Sensitivity of HIV-1 Reverse Transcriptase and Its Mutants to Inhibition by Azidothymidine Triphosphate

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ABSTRACT: HIV-1 reverse transcriptase can catalyze the addition of either azidothymidine monophosphate (AZTMP) or thymidine monophosphate (dTMP) to a primer strand opposite template adenosine bases. The ratio of incorporation of AZTMP to dTMP as catalyzed by HIV-1 reverse transcriptase has been determined to be 0.4 using an RNA–DNA duplex substrate prepared from oligonucleotides with sequences taken from the HIV-1 genome sequence. Slight variations are found for the incorporation ratio of the two nucleotides on other substrates. Substrates containing more than one adenosine in the single-stranded part of the template allow for more chances to incorporate AZTMP and less full-length product. Variations in the intensity of bands on an autoradiograph of a DNA sequencing gel corresponding to different positions of incorporation of AZTMP suggest that not all template adenosine positions offer the same level of discrimination against incorporation of AZTMP. A reverse transcriptase containing a set of four mutations (D67N, K70R, T215Y, K219Q) known to cause resistance to AZT in cell culture assays has a ratio of incorporation that is 0.77 ± 0.03 times the ratio for the wild-type reverse transcriptase opposite one specific template adenosine. In contrast, a hybrid mutant containing the same four mutations that cause resistance to AZT and an additional mutation, Y181C, which by itself causes resistance to the non-nucleoside inhibitor L-697,661 [Sardana et al. (1992), *J. Biol. Chem. 267,* 17526–17530], has a ratio of incorporation that is 1.34 ± 0.01 times that of the wild-type, indicating that the hybrid mutant enzyme is more susceptible to inhibition by AZTTP than the wild-type reverse transcriptase. HIV-1 reverse transcriptase is able to catalyze removal of incorporated AZTMP in the presence of physiological concentrations of pyrophosphate. The second-order rate constant for removal of AZTMP from AZTMP-terminated primers is, however, only 3.4 M^{-1} s^{-1} for the wild-type reverse transcriptase and 1.5 M^{-1} s^{-1} for the AZT-resistant form of reverse transcriptase. The abilities of a number of other site-specific mutants, resistant to either nucleoside or non-nucleoside inhibitors, to incorporate AZTMP have been determined.

Human immunodeficiency virus (HIV)[1] is the etiologic agent of acquired immune deficiency syndrome (AIDS) and contains a reverse transcriptase (RT) that converts the viral RNA genome into a double-stranded DNA copy prior to integration into the host cell genome. The active form of the approved therapeutic, azidothymidine (AZT) is both a substrate and an inhibitor of RT (Reardon & Miller, 1990).

Prolonged therapeutic use of AZT in AIDS patients has allowed the development of strains of HIV resistant to inhibition by AZT (Larder et al., 1989). A number of mutations associated with the emergence of resistant virus have been mapped to the RT gene. Several different sets of mutations have been identified which result in different levels of resistance to inhibition in cell cultures. One set of mutations (D67N, K70R, T215Y, K219Q) results in 120-fold resistance to inhibition by AZT in cell culture (Kellam et al., 1992). Characterization of the purifed RT containing the mutations that give rise to resistance in cell culture has shown that the resistant RT displays only a 2-fold increase in the inhibition constant (K_i) for AZTTP (Lacey et al., 1992).

The development of another class of inhibitors of HIV-1 RT, the non-nucleoside inhibitors (reviewed by De Clercq, 1993), has created the possibility of combination therapies. Combination therapy employing both classes of nucleoside and non-nucleoside inhibitors simultaneously could decrease the likelihood of development of highly resistant strains of virus. The success of combination therapies will depend, in part, on the susceptibility of viral strains that are resistant to one class of inhibitor to inhibition by the other class of inhibitor. Therefore, predictions of the outcome of any therapy based on a combination of AZT and, for example, L-697,661, a non-nucleoside inhibitor (Saari et al., 1991), could be facilitated by a detailed knowledge of the inhibition by AZTTP (or L-697,661) of mutants of RT that are resistant to inhibition by L-697,661 (or AZTTP). Furthermore, an understanding of the resistance to a drug by delineating the sources which give rise to the resistance provides a means of elucidating the true mechanism of action of the drug.

This work examines the ability of HIV-1 RT to catalyze the incorporation of AZTMP and dTMP at specific positions along a synthetic RNA template as a means of judging the susceptibility of RT to inhibition by AZTTP. The probability of incorporation of AZTMP leading to chain termination during reverse transcription is determined to gauge the potency of inhibition by AZTTP. Differences are noted in the ratio of incorporation of AZTMP to dTMP as catalyzed by wild-type RT and site-specific mutants of the enzyme that are resistant to either class of inhibitors. The reversal of incorporation of AZTMP in the presence of physiological concentrations of pyrophosphate can be catalyzed by both

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[1] Abbreviations: AIDS, acquired immunodeficiency syndrome; HIV, human immunodeficiency virus; RT, reverse transcriptase; AZT, 3′-deoxy-3′-azidothymidine; AZTMP, 3′-deoxy-3′-azidothymidine monophosphate; AZTTP, 3′-deoxy-3′-azidothymidine triphosphate; ddI, dideoxyinosine; dNTP, deoxynucleoside triphosphate; EDTA, ethylenediaminetetraacetic acid; EGTA, 1,2-bis(2-aminoethyl)ethanetetraacetic acid; CIC_{95}, the concentration of inhibitor to yield a 95% inhibition of viral growth in cell culture; PEG8000, polyethylene glycol 8000; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PP_i, sodium pyrophosphate.

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wild-type and mutant RTs. The contributions of these mechanisms to the survival of HIV strains resistant to inhibition by AZT are discussed.

MATERIALS AND METHODS

Materials. Plasmids containing the RT gene and its mutants were provided by J. Condra (Merck Research Labs, West Point, PA). Wild-type HIV-1 reverse transcriptase and four of its mutants (the point mutant K103N, the double mutant K103N-Y181C, the quadruple AZT-resistant mutant D67N-R70K-T215Y-K219Q, and the RNase H-deficient mutant D443N) were prepared and purified as described (M. Stahlhut, J. Fu, J. Condra, and D. B. Olsen, manuscript in preparation). Other mutants of RT were expressed in *E. coli* AB1899 as the p66 subunit and purified as the bacterially-processed heterodimeric form with the same procedure; they included the single mutant Y181C and the hybrid mutant D67N-R70K-T215Y-K219Q-Y181C. All enzymes were judged to be >90% pure with SDS-polyacrylamide gel electrophoresis accompanied by Coomassie-blue staining. AZTTP was purchased from Sierra Biomedical Research (Tucson, AZ). Ultrapure dNTPs, poly(rC)–oligo(dG), and poly(rA)–oligo(dT) were purchased from Pharmacia. [α-^{32}P]Deoxynucleotide triphosphates (dNTPs) and [γ-^{32}P]ATP were from New England Nuclear. T4 polynucleotide kinase was supplied by United States Biochemicals. DE81 filter disks were obtained from Whatman. Sep-Pak purification cartridges were from Waters. Sodium pyrophosphate was purchased from Sigma and used without further purification.

Primer/Template. DNA oligonucleotides were synthesized on an Applied Biosystems Model 380B synthesizer and purified by gel electrophoresis. RNA oligonucleotides were synthesized on the same instrument using 2′-silyl-protected phosphoramidites supplied by Milligen and 3′-linked columns from Glen Research. Synthetic RNA was deprotected in 1 M tetrabutylammonium fluoride (Sigma) for 2-3 days and purified as previously described (Olsen et al., 1991). Concentrations of DNA and RNA oligonucleotides were determined with absorbance at 260 nm using extinction coefficients calculated from the sequences. Heteroduplex substrates were prepared immediately prior to use by mixing stoichiometric amounts of the two constituent oligonucleotides for 5 min at 25 °C. DNA oligonucleotides were 5′-end labeled and purified as previously described (Carroll et al., 1993). The sequences of the substrates are given in Figure 1.

Competition for Incorporation of AZTMP and dTMP. The ability of each enzyme to incorporate AZTMP relative to dTMP was measured in reactions that included 250 nM 5′-end-labeled p22/tC5U (primer/template), 250 nM enzyme, 20 μM dGTP, 20 μM dATP, 2 μM dTTP, and from 0 to 12 μM AZTTP in standard reaction buffer (50 mM Tris, pH 7.8, 80 mM KCl, 6 mM $MgCl_2$, 0.2% PEG-8000). Reactions were initiated with the addition of enzyme at 25 °C except where noted. Aliquots were withdrawn at 1.5- and 2-h reaction time and quenched by addition to an equal volume of formamide loading buffer. Analysis of the amount of primer extended in the reaction to 23 bases in length, which corresponds to incorporation of AZTMP, or to lengths greater than 23 bases, which correspond to incorporation of dTMP at the 23rd position of the template, was carried out by electrophoresis on denaturing polyacrylamide gels. Bands corresponding to primer of length 23 or of length greater than 23 were located by autoradiography and quantified by cutting the band from the gel and counting in DuPont Formula 989 scintillation cocktail, or by using a PhosphorImager from

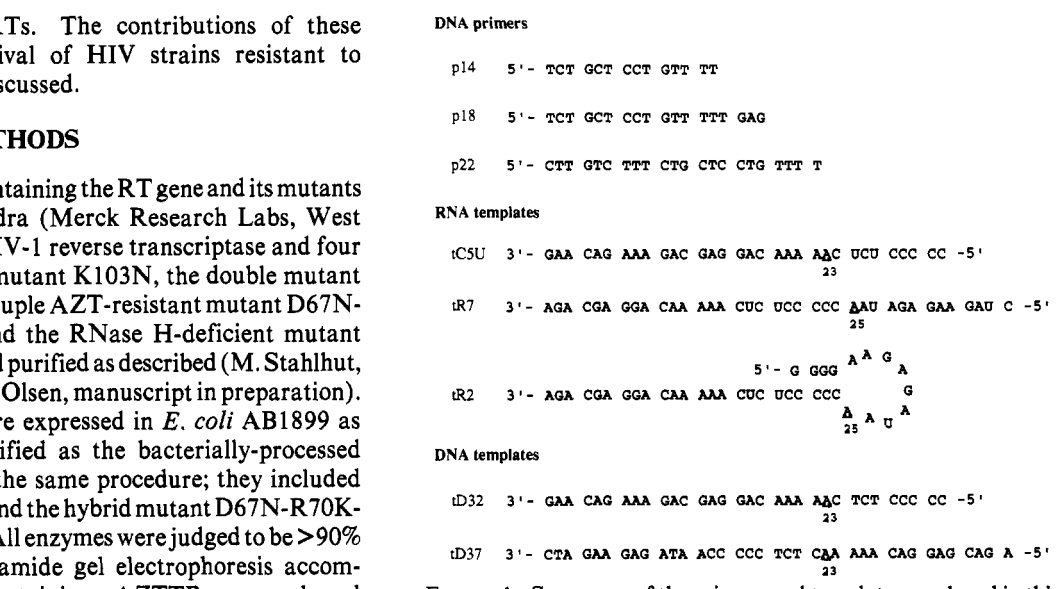

FIGURE 1: Sequences of the primers and templates employed in this study. The sequences of the RNA templates tC5U and tR2 are taken from the sequence of the HIV-1 genome. The template adenosine corresponding to the first position for incorporation of AZTMP is underlined and numbered from the 3′ end.

Molecular Dynamics. Low concentrations of DTT (10–30 μM) were included in the reactions from the enzyme stock solutions. A control reaction with an additional 50 μM DTT showed no change in the ratio of incorporation of AZTMP to dTMP.

Reactions with p18/tR2 and p18/tR7 as primer/template were carried out with the same reaction conditions except the following: the concentrations of dGTP, dATP, and dCTP were 50 μM; the concentration of dTTP was 20 μM; and the concentrations of AZTTP were 0, 5, 10, 15, and 20 μM. The reaction times were 1, 2, and 3 h. For reactions with p18/tR2, bands on the gel corresponding to primer of length 25, the first position for incorporation of AZTMP, 26–33-mers, which includes the other positions for incorporation of AZTMP, and 37-mer and larger, the products of extension of the primer to the end of the template, were excised from the gel and quantified by scintillation counting.

Reactions with p22/tD32 were carried out under the same conditions as those for p22/tC5U except that the concentrations of dGTP and dATP were 25 μM, the concentration of dTTP was 10 μM, and the concentrations of AZTTP were 0, 5, 10, 15, and 20 μM. Reactions with p14/tD37 were carried out under the same conditions as for p22/tD32 except the concentration of dTTP was 20 μM. Bands corresponding to 15-mer, the first position for incorporation of AZTMP, 16–36-mers, which include the other positions for incorporation of AZTMP, and 37-mer and greater, the full length products, were cut from the gel and quantified by scintillation counting. Reactions with p14/tR2 were carried out with 1 μM dTTP, 10 μM dGTP and dATP, and from 0.1 to 2 μM AZTTP. Bands corresponding to 15-mer, the product of incorporation of AZTMP at the first template adenosine position, and 16–37-mers, products from the incorporation of dTMP, were cut from the gel and quantified by scintillation counting.

Removal of Incorporated AZTMP by Pyrophosphorolysis. Reactions were carried out using p22/tC5U as primer/template under the same conditions as above, except that the concentrations of dTTP and AZTTP were 5 μM, and dGTP and dATP were at 50 μM. Sodium pyrophosphate was either 0 or 100 μM. Reactions were initiated by addition of enzyme, and aliquots were withdrawn and quenched at reaction

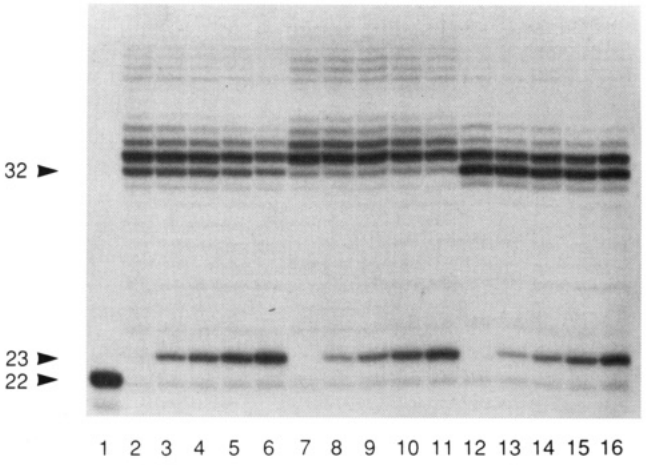

FIGURE 2: Analysis of products from synthesis on p22/tC5U catalyzed by HIV-1 RT and its site-specific mutants. RT-catalyzed reactions included 250 nM enzyme (the double mutant K103N-Y181C, lanes 2–6; wild-type RT, lanes 7–11; AZT-resistant mutant, lanes 12–16), 250 nM [^{32}P]p22/tC5U, 5 μM dTTP, 20 μM dGTP, 20 μM dATP, and 0 (lanes 2, 7, 12), 1.25 μM (lanes 3, 8, 13), 2.5 μM (lanes 4, 9, 14), 5 μM (lanes 5, 10, 15), or 10 μM (lanes 6, 11, 16) AZTTP in reaction buffer. Reactions were carried out at 25 °C for 1.5 h and quenched in gel load buffer. Products were separated by electrophoresis on 20% acrylamide–8 M urea gels, and visualized by autoradiography. The numbers indicate the length of the corresponding oligonucleotide product. The primer is shown in lane 1.

times varying from 1 to 120 min. Bands corresponding to 23- and 32-mers were quantified by cutting and scintillation counting. The second-order rate constants for removal of incorporated AZTMP by wild-type RT and AZT-resistant mutant were determined under the same reaction conditions except that the concentration of sodium pyrophosphate was varied from 50 to 500 μM.

Measurement of k_{cat} and K_M for dTTP with Poly(rA)–Oligo(dT) as Primer/Template. Reactions were carried out at 25 °C in the presence of 50 mM Tris, pH 7.8, 80 mM KCl, 1 mM DTT, 6 mM $MgCl_2$, 0.2% PEG-8000, 100 μM EGTA, 50 μg/mL poly(rA)–oligo(dT), 0.5 nM enzyme, 1–20 μM [α-^{32}P]dTTP. Reactions were initiated with the addition of enzyme and quenched after 15 min by the addition of EDTA to 143 mM (final concentration). Analysis for the amount of product formation was carried out with filter-binding using DE81 filters as described (Bryant et al., 1983). Analysis of the steady-state kinetic parameters was determined by using nonlinear least-squares calculations to fit iteratively the velocity saturation data to the Michaelis–Menten equation.

RESULTS

Ratio of Incorporation of AZTMP to dTMP. The reverse transcriptase from HIV-1 can catalyze the incorporation of either AZTMP or dTMP into a DNA primer during transcription in a template-directed manner. Reaction products resulting from the incorporation of either AZTMP or dTMP were visualized by 5′-end labeling the primer strand with ^{32}P, followed by electrophoretic separation of the products on a sequencing gel. In the case of substrate p22/tC5U (sequences shown in Figure 1), incorporation of AZTMP into the primer strand led to chain termination and the appearance of a product band 23 nucleotides in length, as shown in Figure 2. On the other hand, incorporation of dTMP at the 23rd position allowed for extension of the primer strand to the end of the template, when dATP and dGTP were included in the reaction mixture generating a DNA product 32 nucleotides in length. Due to the ability of HIV-1 RT to extend primers beyond the end of the template (Peliska & Benkovic, 1992) and the long reaction times, part of the 32-mer product was extended to lengths greater than the length of the template. For the purposes of quantification, all products of length greater than 23 bases were grouped together as products resulting from the incorporation of dTMP. Therefore, given a sufficient reaction time for full-length primer elongation in a reaction containing both AZTTP and dTTP, the relative amount of incorporation of AZTMP versus dTMP is reflected in the amounts of the 23-mer and the products of length greater than 23 nucleotides. Under these conditions, the ratio of the rates of incorporation of each base individually governs the ratio of the amount of the two products in the competitive reaction.

The plot of the ratio of the amounts of the AZTMP product to the amounts of the products resulting from incorporation of dTMP against the ratio of the triphosphates is linear, as shown in Figure 3, for the reaction on substrate p22/tC5U. The slope of the line is a measure of probability of incorporating AZTMP relative to dTMP within the range of concentrations of triphosphates tested and is defined as the incorporation ratio, R:[2]

$$\frac{[\text{AZTMP products}]}{[\text{dTMP products}]} = R\left\{\frac{[\text{AZTTP}]}{[\text{dTTP}]}\right\} \quad (1)$$

A greater slope indicates a greater probability of incorporating AZTMP instead of dTMP onto a primer opposite an adenosine on the template. Reaction conditions employed RT at a concentration approximately equivalent to the concentration of the primer/template both to ensure completion of the reaction and to mimic more closely conditions *in vivo*.

For the wild-type RT, the ratio of the two products, in a reaction with a given initial ratio of AZTTP to dTTP remains essentially constant throughout the time course of the reaction (90 min), as shown in Figure 4. The incorporation ratios obtained for the wild type and two of its mutant enzymes with different substrates were determined. The data are shown in Tables 1 and 2. The wild-type RT incorporates AZTMP with 0.40 times the efficiency that it incorporates dTMP on the primer/template p22/tC5U. The AZT-resistant mutant (D67N-R70K-T215Y-K219Q) incorporated AZTMP with 0.30 times the efficiency that it incorporates dTMP, which is 0.75 times the value for the wild-type RT. The double mutant, K103N-Y181C, has an incorporation ratio that is 1.4 times that of the wild-type RT indicating that relative to dTMP it can more easily incorporate AZTMP than can the wild-type enzyme.[3]

Incorporation Ratio on Other Primer/Templates. Other primer/template systems were used to examine the incorporation of AZTMP catalyzed by RT. With thymidine substituting for uracil, p22/tD32 is a DNA–DNA duplex with

[2] Under steady-state conditions, it can be easily shown that

$$\frac{[\text{AZTMP products}]}{[\text{dTMP products}]} = \frac{(k_{cat}/K_M)^{\text{AZTTP}}}{(k_{cat}/K_M)^{\text{dTTP}}}\left\{\frac{[\text{AZTTP}]}{[\text{dTTP}]}\right\}$$

so that a plot of [AZTMP products]/[dTMP products] versus [AZTTP]/[dTTP] is linear with a slope equaling $(k_{cat}/K_M)^{\text{AZTTP}}/(k_{cat}/K_M)^{\text{dTTP}}$, the ratio of the pseudo-second-order rate constants of the enzymic reaction obtained with the respective substrates. This ratio equals the efficiency of AZTMP incorporation versus dTMP incorporation. When the condition of the enzymic assay is not necessarily steady state, as is the case here since the number of incorporation sites is limiting within the time frame of the reaction, the slope may be simply defined as the incorporation ratio of the two substrates. For multiple AZTMP (or dTMP) incorporations, the relation is given in eq 2.

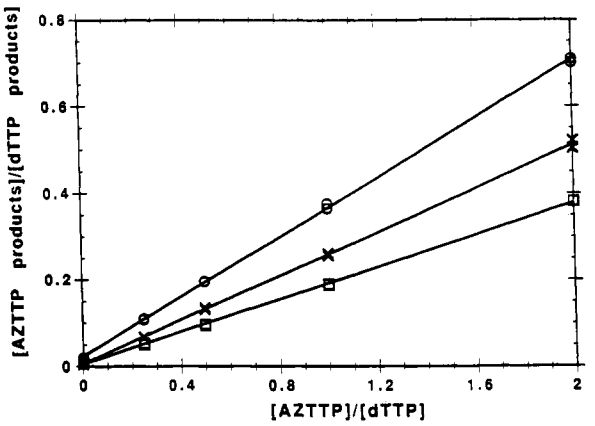

FIGURE 3: Competition for incorporation of AZTMP or dTMP on p22/tC5U as catalyzed by HIV-1 RT and its site-specific mutants. Each enzyme (wild-type, ×; hybrid mutant (D67N-K70R-Y181C-T215Y-K219Q), ○; AZT-resistant mutant, □) present at 250 nM was incubated with 250 nM p22/tC5U in reaction buffer (50 mM Tris, pH 7.8, 80 mM KCl, 6 mM $MgCl_2$, 0.2% PEG8000) in the presence of 10 μM dTTP, 20 μM dGTP, 20 μM dATP and from 0 to 20 μM AZTTP. Aliquots were withdrawn from the reaction at 1.5 and 2 h and quenched into acrylamide gel load buffer. The samples were then separated on a 20% acrylamide–8 M urea gel. The amounts of products corresponding to the incorporation of either AZTMP or dTMP were analyzed as described under Materials and Methods. Both time points are plotted in the figure to demonstrate that the ratio of products is constant during this time interval.

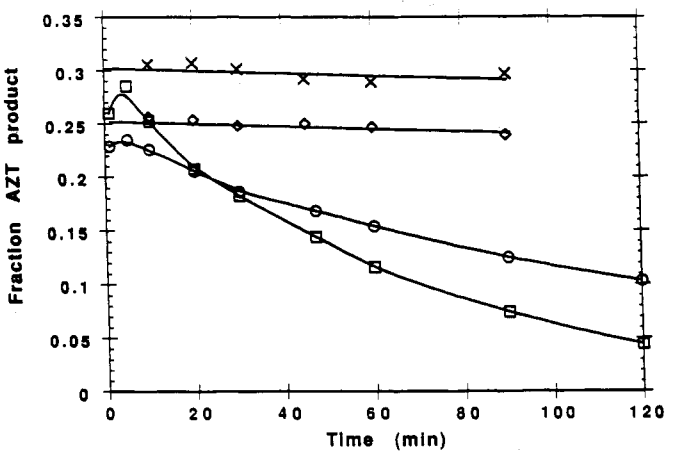

FIGURE 4: Time course of the appearance of the AZTMP-product in the RT-catalyzed polymerization reaction. Reaction conditions were the same for Figure 2 except that the concentration of AZTTP was 10 μM. Reactions were initiated by the addition of enzyme (wild-type RT, ×, □; AZT-resistant mutant, ◇, ○) to 250 nM. Sodium pyrophosphate was not added (×, ◇) or added to 100 μM (○, □). Product analysis was as described in Materials and Methods.

the same sequence as p22/tC5U. The incorporation ratio on p22/tD32 is shown in Table 1 and indicates that there is a slight increase in the incorporation of AZTMP relative to dTMP with a DNA template for the wild-type RT. The reaction on substrate p22/tC5U was repeated at 37 °C, and a slight decrease in the incorporation ratio was detected. The mutant K103N-Y181C and the AZT-resistant mutant showed only modest changes in the incorporation ratio relative to that of the wild-type RT on the other substrates shown in Table 1.

To examine the effect of template sequence on the incorporation of AZTMP versus dTMP, the competition experiment was carried out on the primer/template p18/tR2, which contains six adenosines in the single-stranded portion of the template. To limit the measurement of the incorporation ratio to the first adenosine of the template (adenosine-25), all products of length greater than 25 were combined and treated as products resulting from the incorporation of dTMP at the 25th position of the template. Compared to substrate p22/tC5U, the incorporation ratio for the wild-type RT decreased slightly for the first template adenosine on substrate p18/tR2. The incorporation ratio was determined similarly for mutants K103N-Y181C and the AZT-resistant mutant (D67N-R70K-T215Y-K219Q). The difference between the ability of K103N-Y181C and the wild-type RT to catalyze the incorporation of AZTMP relative to dTMP increased on p18/tR2 relative to p22/tC5U, but the difference between the AZT-resistant mutant and the wild-type RT decreased slightly. The competition experiment was also carried out with substrate p14/tD37. The results are shown in Table 1 and indicate that the level of difference between the mutants and the wild-type RT varies only slightly at adenosine-23 of substrate p14/tD37.

Effect of Multiple Positions for Incorporation of AZTMP or dTMP. The effect of multiple positions for incorporation of AZTMP or dTMP was determined with substrate p18/tR2 and p18/tR7. Qualitatively, as shown in Figure 5A for substrate p18/tR7, the intensities of the bands corresponding to the incorporation of AZTMP vary significantly, and some light bands are followed in the sequence by darker bands. If each template adenosine offered the same level of discrimination against incorporation of AZTMP, the intensity of each succeeding band would decrease monotonically, because the number of primers that are available for extension after each template adenosine would decrease as more primers become terminated with AZTMP. The integration of the amount of products is shown in Figure 5B and indicates that the third position for incorporation of AZTMP (adenosine-28) shows a significantly greater intensity than the first two positions. Therefore, not all template adenosines offer the same level of discrimination against incorporation of AZTMP. The same general pattern of bands was observed with K103N-Y181C, the wild-type RT, and the AZT-resistant mutant. Furthermore, the amount of full-length product at the same ratio of triphosphates increased going from K103N-Y181C to wild-type RT to AZT-resistant mutant (Figure 5A, e.g., lanes 6, 11, and 16).

Quantitatively, the relationship between the ratio of the AZTMP products to the dTMP products is given by eq 1 at *each* adenosine site on the template. Therefore, the plot of the ratio of the two products against the ratio of the concentration of the triphosphates is no longer linear but is a higher order function with respect to the ratio of the concentrations of the triphosphates.

$$\frac{[\text{AZTMP products}]}{[\text{dTMP products}]} = \prod R_n \left\{ \frac{[\text{AZTTP}]}{[\text{dTTP}]} \right\}_n, \quad n = 1, 2, 3, \text{etc.} \quad (2)$$

Substrate p18/tR2 contains six adenosines (p18/tR7 has seven adenosines) in the single-stranded region of the template. To

[3] Incorporation of the HIV viral genome into the host cell DNA requires several hours. For this reason, longer reaction times of *in vitro* RT assays may more appropriately mimic the *in vivo* situation by allowing for pyrophosphorolysis. Longer reaction times also ensure all TTP incorporation products were extended in our assays. When the reaction time is 15 min instead of 90 min, no significant difference in the incorporation ratio was observed. For example, the double mutant, K103N-Y181C, yields an incorporation ratio ([AZTMP]/[dTMP]) that is 1.55 times that of the wild-type RT while the AZT-resistant mutant gives a ratio of 0.69 times that of the wild-type RT.

Table 1: Incorporation Ratios (AZTMP to dTMP) on Different Primer/Templates in Reactions Catalyzed by HIV-1 RT and Its Site-Specific Mutants at 25 °C

primer/template	incorporation ratio[a]			ratio (K103N-Y181C/wt)	ratio (AZT-r/wt)
	K103N-Y181C	wild-type	AZT-resistant mutant[b]		
p18/tR2[c]	0.36	0.16	0.14	2.2	0.88
p22/tD32	0.86	0.54	0.4	1.6	0.74
p14/tD37[c]	1.09	0.69	0.47	1.6	0.68
p22/tC5U	0.55	0.40	0.30	1.4	0.75
p22/tC5U[d]	0.42	0.27	0.22	1.6	0.83

[a] Slope of the plot of the ratio of AZTMP products to dTMP products against the ratio of the concentration of the triphosphates. [b] D67N-K70R-T215Y-K219Q. [c] Incorporation ratio at the first adenosine in the single-stranded region of the template. [d] T = 37 °C.

Table 2: DNA Synthesis by Site-Specific Mutants of HIV-1 RT

enzyme	k_{cat}[a] (s^{-1})	K_M[a] (μM)	k_{cat}/K_M ($\mu M^{-1}\ s^{-1}$)	AZTMP/dTMP incorporation ratio[b] (mutant/wt)
wild-type RT-1	1.9	4.8	0.40	
AZT-r[c]	1.3	4.8	0.27	0.77 ± 0.03[f]
K103N-Y181C	1.8	6.6	0.27	1.45 ± 0.06
AZT-r + Y181C[d]	1.8	16.6	0.11	1.34 ± 0.01
K103N	1.7	4.1	0.41	1.17 ± 0.05
RNaseH deficient[e]	1.6	3.4	0.47	1.00 ± 0.02
Y181C	1.5	8.1	0.19	1.47 ± 0.01

[a] Kinetic parameters were determined with poly(rA)–oligo(dT) as described in Materials and Methods. [b] The incorporation ratios (AZTMP to dTMP) were determined with substrate p22/tC5U as described in Materials and Methods. [c] D67N-K70R-T215Y-K219Q. [d] D67N-K70R-T215Y-K219Q-Y181C. [e] D443N. [f] Standard deviation is from at least three trials. In each experiment the value for the wild-type RT was measured and the ratio taken to eliminate variations from one experiment to the next. The small difference for the AZT-resistant mutant on substrate p22/tC5U from Tables 1 and 2 is due to the fact that the numbers in Table 2 represent averages of at least three trials.

determine the incorporation ratio for all adenosines, all of the product bands shorter than the full-length product were combined as the AZTMP products. As shown in Figure 6, the plot of the ratio of products resulting from the incorporation of AZTMP to those from incorporation of dTMP curves upward with increasing [AZTTP]/[dTTP].

At a ratio of [AZTTP] to [dTTP] of 1, where that term drops out of eq 2, the ratio of the AZTMP products to dTMP products is ~8 for the wild-type RT, a value greater than the ratio of AZTMP products to dTMP products for p22/tC5U. Therefore, as expected, the fraction of total products as AZTMP products increases with the increasing number of adenosine positions in the template. The level of difference between the mutant K103N-Y181C, the AZT-resistant mutant, and the wild-type RT also increases going from substrate p22/tC5U to p18/tR2. At [AZTTP]/[dTTP] of 1 with the reaction on substrate p18/tR2, the ratio of the AZTMP products to dTMP products is 18 for K103N-Y181C and 5 for AZT-resistant mutant to give a ratio of 2.2 for the incorporation ratio for K103N-Y181C to that of the wild-type RT and 0.62 for AZT-resistant mutant to the wild-type RT, respectively. This indicates an increase in the difference between the mutants and the wild-type RT over the difference measured on substrate p22/tC5U. However, the increase in the difference between the mutants and the wild-type RT is not as great as would be expected if each of the six adenosine positions offered the same level of difference in the discrimination against incorporation of AZTMP as that seen for the position of p22/tC5U (e.g., R for AZT-resistant mutant/R for wild-type RT on p22/tC5U = 0.77; $(0.77)^6 = 0.21$). Therefore, not all adenosine positions are equivalent both in terms of the discrimination against AZTMP incorporation and level of difference in incorporation by mutants and wild-type RT.

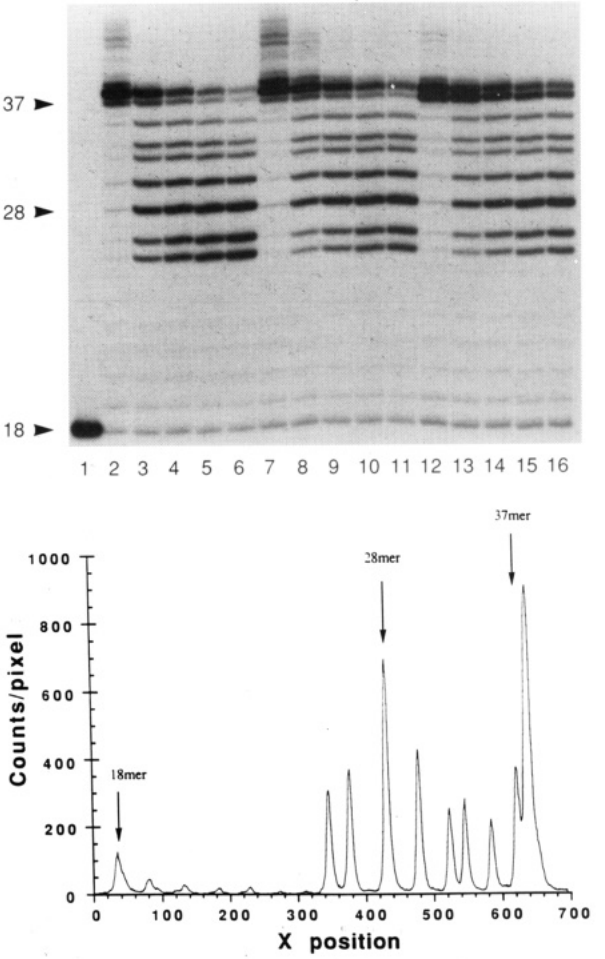

FIGURE 5: Analysis of DNA products from synthesis on p18/tR7 catalyzed by HIV-1 RT. (A, Top) Autoradiograph of products. Reaction conditions were the same as for Figure 2 except for the following. The primer/template was p18/tR7. dNTP concentrations: dTTP, 20 μM; dGTP, dATP, and dCTP, 50 μM; AZTTP, 0 (lanes 2, 7, 12), 5 μM (lanes 3, 8, 13), 10 μM (lanes 4, 9, 14), 15 μM (lanes 5, 10, 15), 20 μM (lanes 6, 11, 16). The reaction was allowed to proceed for 1 hr. Product analysis was performed the same as described in Figure 2. The primer is shown in lane 1. (B, Bottom) Integration of the amounts of products in panel A, lane 3, was performed using a PhosphorImager. The bottom of the gel is to the left of the plot and the full length products correspond to the peaks on the right side of the plot. The numbers and arrows indicate products of specific lengths in nucleotides.

Removal of AZTMP via Pyrophosphorolysis. The ability of HIV-1 RT to reverse the incorporation of AZTMP via pyrophosphorolysis was examined by monitoring the amount of the 23-mer product during the competition experiment on substrate p22/tC5U at various concentrations of added sodium pyrophosphate (PP_i). As shown in Figure 4, in the absence of added PP_i, the concentration of AZTMP-product 23-mer was constant throughout the time course of the reaction.

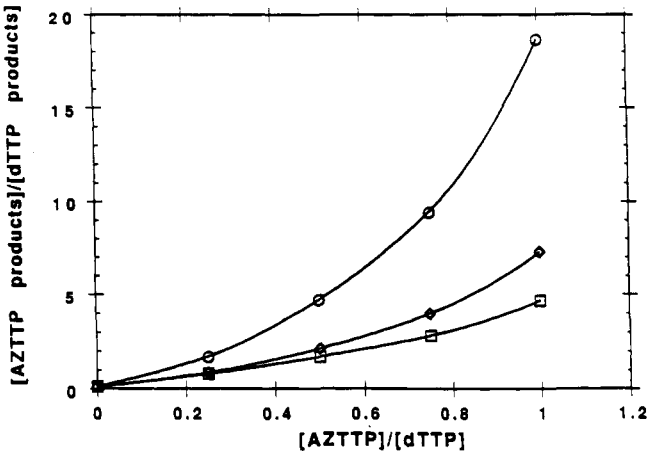

FIGURE 6: Ratio of AZTMP products to dTMP products in HIV-1 RT-catalyzed reactions on substrate p18/tR2. RT at 250 nM (wild-type RT, ◇; K103N-Y181C, ○; AZT-resistant mutant, □) was incubated with 250 nM 5′-[^{32}P]-labeled p18/tR2 in the presence of 20 μM dTTP, 50 μM dGTP, dATP, and dCTP, and either 0, 5, 10, 15, or 20 μM AZTTP in reaction buffer (described in Materials and Methods). The reaction was allowed to proceed for 2 h at 25 °C, at which time an aliquot was removed and quenched in gel load buffer. Product formation was analyzed by denaturing gel electrophoresis. Bands were located by autoradiography. Oligonucleotides from 19 to 36 bases in length were grouped together as products resulting from the incorporation of AZTMP. Those products and the full length 37-mer product were excised from the gel and quantified with scintillation counting.

However, in the presence of 100 μM PP_i the concentration of the 23-mer decreased with longer reaction times *and* a concomitant increase in the full-length product was observed. Reardon has directly demonstrated the reverse of incorporation of AZTMP in the presence of PPi as catalyzed by HIV-1 RT under single-turnover conditions (Reardon, 1993). The most likely explanation for the disappearance of the AZTMP product is the reversal of AZTMP incorporation due to pyrophosphorolysis to regenerate the 22-mer followed by another chance for incorporation of dTMP and by extension of the primer to the end of the template at which point pyrophosphorolysis is probably slower. Under this assumption, the rate of disappearance of the 23-mer is determined by the rate of pyrophosphorolysis of the incorporated AZTMP, the probability of re-incorporating AZTMP, and the probability of incorporating dTMP. The rate of disappearance of the 23-mer band was determined at different concentrations of PP_i from 50 to 500 μM by fitting the time course for disappearance of the 23-mer to a single exponential decay. A replot of the observed rates versus PP_i concentration was linear with slopes of 3.4 M^{-1} s^{-1} for the wild-type RT and 1.5 M^{-1} s^{-1} for the AZT-resistant mutant.

Ability of Site-Specific Mutants of RT To Incorporate AZTMP. The abilities of site-specific mutants of HIV-1 RT to incorporate AZTMP vs dTMP into substrate p22/tC5U were determined. The results are shown in Table 2. The Y181C had a higher incorporation ratio than that of the wild-type RT. A hybrid mutant of RT including the four mutations rendering RT resistant to AZT and Y181C together also incorporated AZTMP with higher relative efficiency than that of the wild-type RT. The single mutant, K103N, had a slightly higher incorporation ratio than that of the wild-type RT. As a negative control, a mutant of RT deficient in RNase H activity due to a mutation in the RNase H active site was determined to have an incorporation ratio not significantly different from that of the wild-type RT. Though the changes in the incorporation ratios are small, repeat experiments have shown that the changes are statistically significant.

The kinetic parameters for synthesis on poly(rA)–oligo(dT) by several site-specific mutants of HIV-1 RT are also shown in Table 2. The efficiencies of catalysis for mutants K103N or the RNaseH deficient mutant are not significantly different from that of the wild-type RT. The single mutant Y181C, the double mutant K103N-Y181C, and the hybrid mutant containing the four AZT-resistance mutations and Y181C together all displayed a slightly reduced efficiency of catalysis compared to that of the wild-type RT, primarily due to a modest increase in the value for K_M.

DISCUSSION

Measurements of viral propagation in cell culture have detected strains of HIV-1 that are resistant to inhibition by AZT (Larder et al., 1989). The mutations responsible for the resistant phenotype map to the RT gene. Several sets of mutations can give rise to resistance to AZT in cell culture, one of which is a mutant containing four amino acid substitutions (D67N-K70R-T215Y-K219Q) that has been shown to be 120-fold resistant (Kellam et al., 1992). However when the RT containing that set of four mutations was purified and the kinetics of synthesis examined, the K_i for AZTTP was increased approximately 2-fold for the AZT-resistant mutant compared to the wild type (Lacey et al., 1992). The quantitative discrepancy between resistance in cell culture and at the level of RT, the presumed final target of inhibition by AZT, leads to questions about the true nature of the mechanism of inhibition by AZT. In view of this discrepancy, we have examined the sensitivity to inhibition by AZTTP of the wild-type RT and several mutant RTs that are resistant to either AZTTP or non-nucleoside inhibitors.

In vivo, RT does not operate under steady-state conditions. The ratio of RT to primer/template is probably close to unity. Therefore, the probability of incorporation of AZTMP leading to chain termination, in the absence of reversal of incorporation, will determine the inhibitory potency of AZTTP rather than the K_i value, as has been discussed (Goody et al., 1991; Reardon, 1992). The incorporation ratio of AZTMP to dTMP at an individual template adenosine position reflects the intrinsic probability of incorporating the analog. The method described here allows for the precise determination of the incorporation ratio of the two nucleotides at any single template adenosine position for a given ratio of concentrations of the two triphosphates.

Small but statistically significant differences were detected in the ratio of incorporation of AZTMP to dTMP catalyzed by the wild-type RT compared to the ratio for the AZT-resistant mutant (D67N-K70R-T215Y-K219Q) or compared to the ratio for a mutant resistant to inhibition by L-697,661 (K103N-Y181C). The AZT-resistant mutant had an incorporation ratio that was 77% of that for the wild-type RT and the K103N-Y181C mutant had a ratio that was 140% that of the wild-type RT.

Significant differences were noted in the ability of other site-specific mutants of RT to incorporate AZTMP vs dTMP. The Y181C mutant and the hybrid mutant containing the AZT-resistant mutant with Y181C together could incorporate AZTMP relative to dTMP more easily than could the wild-type RT, suggesting that such mutants are more sensitive to inhibition by AZTTP than the wild-type RT. The effects do not appear to be strictly additive since the ability of the hybrid containing the AZT-resistant and the Y181C mutations to incorporate AZTMP is greater than the average of the

abilitiesof the AZT-resistant mutant and the Y181C mutant to incorporate AZTMP.

Multiple sites for incorporation of either AZTMP or dTMP allowed for an increase in the level of difference between mutants K103N-Y181C, the AZT-resistant mutant, and the wild-type RT in the amounts of AZTMP products generated, but the increase was less than would have been expected if all sites of incorporation offered the same level of discrimination against incorporation of AZTMP as does the adenosine in p22/tC5U. Variations in the intensity of bands on a DNA sequencing gel corresponding to different sites for incorporation of AZTMP also support the suggestion that different template adenosines offer different levels of discrimination against incorporation of AZTMP.

The correlation between inhibition of the enzyme or its mutants by AZTTP and inhibition of viral propagation in cell culture by AZT is not simple. The Y181C mutation that is resistant to L-697,661 has not been detected in the RT recovered from patients undergoing combination therapy by taking both AZT and L-697,661 (Byrnes et al., 1993), consistent with the observation reported here of the increased ability of Y181C to incorporate AZTMP relative to that of the wild-type RT. However, in cell culture assays the mutant viral strain containing K103N-Y181C in the RT gene is not much more sensitive to inhibition by AZT than the wild-type HIV-1 RT (Saag et al., 1993).

The presence of the Y181C mutation in a mutant viral strain also containing the four AZT-resistance mutations (D67N-K70R-T215Y-K219Q) caused suppression of most of the resistance to AZT (Larder, 1992). The lack of hypersensitivity to inhibition by AZT of the hybrid mutant in cell culture, as might be expected based on the increased inhibition of the mutant RT by AZTTP, indicates that there is not a linear correlation between AZTMP incorporation on p22/tC5U and CIC_{95} in cell culture assays. The lack of a correlation may be due to some threshold effect on inhibition in the cell culture assay or the position for incorporation of AZTMP chosen for study here may not be the important one in the cell. However, the reversal of resistance to AZT due to the presence of Y181C, which engenders a high level of resistance to several nonnucleoside inhibitors, in the AZT-resistant background is encouraging for combination therapies.

Quantitatively, the level of difference in incorporation of AZTMP between the mutants and wild-type RT determined in the present study is not sufficient to explain the resistance observed in cell culture (Kellam et al., 1992) since it should be possible to increase the concentration of AZT outside the cell by 20% to overcome the small difference in the ratio of incorporation of AZTMP to dTMP. The concentration of AZTTP inside MT-4 cell saturates at ~ 5 μM, a level that should be high enough to allow for CIC_{95} of either the wild-type or AZT-resistant strains to be reached (Toyoshima et al., 1991). However, the different levels of discrimination against AZTMP observed in the DNA sequencing gel assay at different positions of the same template suggest the possibility of a unique site or sites for incorporation of AZTMP where larger differences exist between the abilities of the mutant and wild-type to incorporate AZTMP (i.e., important termination hot spots).

The discrepancy between resistance in cell culture assays, or *in vivo*, and at the level of the presumed enzymic target of inhibition has been observed in another system. Mutants of herpes simplex virus can be hypersensitive to inhibition by bromovinyldeoxyuridine. Yet, the viral polymerase containing the mutations thought to be responsible for the hypersensitivity shows an increase in K_i for the inhibitor (Darby et al., 1984).

The wild-type RT was able, not surprisingly, to reverse the incorporation of AZTMP, most probably via pyrophosphorolysis, in the presence of 100 μM PP_i. The physiological concentration of PP_i in T cells has been reported to be 130 μM (Barshop et al., 1991). The repaired primer thus generated could then be extended by incorporation of dTMP, allowing further primer elongation. The reverse reaction catalyzed by the wild-type RT was inefficient. Given sufficient time, there is no doubt that the repair would allow formation of full-length product but with an apparent second-order rate constant (k_{cat}/K_M) of 3.4 M^{-1} s^{-1}, an *in vitro* value consistent with previous measurements (Reardon, 1993), the relevance of reversal of AZTMP incorporation by pyrophosphorolysis to the escape from inhibition of AZT is unclear during viral replication in cell culture or *in vivo* since the rate of repair due to pyrophosphorolysis is much too low to account for productive viral replication. The AZT-resistant mutant of RT could also reverse AZTMP incorporation via pyrophosphorolysis, but at 44% of the efficiency of the wild-type RT. Therefore, a change in the rate of removal of incorporated AZTMP does not appear to contribute towards resistance to inhibition by AZT unless cellular, or viral, factors are involved in this mechanism to alter the rate of pyrophosphorolysis.

CONCLUSION

A correlation can be established for the sensitivity to inhibition of HIV-1 RT and its mutants by AZTTP *in vitro* and the sensitivity to inhibition of HIV-1 to AZT in cell culture. If a mutant enzyme shows decreased ability to incorporate AZTMP on a synthetic primer/template, the corresponding viral strain is also resistant to inhibition by AZT. Quantitatively, the degree of difference in incorporation of AZTMP between mutant and wild-type RTs cannot account for the resistance observed in cell culture. We suggest that cellular or viral factor(s), or a specific site for incorporation of AZTMP in the viral genome, may account for the decreased sensitivity to inhibition by AZT seen in resistant viral strains in cell culture.

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