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Interaction of tRNA-Derivatives and Oligonucleotide Primers with AZT-Resistant Mutants of HIV-1 Reverse Transcriptase

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Abstract—While the molecular basis of HIV-1 AZT resistance has been widely studied, a biochemical explanation of this process is not well known. No significant changes in the binding affinity of reverse transcriptase (RT) mutants for AZT-triphosphate has been found. Here we analyzed the interaction of wild type and AZT-resistant mutant forms of HIV-1 RT with different primers. Site-directed mutagenesis was used to introduce point mutations on the retroviral enzyme. Primers were either synthetic oligonucleotides or $tRNA^{Lys3}$ derivatives containing $d(pT)_n$ or $r(pU)_n$ at the 3′ end. In all cases, determination of kinetic parameters was done in the presence or absence of compounds known to modify protein conformation, such as dimethyl sulfoxide (DMSO), urea, and Triton X-100. Although we found similar K_m values for all RTs, there was generally an increase in the affinity when enzymes were tested in the presence of DMSO, urea, and Triton X-100. Then, we analyzed the nucleation and elongation steps of the polymerization process. The efficiency of formation of the first base pair was determined by measuring K_{m1}, the affinity between RT and the 3′ terminal nucleotide of the primer. An important difference was found: in the presence of DMSO, urea, and Triton X-100, the K_{m1} values for mutated enzymes were higher than those of wild type RTs. Thus, the presence of compounds able to change protein conformation led to a marked destabilization of the interaction of mutated RTs with the 3′ terminal nucleotide of the primer. From these results, it can be hypothesized that resistance to AZT is not due to the direct influence of mutations on RT, but rather to conformational changes of the mutated RT in complex with the template–primer altering the ability of the enzyme to select or reject an incoming dNTP.

Key words: HIV-1 Reverse transcriptase; mutated enzymes; AZT-resistance; oligonucleotide primers; $tRNA^{Lys3}$-derivatives.

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Abbreviations: AIDS, acquired immune deficiency syndrome; HIV-1, human immunodeficiency virus type-1, RT, reverse transcriptase; AZT, 3′-azido-2′,3′-dideoxythymidine; AZT-TP, 3′-azido-2′,3′-dideoxythymidine triphosphate; dNTP, deoxynucleoside triphosphates; ddNTP, dideoxynucleoside triphosphates; DMSO, dimethyl sulfoxide.

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Introduction

The action of most anti-AIDS drugs is based on the inhibition of the viral genome replication catalyzed by RT. AZT was the first drug utilized in HIV-1 infected individuals. This compound must be phosphorylated to become active as an antiviral agent inside the infected cell. Under its 5′-triphosphate form, AZT-TP is incorporated into the DNA chain by RT resulting in termination of DNA synthesis. However, prolonged treatment by AZT induces a number of point mutations in the RT-encoding region of the HIV-1 genome, correlating with the emergence of drug resistance to this virus.[1] The mutations responsible for HIV-1 resistance have been identified at amino acid positions 41 (Met →Leu), 67 (Asp→Asn), 70 (Lys→Arg), 215 (Thr→ Phe/Tyr), and 219 (Lys→Gln).[2] A high level of AZT resistance is observed when at least four mutations are present, while partial resistance is obtained with lesser combinations.[3] Analysis of the crystal structure of HIV-1 RT reveals that all mutations are found in the 'fingers' and 'palm' subdomains of the enzyme, in positions which are able to interact with the template–primer duplex.[4] It has been suggested that resistance to AZT is caused by changes in the mechanism of RT interactions with the template–primer duplex, rather than with the deoxynucleoside 5′-triphosphate precursors.[5]

A peculiar characteristic of HIV-1 resistance to AZT is the fact that while viral replication in infected cells or individuals shows an increasing level of resistance to this drug during treatment, RT isolated from AZT-resistant strains, when tested in vitro, showed the same sensitivity to AZT as that of wild strains.[1] Steady-state kinetic studies using the wild type and the mutant forms of RT presented little differences in kinetic parameters for the mutant RT as compared to the wild enzyme.[6] The same behavior is not observed with other nucleoside analogues. Despite extensive studies, the biochemical mechanism of AZT resistance of RT mutants is still unknown. The changes in affinity of mutated RTs for the specific ligands compared to that of wild-type RT cannot explain the virus resistance to AZT. However, Pokholok et al.[7] have shown that the affinity of a mutated RT (four mutations) for an RNA template is approximately seven times lower than that of the wild-type RT. Using pre-steady-state kinetics analysis in which the individual steps in the overall enzymatic pathway can be directly observed, it has been suggested that the basis of AZT resistance is related to RNA-dependent replication rather than DNA-dependent replication.[8] Another possibility is that mutations may indirectly affect the interaction between RT and template–primer, dNTPs, or AZT-TP, by altering the conformation of the enzyme.

A probable explanation of the paradox concerning the in vitro inhibition of AZT-resistant mutants of RT is that the cell-free test is an oversimplification of the in vivo process. Drug resistance may thus be attributed not only to changes in HIV-1 RT, but also to the interactions that this enzyme may have with viral or cellular factors, obviously absent in the cell-free assays. Additionally, it cannot be excluded that factors such as the gag-derived basic nucleocapsid protein (reviewed in ref. 9), which has been suggested to be involved in genome dimerization, primer annealing, strand transfer, may influence RT activity. In vivo, the nucleocapsid protein (NC) is believed to mediate primer–template annealing. NC can even promote annealing between a cDNA (−) primer containing mutated sequences and the 3′ end of viral genomic RNA. The observation that the NC protein enhances the incorporation of mutations during minus strand DNA elongation favors the notion that NCp is a factor contributing to the high mutation rate of HIV-1.[10] Recently, a report described that in vitro, the NC protein annealed $tRNA^{Lys3}$ onto a non-complementary primer binding site within the HIV genome; moreover, HIV-1 RT was able to extend this complex.[11] Also, other still undefined factors present in the intracellular structure where retroviral replication takes place may modulate HIV-1 polymerase activity during cDNA synthesis. The latter effect may take place when DNA synthesis starts either from the primer $tRNA^{Lys3}$ or from oligonucleotide natural primers.

RT initiates in vivo DNA synthesis from the 3′ end of a host tRNA which is partially annealed to a complementary region near the 5′ end of the viral genome designated the primer binding site or PBS. HIV-1 RT uses $tRNA^{Lys3}$ as primer (reviewed in refs 12–14). By different approaches (gel-shift assay, footprinting, crosslinking) a complex between $tRNA^{Lys3}$ and HIV-1 RT has been identified in vitro.[15–21] We have previously shown that complex formation with the natural primer tRNA induces significant structural changes in HIV-1 RT.[22] Preincubation of RT with tRNA leads to an increase of the catalytic activity on a synthetic template–primer; in this case tRNA is not utilized as a primer for DNA synthesis, but as an enzyme effector.[23] Furthermore, compounds such as DMSO, urea, or Triton X-100 (D/U/T), able to modify protein conformation by altering hydrogen bonds, electrostatic or hydrophobic contacts, interact with HIV-1 RT stimulating its activity.[24] Low concentrations of D/U/T were able to activate RT. D/U/T lead to changes in kinetic and thermodynamic parameters of the enzyme when interacting with templates, primers, or dNTP substrates.

Given the multiple parameters involved in reverse transcription, it seemed interesting to examine the interaction of primers with wild type and mutated RTs. We

used kinetic and thermodynamic methods previously described to study the interaction of HIV-1 RT with different templates and primers.[25,26] In this work, we compared the interaction of primers with wild type and AZT-resistant mutants of HIV-1 RT. Different mutated RTs were obtained by site-directed mutagenesis: (215); (67–215); (67–70–215); (215–219); (67–70–215–219). Primers were either oligonucleotides or $tRNA^{Lys3}$-derivatives containing $d(pT)_n$ or $r(pU)_n$ attached to the 3′ end of tRNA. All determinations were performed in the absence or presence of D/U/T.

Results

Interaction of RT with synthetic oligonucleotides

We have previously shown that the addition of D/U/T enhances the de novo synthesis of DNA catalyzed by HIV-1 RT.[24] To better understand the recognition of primers by AZT-resistant mutated and wild type RTs, we studied their interaction in the absence or presence of D/U/T. As already shown, for primers, the values of Michaelis–Menten constants (K_m) and of dissociation constants of the enzyme×template–primer complexes (K_d) were very close; therefore, they can be used to estimate the primer affinity.[27,28] The K_m in each case was determined using the graphical representation of Eisenthal and Cornish–Bowden[29] as shown in Figure 1. The K_m values of wild type and mutated RTs obtained for the primer $d(pT)_{11}$ are summarized in Table 1. These values were similar, implying that there were no important differences in affinity when comparing wild type or mutated RTs. An important observation, however, was that in all cases the addition of D/U/T increased the affinity for the primer.

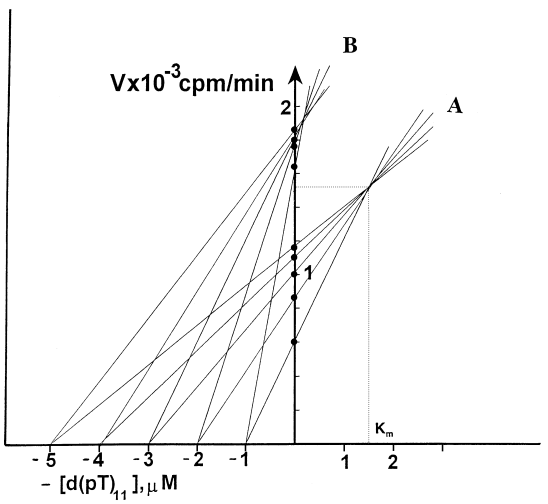

Figure 1. Polymerization catalyzed by wild type HIV-1 RT. Initial rates of polymerization were measured as a function of $d(pT)_{11}$ concentration. The determination of kinetic constants was done according to the representation of Eisenthal and Cornish-Bowden. (A) measured in the absence of DMSO, urea, and Triton X-100; (B) measured in the presence of 0.6% DMSO, 0.0075% urea, and 0.3% Triton X-100.

A similar study was performed next, using different oligoribo- or oligodeoxyribonucleotide primers. The K_m values are shown in Table 2. As described above, the addition of D/U/T increased the affinity of wild type RTs for these primers. This was also the case for the RT mutants, except for $d(pU)_{10}$ where the K_m practically did not change in the presence or absence of D/U/T.

The ability of oligonucleotides to serve as primers is not limited by size: even mononucleotides such as dNMP or dNTP can be used as minimal primers.[25,28] Both the affinity of primers for DNA polymerases and the polymerization rate increased with primer length.

Table 1. Michaelis constants for $d(pT)_{11}$ in the polymerization reaction catalyzed by wild type and mutated RTs

Enzyme[a]	K_m (μm)	
	−D/U/T	+D/U/T[b]
Wild type RT		
Homodimer (p66/p66)	0.80[c]	0.13
Heterodimer (p66/p51)	1.50	0.14
Mutated RT		
215	0.50	0.10
67, 215	0.40	0.09
67, 70, 215	0.26	0.08
215, 219	1.30	0.40

[a]Assays were done in the presence of poly(A) template.
[b]Final concentrations were 0.6% DMSO, 0.0075% urea, and 0.3% Triton X-100.
[c]Values represent the average of at least three independent determinations.

Table 2. Michaelis constants for different primers in the polymerization reaction catalyzed by wild type and mutant RTs

Primer	K_m (μM)					
	Wild type RT[a]		RT 215		RT 67,70,215	
	(−)	(+)	(−)	(+)	(−)	(+)
$d(pT)_{11}$	1.50	0.14	0.50	0.10	0.26	0.08
$[d(pT)_9r(pU)]$	0.90	0.20	0.20	0.16	0.34	0.18
$r(pU)_{11}$	0.80	0.60	0.90	0.39	0.30	0.50
$d(pU)_{10}$	0.45	0.10	0.10	0.18	0.15	0.19

[a]Assays were done in the absence (−) or in the presence (+) of DMSO, urea, and Triton X-100.

These parameters change according to the geometric progression:

$$K_{m(n)} = K_{m1} \times (1/f)^{(n-1)} \quad (1)$$

where K_{m1} represents the K_m value for the 3′ terminal nucleotide of the primer, or the minimal primer (dNTP or dNMP); $K_{m(n)}$, is the Michaelis constant value corresponding to the following nucleotides for primers with n units length (n = 2–10); and f is the factor relating the increase in affinity with primer lengthening by one nucleotide unit.

We used this approach to study the interaction between RTs and primers having different lengths. Experiments were done in the presence or absence of D/U/T. In Figure 2 is shown the dependence of log K_m in the length of the primer: in all cases a linear relationship was found. From the slope of the lines shown on Figure 2 we estimated the dissociation constant, $K_{m(n)}$, and from the intercept at n = 1, the nucleation dissociation constant K_{m1}.

The kinetic and thermodynamic parameters determined for various oligothymidilates are summarized in Table 3. Although in the absence of D/U/T, the K_{m1} values of

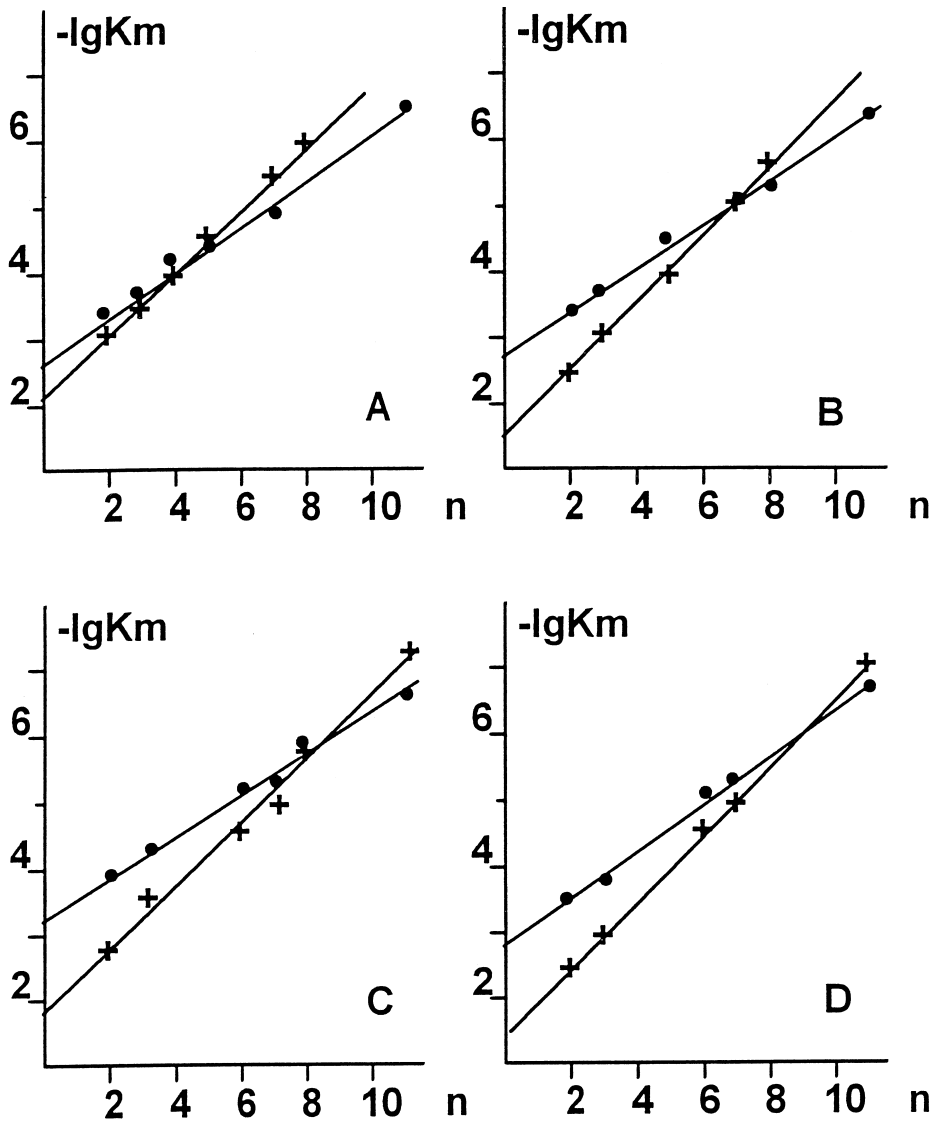

Figure 2. Dependence of the logaritim K_m on the length of thymidilate primers (n) with wild type RT p66/p51 (A) and mutants: 67–215 (B), 67–70–215 (C), 215 (D) in the presence of DMSO, urea, and Triton X-100 (+) or in their absence (●).

Table 3. Kinetic and thermodynamic parameters of the polymerization reaction with oligothymidilate primers

Enzyme	Assay[a]	K_{m1} (mM)	K_{ms} (mM)	F	ΔG1 (kJ/mol)	ΔGs (kJ/mol)	β ($\times 10^3$)
Wild type							
Homodimer (p66/p66)	−	0.50	484.0	2.07	−19.1	−1.80	1.03
	+	1.26	421.7	2.40	−16.8	−2.20	2.99
Heterodimer (p66/p51)	−	1.00	467.7	2.10	−17.4	−1.90	2.14
	+	1.99	372.0	2.70	−15.7	−2.50	5.35
Mutants							
215	−	0.63	426.0	2.30	−18.6	−3.10	1.50
	+	12.60	295.0	3.40	−11.0	−2.15	42.70
67,215	−	0.89	441.6	2.26	−17.7	−2.06	2.02
	+	10.00	312.6	3.20	−11.6	−2.90	32.00
67,70,215	−	0.30	468.0	2.10	−20.4	−1.90	0.68
	+	5.00	330.0	3.03	−13.3	−2.80	15.00

[a]Assays were done in the absence (−) or presence (+) of 0.6% DMSO, 0.0075% urea, and 0.3% Triton X-100.

wild type and mutant enzymes were similar, an important difference between wild type and mutants was found when the determination was done in the presence of D/U/T. Whereas K_{m1} increased only twice for wild type RTs, a very important change was observed for mutated RTs: K_{m1} values increased 10 to 20 times. Thus, the affinity of RT mutants for the 3′ terminal nucleotide of the primer was highly decreased in the presence of D/U/T. The presence of these compounds, which are able to change protein conformation, led to an important destabilization of the interaction between mutant RT and the 3′ terminal nucleotide of the primer.

The efficiency of interaction was then evaluated by measuring the thermodynamic parameters of the process. The free energy change upon primer binding was determined from the data presented in Figure 2, using eq (2):

$$\Delta G = \Delta G_1 + (n-1)\Delta G_s = RT \ln K_{m1} + (n-1)RT \ln K_{ms} \quad (2)$$

where ΔG_1 is the free energy change to bind the first nucleotide (nucleation step) and ΔG_s, that of the following nucleotides (elongation step). The K_{m1} values were obtained by extrapolation of the curves to $n = 1$ in Figure 2 and K_{ms} values were calculated using eq (3):

$$K_{ms} = K_{m(n+1)}/K_{m(n)} = (K_{m1}/K_{m11})^{-1/10} \quad (3)$$

The calculated parameters are summarized in Table 3. The values of ΔG_1 markedly differ for the enzymes investigated, especially in the presence of D/U/T. The values varied from −15.7 to −16.8 kJ/mol for the wild type RT and from −11 to −13 kJ/mol for the mutants. Thus, the interaction of mutated RTs with the 3′-terminal nucleotide of the primer was lower than that of wild-type RT (hetero- and homodimeric forms).

The nucleation coefficient $\beta = K_{m1}/K_{ms}$ reflects the efficiency of formation of the first base pair by the enzyme. In the absence of D/U/T, the β values for all enzymatic forms, including the mutants, were similar. This was not the case in the presence of D/U/T, where β was always higher (Table 3). The most striking observation was that the β values were very different between mutant and wild type RTs. Values increased as much as 28 times for the mutants as compared with wild-type retroviral polymerases. This means that in the presence of D/U/T, there is a decrease in the stabilization and in the nucleation probability for mutated RTs, correlating well with a lower affinity for the 3′-terminal nucleotide of the primer.

The f coefficient reflects the enhancement of affinity with the increase of the primer length by one unit. The K_d inferred from 1/f values reflect the efficiency of interaction between the nucleotides of the primer and the corresponding residues in the complementary template within the enzyme×template–primer complex. The f coefficient values for different RTs are given in Table 3. They were always higher in the presence of D/U/T, implying that these compounds increased the affinity of primers for RT by favoring the template–primer complex interaction. With RT mutants the efficiency of interactions was even higher as judged by the f values obtained.

Pre-steady-state kinetic techniques have been previously employed to determine the mechanism for HIV-1 RT. These studies suggest that catalysis at the active site involves an 'induced fit' model for polymerization.[30,31]

This model describes the reaction pathway for dNTP incorporation for RT in terms of a two-step binding process. The rate-limiting step for each nucleotide incorporation during polymerization by RT is the enzyme conformational change prior to the chemistry step, and following the chemical reaction, the translocation step is fast. Recently, it has been shown that polymerization at pauses sites can be explained assuming that substrates are bound productively at the polymerase site of RT. Other substrates are bound nonproductively and are slowly converted to the productively bound state and are then turned over to products. This slow conversion from the nonproductive to the productive state, without dissociation of the template–primer, accounts for the slow reaction phase from the RT. The shift from the nonproductive to the productive state could involve changes in the structure of the enzyme, the template–primer or both.[32]

In the RT×template–primer complex, the interactions between template and primer are substantially weaker than in the absence of the enzyme. Local melting of the duplex is presumably caused by fixation of the template and the 3′-end of the primer on the enzyme active site.[28,33] However, the enhancement of the f factor ($f = 3.0 - 3.4$) for RT mutants in the presence of D/U/T, implies an increase of the complementary interaction between the template and the primer. Usually, there is a reverse correlation between the K_m and V_{max} values. However, our results show first, that the addition of D/U/T decreased the efficiency of the interaction between RT mutants and the 3′-end of the primer and second, that they increased the template–primer complementary interaction in the RT×template–primer complex. This led to a peculiar situation since we observed a higher rate of polymerization, simultaneously with a higher affinity of the enzyme for the substrate.

Mutations that confer resistance to AZT are located in positions (fingers and palm subdomains) that interact with the template–primer. This region is involved in the appropriate positioning of the template strand. The fingers subdomain could affect the precise positioning or conformation of the template strand and this could change the ability of the enzyme to accept or reject an incoming dNTP. Since the AZT-resistance mutations in the palm subdomain appear to occur at positions that could contact either the template or the primer strand, these mutations could have similar effects. The decision to accept or reject an incoming ddNTP is made based on the precise configuration of both the protein and the nucleic acid at the active site. The position and conformation of the template–primer and its interaction with protein structure will determine the precise recognition of an incoming base. Resistance to ddNTP results from a change in the conformation of the template–primer that alters the ability of the enzyme to select or reject an incoming dNTP.[5] To explain our results it can be hypothesized that conformational changes of mutant RTs in the presence of D/U/T, alter the ability to form a complex with the 3′ terminal nucleotide of the primer.

Interaction of RT with tRNA derivatives

Another possibility that may play an important role in the development of drug resistance could be related to important changes in other steps of the viral replication, such as the tRNA initiation process.

We have shown earlier that complex formation with the natural primer $tRNA^{Lys3}$ increased the activity of HIV-1 RT on poly(A)-oligo(dT).[23] Enzyme activation occurred slowly: the half-time values of the RT activation were within 20–30 min. In the presence of D/U/T, the rate of activation of RT by tRNA was significantly higher.[24] Most probably, D/U/T significantly accelerate and increase the specific changes of the RT conformation induced by tRNA.

To further characterize the interaction of HIV-1 RT with primers similar to the natural one involved in the initiation step of retroviral reverse transcription, we used analogues of $tRNA^{Lys3}$. Different derivatives of $tRNA^{Lys3}$ containing oligothymidilates or oligouridilates at their 3′ end were synthesized. Before determining the kinetic parameters with the RT mutants it was important to ascertain whether the $tRNA^{Lys3}$ derivatives could be used as primers. Results presented in Table 4 show that HIV-1 RT was able to elongate such tRNA derivatives in the presence of a template poly(A). Although the efficiency of polymerization was low, it is important to remark the extremely high affinity of HIV-1 RT for these tRNA derivative primers as compared with the primer oligo $(dT)_{10}$. Then we determined the kinetic parameters of wild type and mutant RTs on the polymerization reaction using poly(A) as template and

Table 4. Michaelis constants and V_{max} values for different tRNA derivatives in the reaction catalyzed by wild type RT p66/p51

Primers	K_m (nM)	V_{max} (%)[a]
$tRNA^{Lys}$-$r(pU)_4$	2.0	1.2
$tRNA^{Lys}$-$r(pU)_{11}$	1.2	1.5
$tRNA^{Lys}$-$r(PU)_6$	2.2	2.1
$d(pT)_{10}$[b]	600	100

[a]V_{max} values are relative to that of primer $d(pT)_{10}$ taken as 100%.
[b]The primer $d(pT)_{10}$ was used as control.

Table 5. Michaelis constant and V_{max} values for $tRNA^{Lys}$-r(pU)$_4$ and d(pT)$_{10}$ primers in the polymerization catalysed by RT and its mutants

Primer	Enzyme	+D/U/T[b]		−D/U/T	
		K_m (nM)	V_{max} (%)[a]	K_m (nM)	V_{max} (%)
	Wild-type:				
d(pT)$_{10}$	p66/p51	600	100	1600	80.4
$tRNA^{Lys}$-r(pU)$_4$	p66/p51	0.20	1.2	0.61	1.10
	Mutants:				
$tRNA^{Lys}$-r(pU)$_4$	67, 70, 215, 219	0.45	0.51	0.85	0.49
$tRNA^{Lys}$-r(pU)$_4$	67, 215, 219	0.40	0.37	0.92	0.31
$tRNA^{Lys}$-r(pU)$_4$	67, 70, 215	0.35	0.80	0.70	0.78
$tRNA^{Lys}$-r(pU)$_4$	67, 215	0.30	0.75	0.75	0.66
$tRNA^{Lys}$-r(pU)$_4$	215	0.27	0.73	0.58	0.70

[a]V_{max} for d(pT)$_{10}$ with wild type RT is taken as 100%. All other values are relative to it.
[b]Assays in the presence of 0.6% DMSO, 0.0075% urea, and 0.3% Triton X-100.

$tRNA^{Lys}$-r(pU)$_4$ as primer (Table 5). As in the case of oligonucleotide primers, the K_m for all RTs were lower in the presence of D/U/T. Thus, the interaction of wild type and mutated RTs with tRNA derivatives follows a pattern similar to that of oligonucleotide primers.

Oligonucleotides having a 'chain terminator' nucleoside analogue

One possible way to inhibit RT by primers is to use oligonucleotides having a very high affinity for the enzyme and a chain terminator nucleoside analogue, like AZT-TP or ddTTP, at the 3′ end. To compare the inhibition of wild-type and RT mutants by oligonucleotides containing a chain terminator at the 3′ end we used [d(pT)9]dd(pT). This oligonucleotide behaved as a competitive inhibitor of the poly(A)-oligo(dT) template–primer in the polymerization reaction. In Table 6 are shown the K_i values obtained in the presence or absence of D/U/T. All enzymes gave K_i values that were not significantly different, either in the presence or in the absence of D/U/T.

Table 6. Inhibition by [d(pT)$_9$]dd(pT) of the polymerization catalyzed by mutants and wild type RT on poly(A)-d(pT)$_{11}$

Enzymes	Ki (nM)	
	+D/U/T	−D/U/T
Mutants		
215	100	75
67, 215	180	60
67, 70, 215	70	90
215, 219	180	170
Wild type		
p66/p66	30	50
p66/p51	75	60

Conclusions

In standard in vitro assays, RT derived from sensitive and AZT-resistant strains appears to be equally susceptible to AZT-TP. The behavior of mutated RTs has failed to show changes in kinetics or susceptibility to AZT-TP sufficient to account for the effects of these mutations on the sensitivity of the virus to AZT.

Here we show that the specific mutations of HIV-1 RT leading to AZT-resistance slightly influenced the enzyme interaction with primers (oligonucleotide or tRNA derivatives) compared with wild type RT. Besides, the addition of compounds inducing protein conformational changes (DMSO, urea, Triton X-100) modified the interaction with primers, leading to an increase in the affinity. The polymerization process was further studied by analyzing the nucleation and the elongation steps. The affinity of mutated RTs for the 3′ terminal nucleotide of the primer was highly decreased in the presence of D/U/T, leading to a marked destabilization compromising the nucleation probability. Our data are more in favor of the possible importance of conformational changes in the RT molecule leading to AZT-resistance, than in the direct influence of the mutations on the binding of the enzyme with the template–primer duplex and/or the precursor deoxynucleotides. It can be speculated that the presence of some natural factors in infected cells, having a similar effect on the enzyme activity as D/U/T may markedly alter the efficiency of the RT mutant forms.

Experimental

Unlabeled nucleotides, polynucleotides, and d(pT)$_{12–18}$ were from Sigma or Pharmacia. [^{3}H]dTTP (600–900 Tbq/mol) was from Amersham or Isotop, Russia. dTTP and

[^{3}H]dTTP were purified by thin-layer chromatography on Kiesselguhr 60 F plates (E. Merck) in dioxane/H_2O/NH_4OH (6/4/1). All oligonucleotides were synthesized as described in refs 34 and 35. Their high degree of purity was confirmed by ion-exchange and reverse-phase chromatography. Oligonucleotide concentration was determined spectrophotometrically using the following absorption coefficients, expressed as $mM^{-1}\,cm^{-1}$: (a) dTTP: 9.6; $d(pT)_4$: 32.0; $d(pT)_5$: 43.5; $d(pT)_6$: 52.0; $d(pT)_7$: 60.5; $d(pT)_8$: 69.0; $d(pT)_{10}$: 87.0; $d(pT)_{11}$: 95.7; [$d(pT_9)$]r(pU): 85.5, determined at 267 nm; (b) $r(pU)_{10}$: 92.4; $r(pU)_{11}$: 101.8, determined at 262.

$tRNA^{Lys3}$ was obtained as described in ref. 36. $tRNA^{Lys\text{-}d(pT)_6}$ and $tRNA^{Lys}$-$r(pU)_n$ derivatives were prepared by the addition of $d(pT)_6$ or $r(pU)_n$ tails to the 3′ end of $tRNA^{Lys}$ by using T4 RNA ligase.

HIV-1 RT heterodimer p66/p51 was expressed and purified from *Eschericha coli*. RT mutants were obtained by oligonucleotide-directed mutagenesis and purified from the same *E. coli* strain producing wild-type RT.[7] RTs were obtained with mutations in the following amino acids: 67 (Asp- > Asn); 70 (Lys- > Arg); 215 (Thr- > Phe); and 219 (Lys- > Glu). The homodimeric RT form, p66/p66, was purified as described earlier, using a protease-deficient yeast strain.[36] One unit of enzyme activity was defined as the amount of RT catalyzing the incorporation of 1 nmol of dNMP into DNA in 1 h at 30 °C. Polymerization was initiated by adding 0.02–2 units of enzyme and carried out for 2–120 min at 30 °C either in the absence or presence of 0.6% DMSO, 0.0075% urea, and 0.3% Triton X-100. The assay mixture contained 50 mM Tris-HCl (pH 8.0), 1 mM dithiotreitol, 80 mM KCl, 4 mM magnesium acetate, 0.5 mM EDTA, 50 μM [^{3}H]dTTP (100–200 Ci/mmol), and 1.7 A_{260} units/mL poly(A). Aliquots of 5–15 μL were taken every 1–10 min and placed onto dry FN-16 paper (1.5×1.5 cm) presoaked in 5% trichloroacetic acid. The filters were washed with the same solution, then with acetone at 0 °C, dried and monitored for radioactivity.

All measurements were performed within the linear regions of the product formation versus time curves. The K_m and V_{max} values were determined from the Eisenthal and Cornish–Bowden double direct linear plots.[29] The error determination of the constant values was within 20–40%.

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