Phenotypic Mechanism of HIV-1 Resistance to 3′-Azido-3′-deoxythymidine (AZT): Increased Polymerization Processivity and Enhanced Sensitivity to Pyrophosphate of the Mutant Viral Reverse Transcriptase†

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ABSTRACT: The multiple mutations associated with high-level AZT resistance (D67N, K70R, T215F, K219Q) arise in two separate subdomains of the viral reverse transcriptase (RT), suggesting that these mutations may contribute differently to overall resistance. We compared wild-type RT with the D67N/K70R/T215F/K219Q, D67N/K70R, and T215F/K219Q mutant enzymes. The D67N/K70R/T215F/K219Q mutant showed increased DNA polymerase processivity; this resulted from decreased template/primer dissociation from RT, and was due to the T215F/K219Q mutations. The D67N/K70R/T215F/K219Q mutant was less sensitive to AZTTP ($IC_{50} \approx 300$ nM) than wt RT ($IC_{50} \approx 100$ nM) in the presence of 0.5 mM pyrophosphate. This change in pyrophosphate-mediated sensitivity of the mutant enzyme was selective for AZTTP, since similar K_m values for TTP and inhibition by ddCTP and ddGTP were noted with wt and mutant RT in the absence or in the presence of pyrophosphate. The D67N/K70R/T215F/K219Q mutant showed an increased rate of pyrophosphorolysis (the reverse reaction of DNA synthesis) of chain-terminated DNA; this enhanced pyrophosphorolysis was due to the D67N/K70R mutations. However, the processivity of pyrophosphorolysis was similar for the wild-type and mutant enzymes. We propose that HIV-1 resistance to AZT results from the selectively decreased binding of AZTTP and the increased pyrophosphorolytic cleavage of chain-terminated viral DNA by the mutant RT at physiological pyrophosphate levels, resulting in a net decrease in chain termination. The increased processivity of viral DNA synthesis may be important to enable facile HIV replication in the presence of AZT, by compensating for the increased reverse reaction rate.

The most widely used clinical therapeutic against the human immunodeficiency virus type 1 (HIV-1)[1] is the nucleoside analogue 3′-azido-3′-deoxythymidine (AZT) (*1*, *2*). This compound, after intracellular conversion to the triphosphate, inhibits the viral reverse transcriptase (RT) partly by acting as a competitive inhibitor with respect to TTP but primarily by acting as a chain terminator of nascent viral DNA synthesis.

Unfortunately, prolonged clinical use of AZT monotherapy invariably results in the appearance of HIV-1 resistant to the drug (*3*−*6*). High-level resistance to AZT is correlated with multiple mutations in RT, namely, D67N, K70R, T215F/Y, and K219Q (*3*), as well as M41L in some cases (*7*). HIV-1 containing these amino acid substitutions in RT replicates readily in the presence of high concentrations of AZT (*3*−*7*). Although the genotypic basis for HIV-1 resistance to AZT is well characterized, the phenotypic mechanism by which mutations in RT lead to high-level viral resistance to AZT has remained elusive. In all previous studies, few if any differences were noted between wt and AZT-mutant RT. For example, virion-associated RT activity from AZT-resistant strains, and recombinant RT containing multiple mutations associated with high-level AZT resistance, is equally sensitive as wild-type (wt) RT to inhibition by AZTTP under standard in vitro RT DNA polymerase assay conditions (*8*, *9*). While slight differences in the ratio of incorporation of dTMP to AZTMP were noted between the D67N/K70R/T215Y/K219Q mutant RT compared to wt enzyme (*10*, *11*), these differences cannot account for the more than 100-fold viral resistance to the drug. The enzyme with AZT-resistance mutations was reported to have a lower catalytic efficiency than wt RT (*12*), which would presumably lead to a decreased replication efficiency for the AZT-resistant virus. However, other observations suggest that AZT-resistant virus may in fact have a replication advantage (*13*−*15*).

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[1] Abbreviations: AZT, 3′-azido-3′-deoxythymidine; DDDP, DNA-dependent DNA polymerase; HIV-1, human immunodeficiency virus type 1; nt, nucleotide; PAGE, polyacrylamide gel electrophoresis; PFA, phosphonoformic acid (Foscarnet); prPBS, oligodeoxynucleotide complementary to the HIV-1 primer binding sequence; RDDP, RNA-dependent DNA polymerase; RT, reverse transcriptase; T/P, template/primer; wt, wild type.

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In an attempt to identify the phenotypic mechanism by which mutations in HIV-1 RT result in resistance to AZT, we characterized a number of enzymological parameters of the D67N/K70R/T215F/K219Q mutant RT, as well as the D67N/K70R and T215F/K219Q mutants, in comparative studies with recombinant wt RT. In this report we show that the D67N/K70R/T215F/K219Q mutant RT possesses increased DNA polymerase processivity, confirming a previous report by Caliendo et al. (*16*); this processivity is increased in the direction of DNA synthesis only. We also show that the increased processivity is associated with a decreased template/primer (T/P) dissociation rate. Alterations in DNA polymerase processivity and T/P dissociation are due to the T215F/K219Q substitutions in RT. Importantly, the mutant RT shows a significant increase in pyrophosphorolysis, the reverse reaction of DNA synthesis. This enhanced pyrophosphorolytic rate of the mutant RT results in removal of chain-terminating 3′-deoxynucleotides, thereby allowing additional DNA chain elongation. This enhanced pyrophosphorolysis results from the D67N/K70R mutations in HIV-1 RT.

MATERIALS AND METHODS

AZTTP was purchased from Moravek Biochemicals. Restriction enzymes were obtained from Boehringer Mannheim, New England Biolabs, and Pharmacia. [^{3}H]- and [α-^{32}P]dNTPs and [γ-^{32}P]ATP were from Amersham. The expression vector pKK223-3, ultrapure dNTPs, and homopolymeric T/P poly(rA)−oligo(dT)$_{12-18}$, poly(rC)−oligo(dG)$_{12-18}$, and poly(dC)−oligo(dG)$_{12-18}$ were obtained from Pharmacia Biotech (Montreal, PQ). $NaPP_i$ and phosphonoformic acid (Foscarnet; PFA) were obtained from Sigma.

The heteropolymeric RNA-dependent DNA polymerase (RDDP) T/P was prepared as described (*17*−*19*) using the T7 polymerase RNA transcript from *Acc*I-linearized plasmid pHIV-PBS (*17*) as template and a synthetic 18 nucleotide (nt) deoxyoligonucleotide (prPBS) that is complementary to the sequence of the tRNA^{Lys3} primer binding as DNA synthesis initiation primer. The heteropolymeric DNA template for measurement of RT DNA-dependent DNA polymerase (DDDP) activity was prepared from the pHIV-PBS plasmid (*17*) by polymerase chain reaction (PCR) amplification of a 530 base pair region using primers pSP72/Acc956 (5′-CTGTCCCAGTATTTGTCT-3′) and pSP72 (5′-TAATACGACTCACTATAGGGAGACCGGCAGATC-3′). The pSP72/Acc956 primer was phosphorylated before use. The resulting PCR product was purified by passage through a NAP-5 column (Pharmacia Biotech) and digested using λ exonuclease (Gibco-BRL) to generate the single-strand DNA (*20*). The synthetic 18 nt deoxyoligonucleotide prPBS was used as DNA synthesis initiation primer.

Cloning, Expression, and Purification of Mutant RT. Plasmid pHIVRTMC containing the D67N/K70R/T215F/K219Q mutations in RT (*3*) was kindly provided by Dr. B. A. Larder. The RT coding region was amplified by PCR using the forward primer RTEco (5′-CT**GAATTC**{ATG}CCCATTAGCCCTATTGAG-3′) and the reverse primer RTZHind66 (5′-CTTCT**AAGC**{**TT**ACTA}TAGTATTTTCCTGATTCCAGC-3′) to obtain the sequence encoding the full-length p66 subunit of RT, and the forward primer RTEco and the reverse primer RTZHind51 (5′-CCCTTCT**AAGC**-**TT**ACTAGAAGGTTTCTGCTCCTAC-3′) to obtain the sequence encoding the truncated p51 subunit of RT. The amplified segments were digested with *Eco*RI and *Hin*dIII, restriction sequences built into the primers (highlighted in boldface), and then cloned into *Eco*RI/*Hin*dIII-digested expression vector pKK223-3. Initiation and termination codons (delineated by braces in the primer sequences) were also included in the primers. Constructs were sequenced to verify the correct nucleotide sequences. The resulting plasmids pRT66-AZT4 and pRT51-AZT4 allowed expression of the p66 and p51 subunits of D67N/K70R/T215F/K219Q RT, respectively.

Plasmids for the expression of the p66 subunits of D67N/K70R and T215F/K219Q mutant RT were constructed by digesting pRT66 and pRT66-AZT4 with *Eco*RV/*Hin*dIII, and then purifying the *Eco*RV/*Hin*dIII fragments and residual digested plasmids by agarose gel electrophoresis. The *Eco*RV/*Hin*dIII fragment from pRT66-AZT4 was inserted into *Eco*RV/*Hin*dIII-digested pRT66 to give pRT66-AZT215/219, and the *Eco*RV/*Hin*dIII fragment from pRT66 was inserted into *Eco*RV/*Hin*dIII-digested pRT66-AZT4 to give pRT66-AZT67/70, which allowed expression of the p66 subunits of T215F/K219Q RT and D67N/K70R RT, respectively. Similar manipulations with pRT51 and pRT51-AZT4 provided plasmids pRT51-AZT215/219 and pRT51-AZT67/70, which express the p51 subunits of T215F/K219Q RT and D67N/K70R RT, respectively.

The p51/p66 heterodimeric forms of wt, D67N/K70R, T215F/K219Q, and D67N/K70R/T215F/K219Q RT were expressed in *E. coli* JM109 and purified by the rapid single-step method we have previously described (*21*). Each of the four RT species had similar (±10%) specific activities when assayed with [^{3}H]TTP and poly(rA)−oligo(dT)$_{12-18}$ as T/P. The purity of these preparations was greater than 95% (as estimated by Coomassie staining), and the preparations were free from detectable nuclease contamination able to degrade ssRNA and/or ssDNA and dsDNA. Several different preparations of wt and mutant RT were used during the course of the present studies, particularly in follow-up and confirmatory experiments. No significant variation in kinetic parameters was noted among different preparations of the same RT species.

Assay of RT DNA Polymerase Activity. HIV-1 RT DNA polymerase activity was determined in a fixed time assay. Briefly, reaction mixtures (20−100 μL total volume) contained 50 mM Tris-HCl (pH 7.8, 37 °C), 60 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT), variable amounts of purified recombinant p51/p66 heterodimeric wt or mutant RT, and template/primer. After preincubation at 37 °C for 5−10 min, polymerization was initiated by the addition of aliquots of appropriate combinations of radiolabeled dNTP substrates for continuous polymerization experiments, or dNTP plus heparin (final concentration of heparin was generally 2 mg/mL), for single processive cycle experiments. Reaction assays were incubated at 37 °C, and then quenched with 500 μL of cold 20 mM sodium pyrophosphate in 10% trichloroacetic acid. After 15 min on ice, the precipitated nucleic acid was filtered on Whatman 934-AH glass fiber filters, washed sequentially with 10% trichloroacetic acid and 95% ethanol, and then analyzed by liquid scintillation spectrometry.

Experiments were carried out in the absence or the presence of various inhibitors, including sodium pyrophos-

Table 1: DNA Polymerization under Continuous and Single Processive Cycle Conditions by wt and Mutant RT

	processivity ratio[a]			
template/primer	wt	D67N/K70R	T215F/K219Q	D67N/K70R/T215F/K219Q
poly(rA)−oligo(dT)$_{12-18}$	0.30 ± 0.02	0.27 ± 0.04 (ns)[b]	0.49 ± 0.03 ($p < 0.01$)	0.51 ± 0.04 ($p < 0.01$)
poly(dC)−oligo(dG)$_{12-18}$	0.12 ± 0.01	0.10 ± 0.01 (ns)	0.29 ± 0.01 ($p < 0.01$)	0.20 ± 0.01 ($p < 0.01$)

[a] The processivity ratio is defined as the picomoles of nucleotides incorporated into newly-synthesized DNA in the presence of the polymerization trap (heparin) to the picomoles of nucleotides incorporated into newly-synthesized DNA in the absence of the polymerization trap. See Materials and Methods for details. The values reported are the means ± SD of four or more separate experiments. [b] Significance with respect to wt RT was calculated by one-way analysis of variance. ns, not significant ($p > 0.05$).

phate. DTT was not included in reactions containing AZTTP or in reactions employing the AZT chain-terminated prPBS primer in order to avoid possible reduction to the 3′-amino-3′-deoxythymidine moiety.

Polyacrylamide Gel Electrophoresis (PAGE) Analysis of RT Polymerization Products Formed under Continuous and Single Processive Cycle DNA Polymerization Conditions. Assay conditions were similar to those described above, except that the concentration of heparin was reduced to 0.2 mg/mL for RDDP reactions, and to 0.4 mg/mL for DDDP reactions. The 18 nt deoxyoligonucleotide primer prPBS was 5′-end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase as previously described (*19*). [^{32}P]prPBS (80 nM) was annealed to the pHIV-PBS RNA transcript (65 nM). RT (10 nM p51/p66 heterodimer) was then preincubated with the T/P for 5 min at 37 °C before initiation of the reaction by the addition of 150 μM each of the dNTPs ± heparin. After 30 min incubation at 37 °C, the reactions were quenched by adding an equivalent volume of sequencing gel loading buffer comprising 98% deionized formamide, 10 mM EDTA, and 1 mg/mL each of bromophenol blue and xylene cyanol. The samples were heated at 100 °C for 5 min, and then analyzed by PAGE using 16% polyacrylamide/7 M urea gels. Electrophoretically resolved products were visualized by autoradiography. Polymerization products were quantified by densitometry.

Analysis of RT−Template/Primer Dissociation. Template/primer dissociation from RT was measured essentially as described (*22*). Briefly, RT (34 nM p51/p66 heterodimer) was incubated for 10 min at 37 °C with poly(rA)−oligo(dT)$_{12-18}$ (0.06 unit) in 50 mM Tris-HCl (pH 7.8, 37 °C) containing 60 mM KCl and 10 mM dithiothreitol, prior to the addition of heparin (final concentration of 5 mg/mL). At various times after the addition of heparin, aliquots were removed and mixed with [^{3}H]TTP (5 μM final concentration) and $MgCl_2$ (10 mM final concentration) for measurement of residual RT RDDP activity.

Assay of RT-Catalyzed Pyrophosphorolysis. The 18 nt prPBS was 5′ end labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase as previously described (*19*). The labeled primer was purified by resolution on PAGE followed by excision and elution of the 18 nt product. The labeled primer was then annealed to the pHIV-PBS RNA transcript (1:1.2 molar ratio of prPBS:pHIV-PBS RNA). In some experiments, this T/P was incubated with RT (200 nM p51/p66 heterodimer) in 50 mM Tris-HCl (pH 7.8, 37 °C) containing 60 mM KCl, 10 mM $MgCl_2$, 50 μM dCTP, and 115 μM AZTTP in order to produce a 20 nt primer, chain-terminated by AZT. The total incubation sample was then dried under reduced pressure, redissolved in 12 μL of sequencing loading buffer comprising 98% deionized formamide, 10 mM EDTA, 1 mg/mL bromophenol blue, and 1 mg/mL of xylene cyanol, and loaded on a 16% polyacrylamide/7 M urea sequencing gel. The 20 nt AZT chain-terminated prPBS primer was excised from the gel, eluted in 0.5 M ammonium acetate, desalted by passage through a NAP 25 column (Pharmacia), and finally annealed to the pHIV-PBS RNA transcript as previously described. Control experiments showed that chain termination was complete. Pyrophosphorolysis assays were carried out both with chain-terminated T/P and with non-chain-terminated T/P. Briefly, T/P (20 nM) was preincubated at 37 °C for 5 min with HIV-1 RT (30−60 nM of heterodimer) in a 5 μL total volume of 50 mM Tris, pH 8.1, containing 60 mM KCl and 10 mM $MgCl_2$ to allow the formation of the RT−T/P binary complex. Pyrophosphorolysis was then initiated by the addition of various concentrations of sodium pyrophosphate. After varying incubation times, the reaction was stopped by the addition of an equivalent amount of sequencing gel loading buffer. The samples were heated at 100 °C for 5 min, and then run on a 16% polyacrylamide/7 M urea sequencing gel. The resolved products were visualized by autoradiography and quantitated by densitometry.

Analysis of Chain Termination of RT-Catalyzed DNA Synthesis. Assay conditions were similar to those described above, except for the addition of various concentrations of AZTTP. The ^{32}P-labeled prPBS primer, annealed to the pHIV-PBS RNA transcript (20 nM), was preincubated for 5 min at 37 °C with RT (6 nM p66/p51 heterodimer). DNA polymerization was initiated by the addition of 8.3−25 μM each of the dNTPs, in the absence and the presence of 1 μM AZTTP, and in the absence or the presence of varying concentrations of PP_i. The samples were incubated at 37 °C for varying times, and then quenched with an equivalent volume of sequencing loading buffer. The samples were heated at 100 °C for 5 min, and then resolved by PAGE on a 10% polyacrylamide/7 M urea sequencing gel. Polymerization products were visualized by autoradiography and quantitated by densitometry.

RESULTS

Processivity of DNA Polymerization by wt and Mutant RT. DNA synthesis by the D67N/K70R/T215Y/K219Q mutant RT was previously shown to be more processive than that catalyzed by wt RT (*16*). As seen in Table 1 and in Figure 1, under our assay conditions the D67N/K70R/T215F/K219Q mutant RT showed increased processivity relative to wt RT, confirming the results of Caliendo et al. (*16*). Increased polymerase processivity was noted on both RNA and DNA templates. We found that the T215F/K219Q RT also showed increased DNA polymerase processivity, whereas the processivity of the D67N/K70R mutant was similar or slightly

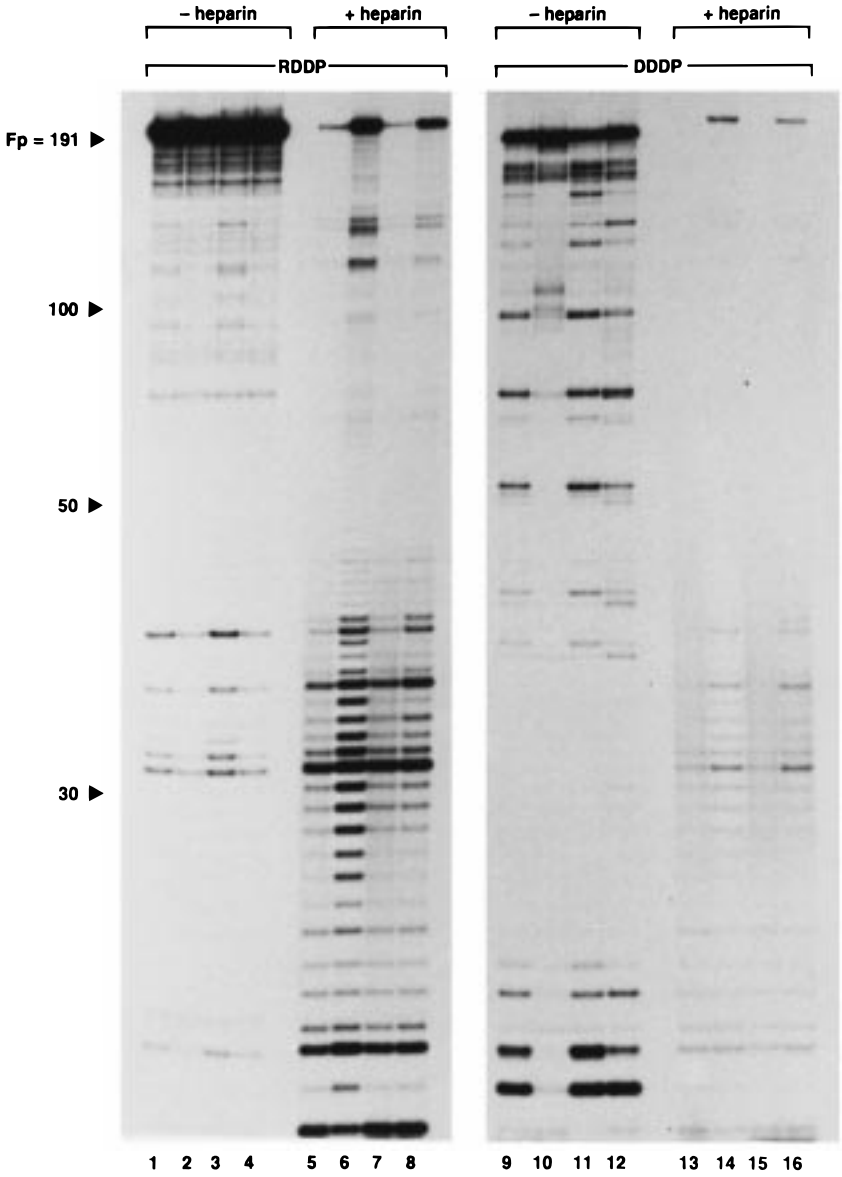

FIGURE 1: DNA polymerization processivity of wt and mutant RT. DNA synthesis primed by the 18 nt prPBS primer on the pHIV-PBS heteropolymeric RNA template ('RDDP', lanes 1−8) or the analogous heteropolymeric DNA template ('DDDP', lanes 9−16) was determined under continuous ('− heparin', lanes 1−4, 9−12) or single processive cycle conditions ('+ heparin', lanes 5−8, 13−16), as described under Materials and Methods. wt RT (lanes 1, 5, 9, 13); D67N/K70R/T215F/K219Q RT (lanes 2, 6, 10, 14); D67N/K70R RT (lanes 3, 7, 11, 15); T215F/K219Q RT (lanes 4, 8, 12, 16). Size markers are indicated on the left of the figure; the expected full-length product is also indicated (Fp = 191).

decreased compared to the wt enzyme (Table 1; Figure 1). Interestingly, the processivity of pyrophosphorolysis catalyzed by the T215F/K219Q and D67N/K70R/T215Y/K219Q enzymes was similar to that of wt RT (data not shown).

T/P dissociation from both the D67N/K70R/T215F/K219Q and the T215F/K219Q mutants was significantly decreased relative to that from wt RT (Table 2), whereas the D67N/K70R mutant showed a statistically significant increased rate of T/P dissociation compared to wt enzyme. Thus, the DNA polymerase processivity of the wt and mutant RT correlates with the ability of these enzymes to remain bound to the T/P.

Sensitivity of wt and Mutant RT to Pyrophosphate (PP_i) and Phosphonoformate (PFA). Pyrophosphate is a product inhibitor of HIV-1 RT (*23, 24*). In good agreement with these previous studies, we found that PP_i inhibited wt RT with an IC_{50} of approximately 1 mM; the T215F/K219Q mutant was as sensitive to PP_i as wt RT (Table 3). In contrast, both the D67N/K70R and the D67N/K70R/T215F/K219Q mutant enzymes were up to 4-fold more sensitive to PP_i inhibition than either wt or the T215F/K219Q mutant

Table 2: Template/Primer Dissociation Rates for wt and Mutant RT

RT	$k_{dissociation}$ (min^{-1})[a]
wt	0.042 ± 0.005
D67N/K70R	0.054 ± 0.002 ($p < 0.05$)[b]
T215F/K219Q	0.018 ± 0.006 ($p < 0.001$)
D67N/K70R/T215F/K219Q	0.027 ± 0.006 ($p < 0.01$)

[a] Values are means ± SD of three to five separate determinations for each enzyme. Dissociation rates were calculated as described under Materials and Methods. [b] Significance with respect to wt RT was calculated by one-way analysis of variance.

Table 3: Sensitivity of wt and Mutant RT to Pyrophosphate and Foscarnet (PFA)[a]

RT	IC_{50} (μM)	
	$NaPP_i$	foscarnet (PFA)
wt	1200 ± 200	0.26 ± 0.04
D67N/K70R	300 ± 50 ($p < 0.001$)[b]	0.11 ± 0.02 ($p < 0.001$)
T215F/K219Q	980 ± 175 (ns)	0.19 ± 0.03 (ns)
D67N/K70R/ T215F/K219Q	450 ± 45 ($p < 0.001$)	0.14 ± 0.02 ($p < 0.01$)

[a] RT activity was measured using 10 μM [^{3}H]TTP and 45 nM poly(rA)−oligo(dT)$_{12-18}$, in the absence and the presence of varying concentrations of inhibitor, as described under Materials and Methods. The results are the means ± SD from three or more independent experiments. [b] Significance with respect to wt RT was calculated by one-way analysis of variance. ns, not significant.

RT (Table 3). PP_i inhibition of the wt and the mutant RTs was mixed noncompetitive (data not shown), implying that the increased sensitivity of the mutant RTs to PP_i was not due to a change in the inhibition kinetic mechanism. Phosphonoformate (PFA; foscarnet) is a pyrophosphate analogue that inhibits by binding to the pyrophosphate binding site of RT (*25*, *26*). Both the D67N/K70R and the D67N/K70R/T215F/K219Q mutant enzymes were more sensitive to inhibition by PFA than either the wt or the T215F/K219Q mutant enzyme (Table 3).

Effect of $NaPP_i$ on the Rate of Pyrophosphorolysis by wt and Mutant RT. The reverse reaction of DNA polymerization, pyrophosphorolysis, is given in eq 1:

$$T/P_n + PP_i \leftrightarrow T/P_{n-1} + dNTP \quad (1)$$

The differences in PP_i sensitivity of the wt and mutant RTs prompted us to examine pyrophosphorolysis catalyzed by each of the enzyme species. Two types of primer were used in these experiments, one with a normal 2′-deoxyribonucleotide as the 3′-terminal nucleotide and the other with AZT as the 3′-terminus. In the absence of added dNTP, addition of PP_i to RT reaction mixtures resulted in reduction of the AZT chain-terminated primer to shorter products; the extent of pyrophosphorolysis increased with increasing concentrations of PP_i (Figure 2). The pyrophosphorolytic activity of the D67N/K70R/T215F/K219Q RT was substantially greater than that of the wt enzyme (Figure 2), whereas the specific activities of these two enzymes for DNA synthesis (forward reaction) were essentially identical in the absence of polymerization traps. The rate of the forward (DNA synthesis) reaction was significantly greater (nearly 100-fold) than that of the reverse (pyrophosphorolysis) reaction with all of the RT species studied, wt or mutant (data not shown).

The time dependence of pyrophosphorolysis of AZT-terminated primers was investigated at a fixed concentration of 125 μM PP_i, in the absence of added dNTPs. The rates of pyrophosphorolysis by the various RT species, as determined by the loss of the 20 nt AZT-chain terminated primer substrate, were D67N/K70R > D67N/K70R/T215F/K219Q > T215F/K219Q ≈ wt (Figure 3A,B). Similar results were noted in experiments using non-chain-terminated primers (data not shown).

Effect of PP_i on AZTTP Inhibition of RT-Catalyzed DNA Synthesis. In the absence of PP_i, AZTTP was equally potent at inhibiting DNA synthesis catalyzed by each of the RT species (IC_{50} 30−40 nM), consistent with earlier observations

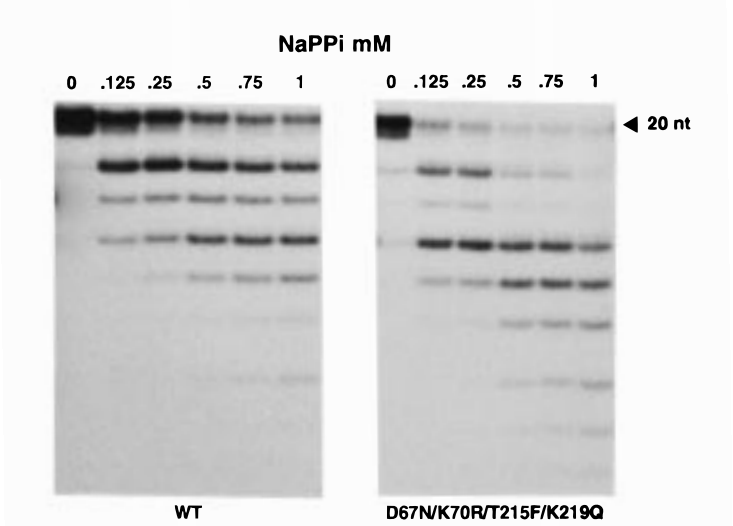

FIGURE 2: Effect of pyrophosphate concentration on pyrophosphoroloysis catalyzed by wt and mutant RT. Pyrophosphorolysis reactions were carried out as described under Materials and Methods, and were allowed to proceed for 80 min at 37 °C in the presence of varying concentrations of $NaPP_i$ using 10 nM heteropolymeric T/P (with the 20 nt AZT-terminated prPBS primer) and 80 nM RT heterodimer.

from our laboratory (*8*) and from others (*9*). The in vitro inhibition by AZTTP was diminished in the presence of PP_i; this effect was significantly more pronounced for the D67N/K70R and D67N/K70R/T215F/K219Q compared to the T215F/K219Q and wt enzymes (Table 4). AZTTP inhibition of the T215F/K219Q mutant was also reduced in the presence of PP_i although this effect was less pronounced than for the other mutants.

The pyrophosphate-mediated change in sensitivity of the mutant enzyme was selective for AZTTP, since the K_m for dTTP and the IC_{50} for ddCTP and ddGTP were unchanged in the absence or in the presence of pyrophosphate (Table 4).

Effect of PP_i on Chain Termination by AZTTP. When RT-catalyzed DNA synthesis was carried out in the presence of 1 μM AZTTP, but in the absence of PP_i, several additional polymerization products are noted, due to chain termination by the dideoxynucleotide triphosphate. This is especially apparent in a region of the pHIV-PBS RNA template between 11 and 19 nt downstream from the 3′-terminus of the prPBS primer. The template sequence (3′-AAAAGG-TAA-5′) in this region allows possible AZTTP chain termination at six of the nine dNTP incorporation events. At relatively low dNTP concentrations (8.3 μM), significant chain termination is noted at each of the six possible sites in this sequence (Figure 4). The intensity of these chain termination products decreased with increasing concentrations of PP_i. The average intracellular concentration of PP_i is about 150 μM (*27*). At this concentration of PP_i, the inhibition of chain termination in vitro was most noticeable with the D67N/K70R and the D67N/K70R/T215F/K219Q mutants in vitro (Figure 5). The T215F/K219Q mutant also showed a significant decrease in chain termination, compared to wt RT (Figure 5), although this decrease was less than that noted for the other mutant RTs. No differences in chain termination by ddCTP were noted in reactions catalyzed by the wt and the D67N/K70R/T215F/K219Q enzymes in the presence of PP_i (data not shown).

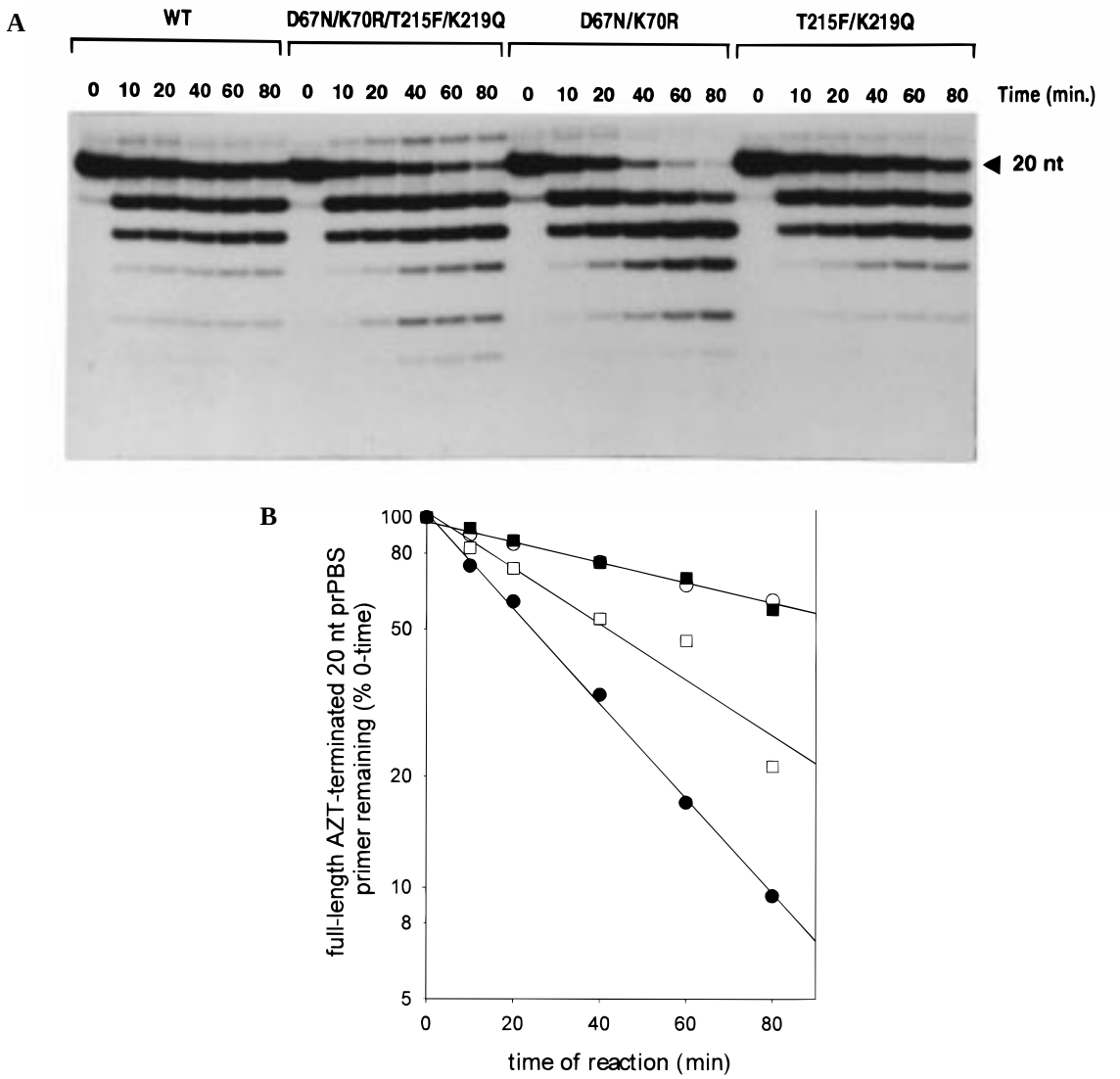

FIGURE 3: (A) Time course of pyrophosphorolysis catalyzed by wt and mutant RT. Reactions were carried out in the presence of 1 mM $NaPP_i$ as described under Materials and Methods, using 20 nM heteropolymeric T/P (with the 20 nt AZT-terminated prPBS primer) and 50 nM RT heterodimer. (B) Rates of pyrophosphorolytic removal of chain-terminating AZT by wt and mutant RT. The plot illustrates the residual amount of the 20 nt AZT-terminated prPBS primer band in Figure 3A as a function of time of pyrophosphorolysis. Band intensities were determined by densitometry. (○) wt RT; (□) D67N/K70R/T215F/K219Q RT; (●) D67N/K70R RT; (■) T215F/K219Q RT.

The net result of the PP_i-mediated decrease in chain termination is increased levels of full-length DNA synthesis in the presence of AZTTP (Figure 6). This is especially noticeable in reactions catalyzed by the D67N/K70R/T215F/K219Q enzyme (Figure 6B).

DISCUSSION

High-level resistance of HIV-1 to AZT requires multiple mutations in the viral reverse transcriptase. These mutations localize to two separate subdomains of RT. In the present work, we have demonstrated that these two sets of mutations impart distinct phenotypic alterations in RT activity.

Mutations T215F/K219Q occur in the "palm" subdomain, and in the p66 subunit lie on the surface of the nucleic acid binding cleft, contacting the template about eight nucleotides from the 3′-terminus of the primer (*28*). We found that these mutations result in increased processivity of DNA synthesis and decreased T/P dissociation from the mutant enzyme, entirely consistent with their role in interacting with the template nucleic acid strand. It has been proposed that these mutations alter the interaction between RT and the template strand, thereby allowing the RT to discriminate between normal and modified nucleotides (*28, 29*). As well, the increased bulk of the amino acid side chain due to the T215Y/F mutation has been suggested to confer AZT resistance by displacing or repositioning the template/primer in the nucleic acid binding cleft (*30, 31*), thereby diminishing the ability of modified nucleotides such as AZTTP to position appropriately to allow phosphodiester bond formation with the 3′-hydroxyl of the primer terminal nucleotide. However, the wt and the T215F/K219Q RT are equally sensitive to AZTTP in vitro in assays employing either homopolymeric or heteropolymeric T/P in the absence of PP_i (*8, 9*). It is therefore difficult to assign AZT resistance as due to T/P repositioning effects alone. However, the T215F/K219Q RT is less sensitive to AZTTP inhibition in vitro in the presence of PP_i. Perhaps under these conditions T/P repositioning plays some role in resistance. The T215F/K219Q mutant is also significantly more processive in DNA synthesis than the wt RT. It is, however, difficult to ascribe AZT resistance

Table 4: Effect of Pyrophosphate on the Interaction of dNTP and ddNTP with wt and Mutant Reverse Transcriptases[a]

		IC_{50} (nM)			
inhibitor	$[PP_i]$ μM	wt	D67N/K70R/T215F/K219Q	D67N/K70R	T215F/K219Q
AZTTP	0	33 ± 3	29 ± 7 (ns)[b]	43 ± 15 (ns)	36 ± 2 (ns)
	150	46 ± 5	83 ± 9 ($p<0.01$)	93 ± 8 ($p<0.001$)	68± 12 ($p<0.05$)
	500	100 ± 10	280 ± 55 ($p < 0.001$)	270 ± 38 ($p<0.001$)	167 ± 30 ($p<0.01$)
ddGTP	0	154 ± 25	126 ± 13 (ns)	nd[c]	nd
	150	177 ± 54	181 ± 53 (ns)	nd	nd
	500	230 ± 58	185 ± 35 (ns)	nd	nd
ddCTP	0	200 ± 32	172 ± 18 (ns)	nd	nd
	150	210 ± 12	195 ± 26 (ns)	nd	nd
		K_m (μM)			
substrate	$[PP_i]$ (μM)	wt	D67N/K70R/T215F/K219Q	D67N/K70R	T215F/K219Q
dTTP	0	6.6 ± 2.1	6.9 ± 2.4 (ns)	nd	nd
	150	7.7 ± 1.7	5.6 ± 2.6 (ns)	nd	nd
	500	6.7 ± 0.4	5.8 ± 1.6 (ns)	nd	nd

[a] Values are the means ± SD of three or more independent determinations. [b] Significance with respect to wt RT at the same concentration of pyrophosphate was determined by one-way analysis of variance. ns, not significant ($p > 0.05$). [c] Not determined.

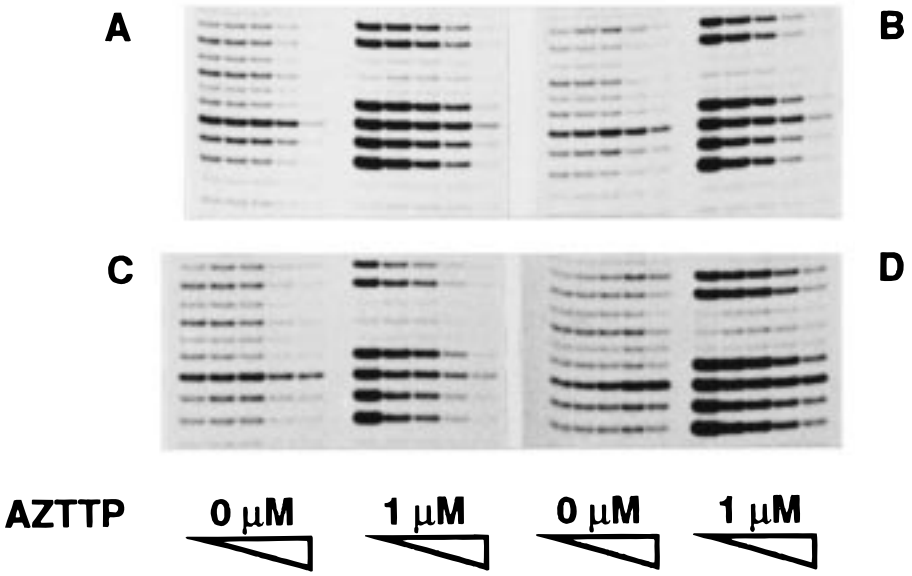

FIGURE 4: Effect of increasing PP_i concentration on chain termination by AZTTP during RT RDDP DNA synthesis in the region 11−19 nt downstream from the 3′-terminus of the prPBS primer. Reactions were carried out as described under Materials and Methods at 37 °C for 15 min using the pHIV-PBS heteropolymeric RNA template primed by the 18 nt ^{32}P-labeled prPBS primer, in the presence of 8.3 μM each of dATP, dCTP, dGTP, and TTP. The figure illustrates DNA polymerization products corresponding to the template sequence 3′-AAAAGGTAA-5′. In each of panels A−D, the left set of lanes is DNA synthesized in the absence of AZTTP, and the right set of lanes is DNA synthesized in the presence of 1 μM AZTTP. Each set of lanes shows the DNA products formed in the presence of increasing concentrations of PP_i (0, 150, 300, 600, and 1200 μM PP_i, indicated by the expanding line beneath each set of lanes). (A) wt RT; (B) D67N/K70R/T215F/K219Q RT; (C) D67N/K70R RT; (D) 215F/K219Q RT.

solely to this higher level of DNA polymerase processivity. Indeed, studies with recombinant RT mutated in helix H of the "thumb" subdomain showed that resistance to AZTTP is inversely proportional to the processivity of RT (*32*, *33*).

Mutations D67N/K70R are in the "fingers" subdomain and in the p66 subunit form part of the flexible $\beta3-\beta4$ loop. A large number of mutations associated with resistance to ddN occur in this region (*28*). In the present study, we have shown that the D67N/K70R mutations confer an increased sensitivity of RT to PP_i and, perhaps more importantly, correlate with an increased rate of pyrophosphorolysis, the reverse reaction of DNA synthesis. Interestingly, R72, another residue in the $\beta3-\beta4$ loop, has been implicated in PP_i binding/release from HIV-1 RT (*34*). Furthermore, R754 of the Klenow fragment, the equivalent of R72 of HIV-1 RT, has been implicated in dNTP binding as well as for enzyme translocation along the template (*35*, *36*). Immunochemical studies from our laboratory also suggest that residues 65−73 of HIV-1 RT may contribute to dNTP binding (*37*). The increased sensitivity to PP_i conferred by the D67N/K70R mutations might imply increased binding of the compound to the enzyme. Interference between PP_i and AZTTP for interaction at or near the same region on RT might then result in a decreased binding of the latter, consistent with our observations of significant increases in IC_{50} for AZTTP against the D67N/K70R and the D67N/K70R/T215F/K219Q mutants. However, neither the K_m for dTTP nor the IC_{50} for ddCTP or ddGTP is affected by PP_i with these mutants. In addition, we have shown that the K65R mutation in ddI/ddC/3TC cross-resistant RT contrib-

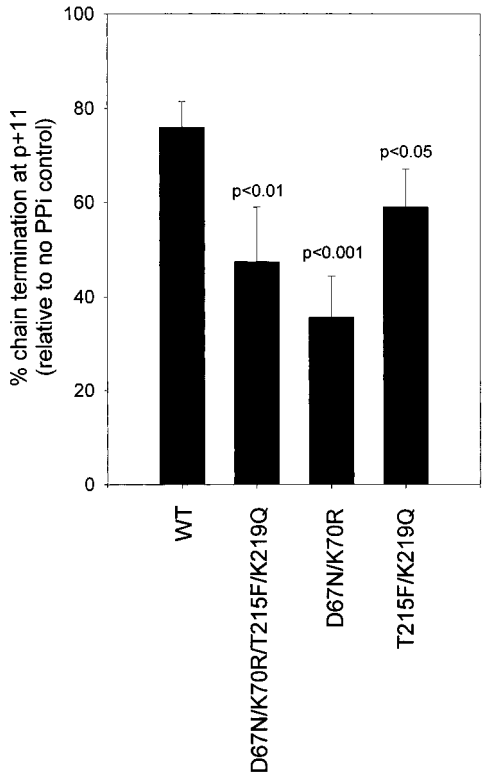

FIGURE 5: Effect of physiological concentrations of PP_i (150 μM) on chain termination by AZTTP in reactions catalyzed by wt and mutant RT. Reaction conditions were similar to those in the legend to Figure 4. The amounts of chain termination product 11 nt downstream from the 3′-terminus of the prPBS primer were quantitated by densitometry. The graph illustrates the intensity of the chain-termination product in the presence of 1 μM AZTTP + 150 μM PP_i as a percentage of that formed in the presence of 1 μM AZTTP alone, after correction for the amount of product formed in the absence of AZTTP. The data are means ± SD from three independent determinations.

utes to increased DNA polymerase processivity of that mutant RT (*38*), suggesting that residues 65−73 of the HIV-1 RT fingers subdomain may be important in multiple functions of RT.

Several studies indicate that AZT-resistant HIV-1 possesses an increased susceptibility to PFA, and that PFA-resistant HIV becomes resensitized to the compound upon introductions of mutations associated with AZT resistance (*3, 39*−*41*). These data suggest a mutually exclusive relationship between resistance to AZT and to PFA. Our results correlate well with these findings, since each of the mutants was more susceptible to PFA inhibition than wt RT (Table 3).

Intracellular concentrations of PP_i average 150 μM (*27*). At these concentrations of PP_i, the D67N/K70R and the D67N/K70R/T215F/K219Q mutants show significantly higher rates of pyrophosphorolysis than either the wt or the T215F/K219Q RT. With AZT-terminated primers, this results in pyrophosphorolytic release of AZTTP from the 3′-end of the primer DNA chain. In the presence of dNTPs, this pyrophosphorolysis is accompanied by renewal of primer extension (viral DNA synthesis). Since the rate of DNA synthesis (forward reaction) is substantially greater than the rate of pyrophosphorolysis (reverse reaction), the result is net DNA synthesis. This is especially notable for the D67N/K70R/T215F/K219Q enzyme. With this mutant, the combination of increased pyrophosphorylytic release of 3′-terminal AZTTP due to the D67N/K70R mutations, coupled with the increased DNA polymerase processivity due to the T215F/K219Q mutations, enables facile formation of full-length viral DNA products in the presence of AZTTP and PP_i (Figure 6).

If increased pyrophosphorolysis was the sole mechanism for AZT resistance, then one would expect that AZT-resistant HIV should exhibit cross-resistance to all chain-terminating dideoxynucleoside inhibitors. In general, AZT-resistant virus remains sensitive to other ddN inhibitors (*3, 7*). We found no differences in inhibition of wt and mutant RT by ddCTP and ddGTP, either in the absence or in the presence of PP_i. We suggest that decreased chain termination by AZTTP in reactions catalyzed by the mutant enzymes results from a combination of the selectively decreased binding of AZTTP in the presence of PP_i combined with pyrophosphorolytic removal of any AZTMP that is incorporated. The interaction with other ddNTPs is apparently not affected by PP_i; thus, the extent of chain termination by these inhibitors is similar to wt.

Our studies enable formulation of a working model for the phenotypic mechanism of HIV-1 high-level resistance to AZT arising from the D67N/K70R/T215F/K219Q mutations in RT. The antiviral efficacy of ddN inhibitors such as AZT is due primarily to chain termination of the nascent viral DNA. The D67N/K70R mutations confer increased sensitivity to PP_i to RT, leading to a selectively decreased binding of AZTTP and an increased pyrophosphorolytic cleavage of the 3′-terminal chain-terminating nucleotides. This pyrophosphorolytic removal of chain-terminating AZT may be augmented by the increased enzyme residence time noted for the interaction of the D67N/K70R/T215F/K219Q mutant with AZT-terminated primers (*42*). In a sense, one may consider this pyrophosphorolytic removal of the chain-terminator as a type of "proof-reading" activity expressed by the mutant enzyme. However, an increase in the reverse rate of the RT mechanism without a corresponding compensation in the forward direction would confer a replicative disadvantage to the mutant. This does not appear to be the case with AZT-resistant HIV-1, since this virus may in fact have a replication advantage (*13*−*15*). We suggest that the increased DNA synthesis processivity provided by the T215F/K219Q mutations compensates for the potential replication disadvantage that might be conferred by other mutations that increase the rate of the reverse reaction, pyrophosphorolysis. Accordingly, high-level viral resistance to AZT requires multiple mutations in different subdomains of the viral RT.

The magnitudes of each of the multiple phenotypic changes in RT due to AZT-resistance mutations, namely, differences in each of AZTTP binding, pyrophosphorolytic cleavage, and processivity, are relatively modest (2−10-fold) compared to the 100−200-fold resistance exhibited by AZT-resistant HIV. However, the interplay among the observed phenotypic changes need not be additive. These phenotypic changes may interact in some 'synergistic' manner to provide high-level resistance. This is illustrated very well in Figure 6, in which the amount of full-length DNA synthesized by

[2] G. Borkow, D. Arion, M. A. Wainberg, and M. A. Parniak, submitted for publication.

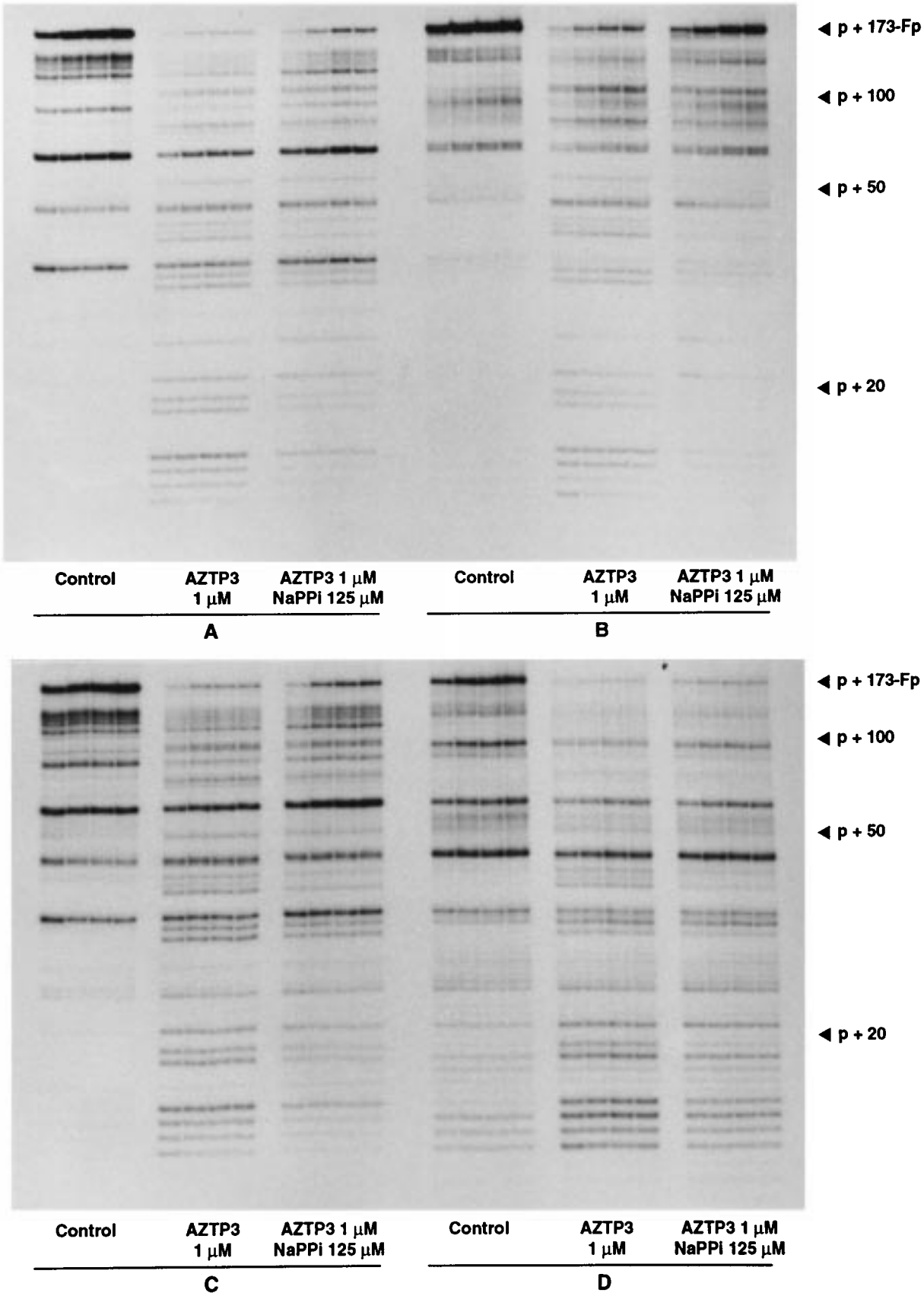

FIGURE 6: Effect of AZTTP ± PP_i on the extent of DNA polymerization product formation during RT RDDP activity on a heteropolymeric T/P. Reactions were carried out at 37 °C using the pHIV-PBS heteropolymeric RNA template primed by the 18 nt ^{32}P-labeled prPBS primer and 25 μM each of dATP, dCTP, dGTP, and TTP. In each of panels A−D, the left set of lanes is DNA synthesized in the absence of AZTTP and the absence of PP_i, the middle set of lanes is DNA synthesized in the presence of 1 μM AZTTP only, and the right set of lanes is DNA synthesized in the presence of 1 μM AZTTP + 125 μM PP_i. Each set of lanes corresponds to 30, 60, 90, and 120 min of reaction time. The full-length product is 191 nt (18 nt primer plus 173 nt RT-catalyzed extension). (A) wt RT; (B) D67N/K70R/T215F/K219Q RT; (C) D67N/K70R RT; (D) 215F/K219Q RT.

the D67N/K70R/T215F/K219Q mutant in the presence of high levels of AZTTP and physiological levels of PP_i is quite similar to that synthesized by RT in the absence of the chain terminator.

Our studies imply a phenotypic role for the K70R/T215Y/F mutations often observed with HIV-1 resistance to AZT. We do not yet know the contribution, if any, of the M41L mutation to either polymerase processivity or pyrophosphorolysis. A previous study failed to observe increased in vitro pyrophosphorolysis with mutant RT (*10*). We cannot presently account for the discrepancy between these data and our observations. However, alterations in pyrophosphorolytic rate by the mutant RT are entirely consistent with other recent observations from our laboratory showing that combinations of AZT and UC781, a potent nonnucleoside inhibitor of HIV-1 RT (*43*), act synergistically to inhibit replication of AZT-resistant strains of HIV-1,[2] implying that UC781 "restores" the antiviral activity of AZT against resistant virus. UC781 inhibits RT-catalyzed pyrophosphorolysis;[2] we propose that this inhibition enables AZT to function as a chain-terminator when used in combination with the nonnucleoside inhibitor against AZT-resistant virus.

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