Mutational analysis of Lys⁶⁵ of HIV-1 reverse transcriptase

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Amino acid Lys⁶⁵ is part of the highly flexible $\beta 3$ – $\beta 4$ loop in the fingers domain of the 66 kDa subunit of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT). Recent crystal data show that the ϵ -amino group of Lys⁶⁵ interacts with the γ -phosphate of the bound deoxynucleoside triphosphate ('dNTP') substrate [Huang, Chopra, Verdine and Harrison (1998) *Science* **282**, 1669–1675]. In order to biochemically define the function of RT Lys⁶⁵, we have used site-specific mutagenesis to generate RT with a variety of substitutions at this position, including K65E, K65Q, K65A and K65R. Kinetic analyses demonstrate that if Lys⁶⁵ in RT is substituted with an amino acid other than arginine the enzyme exhibits dramatic decreases in the binding affinity (K_m) for all dNTP substrates, in RT catalytic efficiency (k_{cat}/K_m) and in the mutant enzyme's ability to carry out pyrophosphorolysis, the reverse reaction of DNA synthesis. The pH optimum for the DNA polymerase activity of K65E RT was 6.5, compared to 7.5 for the wild-type enzyme, and 8.0 for the K65R, K65A and K65Q mutants. Molecular modelling

studies show that mutations of Lys⁶⁵ do not affect the geometry of the loop's α -carbon backbone, but rather lead to changes in positioning of the side chains of residues Lys⁷⁰ and Arg⁷². In particular, Glu in K65E can form a salt bridge with Arg⁷², leading to the diminution of the latter residue's interaction with the α -phosphate of the dNTP residue. This alteration in dNTP-binding may explain the large pH-dependent changes in both dNTP-binding and catalytic efficiency noted with the enzyme. Furthermore, the K65A, K65Q and K65E mutant enzymes are 100-fold less sensitive to all dideoxynucleoside triphosphate ('ddNTP') inhibitors, whereas the K65R mutation results in a selective 10-fold decrease in binding of ddCTP and ddATP only. This implies that mutations at position 65 in HIV-1 RT influence the nucleotide-binding specificity of the enzyme.

Key words: ddI/ddC cross-resistance, nucleoside inhibitor resistance, pyrophosphorolysis.

INTRODUCTION

Due to the essential role of the human immunodeficiency virus type-1 (HIV-1) reverse transcriptase (RT) in viral replication, much of the anti-HIV-1 chemotherapeutic strategy has been targeted against this enzyme [1]. Dideoxynucleoside (ddN) compounds such as 2',3'-dideoxy-3'-thiacytidine (3TC), 2',3'-dideoxycytidine (ddC), and 2',3'-dideoxyinosine (ddI) are effective inhibitors of viral RT-catalysed proviral DNA synthesis. These antiviral agents, once phosphorylated to their active triphosphate forms by the host cell's metabolism, inhibit viral replication by either acting as competitive inhibitors with respect to the appropriate dNTP substrate, or by effectively terminating further DNA polymerization upon their incorporation into the nascent proviral DNA chain [2,3]. Unfortunately, HIV-1 develops resistance to ddN anti-viral treatment under selective drug pressure. This resistance is directly correlated with mutations in viral RT [4].

Several ddN resistance mutations arise in the flexible $\beta 3$ – $\beta 4$ loop situated in the fingers domain of the 66 kDa subunit of HIV-1 RT [5]. Five residues that form part of this loop (Ile⁶³, Lys⁶⁴, Lys⁶⁵, Trp⁷¹ and Arg⁷²) are conserved in all retroviral RTs [6]. In the RT–template/primer (T/P)–dNTP ternary complex [7], the guanidinium group of Arg⁷² and the ϵ -amino group of Lys⁶⁵ make salt bridges with the α - and γ -phosphates of the bound dNTP substrate, respectively. These interactions help position the dNTP for base-pairing with the complementary template residue, and for catalysis of phosphodiester bond formation with the 3'-OH of the primer terminal nucleotide. While the available structural data have provided considerable

information about RT molecular structure and the amino acid residues critical for catalysis, it has not yielded insight into how selectivity in resistance is conferred.

The K65R mutation in RT confers viral cross-resistance to 3TC, ddC and ddI [8]. This mutation provides approximately 5-fold viral-resistance to ddI and ddC, and 20-fold viral-resistance to 3TC [8]. Studies with recombinant RT have shown that the K65R mutation results in a measurable change in the *in vitro* affinity of the mutant RT for ddCTP and ddATP but not for either ddTTP or ddGTP [9,10]. How is this selectivity in resistance conferred? In order to address this question and to better understand the limits of genetic and physical flexibility of HIV-1 RT we have used site-specific mutagenesis to generate enzymes with a variety of substitutions at this position. Our data show that a positively charged residue at position Lys⁶⁵ in HIV-1 RT is essential for the correct catalytic functioning of the enzyme. This implies that HIV is severely curtailed in the choice of mutations at this position in the development of resistance to various ddNs. Furthermore, mutations at this codon dramatically influence the nucleotide-binding selectivity of HIV-1 RT.

EXPERIMENTAL

Materials

High purity dNTPs, ddNTPs, [³H]dNTPs and [γ -³²P]ATP were purchased from Amersham Pharmacia Biotech (Baie d'Urfe, Quebec, Canada). The homopolymeric T/Ps, poly(rC)-oligo(dG)_{12–18}, poly(rI)-oligo(dC)_{12–18} and poly(rA)-oligo(dT)_{12–18} were acquired from Pharmacia Biotech (Montreal, PQ, Canada).

Abbreviations: 3TC, 2',3'-dideoxy-3'-thiacytidine; ddC, 2',3'-dideoxycytidine; ddI, 2',3'-dideoxyinosine; ddN, dideoxynucleoside; HIV-1, human immunodeficiency virus type 1; pPBS, synthetic 18 nt primer; RT, reverse transcriptase; T/P, template/primer; wt, wild-type.

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The heteropolymeric T/P was prepared as described previously [11], using the T7 polymerase RNA transcript from *AccI*-linearized plasmid pHIV-PBS as the template and a synthetic 18 nt primer (prPBS) that is complementary to the sequence of the tRNA^{Lys3} primer as the DNA synthesis initiation primer. All other reagents were of the highest quality available and were used without further purification.

Site-specific mutagenesis, expression and purification of mutant RT

The mutations K65A, K65Q, K65R and K65E were introduced into the 66 kDa subunit of HIV-1 RT using the phosphorothioate technique described by Taylor et al. [12]. Phosphorylation of the mutagenic primers, annealing, extension and ligation were carried out in accordance with the manufacturer's protocol (Amersham Pharmacia Biotech) supplied with the *in vitro* mutagenesis kit (Sculptor®). Following positive identification of the mutant in M13 by DNA sequencing, the 66 kDa RT gene was subcloned into the pET-28a expression vector. The recombinant plasmid was then introduced into *Escherichia coli* JM109 for induction and isolation of the enzyme [13].

Assay of RT activity

HIV-1 RT polymerase activity was determined by a fixed time assay. Briefly, the reaction mixtures (50 μ l, total volume) contained 50 mM Tris/HCl (pH 7.5, 37 °C), 60 mM KCl, 8 mM MgCl₂, 10 mM dithiothreitol, 10–20 ng of wild-type (wt) or mutant RT, 1–80 nM T/P and 0.2–200 μ M [³H]dNTP substrate. Reaction assays were incubated at 37 °C and then quenched with 0.5 ml of ice-cold 20 mM sodium pyrophosphate in 10% (w/v) trichloroacetic acid. Following 20 min of incubation on ice, the precipitated nucleic acid was filtered on Whatman 934-AH glass-fibre filters and then washed sequentially with 10% trichloroacetic acid and ethanol. Radioactivity was determined by liquid scintillation analysis. Kinetic parameters of the recombinant enzymes were calculated as described previously [9].

RT DNA polymerase pH-dependent activity profiles were carried out essentially as described above, except that the Tris buffer was substituted with either 50 mM Bis-Tris propane (pH 6.5–9.0) or 50 mM Mes (pH 5.5–6.5).

Assay of RT-catalysed pyrophosphorolysis

The 18 nt prPBS was 5' end-labelled using [γ -³²P]ATP and T4 polynucleotide kinase (Pharmacia Biotech) as described previously [10]. The labelled primer was purified by electrophoretic resolution on a polyacrylamide gel, followed by excision and elution of the 18 nt product. The labelled primer was then annealed to the pHIV-PBS RNA transcript (1:12 molar ratio of prPBS/pHIV-PBS RNA). Recombinant HIV-1 RT (20–30 nM) was preincubated with 50 nM T/P for 5 min in 50 mM Tris/HCl (pH 7.5) containing 60 mM KCl and 10 mM MgCl₂ to allow the formation of the RT-T/P binary complex. Pyrophosphorolysis was initiated by the addition of 150 μ M sodium pyrophosphate. Aliquots (5 μ l) were removed after various time intervals and the reaction was quenched by the addition of an equivalent amount of sequencing loading buffer [98% (v/v) deionized formamide/10 mM EDTA/1 mg/ml Bromophenol Blue/1 mg/ml xylene cyanol]. Samples were loaded onto a 16% acrylamide/7 M urea sequencing gel and electrophoresed for approximately 3 h at 2000 V. The resolved products were visualized by autoradiography and quantified by densitometry.

Molecular modelling of RT mutant enzymes

Molecular models of the Lys⁶⁵ HIV-1 RT mutants were constructed using the X-ray crystallographic co-ordinates for the RT-T/P-dNTP ternary complex reported by Huang et al. ([7]; Brookhaven Protein Databank entry 1rtd). Introduction of the mutations into wt RT and energy minimization experiments were carried out using Sybyl 6.2 (Tripos, St. Louis, MO, U.S.A.). Charges were calculated using the Kollman-All-Atom charge method, and iterative minimizations were carried out using the Kollman-All-Atom forcefield until the energy difference between iterations was less than 0.04 kJ (0.01 kcal)/mol per Å.

RESULTS

pH dependence of wt and Lys⁶⁵ mutant RT

Initial experiments indicated that the K65E mutant RT displayed exceptionally low DNA polymerase activity under our standard assay conditions [50 mM Tris/HCl (pH 7.5)/60 mM KCl/10 mM MgCl₂]. Since the ϵ -amino group of Lys⁶⁵ makes a salt bridge interaction with the γ -phosphate of the dNTP [7], we investigated whether these mutant enzymes displayed altered pH activity profiles compared to wt HIV-1 RT (Figure 1). The K65R, K65A and K65Q mutant enzymes exhibited a moderate shift in pH optimum from pH 7.5 (for wt RT) to pH 8.0. In contrast, the K65E RT DNA polymerase activity optimum was shifted to pH 6.5.

Steady-state kinetic parameters of wt and mutant RT

Analysis of the steady-state kinetic parameters of the Lys⁶⁵ mutant RT indicates that substitutions at this position are phenotypically expressed at the level of dNTP recognition (Table

Figure 1 DNA polymerization pH-dependence of wt and mutant RTs

RT activity assays were carried out as described in the Experimental section, using poly(rA)-oligo(dT)_{12–18} as T/P. ○, wt RT; △, K65R RT; ■, K65E RT; □, K65A RT; ●, K65Q RT.

Table 1 Steady-state kinetic parameters for wt and mutant RT

RT activity assays were carried using 50 mM Tris/HCl (pH 7.5) containing 60 mM KCl and 8 mM MgCl₂ or 50 mM Bis-Tris propane (pH 6.5) containing 60 mM KCl and 8 mM MgCl₂ for the K65E RT. Values are means $\pm$ S.E.M. of at least three separate determinations.

Variable dNTP	Saturating T/P	RT	K_m (μ M)	$10^{-3} \times k_{cat}$ (s ⁻¹)	$10^{-3} \times k_{cat}/K_m$ (s ⁻¹ · M ⁻¹)
dCTP	Poly(rl)-oligo(dC) ₁₂₋₁₈	wt	10.6 $\pm$ 3.5	32 $\pm$ 6	3.00
		K65R	25.1 $\pm$ 3.1	15 $\pm$ 5	0.60
		K65E (pH 7.5)	147.0 $\pm$ 5.0	7 $\pm$ 1	0.05
		K65E (pH 6.5)	45.5 $\pm$ 7.3	11 $\pm$ 2	0.24
		K65A	30.9 $\pm$ 1.0	17 $\pm$ 3	0.55
		K65Q	26.3 $\pm$ 3.1	10 $\pm$ 1	0.38
dTTP	Poly(rA)-oligo(dT) ₁₂₋₁₈	wt	3.8 $\pm$ 1.1	68 $\pm$ 4	17.90
		K65R	4.9 $\pm$ 2.8	65 $\pm$ 3	13.20
		K65E (pH 7.5)	41.2 $\pm$ 3.3	11 $\pm$ 5	0.27
		K65E (pH 6.5)	27.0 $\pm$ 4.0	32 $\pm$ 9	1.90
		K65A	23.3 $\pm$ 0.8	38 $\pm$ 7	1.63
		K65Q	21.9 $\pm$ 2.4	36 $\pm$ 6	1.64
dGTP	Poly(rC)-oligo(dG) ₁₂₋₁₈	wt	1.8 $\pm$ 0.3	28 $\pm$ 1	15.40
		K65R	2.8 $\pm$ 1.0	31 $\pm$ 4	11.90
		K65E (pH 7.5)	9.9 $\pm$ 2.3	19 $\pm$ 3	1.90
		K65E (pH 6.5)	3.5 $\pm$ 0.9	21 $\pm$ 2	6.00
		K65A	2.6 $\pm$ 0.7	20 $\pm$ 6	7.60
		K65Q	2.9 $\pm$ 1.2	23 $\pm$ 3	7.90

Table 2 ddNTP inhibition of wt and mutant RT

RT activity assays were carried out in 50 mM Tris/HCl (pH 7.5) containing 60 mM KCl and 8 mM MgCl₂ or 50 mM Bis-Tris propane (pH 6.5) containing 60 mM KCl and 8 mM MgCl₂ for K65E RT determinations. The pHIV-PBS heteropolymeric RNA template annealed to the prPBS primer was used as the T/P. Values are means $\pm$ S.E. of at least three separate determinations.

RT	IC ₅₀ (μ M)			
	ddATP	ddCTP	ddGTP	ddTTP
WT	4.8 $\pm$ 1.4	6.3 $\pm$ 1.2	3.2 $\pm$ 0.3	2.9 $\pm$ 1.0
K65R	61.6 $\pm$ 3.2	50.4 $\pm$ 4.6	5.4 $\pm$ 2.2	7.0 $\pm$ 0.5
K65E (pH 7.5)	136.0 $\pm$ 14.0	148.0 $\pm$ 9.0	121.0 $\pm$ 1.0	160.0 $\pm$ 3.0
K65E (pH 6.5)	143.0 $\pm$ 9.0	130.0 $\pm$ 7.0	117.0 $\pm$ 5.0	154.0 $\pm$ 5.0
K65A	147.0 $\pm$ 10.0	146.0 $\pm$ 9.0	125.0 $\pm$ 5.0	148.0 $\pm$ 5.0
K65Q	127.0 $\pm$ 11.5	147.0 $\pm$ 8.0	96.0 $\pm$ 4.5	95.0 $\pm$ 1.0

1). T/P interactions with mutant RT were not significantly affected for all T/Ps tested [e.g. K_m values for poly(rA)-oligo(dT)₁₂₋₁₈ were 10.0 $\pm$ 1.2, 12.4 $\pm$ 0.7, 7.8 $\pm$ 0.9, 8.8 $\pm$ 0.9 and 15.0 $\pm$ 3.7 nM for wt, K65R, K65E, K65A and K65Q enzymes, respectively].

The K65R mutation in HIV-1 RT resulted in a selective 10–12-fold decrease in the IC₅₀ for ddCTP and ddATP (Table 2), while providing only a 2-fold increase in the K_m for dCTP (Table 1). In contrast, the K65A, K65Q and K65E mutations resulted in nearly a 100-fold increase in IC₅₀ for all ddNTP inhibitors (Table 2). However, these mutations also resulted in significantly decreased affinity (K_m) for all dNTP substrates, as well as considerable diminution in RT catalytic efficiency (k_{cat}/K_m). These effects were most pronounced with the K65E mutant enzyme, which displayed a 10-fold increase in K_m for all dNTP substrates and a 60-fold decrease in catalytic efficiency (k_{cat}/K_m) compared to wt RT.

Interestingly K_m values for both dTTP and dGTP for the K65E RT were decreased 4-fold at pH 6.5 compared with those

values determined for the same enzyme at pH 7.5. However IC₅₀ values for ddNTP inhibition at pH 6.5 remained unchanged with respect to those values obtained at pH 7.5 (Table 1).

Pyrophosphorolytic activity of wt and mutant RT

Pyrophosphorolysis is the reverse reaction of DNA polymerization. The DNA primer is sequentially cleaved from the 3' terminus in the presence of pyrophosphate, resulting in the generation of dNTP. Previous studies have shown that an R72A mutation decreases this activity in HIV-1 RT [13]. Since both Arg⁷² and Lys⁶⁵ in HIV-1 RT are involved in dNTP phosphate recognition and pyrophosphate removal [7,16], we examined whether the pyrophosphorolytic activities of the mutant enzymes were adversely affected by the amino acid substitutions. The results indicate that the pyrophosphorolytic activities of all mutant RTs are decreased compared to wt RT (Figure 2). While the K65R RT displayed a moderate decrease in this activity, the K65A, K65Q, and in particular the K65E, enzymes all displayed significantly reduced levels of pyrophosphorolysis. Furthermore, all Lys⁶⁵ mutant enzymes were less susceptible to inhibition by sodium pyrophosphate when compared with wt RT (results not shown).

Molecular modelling of mutant RT

Molecular modelling studies (Figure 3) suggest that mutations at position Lys⁶⁵ do not affect the geometry of the α -carbon backbone of the β 3– β 4 loop, but rather lead to a repositioning of the residue side chains at positions 65, 70 and 72. The K65R mutation results in only subtle differences in the interaction of these residues with the dNTP phosphate backbone when compared with wt RT. However, the introduction of the longer side chain of arginine at position 65 forces the dNTP phosphate backbone to adopt a slightly different conformation (Figure 3B). The K65A and K65Q mutations in HIV-1 RT (Figures 3D and 3E, respectively) completely eliminate the electrostatic interaction between this residue and the γ -phosphate of the dNTP substrate. In both of these models only Arg⁷² in the β 3– β 4 loop shows significant interaction with the phosphate backbone of the dNTP

Figure 2 Time course of pyrophosphorolysis catalysed by wt and mutant RT

Reactions were carried out in the presence of 150 μ M pyrophosphate as described in the Experimental section, using 50 nM heteropolymeric T/P (with the 18 nt prPBS primer) and 20 nM RT heterodimer.

Figure 3 Molecular modelling of the interaction of the $\beta 3$ – $\beta 4$ loop of the Lys⁶⁵ mutant RT with the dNTP substrate

The models were constructed using the X-ray crystallographic co-ordinates for the RT–dNTP–T/P ternary complex reported by Huang et al. ([7]; Brookhaven Protein Databank entry 1rtf). The aspartic acid triad (Asp¹¹⁰, Asp¹⁸⁵ and Asp¹⁸⁶) which co-ordinates the metal ions and constitutes part of the RT active-site is also indicated. (A) WT RT, (B) K65R RT, (C) K65E RT, (D) K65Q RT and (E) K65A RT.

substrate. This allows the phosphate moieties of the respective mutant enzymes to assume slightly different orientations at the active sites. Interestingly, K65E can form a salt bridge with

Arg⁷² (Figure 3C). This leads to the diminution of the latter residue's interaction with the α -phosphate of the dNTP substrate. There is, however, some compensation through Lys⁷⁰, which can

now interact with the γ -phosphate of the dNTP substrate. The almost complete loss of interaction of residues 65 and 72 with the dNTP phosphate backbone is consistent with the low polymerase activity exhibited by this enzyme at pH 7.5.

DISCUSSION

A positively charged amino acid at position Lys⁶⁵ is essential for a catalytically functional RT enzyme

The experimental data demonstrate that if Lys⁶⁵ in RT is mutated to any amino acid other than arginine, a relatively conservative substitution, the enzyme exhibits dramatic decreases both in the binding affinity (K_m) for all dNTP substrates, and in RT catalytic efficiency (k_{cat}/K_m). Site-directed mutagenesis studies on the analogous residue in *E. coli* DNA polymerase I (Arg⁷⁵⁴) or the Klenow fragment (Arg⁶⁵⁹) exhibited similar effects on the $K_{m(dNTP)}$ and $K_{m(PPi)}$ with little or no effects on DNA-binding [14,15]. This implies that HIV-1 is severely curtailed in the choice of mutations at this position in the development of resistance to ddN inhibitors. Recent crystal data show that Lys⁶⁵ interacts with the γ -phosphate of the bound dNTP (or ddNTP) substrate [7]. This interaction is essential for the correct positioning of the dNTP substrate for catalysis of the phosphodiester bond formation. Molecular modelling of the K65A and K65Q RTs demonstrates that these mutations completely eliminate the salt-bridge interaction between this residue and the γ -phosphate of the dNTP substrate. The loss of this critical interaction in dNTP-binding, coupled with the observation that the mutations result in the dNTP adopting a slightly different orientation at the active-site, could account for the decreased DNA polymerization and pyrophosphorolysis activities of these mutant enzymes.

In the K65E mutant enzyme the glutamic acid residue can form a salt bridge with Arg⁷², leading to the diminution of the latter residue's interaction with the α -phosphate of the dNTP. This observation is consistent with the dramatically altered binding affinity for dNTP substrates of K65E RT, and its low DNA polymerase activity at pH 7.5. Our pH studies indicate that the K65E RT displays moderate DNA polymerization activity at pH 6.5. At this pH it is possible that the deleterious interaction between Glu⁶⁵ and Arg⁷² is attenuated due to the protonation of Glu⁶⁵. This might then permit Arg⁷² to interact more favourably with the α -phosphate of the dNTP substrate, resulting in some restoration of RT polymerase activity.

dNTP-binding to DNA polymerases is mediated primarily through interactions of the enzyme with the phosphate component of the nucleotide [17,18]. Our observation that the K65E mutant RT $K_{m(dNTP)}$ values are decreased at pH 6.5, compared with those values calculated at pH 7.5, provides further evidence that at the lower pH value the residues in the $\beta 3$ – $\beta 4$ loop are more amenable to nucleotide-binding. Interestingly the $K_{m(dNTP)}$ values for the K65E RT at pH 6.5 are similar to those determined for the K65Q and K65A mutant enzymes at pH 7.5. Molecular modelling studies in which the glutamic acid residue in the K65E mutant enzyme is not assigned a charge yield a structure similar to those observed for the K65A and K65Q mutant enzymes (results not shown).

Lys⁶⁵ influences nucleotide-binding specificity of the enzyme

In general, specific mutations in RT seem to confer specificity in resistance, i.e. the K65R mutation provides cross-resistance to ddI and ddC, but does not confer resistance to either ddG or ddT. In contrast to the K65R mutant RT, the K65A, K65Q and K65E enzymes exhibit decreased affinity for all dNTPs and ddNTPs. This suggests that the residue at position 65 in HIV-1

RT influences the nucleotide-binding specificity of the enzyme. Interestingly the K65A [16] and K65R [24] RT mutant enzymes also display increased fidelity (ability to discriminate between the correct versus the incorrect nucleotide).

Recent crystallographic studies reveal that in the *Thermus aquaticus* DNA polymerase I, the phosphate backbones of the four nucleotides all interact slightly differently with the O-helix in the fingers domain of the enzyme [17]. Since the initial dNTP-binding is mediated primarily through interactions of the enzyme with the phosphate component of the nucleotide [17,18], it follows that mutations in one of the phosphate contacting residues would lead to a considerable change in the positioning of specific dNTP substrates for catalysis. Therefore other specific ddN mutations in the $\beta 3$ – $\beta 4$ loop of HIV-1 RT (e.g. T69D and L74V) may arise due to the different orientations that the respective ddNTPs adopt on their binding to the enzyme.

It should be noted that all experiments reported in the present work were carried out using homopolymeric T/Ps rather than heteropolymeric T/Ps such as those that are used during HIV replication. However, we feel that the T/P identity does not impact on our conclusions, since we have previously shown that the alterations in dNTP and ddNTP affinity, due to the K65R mutation, are similar whether homopolymeric or heteropolymeric T/Ps are used [9].

Mechanism of resistance of K65R RT to ddN

HIV-1 RT DNA polymerase activity follows an ordered sequential mechanism [19,20]. T/P binds to free RT to form the RT–T/P binary complex, which interacts with dNTP to form the RT–T/P–dNTP ternary complex. Upon binding of the complementary dNTP to form the ternary complex, RT undergoes a conformational change which positions the substrates to enable nucleophilic attack of the 3'-OH of the primer terminal nucleotide on the α -phosphate of the bound dNTP. Discrimination between dNTP and ddNTP can occur at two levels: initial dNTP-binding prior to the conformational change; and the catalytic incorporation step.

Rapid kinetic studies have indicated that RT is less efficient at catalysing the incorporation of bound ddNTP into the nascent DNA compared to the corresponding dNTP substrate [19,21]. This bias is a result of the bound ddNTP adopting an orientation in the dNTP-binding-pocket that is unfavourable for catalysis [22]. The mutation K65R in HIV-1 RT increases the K_m and decreases the k_{cat}/K_m for dCTP (the present study; [8]) and dATP [8]. This loss of affinity and catalytic efficiency of the mutant enzyme will inevitably increase the already existing bias for ddCTP and ddATP, resulting in low-level resistance of the mutant virus to these ddN inhibitors. The modest loss of affinity for the dNTP substrate could be compensated for by the increase in DNA synthesis processivity of the K65R mutant enzyme [23].

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REFERENCES

- 1 Parniak, M. A. and Sluis-Cremer, N. (1999) HIV: Molecular Mechanisms and Clinical Applications Advances in Pharmacology (Jeang, K.-T., ed.), in the press
- 2 Furman, P. A., Fyfe, J. A., St Clair, M. H., Weinhold, K., Rideout, J. L., Freeman, G. A., Lehrman, S. N., Bolognesi, D. P., Broder, S., Mitsuya, H. and Barry, D. W. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 8333–8337
- 3 Goody, R. S., Muller, B. and Restle, T. (1991) FEBS Lett. **291**, 1–5
- 4 De Clercq, E. (1994) Biochem. Pharmacol. **47**, 155–169

- 5 Tantillo, C., Ding, J., Jacobo-Molina, A., Nanni, R. G., Boyer, P. L., Hughes, S. H., Pauwels, R., Andries, K., Janssen, P. A. J. and Arnold, E. (1994) *J. Mol. Biol.* **243**, 369–387
- 6 Boyer, P. L., Ferris, A. L. and Hughes, S. H. (1992) *J. Virol.* **66**, 1031–1039
- 7 Huang, H., Chopra, R., Verdine, G. L. and Harrison, S. C. (1998) *Science* **282**, 1669–1675
- 8 Gu, Z., Gao, Q., Fang, H., Salomon, H., Parniak, M. A., Goldberg, E., Cameron, J. and Wainberg, M. A. (1994) *Antimicrob. Agents Chemother.* **38**, 275–281
- 9 Gu, Z., Fletcher, R. S., Arts, E. J., Wainberg, M. A. and Parniak, M. A. (1994) *J. Biol. Chem.* **269**, 28118–28122
- 10 Gu, Z., Arts, E. J., Parniak, M. A. and Wainberg, M. A. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 2760–2764
- 11 Arts, E. J., Li, X., Gu, Z., Kleiman, L., Parniak, M. A. and Wainberg, M. A. (1994) *J. Biol. Chem.* **269**, 14672–14680
- 12 Taylor, J. W., Ott, J. and Eckstein, F. (1985) *Nucleic Acids Res.* **13**, 8764–8785
- 13 Sarafianos, S. G., Pandey, V. N., Kaushik, N. and Modak, M. J. (1995) *J. Biol. Chem.* **270**, 19729–19735
- 14 Astatke, M., Grindley, N. D. F. and Joyce, C. M. (1995) *J. Biol. Chem.* **270**, 1945–1954
- 15 Polesky, A. H., Dahlberg, M. E., Benkovic, S. J., Grindley, N. D. F. and Joyce, C. M. (1992) *J. Biol. Chem.* **267**, 8417–8428
- 16 Harris, D., Kaushik, N., Pandey, P. K., Yaddav, P. N. S. and Pandey, V. N. (1998) *J. Biol. Chem.* **273**, 33624–33634
- 17 Li, Y., Kong, Y., Korolev, S. and Waksman, G. (1998) *Protein Sci.* **7**, 1116–1123
- 18 Li, Y., Korolev, S. and Waksman, G. (1998) *EMBO J.* **17**, 7514–7525
- 19 Reardon, J. E. (1992) *Biochemistry* **31**, 4473–4479
- 20 Reardon, J. E. (1993) *J. Biol. Chem.* **268**, 8743–8751
- 21 Krebs, R. A., Immendorfer, U., Thrall, S. H., Wohrl, B. M. and Goody, R. S. (1997) *Biochemistry* **36**, 10292–10300
- 22 Astatke, M., Grindley, N. D. F. and Joyce, C. M. (1998) *J. Mol. Biol.* **278**, 147–165
- 23 Arion, D., Borkow, G., Gu, Z., Wainberg, M. A. and Parniak, M. A. (1996) *J. Biol. Chem.* **271**, 19860–19864
- 24 Shah, F., Curr, K., Parniak, M. A. and Prasad, V. R. (1999) in *Retroviruses* (Bates, P. and Malin, M., eds.), pp. 22, Cold Spring Harbor Laboratories, New York

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