., Boston, MA 02115.

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[1] Abbreviations used: AIDS, acquired immunodeficiency syndrome; AZT, 3′-azido-3′-deoxythymidine; AZTDP, AZT 5′-diphosphate; AZTMP, AZT 5′-monophosphate; AZTTP, AZT 5′-triphosphate; dAMP, 2′-deoxyadenosine 5′-monophosphate; dATP, 2′-deoxyadenosine 5′-triphosphate; dNMP, 2′-deoxynucleoside 5′-monophosphate; dNTP, 2′-deoxynucleoside 5′-triphosphate; dTMP, 2′-deoxythymidine 5′-monophosphate; dTTP, 2′-deoxythymidine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid; HIV-1, human immunodeficiency virus type 1; MT, mutant or AZT resistant; RT, reverse transcriptase; Tris, tris(hydroxymethyl)aminomethane; WT, wild type.

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steady-state kinetic analysis. Previous mechanistic studies on RT have shown that the rate-determining step in the overall enzymatic pathway is the final dissociation of product from the enzyme (Reardon & Miller, 1990; Reardon, 1993; Kati et al., 1992; Hsieh et al., 1993). Thus K_i or K_m and k_{cat} values obtained from such investigations are a complex combination of the kinetic parameters from the overall kinetic pathway and provide limited information about events occurring at the active site of the protein (Johnson, 1992). For this reason, a comparative study of AZT resistant (mutant) and wild type enzyme, subtle changes in dissociation constants (K_ds), and/or polymerase rate constants (k_{pol}s) that are occurring at the active site may be difficult to observe using a steady-state analysis. Thus a transient (pre-steady-state) kinetic analysis in which the individual steps in the overall pathway can be directly observed would provide a more detailed analysis of the events occurring at the active site of RT and allow more subtle changes in the reaction pathway for wild type and mutant proteins to be detected.

The crystal structure of wild type RT (Kohlstaedt et al., 1992; Jacobo-Molina et al., 1993) indicates that the mutations developed in RT with the nucleotide inhibitors (including the AZT resistant RT) reside in the polymerase subunit of the heterodimeric (p66/p51) protein, in a domain designated as the "fingers". It has been suggested that these mutated residues may interact with the template strand of the template−primer substrate for RT. On the basis of our model of an induced fit mechanism for RT (Kati et al., 1992; Kerr & Anderson, 1997), it is conceivable that the mutated residues in the fingers domain of RT might affect the transition of the ternary complex, (enzyme−template/primer−AZTTP), on going from an "open" to a "closed activated" state. It has been suggested that the mechanistic basis of some nucleoside analog resistance may be related to template−primer repositioning or altered conformation of the ternary complex (Boyer et al., 1994; Tantillo et al., 1994). Thus changes or variations in the activated ternary complex may be manifested in terms of alterations in nucleotide dissociation constant, K_d, polymerase rate, k_{pol}, or the nature and kinetics of RNAse H cleavage. These effects, especially if subtle, may be obscured in a steady-state analysis but would be discernible under a transient kinetic analysis. In order to explain the discrepancy between the 100-fold decrease in AZT sensitivity for a drug resistant virus and minimal change in the apparent binding constant for AZTTP, we have employed transient kinetic techniques in an effort to more fully define the mechanism of AZT resistant HIV-1 RT. In this work we present evidence implicating the RNA dependent DNA replication event to play a role in the generation of AZT resistance.

MATERIALS AND METHODS

Overexpression and Purification of Recombinant Wild Type and AZT Resistant (Mutant) HIV-1 RT. In these experiments, the physiologically relevant heterodimeric p66/p51 wild type and AZT resistant mutant (D67N, K70R, T215Y, K219Q) RT having an N-terminal histidine tag were purified from clones generously provided by Dr. Stephen Hughes and Dr. Paul Boyer (Frederick Cancer Research and Development Center, MD). The clones were transformed into competent BL21 cells (Novagen) following the manufacturer's directions. The cells were grown up in LB broth containing ampicillin (100 mg/L) at 37 °C to an optical density of 0.6 unit at 595 nm. The cultures were then induced with IPTG (1 mM) and the cells allowed to grow for an additional 5−6 h. Purification for both wild type and mutant RT followed the following identical steps. All purification steps were done at 0−4 °C. Cells were harvested by centrifugation at 4 °C and resuspended in 1× binding buffer (20 mM Tris·HCl pH 7.9, 500 mM NaCl; 5 mM imidazole). The cells were lysed on a french pressure cell press (SLM Instruments) at 20 000 psi. The lysed cells were centrifuged at 15 000 rpm. The supernatant was decanted and the pellet extracted once more with 1× binding buffer and spun down. The two supernatants were combined and nucleic acids precipitated by addition of 5.0% poly(ethyleneimine) to a final concentration of 0.3% followed by centrifugation at 10 000 rpm. The supernatant was then treated with 60% ammonium sulfate and centrifuged. The resulting protein pellet was resuspended and dialyzed against 1× binding buffer. Crude RT was purified by column chromatography over a nickel column (His-bind resin, Novagen, 20 mL bed volume) according to the manufacturer's protocol. Fractions showing RT (from SDS-PAGE analysis) were pooled and dialyzed against buffer A (50 mM Tris·HCl, pH 7.8, 2 mM dithiothreitol, 2 mM EDTA, and 10% glycerol) and subsequently loaded onto a Q-Sepharose (Pharmacia) column (40 mL bed volume) equilibrated in buffer A. RT was eluted using a linear gradient from 10 to 300 mM NaCl in buffer A. The pooled RT fractions were combined and purified further by strong cation exchange FPLC using a Mono S 10/10 column (Pharmacia) as previously described (Stahlhut et al., 1994). Pure heterodimeric RT was dialyzed against buffer A containing 50 mM NaCl, concentrated using a centricon pressure cell (Amicon), aliquoted, and stored at −70 °C. Enzyme concentration was estimated by measuring the UV absorbance at 280 nm using an extinction coefficient of 260 450 M^{-1} cm^{-1} as described previously (Kati et al., 1992). Concentrations of RT used in subsequent experiments were determined by an active site titration method as previously described (Kati et al., 1992). The preparation of RT with this purification procedure gave burst amplitudes of 40−45%, and the experiments described here were performed using the corrected active site concentration. This his-tagged version of RT was kinetically indistinguishable from other recombinant forms of the protein without the tag prepared in our laboratory (Kati et al., 1992; Kerr & Anderson, 1997).

Nucleotide Triphosphates and Other Materials. AZTTP and dTTP were obtained from Moravek Biochemicals (Brea, CA) and Pharmacia LKB Biotechnology Inc., respectively. [^{32}P]ATP was obtained from Amersham Co. Biospin columns (for purification of labeled oligomers) were obtained from BioRad.

Synthetic Oligonucleotides. The DNA 45-mer and DNA 22-mer (Table 1) were synthesized on an Applied Biosystems 380A DNA synthesizer from the Yale DNA Synthesis Facility and purified by denaturing polyacrylamide gel electrophoresis (16%) as previously described (Kati et al., 1992). RNA 45-mer (Table 1) was obtained as the deprotected gel and HPLC purified material from New England Biolabs, Inc. The heteroduplex RNA/DNA and homoduplex DNA/DNA 45/22-mer template/primer strands were annealed using equimolar ratios of pure template/primer at 80 °C for 4 min and 50 °C for 30 min as previously described (Kati et al., 1992). The concentrations of the oligomers were

Table 1: Oligonucleotide Substrates

RNA/DNA 45/22-mer template-primer
CGG AGC GUC GGC AGG UUG GUU GAG UUG GAG CUA GGU UAC GGC AGG-5'*
*5'-GCC TCG CAG CCG TCC AAC CAA C
DNA/DNA 45/22-mer template-primer
CGG AGC GTC GGC AGG TTG GTT GAG TTG GAG CTA GGT TAC GGC AGG-5'*
*5'-GCC TCG CAG CCG TCC AAC CAA C

* Indicates 5'-[^{32}P]-labeling of the oligomer.

estimated from measuring the UV absorbance at 260 nm using extinction coefficients of 507 960 and 491 960 M^{-1} cm^{-1} for the RNA 45-mer and DNA 45-mer, respectively, and 218 390 M^{-1} cm^{-1} for the DNA 22-mer.

Buffers. All experimental procedures involving kinetic experiments of both wild type and mutant RT were done in 50 mM Tris-Cl pH 7.5, 50 mM NaCl, 10 mM Mg^{2+}, at 37 °C and used sterile buffers, reagents, and labware wherever possible.

5′-[^{32}P]-Labeling of the 45/22-mers. The RNA and DNA 45-mer and DNA 22-mer strands were 5′-labeled with [^{32}P]-ATP using T4 polynucleotide kinase (New England Biolabs, Inc.) according to previously described procedures (Kati et al., 1992).

Fluorescence Titration Experiments. Equilibrium fluorescence measurements were conducted using an SLM 4800C spectrofluorometer (Urbana, IL). The buffer contained 50 mM Tris·HCl, pH 7.8, 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol in a total volume of 0.5 mL. The excitation wavelength was 290 nm (8 nm slit width) and emission wavelength was 334 nm (8 nm slit width). The decrease in fluorescence at 334 nm was monitored. Experiments were carried out as previously described (Anderson et al., 1988). Briefly, in a typical experiment, 0.5 μL aliquots (from a 10 μM annealed DNA/DNA 45/22-mer template/primer stock solution) were added to a 0.5 mL solution containing either wild type or AZT resistant RT (100 nM). The solution was maintained with continuous stirring during all measurements, and the fluorescence intensity was recorded as an average of five 10 s readings. Template/primer was added until no further decrease in fluorescence was observed. The observed fluorescence (corrected for dilution) was then plotted versus total template/primer concentration, and the data was fit to a quadratic equation, analogous to that described previously (Anderson et al., 1988; Müller et al., 1991) by nonlinear regression. All experiments were done at room temperature.

Rapid Quench Experiments. Rapid chemical quench experiments were carried out at 37 °C, in an apparatus designed by Johnson (Johnson, 1986) and built by Kintek Instruments (State College, PA). The apparatus was modified to allow small reaction volumes of 15 μL. Experiments were carried out as described (Kati et al., 1992) at 37 °C. Briefly, 15 μL of substrate (RNA/DNA or DNA/DNA) preincubated with enzyme (wild type or mutant RT) was loaded in one sample loop while the other sample loop contained the nucleotide (AZTTP or dTTP) to be incorporated (preincubated with Mg^{2+}). Reactions were initiated by rapidly mixing the two volumes together for times ranging from milliseconds to several seconds and were terminated by quenching with 0.3 M EDTA (final concentration). All concentrations reported are final concentrations after mixing.

Product Analyses. The products were analyzed on a 16% polyacrylamide gel in 8 M urea in which elongation products from the primer strand and degradation products from the template strand could be resolved and quantitated. The products and substrates were quantitated by scanning the gel using either a GS-250 Molecular Imager System (BioRad) or Betascope (Betagen).

Data Analyses. Data were fit by nonlinear regression analysis using commercially available programs, KaleidaGraph for the Macintosh computer or GRAFIT for the IBM PC computer. The data were fit to a burst equation, product $= A(1 - \exp(-kt)) + mt$, where A is the amplitude of the burst, k is the observed first-order burst rate constant, and m is the linear steady-state rate constant, according to the mechanism previously described (Kati et al., 1992). The concentration dependence of the burst rate were fit to $k = k_{pol}[dNTP]/(K_d + [dNTP])$, where k is the observed pre-steady-state burst rate, k_{pol} is the maximum rate of incorporation, and K_d is the equilibrium dissociation constant for the dNTP.

RESULTS

The present investigation describes the kinetics of single nucleotide incorporation of dTMP and AZTMP opposite a template deoxyadenosine for DNA dependent DNA polymerization and template adenosine for RNA dependent DNA polymerization with wild type and AZT resistant (mutant) HIV-1 RT. Pre-steady-state burst experiments were conducted to determine the kinetic parameters of polymerization, k_{pol}, equilibrium dissociation, K_d, constants, and selectivity ratios (k_{pol}/K_d) for the deoxynucleotide substrates, dTTP and AZTTP, with defined homoduplex DNA/DNA 45/22-mer as well as an analogous heteroduplex RNA/DNA 45/22-mer oligomer substrates. Additionally, RNase H cleavage activity was characterized by determining the nature and kinetics of cleavage products. Pre-steady-state burst experiments also provided the linear steady-state rates.

Equilibrium Binding of DNA/DNA 45/22-mer to Wild Type and Mutant RT. The binding constant, K_d, for the oligomer substrate to RT was determined by monitoring the fluorescence due to the intrinsic quenching of tryptophan fluorescence of RT upon binding the substrate. Figure 1 (A and B) shows the decrease in fluorescence emission of wild type and mutant RT upon binding a D45/D22-mer substrate, respectively. The fluorescence change at 334 nm was titrated and the data fit to a quadratic equation to determine the K_d. For wild type the K_d for the D45/D22-mer substrate was 16 ± 7 nM while for mutant RT the K_d was 19 ± 8 nM.

Incorporation of dNMP into 45/22-mer Template Primer by Wild Type HIV-1 RT. Pre-steady-state burst experiments were performed by mixing a preincubated solution of RT (100 nM) and 5′-[^{32}P]-labeled RNA/DNA 45/22-mer (300 nM) with Mg^{2+} (10 mM) and various concentrations of dNTP under rapid quench conditions. Table 1 shows the templates (RNA and DNA 45-mers) and primer (DNA 22-mer) used in these experiments. Figures 2A and 2B shows the pre-steady-state kinetics for the incorporation of AZTMP into the heteroduplex RNA/DNA 45/22-mer (300 nM) template−primer by either wild type (panel A) or mutant (panel B) RT. The solid line represents the best fit of the data to a burst equation with $k = 20.4$ s^{-1} and $m = 0.04$ s^{-1} for wild type RT (panel A) and $k = 12.4$ s^{-1} and $m = 0.06$ s^{-1} for

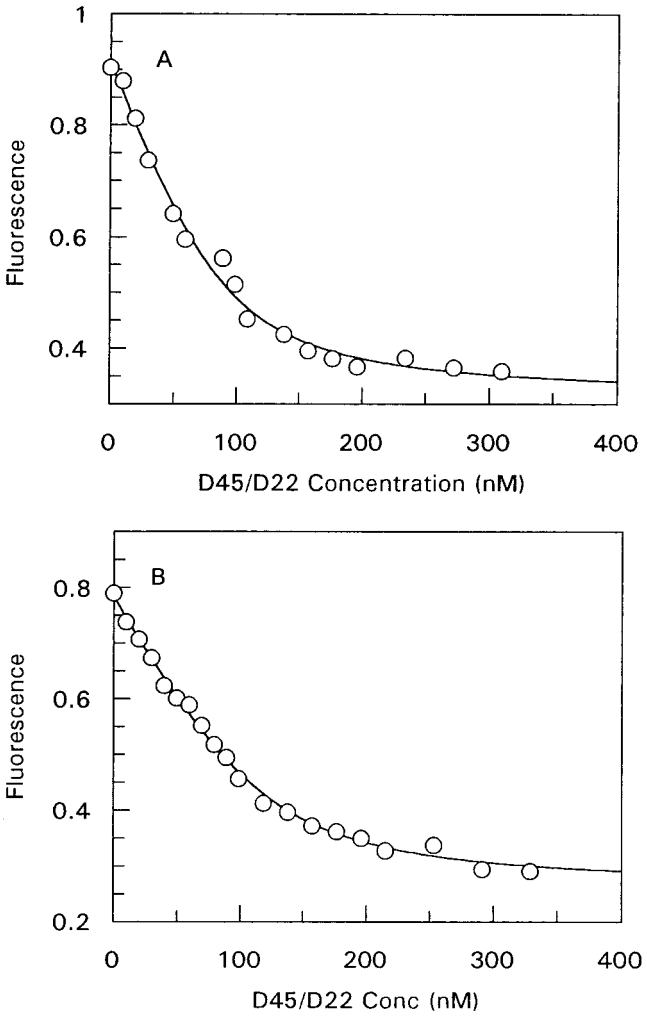

FIGURE 1: K_d for interaction of wild type and mutant HIV-1 RT with DNA/DNA 45/22-mer as determined by fluorescence tittration. The decrease in intrinsic protein fluorescence was monitored as the template−primer substrate was added in small increments (0.5 μL) to a solution of enzyme (50 nM) until no further change was noted. (A). The curve shows the best fit of the data to the quadratic equation for wild type RT with a K_d of 36 ± 7 nM. (B). The curve shows the best fit of the data to the quadratic equation for mutant RT with a K_d of 29 ± 8 nM.

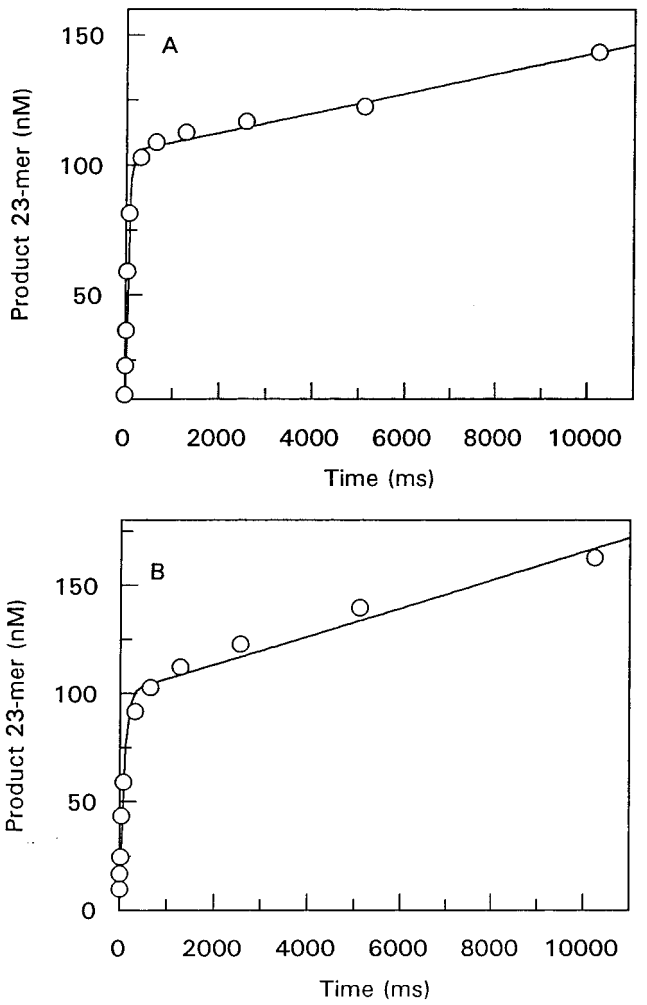

FIGURE 2: Pre-steady-state kinetics of incorporation of AZTMP with wild type and mutant HIV-1 RT. (A) Pre-steady-state kinetics of incorporation of AZTMP into heteroduplex RNA/DNA 45/22-mer (template−primer) were measured by mixing a preincubated solution of wild type RT (100 nM) and 5′-[^{32}P]-labeled RNA/DNA (300 nM) with Mg^{2+} (10 mM) and AZTTP (100 μM) under rapid quench conditions. The reactions were quenched with 0.3 M EDTA at the indicated times, and the product 23-mer was separated and quantitated by sequencing gel analysis. The solid line represents the best fit of the data (○) to a burst equation with rate constants equal to 20.4 and 0.04 s^{-1}, for the exponential and linear phases, respectively. (B) Pre-steady-state kinetics of incorporation of AZTMP into heteroduplex RNA/DNA 45/22-mer (template−primer) as described in A using AZT resistant mutant RT. The solid line represents the best fit of the data (○) to a burst equation with rate constants equal to 15 and 0.06 s^{-1}, for the exponential and linear phases, respectively.

mutant RT (panel B). Analogous experiments were conducted with varying concentrations of dNTP (AZTTP or dTTP) for both wild type and mutant RT with both homoduplex (DNA/DNA) and heteroduplex (RNA/DNA) template−primers to obtain the dNTP concentration dependence of the observed burst (polymerization) rate and the steady-state (product dissociation) rate for each substrate. Table 2 summarizes the data. All errors were ≤15%.

Determination of the Dissociation Constants for Nucleotide Binding and Maximum Rates of Nucleotide Incorporation. The dissociation constants for deoxynucleotide binding, K_d, and maximum rates of polymerization, k_{pol}, were obtained by examining the concentration dependence on the burst rates. Figure 3 shows the AZTTP concentration dependence on the observed polymerization rate constant for incorporation into an RNA/DNA 45/22-mer template−primer using wild type and mutant RT. The data were fit to the hyperbolic equation. A K_d value of 13 μM and a maximum rate of incorporation (k_{pol}) of 22.5 s^{-1} was determined for wild type RT. A K_d of 32 μM and k_{pol} of 15 s^{-1} was determined for the mutant RT. Similar curves were generated for each nucleotide (dTTP and AZTTP) and template−primer with wild type and mutant RT, respectively. The data is summarized in Table 2. All errors were ≤15%.

RNase H Activity of Wild Type and Mutant RT. Since the RNA template strand was 5′-labeled, it was possible to determine the cleavage rates for the RNase H activity (Kati et al., 1992) of either wild type or mutant enzyme. The RNase H rates (Table 2) ranged from 7 to 15 s^{-1} depending on the incoming nucleotide (AZTTP or dTTP, respectively) for the wild type enzyme and were between 10 to 20 s^{-1} for the mutant with either AZTTP or dTTP, respectively, consistent with wild type RT (Kati, et al., 1992; Kerr and Anderson, 1997). With the RNA/DNA 45/22-mer template−primer, the RNase H activity of both wild type and mutant RT indicated identical cleavage products (Figure 4) corre-

Table 2: Comparison of Kinetic Parameters for Wild Type (WT) and AZT Resistant (MT) HIV-1 RT

		$k_{steady\ state}$ (s^{-1})[a]		RNase H rate (s^{-1})[b]		k_{pol} (s^{-1})		K_d (μM)		k_{pol}/K_d ($\mu M^{-1}\ s^{-1}$)	
dNTP	template−primer	WT	MT	WT	MT	WT	MT	WT	MT	WT	MT
dTTP	RNA/DNA	0.06	0.05	15	20	61	44	24	38	2.5	1.15
AZTTP	RNA/DNA	0.04	0.065	7	10	22.5	15	13	32	1.73	0.47
dTTP	DNA/DNA	0.15	0.1			16.7	4	19	10	0.88	0.4
AZTTP	DNA/DNA	0.02	0.07			0.7	1.8	2	7	0.35	0.25

[a] Steady-state (dissociation) rate at maximum dNTP concentration. [b] Average value of the burst rate of RNA degradaton by RT. Standard errors for all the above values were ≤15%.

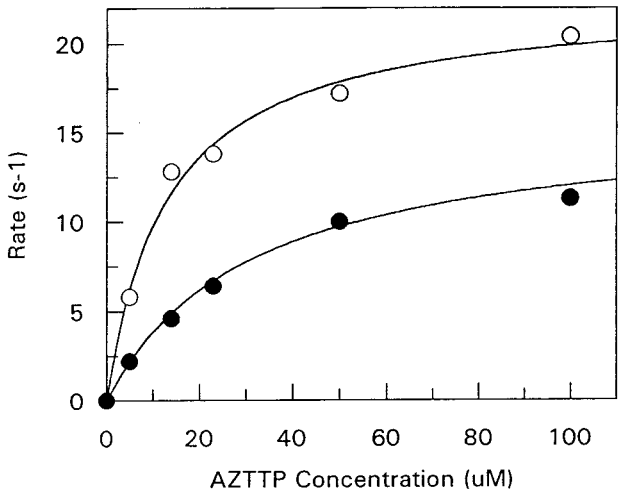

FIGURE 3: Determination of k_{pol} and K_d for AZTTP incorporation with wild type and mutant RT. The AZTTP concentration dependence on the polymerase rate for incorporation into RNA/DNA 45/22-mer template−primer using wild type (○) and mutant (●) HIV-1 RT. The polymerase rates were plotted against AZTTP concentration and the data (○, ●) were fit to a hyperbola (solid line) to yield a K_d value of 13 μM and a maximum rate of incorporation (k_{pol}) of 22.5 s^{-1} for the wild type RT and a K_d of 32 μM and k_{pol} of 15 s^{-1} for the mutant, respectively.

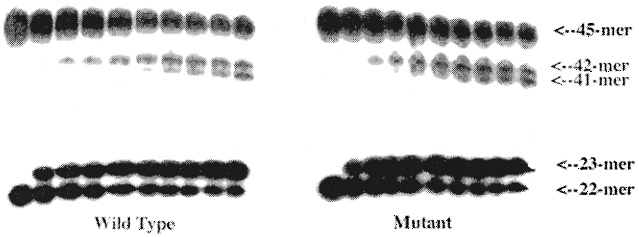

FIGURE 4: Gel analysis comparing the nature and kinetics of RNase H activity for wild type and mutant RT. Autoradiogram of an experiment showing time course of AZTMP (100 μM) incorporation into RNA/DNA 45/22-mer template−primer (100 nM) using wild type (left) and mutant (right) HIV-1 RT (250 nM). Polymerization to a 23-mer and RNase H cleavage products of 42- and 41-mers are clearly visible for both proteins.

sponding to a 42- and 41-mer. From this, the distance between the two sites of activity (polymerase and RNase H) corresponded to a length of 18−19 nucleotides, identical to what was observed previously with a 45/25-mer RNA/DNA template−primer (Kati et al., 1992) and in full agreement to the structural studies of RT (Kohlstaedt et al., 1992).

DISCUSSION

Steady-state kinetic studies examining AZT resistant RT have been unable to completely resolve the discrepancy observed with AZT resistance in the clinic (or cell culture) to that using purified AZT resistant RT (Lacey et al., 1992; Carroll et al., 1994; Caliendo et al., 1996). In this investigation we have determined the rate of single nucleotide incorporation with defined template−primers for wild type and AZT resistant HIV-1 RT using pre-steady-state kinetic techniques which allow for the direct observation of catalytic events occurring at the active site of the enzyme. Pre-steady-state kinetic methods have been employed previously by our laboratory and others in extracting mechanistic information from wild type HIV-1 RT (Kati et al., 1992; Spence et al., 1995; Reardon, 1992 and 1993; Hsieh et al., 1993). The pre-steady-state kinetic parameters, k_{pol} and K_d, for single nucleotide incorporation into a defined template−primer by either wild type of mutant RT allow a direct comparison of each protein's selectivity and specificity for incorporation of dNMP (dTMP or AZTMP) at the active site. By analyzing the relative rates of polymerization and equilibrium dissociation constants of dTTP, the natural dNTP, and AZTTP (competitive inhibitor), the overall intrinsic selectivity of each protein, wild type or mutant, for AZTMP incorporation in relation to the uptake of the natural nucleotide may be assessed. This analysis provides an unambiguous picture of the specificity of nucleotide uptake or incorporation on a mechanistic level.

The affinity of the oligomer substrates to either wild type or mutant RT is similar as determined by fluorescence titration (Figures 1A and 1B). The nanomolar K_d values suggest that the mutated residues are not essential for forming a tight binary complex between enzyme and oligonucleotide substrate. The kinetic parameters summarized in Table 2 show that the mutant RT is overall less efficient than wild type RT. The polymerization rate for mutant RT for dTTP with either template−primer (RNA or DNA oligomer substrate) is consistently slower than that of the wild type polymerization rates. Curiously, for AZTTP the polymerization rate of the mutant for the DNA/DNA substrate is faster than that of the wild type. While the K_d for dTTP with the DNA/DNA substrate shows slightly higher affinity with the mutant compared to wild type, the K_d for AZTTP for the identical substrate shows a 3.5-fold larger value for the mutant compared to wild type RT, indicating a decreased affinity for AZTTP for the mutant with this substrate. However, when the ratio of k_{pol} to K_d is calculated for this DNA/DNA template−primer, a measure of the selectivity, the mutant RT shows a 2.2-fold decrease in selectivity for the natural dTTP compared to only a 1.4-fold decrease for AZTTP to that of wild type RT. Hence, comparison of only K_d values of AZTTP, while showing decreased affinity of AZTTP for the mutant, are not sufficient in themselves to explain the selectivity of one protein (wild type) over that of the other (mutant) to AZTTP, but the overall selectivity

in relation to the natural nucleotide needs to be assessed. The trend is more straightforward for the results with the heteroduplex, RNA/DNA template−primer. With either nucleotide, dTTP or AZTTP, the polymerization rate constant for the wild type is approximately 1.5-fold faster than that of the mutant RT. The equilibrium dissociation constants for both dTTP and AZTTP with this RNA/DNA template−primer are also straightforward. In either case the mutant RT has a lower affinity for the dNTP—1.5-fold lower affinity for dTTP and 2.5-fold lower for AZTTP. The RNA template shows a 2-fold decrease in selectivity for dTTP and a 4-fold decrease in selectivity for AZTTP, by comparing the mutant to the wild type RT thus indicating subtle differences related to RNA directed DNA polymerization.

Pre-steady-state kinetic techniques have been previously employed (Kati et al., 1992; Spence et al., 1995; Rittinger et al., 1995; Reardon, 1992; Hsieh et al., 1993) to determine mechanistic information for HIV-1 RT. We have previously proposed (Kati et al., 1992) an induced fit model involving a two-step mechanism for RT, whereby the enzyme goes from an "open" to a "closed" activated state (involving a rate-limiting conformational change), which is followed by catalysis and product dissociation. While this model has been challenged (Reardon, 1993), other transient kinetic studies on HIV-1 RT have further substantiated this two-step model (Hsieh et al.,1993; Johnson 1993; Spence et al., 1995; Rittinger et al., 1995).

The pre-steady-state and steady-state dissociation rates for wild type RT were in general agreement to previously reported values (Kati et al., 1992, Reardon, 1992, 1993; Hsieh, et al., 1993). This study provides the first pre-steady-state kinetic analysis comparing wild type and AZT resistant mutant RT. The RNase H activity of the mutant enzyme showed faster kinetics than the wild type; however, in either case, the activities were slower than the polymerase rates indicating sufficient time for DNA synthesis prior to the degradation of the RNA template, corroborating earlier studies (Kati et al., 1992).

The present study provides a detailed mechanistic analysis of the events occurring at the active site of RT. From these studies, it is possible to infer that RNA dependent DNA polymerization function of RT may play a more important role in the generation of resistance to AZTTP compared to DNA dependent DNA polymerization. Verification of this implication, however, requires analysis of multiple sequences to establish the generality of our observations. This suggestion is consistent with the postulations from structural studies suggesting the RNA template to be in contact with the mutated residues (Kohlstaedt et al., 1992) in RT. Previous studies (Tantillo et al. 1994; Boyer et al., 1994) using molecular modeling and inhibition assays have postulated nucleoside resistance to be related to differences in the exact positioning of template−primer due to altered interactions of the protein and substrate, especially those involving a template overhang. However from our transient kinetic functional studies we see essentially no difference in the nature of the rate-limiting conformational change, apart from the decreased polymerization rate constants for nucleotide incorporation for wild type and mutant RT. Furthermore, no differences were seen in the cleavage patterns of the degraded RNA template for mutant RT and wild type (Figure 4). These results do not support the suggestion of an altered binding mode for the mutant compared to wild type RT.

The small but significant 4-fold decrease in sensitivity to AZTTP by the mutant RT compared to wild type RT, for the RNA/DNA template−primer, while unable to account for the 100-fold decrease in sensitivity of AZT observed in the clinic and cell culture studies does provide a basis for future investigations to more fully account for the clinical observations. It remains a possibility that the decrease in sensitivity observed in this study, an isolated system involving only substrate and protein, may be augmented, *in vivo*, by cellular and other viral factors, such as nucleocapsid proteins, that are involved in viral replication. Recent studies suggesting decreased levels of thymidine kinase in virally infected cells (Avramis et al., 1993; Wu et al., 1995; Jacobsson et al., 1995) could also contribute to the overall decreased sensitivity of AZT. Furthermore, studies showing metabolites of AZT (AZTMP, AZTDP, and AZTTP) to be inhibitors of HIV-1 integrase (Mazumder et al., 1994) suggest development of integrase mutants to be another factor in the overall development of clinical resistance to AZT. An additional possibility may be that more dramatic changes are noted in other parts of viral replication such as the $tRNA_{lys}$ initiation step and may also play an important role in the development of drug resistance. This possibility is currently under study in our laboratory.

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