Enhanced Binding of Azidothymidine-resistant Human Immunodeficiency Virus 1 Reverse Transcriptase to the 3'-Azido-3'-deoxythymidine 5'-Monophosphate-terminated Primer

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters

Citation
Canard, Bruno, Simon R. Sarfati, and Charles C. Richardson. 1998. "Enhanced Binding of Azidothymidine-Resistant Human Immunodeficiency Virus 1 Reverse Transcriptase to the 3'-Azido-3'-Deoxythymidine 5'-Monophosphate-Terminated Primer." Journal of Biological Chemistry 273 (23): 14596–604. https://doi.org/10.1074/jbc.273.23.14596.

Citable link
http://nrs.harvard.edu/urn-3:HUL.InstRepos:41483436

Terms of Use
This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA
Enhanced Binding of Azidothymidine-resistant Human Immunodeficiency Virus 1 Reverse Transcriptase to the 3'-Azido-3'-deoxythymidine 5'-Monophosphate-terminated Primer*

(Received for publication, February 2, 1998)

Bruno Canard‡‡, Simon R. Sarfati‡†, and Charles C. Richardson‡‡

From the ‡Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115 and the †Institut Pasteur, 28 rue du Dr Roux, 75724 Paris, France

Human immunodeficiency virus type 1 is resistant to 3'-azido-3'-deoxythymidine (AZT) when four amino acid substitutions (D67N, K70R, T215F, and K219Q) are present simultaneously in its reverse transcriptase. Wild-type and AZT-resistant reverse transcriptases show identical binding to a 3'-azido-3'-deoxythymidine 5'-monophosphate (AZTMP)-terminated primer/RNA template. On DNA templates, the equilibrium dissociation constant (KD) for primer/template and AZT-resistant reverse transcriptase (RT) (KD = 4.1 nM) is similar to that of the wild-type enzyme (KD = 6.2 nM). However, koff is 4-25-fold lower for the AZT-resistant enzyme than for the wild-type enzyme, depending on the nucleotide and the template. The kinetic decay of a wild-type RT/prime/ AZTMP-terminated DNA template complex is biphasic. Seventy percent of the initial complex decays with a rate constant greater than 0.05 s^{-1} and 30% with a rate constant of 0.0017 s^{-1}. Decay of an AZT-resistant RT/ AZTMP-terminated primer/DNA template complex is monophasic, with a rate constant of 0.0018 s^{-1}. The last two nucleotides at the 3' end of the AZTMP-terminated DNA primer in complex with AZT-resistant RT, but not wild-type RT, and a DNA template are protected from exonuclease digestion, suggesting that enhanced binding of the 3' end of the AZTMP-terminated DNA primer to reverse transcriptase is involved in the mechanism of AZT resistance by human immunodeficiency virus type 1.

Reverse transcriptase (RT) of the human immunodeficiency virus type 1 (HIV-1) is a DNA- and RNA-dependent DNA polymerase that synthesizes a double-stranded DNA from the viral (+) RNA genome (1). Most of the replicative steps of the HIV-1 genome are dependent on the RT encoded by the viral pol gene. This dependence of the replication cycle on RT has directed much of the anti-HIV-1 chemotherapy strategy toward this enzyme (2). Nucleoside analogues were among the first RT inhibitors described (3, 4). Once taken up by the infected eukaryotic cell, most nucleoside analogues require phosphorylation of their 5'-hydroxyl group by cellular kinases to convert them to the 5'-triphosphate, substrates for RT. Most of these analogues do not possess a free 3'-hydroxyl group, and chain termination occurs once they are added to the nascent viral DNA. Termination is believed to account for the observed inhibition of viral growth. It is not known whether incorporation of the analogue in vivo occurs during DNA- or RNA-dependent DNA synthesis, or both.

3'-Azido-3'-deoxythymidine (AZT) is a prototype of the chain-terminating analogues (4). 3'-Azido-3'-deoxythymidine 5'-triphosphate (AZTTP) is over a thousandfold more efficient as a substrate for RT than for human polymerases (5–7), and is as good a substrate as the natural substrate dTTP for RT. Since AZTTP and dTTP pools are comparable in cultured cells (8), one would expect that replication and hence viral growth would be inhibited completely (9). However, decreased sensitivity to AZT appears gradually over time together with the rapid emergence of mutations in the viral pol gene (2, 10). This decreased sensitivity of the virus to AZT is called AZT resistance, and correlates with a specific pattern of mutations in the pol gene (11, 12). One particular set of amino acid changes, D67N, K70R, T215F/Y, and K219Q, gives rise to a 120-fold AZT-resistant virus; the corresponding RT will be referred to as AZT-resistant RT throughout this paper.

Decreased recognition of AZTTP in favor of dTTP (AZTTP discrimination) by AZT-resistant RT occurs only to a modest level (4-fold), if at all (13–16). When pools of dTTP and AZTTP are comparable, AZTTP discrimination can be reflected by the ratio of dTTP over AZTTP incorporation efficiencies (kcat/Km)AZTTP/kcat/Km dTTP during a single nucleotide incorporation event. Kerr and Anderson (15) found similar kcat values for wild-type and AZT-resistant RT. Comparison of Km values for substrates and inhibitors can be misleading because Km values are composed of several kinetic constants (17, 18). However, if AZT resistance arises through a more stringent selection of dTTP relative to AZTTP, RT should be able to discriminate several hundredfold against AZTTP during the more than 4000 events of incorporation of T opposite A along the HIV-1 genome (9). It is unlikely that this high degree of discrimination would not be reflected in KmAZTTP.

A possible alternative mechanism to achieve resistance to AZT would be to remove AZTMP once it has been incorporated at the 3' end of the primer. Such a model of AZT resistance involving repair of the AZTMP-terminated DNA chain does not imply a high degree of discrimination during the incorporation of the inhibitor AZTTP relative to the natural substrate dTTP. If repair is extremely efficient without any nucleotide discrimination, the synthesis of viral DNA would be simply delayed.
Thus, the combined effect of a modest discrimination of the inhibitor in conjunction with repair of the inhibitor-terminated DNA chain is an attractive possibility to account for the 120-fold increase in IC_{50} provided by the set of amino acid changes described above. Since RT does not have a proofreading 3' to 5' exonuclease activity, one must invoke another enzyme to move the chain-terminating AZT. Although eukaryotic cells have a variety of 3' to 5' exonucleases, one obvious candidate for repair of prematurely terminated DNA chains is by RT itself, through pyrophosphorolysis, the reversal of nucleotide polymerization. In this reaction, RT in complex with an AZTMP-terminated primer/template binds pyrophosphate (PPi) and catalyzes the formation of AZTTP at the expense of pyrophosphate. Intracellular PPi concentrations in human lymphocytes are around 130 μM (19), but the K_{d} (PPi) of RT is several μM, making pyrophosphorolysis an unfavorable reaction compared with nucleotide polymerization (18, 20). However, once the chain-terminator has been added at the 3' end of the primer, nucleotide polymerization can no longer occur and hence does not compete with pyrophosphorolysis. Therefore, the removal of the chain-terminating AZTMP through pyrophosphorolysis depends on the presence of RT in complex with AZTMP-terminated primer/template and PPi, concentration. In this case, the dNTP concentration is relevant only if dNTPs compete with PPi for the same binding site as true competitive inhibitors. Thus, any factor increasing the lifetime of RT in complex with AZTMP-terminated primer/template should favor pyrophosphorolysis. In case repair is not provided by RT itself, a stable complex made of RT bound to the AZTMP-terminated primer/template would impede access of auxiliary proteins to the termined primer.

With these observations in mind, we compared binding of wild-type and AZT-resistant RT to various primer/templates having either a hydroxyl or an azido group at the 3' end of the primer. We show that AZT-resistant RT has a slower off-rate than wild-type RT from its primer/template, and that the presence of AZTMP at the primer 3' end is responsible for additional specific nucleic acid/protein contacts.

**EXPERIMENTAL PROCEDURES**

**Proteins and Reagents**—The gene encoding bacteriophage T4 polynucleotide kinase was mutated so as to eliminate phosphatase activity. The phosphatase-free T4 polynucleotide kinase was purified and provided by Dr. C. & P. Tabor, Howard Medical School, Harvard University, Cambridge, MA. E. coli Xeroxishivochilous exonuclease III (Exo III) was purchased from New England Biolabs. 3'-Deoxyribonucleoside 5'-triphosphates (dNTPs) and all 32P-labeled nucleotides were from Amersham Pharmacia Biotech. Oligonucleotides were 5' 32P-labeled using phosphatase-free bacteriophage T4 polynucleotide kinase nii purified and provided by S. Tabor, p-Azidophenacyl bromide was from Sigma. The RNA template and DNA oligonucleotide containing a unique phosphorothioate linkage at nucleotide position 15 were obtained from Oligos, Etc. DNA primers were synthesized by the Biopolymers Laboratory, Harvard Medical School. RNA and DNA primers were purified by electrophoresis through an 18% polyacrylamide gel, and oligonucleotides were extracted from the gel using standard methods.

The HIV-1 RT gene cloned into a M13 filamentous bacteriophage vector to give mpRT4 has been described (21). The RT gene was excised from the mpRT4 construct and subcloned into a plasmid expression vector as follows. mpRT4 DNA was digested with EcoRI restriction enzyme, and the resulting 5'-overhangs were eliminated using dNTPs and the Klenow fragment of E. coli DNA polymerase I. The DNA was then digested with HindIII and subcloned into the plasmid vector pTRE99a (Amersham Pharmacia Biotech) at its dephosphorylated Ncol and HindIII restriction sites to give p66-WT. E. coli XLI-Blue (Stratagene) harboring p66-WT was used to express the RT gene. mpRT4 was also used as the starting material to construct a RT gene carrying four mutated codons producing the amino acid changes D67N, K70R, T215F, and K219Q, using the single-stranded template method. The resulting RT gene was subcloned into the same expression vector as p66-WT to produce p66-Res. To construct a gene encoding the p51 subunit counterpart of either p66-WT or p66-Res, p66-WT or p66-Res DNA was digested with KpnI and HindIII, dephosphorylated, and ligated with a double-stranded, phosphorylated synthetic oligonucleotide. The latter oligonucleotide was designed so as to maintain the correct reading frame of the RT gene, contain KpnI and HindIII restriction site sequences to be ligated at each end, and introduce a stop codon at the downstream position for a 55-kDa protein containing 440 amino acids. All plasmid constructs containing the RT genes were verified by restriction enzyme analysis and by DNA sequencing.

**Expression and Purification of Enzymes**—Ten-liter cultures of bacteria harboring p66-WT or p66-Res plasmids were grown up at 37 °C in 100 ml of a 16-g/liter yeast extract, 5% beef extract, 5% sucrose containing 100 μg/ml ampicillin. Bacteria were grown to an A_{600} of 1, and isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM. After 3 h of incubation, the cells were harvested by centrifugation. All subsequent steps were at 4 °C. The pellet (about 100 g of wet cells) was resuspended in 100 ml of 10% sucrase, 40 mM Tris-HCl, pH 8.0, 25 mM EDTA. Lysozyme was added to 1 mg/ml together with 0.5 mM phenylmethylsulfonyl fluoride and 1 mM benzamidine. After a 15-min incubation, cells were lysed with an equal volume of solution containing 0.4% Nonidet P-40, 10 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine. Following another 15-min incubation with gentle agitation, the mixture was brought to 0.5 M NaCl and sonicated six times for 20 s at maximum output in a BQ sonifier. The sonog 450 was brought to 0.5 M NaCl and sonicated six times for 20 s at maximum output in a BQ sonifier. The solution was adjusted to 100,000 g x 1 h in a Beckman SW27 swinging bucket rotor, and the supernatant fluid was collected to yield fraction I (200 ml, about 20 mg/ml protein).

Fraction I was diluted 7.5-fold with 50 mM KCl, 40 mM potassium phosphate, pH 7.4, 1 mM EDTA, 0.1% Nonidet P-40, 1 mM DTT, 10% glycerol, and the protein was adsorbed to 100 ml of settled phosphocel- lulose (Whatman) pre-equilibrated in 200 ml of the same buffer by gentle stirring for 30 min. The phosphocellulose was washed three times with 400 ml of the same buffer by low speed centrifugation (3,000 × g), the slurry was poured into a column (12 cm × 20 cm), and the protein was eluted using a gradient (2 liters) from 50 mM to 1 M KCl in the same buffer. Fractions were analyzed by SDS-PAGE electrophoresis using a PhastSystem (Amersham Pharmacia Biotech), and proteins were visualized by silver staining. Fractions containing p66 were combined in a dialysis bag (molecular mass cut-off = 3500 Da), concentrated by coating the bag with solid polyethylene glycol for 8 h, and dialyzed against buffer A (10% glycerol, 20 mM potassium phosphate, pH 7.4, 1 mM DTT, 0.25 mM sodium citrate). The dialysate (100 ml, 1.2 mg/ml protein) was designated fraction II.

Fraction II was loaded onto a ceramic type II hydroxyapatite (Bio- Rad) column (3 cm × 13 cm) that had been manually packed and connected to a fast performance liquid chromatography (FPLC) apparatus (Amersham Pharmacia Biotech). Elution was with a gradient (400 ml) from 20 to 500 mM potassium phosphate in buffer A, eluted at approximately 150 mM KCl. The combined fractions (30 ml) were brought to 1 M (NH_{4})_{2}SO_{4} by the addition of buffer A containing 2.4 M (NH_{4})_{2}SO_{4} to a final volume of 30 ml. The solution (50 ml, 0.8 mg/ml protein) was designated fraction III.

Fraction III was loaded onto a phenyl-Superose column (Amersham Pharmacia Biotech, model HR10/10), washed with 10 ml of 1 M (NH_{4})_{2}SO_{4} in buffer A and eluted with a decreasing (NH_{4})_{2}SO_{4} gradient (200 ml) from 1 M to 0 in buffer A. Fractions containing p66 were combined and concentrated by dialysis against 50% glycerol in buffer A to yield fraction IV (1 mg/ml, 15 mg total).

The purified enzyme (fraction IV) was homogeneous as judged by SDS-PAGE electrophoresis analysis. The AZT-resistant p66 subunit as well as all p51 proteins were purified following the same procedure. Typically, the yield for p51 subunits was twice that obtained for the p66 subunits. The protein concentrations of the final enzyme preparations were determined spectrophotometrically using the absorption coefficients E_{280} of Kuti et al. (22), which are 136,270 and 124,180 M^{-1} cm^{-1} for p66 and p51 subunits, respectively. p66/p51 heterodimers were reconstituted as described in Ref. 23.

**Polymerase Assays**—RT activity was determined either by monitoring the formation of radioactively labeled nucleic acid product using iodine exchange paper discs (21) (assay 1) or by measuring the incorporation of a single nucleotide using the gel assay described below (assay 2). Polymerase activities during enzyme purification and re-association were determined using assay 1. Reactions were carried out in RT buffer (50 mM Tris-HCl, pH 8.0, 50 mM KCl, 10 mM MgCl_{2}, 0.05% Triton X-100) in the presence of 50 μM poly(rA)oligo(dT), 30 μM each of dATP, dCTP, dGTP, and (3H)dTTP (36 μCi/μmol). Control experiments showed that the presence of dGTP, dATP, and dCTP in assays using
poly(A)olig(T)D did not affect the reaction. Hence, for convenience all reaction mixtures for RT activity using assay 1 contained the four dNTPs. After addition of RT, aliquots were withdrawn at several time points and spotted onto DE-51 paper discs that had been previously soaked in 50 mM EDTA. Paper discs were washed three times for 10 min in 1 M ammonium formate, pH 8.0, 2.5 M ammonium acetate, pH 8.0, 2.5 M ammonium formate, and 0.5 M ammonium acetate, and the radioactivity bound to the filters was determined by liquid scintillation counting. One unit of enzyme catalyzes the incorporation of 1 nmol of DE-51- absorbable dTMP in 10 min at 37 °C.

For assay 2, the incorporation of a single nucleotide was monitored using a primer extension reaction followed by polyacrylamide gel electrophoresis. Except when indicated, only one nucleoside triphosphate was present in the reaction. A 21-mer DNA primer (5′-ATACCTTAAAC-CAATAGTGACC-3′) was 5′ end-labeled with γ-[^32]P]ATP using phosophatase-free T4 polynucleotide kinase. Duplex DNA substrates were prepared by annealing the labeled primer (2.5 μM) to a 31-mer DNA template (5′-TTTTTTTTTTAGATAGATTTTTAGAT-3′) at a 1:1.5 primer/template molar ratio (determined spectrophotometrically) in 10 mM Tris-HCl, pH 8.0, 50 mM KCl by heating to 80 °C and slow cooling to room temperature over at least a 1-h period. The template base directing incorporation of the nucleotide under study is shown above in bold, and was either C or A, for dGTP- or thymine-containing nucleotides, respectively. Annular primer/templates were diluted to the desired concentration immediately prior to use. RT and primer/ template mixes in RT buffer at 0 °C at a concentration of 1–5 μM and 100 μM, respectively. After incubation for 2 min at 37 °C, the reaction was initiated by the addition of AZTTP (in 5 mM MgCl₂) to yield the indicated concentration. At various times, aliquots (5 μl) were withdrawn and the reaction was stopped by the addition of an equal volume of gel loading buffer (50 mM EDTA, 0.01% xylene cyanol, and bromphenol blue in formamide). Aliquots were heated to 70 °C for 2 min and loaded onto an 18% polyacrylamide denaturing gel. Single nucleotide incorporation was measured using photostimulable plates on a Fuji Imaging apparatus. The amount of radioactivity in the product relative to the total amount of radioactivity per lane was determined. Initial velocities of AZTTP incorporation were determined at up to 12 different concentrations of AZTTP ranging from 50 nM to 100 μM, and used to determine Henri-Michaelis-Menten constants Kₘ and kₐ for the RT of interest. Data analysis was performed using non-linear regression and the Kaleidagraph 3.0 software for MacIntosh (Abelbeck software, Inc).

**Determination of Equilibrium Dissociation (Kₒ) and On-rate (kₒ) Constants for DNA Template and RT Dimers—**RT was titrated with the same 5′-labeled primer/template as above for polymerase assay 2 to determine the equilibrium dissociation constant (Kₒ) (17, 20, 22). RT (40–200 μM final concentration) was allowed to bind to different concentrations of primer/template in RT buffer at 0 °C. After incubation for 1–2 min, the reaction was divided into three portions. Each of the three reactions was incubated at 23 °C for 2 min before addition of dNTP-Mg²⁺ to yield the desired final concentration in 5 mM MgCl₂. For each reaction, after rapid manual mixing of the dNTP with the RT primer/template, two aliquots were withdrawn and quenched at times 5 and 15 s, 5 and 30 s, or 10 and 30 s for the three reactions, respectively, using formamide gel loading buffer. Products were analyzed by 18% polyacrylamide gel electrophoresis as described above and quantitated using photostimulable plates on a Fuji Imaging apparatus.

The time course of product formation was biphasic, with an initial burst of product formation too fast for manual sampling, followed by a slower, linear phase. The burst amplitudes were determined by linear extrapolation of the slow phase on the ordinate axis. Comparisons with experiments conducted using a rapid quench apparatus (Kintech) gave essentially the same burst amplitude values. It was found that the manual initial mixing of reagents was the limiting step to obtain comparable pre-steady-state values. However, after 3–4 s, the linear time course was indistinguishable from time points obtained with the rapid quench apparatus, yielding an accurate extrapolation of burst amplitude values. Burst amplitudes were plotted against primer/template concentration. The equilibrium constant Kₒ for binding of the enzyme to the primer/template, and total active enzyme Eₙ were determined from the fit of the data to the quadratic equation shown below, where Dₙ is total primer/template concentration.

\[
[E \cdot DNA] = 0.5(Kₒ + Eₙ + Dₙ) - 0.5(Kₒ + Eₙ + Dₙ) - 4 Eₙ \cdot Dₙ \tag{1}
\]

This experiment was also used to determined the on-rate constant kₒ as follows (17). Total enzyme and DNA concentrations can be written as shown by the following two equations, where [E-DNA] is determined as the value of the burst amplitude.

\[E = [E \cdot DNA] + [E]_{rev} \tag{2}
\]

\[DNA = [E \cdot DNA] + [DNA]_{rev} \tag{3}
\]

The definition of Kₒ is given below.

\[Kₒ = kₒ/kₐ = [E]_{rev} \cdot [DNA]_{rev}/[DNA \cdot E] \tag{4}
\]

This equation can be re-arranged as shown below, where kₒ/[DNA] is the post-burst rate of product formation, i.e. the slope corresponding to the second, steady-state phase.

\[kₒ/[DNA] = [DNA]_{rev}/[DNA \cdot E] \cdot kₐ \tag{5}
\]

Thus, a plot of this post-burst rate divided by the free enzyme concentration (determined with Equation 2) versus the free primer/template concentration ([DNA]rev, determined with Equation 3) gives a straight line with a slope equal to kₒ/[DNA]. Values of the on-rate constant kₒ were determined for wild-type and AZT-resistant enzymes on DNA templates.

**Synthesis and Purification of Primers Containing a p-Azidophenacyl Photo-activatable Probe—**Upon UV irradiation at 365 nm, aromatic azido groups react with a variety of protein side chains, such as carboxylic acids and aromatic rings, to form a covalent bond. Attachment of a photo reactive p-azidophenacyl moiety to DNA primers was as described (24, 25). Phosphorylated p-specific oligonucleotides were dissolved in 0.3 ml of 150 mM potassium phosphate buffer, pH 7.0, mixed with 0.75 ml of p-azidophenacyl bromide (5 mg/ml in methanol), and incubated for 3 h at room temperature. Reaction products were precipitated with excess ethanol, redissolved in water, and lyophilized. The modified oligonucleotides were purified from unreacted reagents using C₁₈ reversed-phase HPLC. Annealing of the various primer/templates was as described (22). The enzymatic phosphorylation of the 5′ end of the oligonucleotide primer was not affected by the photoprobe (data not shown).

**DNA/Protein Cross-linking Assays Using Photoprobe-coupled Oligonucleotide—**The p-azidophenacyl-modified primer was labeled and annealed in a fashion similar to that of unmodified primers (see below). RT (250 nM) was mixed (1:1 v/v) with an equimolar amount of 5′-32P-labeled p-azidophenacyl-modified oligonucleotide annealed to a DNA or RNA template on parafilm laid on a flat, cold (4 °C) surface in a 10-μl final volume in 10% glycerol, 5 μg/ml bovine serum albumin, 5 mM MgCl₂, 25 mM potassium phosphate buffer, pH 7.4, 1 mM DTT, and KCl as indicated. The mixture was irradiated (λ = 365 nm) with a two-bulb 15-watt UV lamp at a distance of 2 cm to yield a UV irradiation dose of 0.1–0.2 watt/cm². The mixture was heated in SDS-PAGE loading buffer containing 80% formamide, 4% SDS, and 1 mM β-mercaptoethanol at 95 °C for 3 min and loaded onto a SDS-polyacrylamide gel containing 7% urea. Gels were covered with cellophane wrap, radioactive signals were measured using a Fuji imaging apparatus, and the gel was exposed to x-ray films. Measurements of radioactive label incorporated into protein products indicated that about 10% of the input protein was labeled.

**Exonuclease III Footprinting of RT/Primer/DNA Template Complexes—**Duplex DNA substrates were prepared by annealing the labeled primer (5′-ATACCTTTAACATATGACC-3′, 2.5 μM) with a 31-mer DNA template (5′-TTTTTTTTTTAGATAGATTTTTAGAT-3′) at a 1.15 primer/template molar ratio (determined spectrophotometrically) in 10 mM Tris-HCl, pH 8.0, 50 mM KCl by heating to 80 °C and slow cooling to room temperature over a period of at least 1 h. Immediately prior to the assay, the primer/template was diluted in RT buffer (50 mM Tris-HCl, pH 8.0, 50 mM KCl, 10 mM MgCl₂, 0.05% Triton X-100). RT (100 nM) was allowed to bind to the 5′-32P-labeled primer/ DNA template (100 nM), and 25 units of Exo III were added at time 0. When present, AZTTP (10 μM) was incorporated into the primer/template prior to Exo III digestion to yield a 3′-AZTMP-terminated 22-mer primer annealed to the 31-mer template. Aliquots were withdrawn at indicated times, and the reaction was quenched by the addition of formamide gel loading buffer. Products were analyzed by electrophoresis through a 12% polyacrylamide denaturing gel. Autoradiography and measurements of products using photostimulable plates were as described above.

**RESULTS**

We have compared binding of wild-type and AZT-resistant RT to various primer/templates in which the 3′ end of the DNA primer is either a hydroxyl or an azido group, and the template is either DNA or RNA.
Active-site Titration and Affinity of RT for Primer/DNA Templates—The binding affinities of wild-type and of AZT-resistant RT for a primer/DNA template have been determined using active-site titration procedures (17, 20, 22). Since the rate-limiting step is the release of the enzyme from the primer/template, the ternary complex accumulates much faster than it undergoes catalysis, and a burst of product is observed in the early course of the reaction. This burst is related to the concentration of the ternary complex. Hence, at early times during the reaction course, one can measure the ternary complex and obtain the equilibrium constant \( K_D \) of RT for its primer/template as well as the proportion of enzyme \( E_0 \) which is catalytically productive in the ternary complex. When the enzyme is allowed to bind to the primer/template and this binary complex is rapidly mixed with an excess of a single dNTP, the amplitude of the burst can be described as a function of the total enzyme concentration \( (E_0) \), the dissociation constant \( (K_D) \) of enzyme from its nucleic acid substrate and total nucleic acid concentration \( (D_0) \). Consequently, we assayed the polymerase activity of RT in this manner with a single dNTP present in the reaction in order to determine the equilibrium constant of dissociation \( (K_D) \) of wild-type and AZT-resistant RT from their primer/templates. Single turnover incorporation products were analyzed and measured by denaturing gel electrophoresis.

From the data obtained from the experiments shown in Fig. 1 (A and B), the \( K_D \) for binding DNA by the wild-type and AZT-resistant RT was 6.2 and 4.1 nM, respectively. This result is in agreement with the value that has been reported previously for a similar primer/template of defined sequence (17, 22). Depending on the enzyme preparation, 0.4–0.6 active site/dimer were found for the wild-type and 0.8–1.4 for the AZT-resistant RT, as judged by active-site titration regardless of the template used (Fig. 1B).

Values of \( k_{\text{cat}} \) were determined as described under “Experimental Procedures” to yield 6.27 ± 0.04 \( \times 10^6 \) M\(^{-1}\) s\(^{-1}\) and 2.7 ± 0.4 \( \times 10^5 \) M\(^{-1}\) s\(^{-1}\) for the wild-type and the AZT-resistant enzyme, respectively (Fig. 1C). From these values and the values of \( K_D \) determined above, \( k_{\text{off}} \) can be calculated from Equation 4 to yield off-rate constants of 0.04 s\(^{-1}\) and 0.011 s\(^{-1}\) for the wild-type and the AZT-resistant enzyme, respectively. Thus, the AZT-resistant RT might have an on-rate slightly lower than the wild-type enzyme. This finding deserves confirmation with more data points since the on-rate is generally diffusion-limited, and thus should be the same for both enzymes. Likewise, the AZT-resistant RT might have an off-rate slower than the wild-type enzyme, and this point was studied further.

Determination of the Catalytic Constant \( k_{\text{cat}} \)—For wild-type RT, \( k_{\text{cat}} \) represents the rate-limiting step of nucleotide polymerization when a single nucleotide is added to a primer. \( k_{\text{cat}} \) is equal to the off-rate constant \( k_{\text{off}} \) from the primer/template. It is thus important to determine if \( k_{\text{cat}} \) still represents the off-rate \( k_{\text{off}} \) for the AZT-resistant RT. We have measured the catalytic constant \( k_{\text{cat}} \) of the two RTs under steady-state conditions with various primer templates using assay 2.

The values of \( k_{\text{cat}} \) are shown in Table I. For the wild-type enzyme, \( k_{\text{cat}} \) values for the incorporation of various dNTPs are in the vicinity of 0.05 s\(^{-1}\) when DNA is the template, whereas they are 10-fold lower (0.004 s\(^{-1}\) when RNA is the template. There is no difference when AZTTP is the nucleotide substrate; \( k_{\text{cat}} \) equals 0.077 s\(^{-1}\) and 0.005 s\(^{-1}\) on DNA and RNA templates, respectively. When these measurements are performed using AZT-resistant RT, \( k_{\text{cat}} \) values for the incorporation of various dNTPs are around 0.008 s\(^{-1}\) on DNA templates, and 0.001 s\(^{-1}\) on RNA templates, 4–6-fold lower than values obtained with wild-type RT. When AZTTP is the nucleotide substrate, \( k_{\text{cat}} \) values are 0.003 s\(^{-1}\) and 0.001 s\(^{-1}\) when DNA and RNA is the template, respectively. Thus, the presence of the AZT resistance mutations decreases \( k_{\text{cat}} \) values. This decrease is maximum (from 0.077 to 0.003, i.e. 25-fold) when AZTTP is the substrate and the template is DNA.

Cross-linking of the Primer/Template to Wild-type and AZT-resistant RT—\( k_{\text{cat}} \) values are 4–25-fold lower for AZT-resistant compared with wild-type RT. In order to determine if \( k_{\text{cat}} \) equals \( k_{\text{off}} \) for the AZT-resistant RT also, we have examined

FIG. 1. Active-site titration of wild-type and AZT-resistant RT with a primer/template. A, time course of product formation as a function of primer/template concentration. Wild-type RT (40 nM) was preincubated in RT buffer (see “Experimental Procedures”) at 23 °C in the presence of 5 mM MgCl\(_2\) with increasing amounts of 5'-labeled primer/DNA template. The enzyme/primer/template complex was then challenged with AZTTP (final concentrations, 500 \( \mu \)M in 5 mM MgCl\(_2\)), quenched with formamide gel loading buffer after 5–30-s time intervals, and the products were analyzed by polyacrylamide gel electrophoresis. Individual time points of product formation corresponding to the different primer/template concentrations are plotted. The amount of product corresponding to the initial burst was determined by non-linear regression analysis of individual time courses. Extrapolation of the straight time course lines to the y axis allowed us to determine burst amplitude values. Burst amplitude as a function of primer/template concentration for wild-type and AZT-resistant (AZT-Res) RT. Burst amplitude values determined in A were plotted as a function of primer/template concentration. For AZT-resistant RT (50 nM), the nucleotide used to obtain the primary data set as in A was dTTP. Solid circles, AZT-resistant RT; empty squares, wild-type RT. The lines represent best fits to the quadratic equation for burst amplitude (Equation 1 under “Experimental Procedures”). From these fits, both \( E_0 \) and \( K_D \) were determined at 50.4% active enzyme and 6.2 ± 0.7 nM for the wild-type enzyme, respectively, and 72% active enzyme and 4.1 ± 1.6 nM for the AZT-resistant RT, respectively. C, determination of the on-rate \( k_{\text{on}} \) of wild-type and AZT-resistant RT on DNA. Data in A and B were used to determine the on-rate constant \( k_{\text{on}} \), as described under “Experimental Procedures.” Because this analysis is valid only when a substantial amount of free enzyme remains, the five and seven lower primer/template concentrations were used for AZT-resistant and wild-type RT, respectively. Data from A and B were used to calculate \( k_{\text{on}} E_0 \) and \( P/T_{\text{free}} \) and generate C. The slope of the linear fit gave \( k_{\text{on}} \) values of 6.27 ± 0.04 \( \times 10^5 \) M\(^{-1}\) s\(^{-1}\) and 2.7 ± 0.4 \( \times 10^4 \) M\(^{-1}\) s\(^{-1}\) for the wild-type and AZT-resistant RT, respectively.
both the binding of RT to a primer/template and the kinetics of release of RT from the RT/primer/template complex. If $k_{cat}$ equals $k_{off}$, it would indicate that primer/template binding properties differ for the two enzymes. Such a difference could either originate from a different manner of binding to the primer/template, or binding in a similar manner but with different kinetic properties. In order to address these issues, we have examined the binding of RT to a primer/template by cross-linking the enzyme to the primer. In this system, a 21-mer DNA primer has a photoreactive group at nucleotide position 15 relative to the 3′ end (26). This primer, labeled at its 5′ end with $^{32}$P, is first annealed to a 31-mer DNA or RNA template, and then RT is allowed to bind. Upon UV irradiation, the primer will be cross-linked to the p66 and p51 subunits of RT at amino acid residues sufficiently close to the photoreactive group. The nucleotide position 15 was chosen because the photoreactive group cross-links efficiently to either p66 when RNA is the template or p51 when DNA is the template (26). The extent and ratio of cross-linking of the labeled primer to p66 and p51 can be determined by denaturing polyacrylamide gel electrophoresis, where the 51- and 66-kDa subunits of RT cross-linked to the primer have apparent molecular masses of 57 and 72 kDa, respectively. It has been shown that both KCl concentration and template excess can affect primer/template binding by RT (27, 28).

In the previous section, we presented a kinetic analysis of the dissociation of the RT/primer/template complex in the absence of KCl (Fig. 4). We observed that the dissociation of wild-type RT was biphasic, while the dissociation of AZT-resistant RT was monophasic. We also showed that the dissociation rates of both enzymes were affected by KCl concentration, with wild-type RT being more sensitive to KCl than AZT-resistant RT.

To further investigate the effects of KCl on the dissociation of the RT/primer/template complex, we performed a kinetic analysis of the dissociation of the complex in the presence of varying KCl concentrations. The results of this analysis are presented in Table I.

**Table I**

| Kinetic constants $k_{cat}$ and amplitude for the dissociation of wild-type and AZT-resistant RT dimers from primer/DNA and primer/RNA templates |

When two values are present per row, the upper and lower values correspond to the first and second kinetic constant $k_{cat}$ of a biphasic curve fit, respectively (see “Experimental Procedures”). A single value per row is the kinetic constant $k_{cat}$ of a first-order curve fit.

| DNA template | RNA template |
|--------------|--------------|
| $k_{cat}^{a}$ | $k_{cat}^{a}$ |
| Amp. | $k_{cat}^{a}$ |
| $s^{-1}$ | $s^{-1}$ | % | % |
| WT-RT | WT-RT |
| 3′-OH | 0.057 | 0.0014 | 76 | 24 | 0.050 | 0.0098 |
| 3′-AZTMP | >0.05 | 0.0017 | 70 | 30 | 0.077 | 0.077 |
| Res-RT | Res-RT |
| 3′-OH | 0.047 | 0.00035 | 38 | 62 | 0.008 | 0.0015 |
| 3′-AZTMP | 0.0018 | 100 | 100 | 0.003 |

| DNA template | RNA template |
|--------------|--------------|
| $k_{cat}^{a}$ | $k_{cat}^{a}$ |
| Amp. | $k_{cat}^{a}$ |
| $s^{-1}$ | $s^{-1}$ | % | % |
| WT-RT | WT-RT |
| 3′-OH | 0.057 | 0.0014 | 76 | 24 | 0.050 | 0.0098 |
| 3′-AZTMP | >0.05 | 0.0017 | 70 | 30 | 0.077 | 0.077 |
| Res-RT | Res-RT |
| 3′-OH | 0.047 | 0.00035 | 38 | 62 | 0.008 | 0.0015 |
| 3′-AZTMP | 0.0018 | 100 | 100 | 0.003 |

$^{a}$ Values of correlation coefficients for both amplitude (Amp.) and $k_{cat}$ determination corresponding to Figs. 3 and 4 were found between 0.94 and 0.99.

**Fig. 2.** Cross-linking to RT p66 and p51 subunits of 5′-azido-32P-end-labeled primer annealed to DNA or RNA templates. A, wild-type RT was bound to a 5′-azido-32P-end-labeled 21-mer primer annealed to RNA templates, in the presence or absence of a 20-fold excess of template and KCl as indicated (see “Experimental Procedures”). The primer had a photoreactive $p$-azidophenacyl probe attached to the phosphate group at nucleotide position 15 from the 3′ end. The labeled primer was cross-linked to RT by UV irradiation (365 nm), the mixture was boiled for 3 min, subjected to denaturing gel electrophoresis, and the amount of label was quantitated using photostimulable plates. B, AZT-resistant (AZT-Res) RT was bound to the same primer/template as in A, and the cross-linking to the two subunits was analyzed as in A.

**Fig. 3.** Stability of a RT/primer/template complex—The results presented in the previous section indicated that $k_{cat}$ values are different between wild-type and AZT-resistant RT, and that the presence of the AZT resistance mutations alters binding efficiency or on the distribution of the label, but the total amount of label in both subunits decreased dramatically in the presence of 150 mM KCl (lanes 3 and 4). With RNA templates, 90% of the label was found on the p66 subunit regardless of KCl or template concentration (lanes 5–8), to yield the same cross-linking pattern as did the wild-type enzyme in the presence of 150 mM KCl when RNA was the template (Fig. 2A, lanes 7 and 8). In view of the effect seen with KCl we also carried out cross-linking of the primer on DNA templates to the AZT-resistant RT in the presence of increasing concentrations of KCl (0–150 mM). The total amount of cross-linked primer was inversely proportional to KCl concentration in an approximately linear fashion (data not shown). Thus, varying the KCl concentration reveals different cross-linking patterns between the two enzymes. Because hydrophobic and/or ionic interactions between primer/template and RT might be influenced by KCl concentration, we conclude that wild-type RT and AZT-resistant RT do not interact identically with their primer/templates. All subsequent experiments using wild-type and AZT-resistant RT were performed in the presence of 50 mM KCl to allow comparison with our previous kinetic data and results obtained by others.
properties of RT to its primer/template. In order to determine if these differences are related, we designed an alternate method to measure the off-rate constant $k_{o5}$ of the enzyme from the primer/template and to compare $k_{o5}$ to $k_{cat}$. In these experiments, wild-type or AZT-resistant RT was bound to a 5'-32P-end-labeled primer annealed to a RNA template. At time 0, a large excess of DNA was added to serve as a trap for free RT; at the indicated times, aliquots were cross-linked under UV (365 nm) for 25 s and subjected to denaturing gel electrophoresis (see "Experimental Procedures"). The total amount of label attached to RT subunits in each lane was measured using photostimulable plates. For both left and right panels, prior to addition of the trap, RT was incubated with primer/template in the presence of 10 $\mu$M AZTTP, giving rise to a 22-mer primer having a terminal 3'-azidophenacyl probe as in Fig. 2. At time 0, excess calf thymus DNA was added to serve as a trap for free RT; at the indicated times, aliquots were cross-linked under UV (365 nm) for 25 s and subjected to denaturing gel electrophoresis (see "Experimental Procedures"). The total amount of label attached to RT subunits in each lane was measured using photostimulable plates. For both left and right panels, prior to addition of the trap, RT was incubated with primer/template in the presence of 10 $\mu$M AZTTP, giving rise to a 22-mer primer having a terminal 3'-AZTMP. Time course of the decay of the RT/primer/RNA template complex.

The amount of radioactivity attached to RT subunits measured in each lane in left (wild-type RT) and right (AZT-resistant RT) panels in A served to construct left and right plots, respectively. Data were fitted to a double exponential (3'-OH, open circles) or a single exponential (3'-azido, solid circles) as described under "Experimental Procedures." Total counts per lane are absolute values and are not comparable between panels because they do not come from the same exposure to photostimulable plates.

In contrast to the results obtained with an RNA template, wild-type and AZT-resistant enzymes had different primer/template binding properties when DNA was the template (Fig. 4). Approximately 75% of the wild-type RT complex decayed during the first rapid phase regardless of the presence of AZTMP (Fig. 4, A and B, left panels). The second phase had an off-rate constant around 0.0013 s$^{-1}$ (Table I). AZT resistance mutations did not result in kinetic differences between the two enzymes (Fig. 3A, right panel). Virtually identical off-rate values were obtained under comparable conditions (Fig. 3B, right panel; Table I). As shown previously (Fig. 2), the majority of the label was found on the p66 subunit for both RTs.

The stability of a RT/primer/RNA template complex is shown in Fig. 3. On RNA templates, both wild-type and AZT-resistant RTs bound to the primer in a similar manner to their primer/template complexes. Both enzymes bound relatively tightly as judged by the slow decrease of label over the 10-min period. For the wild-type enzyme (Fig. 3, left panel), there was no difference when the 3' end of the primer was either AZTMP or dCMP. When dCMP terminated the primer, the decay of wild-type RT from the RT/primer/template complex was biphasic (Fig. 3B), with an initial phase too fast to be measured precisely in this experiment, followed by a slower phase with an off-rate constant around 0.0008 s$^{-1}$ (Table I). The amplitude of the first phase indicated that approximately 40% of the enzyme in the complex decayed during the early rapid phase. In the presence and after incorporation of AZTTP, the initial rapid phase disappeared and the decay of the complex could be fitted to a single exponential (Fig. 3B, left panel) to yield a similar off-rate value of 0.0013 s$^{-1}$ (Table I). AZT resistance mutations did not result in kinetic differences between the two enzymes (Fig. 3A, right panel).
The values of $k_{\text{cat}}$ calculated here are in good agreement with the calculated values of $k_{\text{cat}}$ for wild-type and AZT-resistant RT, respectively (Table I). $k_{\text{cat}}$ values fall between the two values of $k_{\text{cat}}$ when the decay is biphasic, or very close to them when the decay is a single exponential (Table I).

We conclude that the rate-limiting step under steady state conditions of a single nucleotide incorporation is the release of RT from its primer/template. In other words, $k_{\text{off}}$ equals $k_{\text{cat}}$ for both wild-type and AZT-resistant enzyme. The presence of these AZT resistance mutations confer a tighter binding of RT to its primer/DNA template. The presence of the 3’-terminal AZTMP confers an additional specificity of binding to the AZT-resistant enzyme on DNA templates. The AZT-resistant RT has very similar binding properties on DNA and RNA templates, whereas the wild-type RT has a much looser binding on DNA templates than on RNA templates.

Mapping the Interactions of the 3’ End of the Primer with RT—In order to examine the basis of the enhanced binding of the AZT-resistant RT to a primer/DNA template, we determined the footprint of RT on a RT/primer/DNA template complex. In this experiment, RT was bound to a primer/DNA template in which the 5’-32P-labeled primer bears either a hydroxyl (dCMP) or an azido (AZTMP) group at its 3’ end. In order to introduce an AZTMP at the 3’ end of the primer, the primer/template/RT complex was first incubated in the presence of 10 μM AZTTP. In this manner, more than 90% of the 21-mer primer gave rise to an AZTMP-terminated 22-mer product. At time 0, an excess of Exo III was added and aliquots were quenched using denaturating gel loading buffer at the indicated times. Samples were subjected to denaturing 15% polyacrylamide gel electrophoresis, and the gel was dried and exposed to x-ray films. A, time course of Exo III degradation of the 5’-32P-end-labeled 21-mer primer (control, RT omitted). B, time course of protection from Exo III degradation of the 5’-32P-end-labeled 21-mer primer by wild-type RT. C, time course of protection from Exo III degradation of the 5’-32P-end-labeled 21-mer primer by AZT-resistant RT. D, time course of protection from Exo III degradation of the 5’-32P-end-labeled 3’-AZMP-terminated 22-mer primer by wild-type RT. E, time course of protection from Exo III degradation of the 5’-32P-end-labeled 3’-AZMP-terminated 22-mer primer by AZT-resistant RT. The arrow in panels B–E points toward the 19-base-long primer discussed under “Results.”
AZT Resistance of HIV-1 RT

14603

without translocation of RT on DNA.

We conclude that AZT-resistant RT has an increased affinity for the 3′ end of the primer relative to wild-type RT, and that this affinity is further enhanced on AZTMP-terminated DNA chains. This conclusion is in agreement with the data obtained in the previous section in which the stability of the complex was measured using cross-linking of the primer to RT.

**DISCUSSION**

In HIV-1-infected cells, replication of the viral genome is dependent on the virus-encoded RT. For this reason, considerable efforts have been devoted to identifying inhibitors of RT. Since nucleotide analogues usually act as competitive inhibitors, most attempts to understand the mechanism of drug resistance have logically focused on nucleotide recognition. Previously, no obvious differences had been found for dTTP and AZTTP in nucleotide incorporation parameters, such as $K_m$ and $K_v$ values, sufficient to explain a >100-fold resistance to the analogue of the AZT-resistant virus in cultured cells (13, 14).

Pre-steady state kinetic studies have found that AZT-resistant RT is able to discriminate only 4-fold AZTTP when RNA is the template (15, 16). Consequently, it would appear that either undetectable changes in biochemical properties of RT in vitro translate into large phenotypic changes in AZT resistance in vivo, or as yet unknown cellular factor(s) are involved in AZT resistance (13).

The results presented here show that a clear difference exists in primer/template binding of the AZT-resistant RT compared with the wild-type enzyme. In addition, the nature of the template, either DNA or RNA, influences the binding of the RT to the primer/template. Wild-type and AZT-resistant RT show a similar binding to primer/RNA template tighter than to a primer/DNA template. Although this is in agreement with the results of Kerr and Anderson (15), these authors report values of $k_{cat}$ representing the dissociation rate constant significantly higher than the values reported here. These lower values might be due to the fact that our experiments were performed at 23 °C instead of 37 °C. However, RT used in the experiments of Kerr and Anderson (15) carries a polyhistidine extension at the N terminus of the p66 subunit. Examination of the 3α-structure of RT (29) shows that the p66 N-terminal peptide chain runs in the direction of the domain carrying mutations at amino acid 215 and 219, and proline 1 in the peptidic sequence of RT is located only 7.4 Å from threonine 215. A peptide made of 6 histidines would cover 15 Å when fully extended, and 9 Å when under a hypothetical helical conformation. Thus, it remains possible that the histidine tag on the RT used by Kerr and Anderson (15) interfered with the binding properties of RT to its primer/template.

The release of RT from the RT/primer/template complex is biphasic except when a 3′-azido group is present at the 3′ end of the primer. Therefore, the half-life of the RT/primer/template complex, which refers to a first order, monomolecular decay, is not appropriate for describing the kinetics of decay of the complex. Biphasic binding of wild-type RT to its primer/template is in agreement with results presented by others (27, 30, 31). We show here that the presence of an AZTMP-terminated DNA primer converts the biphasic pattern of decay to a monomolecular one. The presence of the next correct dNTP does not increase the stability of the complex (data not shown), indicating that the AZTMP-terminated DNA primer is sufficient to induce this switch from a biphasic to a monomolecular decay.

Binding of AZT-resistant RT to primer/DNA templates is virtually identical to binding to primer/RNA templates. With either complex a biphasic decay was observed unless a 3′-azido group was present at the 3′ end of the primer, in which case the decay was monomolecular. This result is in sharp contrast with the wild-type enzyme; 75% of the wild-type RT in the RT/primer/DNA template complex decayed at least 10-fold faster in the initial stages of the reaction than during the second slow phase. Furthermore, the first rapid phase of decay was not suppressed by the presence of AZTMP-terminated DNA. We ruled out an active displacement of 75% of the RT from the RT/primer/DNA template complex during the initial phase by the DNA trap. Indeed, displacement does not occur when AZT-resistant RT is bound to the primer/DNA template (Fig. 2A, lanes 1 and 2). The binding differences between the two RTs when DNA is the template are further demonstrated in a nuclease assay. Footprinting experiments indicate that the last 2–4 bases of the AZTMP-terminated DNA primer are bound more tightly to the AZT-resistant enzyme than to the wild-type enzyme. Overall, these results indicate that the wild-type enzyme and the AZT-resistant enzyme binds to the primer differently, depending on whether the template is DNA or RNA. In this scheme, the AZT resistance mutations might add novel contacts to the 3′ end of the primer on DNA templates to yield a tighter contact than that observed when the primer is annealed to a RNA template. Since the result shown in Fig. 2 as well as in previous work (26) have shown that primer/DNA templates are not positioned the same as primer/RNA templates at the RT surface, we do not believe that the tighter binding of primer/DNA templates by AZT-resistant RT is due to positioning the primer/DNA template like a primer/RNA template.

A tighter binding of the 3′ end of the AZTMP-terminated primer suggests that repair accounts at least in part for AZT resistance, and argues against any other enzyme than RT itself to mediate this process. In this model, AZTTP would be discriminated against modestly by RT, 1–4-fold (14, 15) to 6-fold.2 Removal of the terminal 3′-AZTMP would provide RT with another chance to incorporate dTMP. Since RT does not have an inherent 3′ to 5′ exonuclease activity, pyrophosphorolysis, the reversal of nucleotide polymerization, must account for the removal of AZTMP. For wild-type RT, the rate constant for pyrophosphorylolytic excision of AZTMP from the 3′-primer terminus is comparable to the rate constant for dissociation (18). Consequently, Reardon (18) has pointed out that millimolar concentrations of intracellular inorganic pyrophosphate would be required for pyrophosphorolysis repair of AZTMP-terminated DNA. Thus, a lower off-rate constant of RT from the 3′-AZTMP terminated primer should increase the likelihood of repair at lower concentrations of pyrophosphate. This is pre-

---

2 B. Canard, S. R. Sarfati, and C. C. Richardson, unpublished results.
cisely what we observe; the presence of the four mutations decreases $k_{\text{cat}}$ 25-fold for AZT-resistant RT relative to wild-type RT on AZTMP-terminated primer/DNA templates. Thus, enhanced repair of the AZTMP-terminated DNA chain through an increased stability of the RT3′-AZTMP primer/template complex and pyrophosphorolysis is an attractive possibility.

Finally, this model of AZT resistance makes several predictions that are fully consistent with existing data regarding HIV-1 chemotherapy. First, pyrophosphorolysis must infer a certain degree of AZT resistance to wild-type RT. This conclusion is consistent with the failure of AZTTP to block completely HIV-1 replication. AZTTP and dTTP are equally good substrates for RT, and AZTTP is present at the micromolar level in the cell (19). Hence, even if dTTP is in excess over AZTTP, an exceedingly small quantity of replicating HIV-1 genomes should avoid premature AZTMP termination during the >4000 events of insertion of a thymine opposite to a template adenine (17). Yet, viral load hardly decreases ($<$1 log$_{10}$) after initiation of AZT therapy and then quickly return to its original value prior to the appearance of the first AZT resistance mutations (34). Thus, one can conclude that wild-type HIV-1 has a natural resistance to AZT and that this resistance is not accounted solely by nucleotide selection at the RT active site.

The second prediction is that any drug-mediated effect on pyrophosphorolysis should interfere with AZT resistance. Precisely such an effect is observed with the pyrophosphate analogue phosphonoformate (PFA). When PFA is used to inhibit HIV-1 in cultured cells, it is possible to select for PFA-resistant isolates of the virus. PFA-resistant mutations map to the pol gene, and include amino acids 88, 89, 90, 92, 113, and 114. Moreover, viruses having these mutations are now hypersensitive to AZT, whereas 3TC resistance results but AZT resistance is suppressed (35). Resistance to both PFA and AZT are difficult to achieve and might even be exclusive (35). This finding is also reminiscent of the difficulty for the virus to develop resistance to both AZT and the nucleotide analogue 3′-thiacytidine (3TC) (36). It is interesting to note that the AZT-resistant enzyme has a higher processivity of nucleotide polymerization (37) and primer/template affinity (this work), whereas 3TC-resistant RT (M184LV) is a less processive enzyme (38). As in the case of PFA and AZT, this suggests that the mechanism of resistance would be different for AZT and 3TC, accounting for the difficulty in obtaining dual resistance to these analogues (36). Consistent with this observation, 3TC resistance can be totally accounted for by a loss of affinity for RT of the 5′-triphosphate form of 3TC (16). It would be interesting to study the pyrophosphorolysis reaction using PFA- and 3TC-resistant or AZT-resistant RTs in order to determine the validity of the proposed model.

The third consequence of the model is the issue of cross-resistance. Amino acid changes altering the selection of nucleotide analogue are expected to be specific and dictated in part by the chemical properties of the analogue. Thus, these amino acids are less likely to yield to cross-resistance to other nucleotide analogues. However, if resistance to two different drugs arises as a result of effects on pyrophosphorolysis, then cross-resistance may occur. The fourth consequence of the model is related to the pattern of acquisition of the AZT resistance mutations over time. It is clear that this pattern follows a defined order important to obtain full AZT resistance. One logical explanation for this ordered pattern is that the enzyme must acquire at least two new properties, and that acquisition of a single property (e.g., discrimination) might decrease the other (e.g., pyrophosphoroly-
Enhanced Binding of Azidothymidine-resistant Human Immunodeficiency Virus 1 Reverse Transcriptase to the 3'-Azido-3'-deoxythymidine 5'-Monophosphate-terminated Primer
Bruno Canard, Simon R. Sarfati and Charles C. Richardson

J. Biol. Chem. 1998, 273:14596-14604.
doi: 10.1074/jbc.273.23.14596

Access the most updated version of this article at http://www.jbc.org/content/273/23/14596

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 37 references, 25 of which can be accessed free at http://www.jbc.org/content/273/23/14596.full.html#ref-list-1