Structural Basis for the Role of the K65R Mutation in HIV-1 Reverse Transcriptase Polymerization, Excision Antagonism, and Tenofovir Resistance

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K65R is a primary reverse transcriptase (RT) mutation selected in human immunodeficiency virus type 1-infected patients taking antiretroviral regimens containing tenofovir disoproxil fumarate or other nucleoside analog RT drugs. We determined the crystal structures of K65R mutant RT cross-linked to double-stranded DNA and in complexes with tenofovir diphosphate (TFV-DP) or dATP. The crystals permit substitution of TFV-DP with dATP at the dNTP-binding site. The guanidinium planes of the arginines K65R and Arg72 were stacked to form a molecular platform that restricts the conformational adaptability of both of the residues, which explains the negative effects of the K65R mutation on nucleotide incorporation and on excision. Furthermore, the guanidinium planes of K65R and Arg72 were stacked in two different rotameric conformations in TFV-DP- and dATP-bound structures that may help explain how K65R RT discriminates the drug from substrates. These K65R-mediated effects on RT structure and function help us to visualize the complex interaction with other key nucleotide RT drug resistance mutations, such as M184V, L74V, and thymidine analog resistance mutations.

During HIV-1 replication, the enzyme reverse transcriptase (RT) converts the single-stranded viral genomic RNA into double-stranded DNA (dsDNA). Because of its essential role in the viral life cycle, RT is an important target for antiviral agents. Both nucleoside and nucleotide RT inhibitors (NRTIs) and non-nucleoside RT inhibitors are widely used as components of antiretroviral therapy for HIV-1 infection. The approved NRTIs are nucleoside or nucleotide analogs that are phosphorylated to their triphosphate or diphosphate active metabolites in cells (Fig. 1). NRTIs, once incorporated into the viral DNA, act as DNA chain terminators due to the lack of a 3′-OH group. RT with resistance mutations must maintain an adequate rate of nucleotide incorporation while recognizing dNTPs and enhancing discrimination against NRTIs or increasing the excision of incorporated NRTIs.

In HIV-1 RT, NRTI resistance mutations have a broad spatial distribution in and adjacent to the nucleotide substrate binding region (1). Different NRTI resistance mutations or sets of mutations are selected in response to treatment with different NRTIs. K65R emerges in response to treatment with tenofovir (TFV) disoproxil fumarate, abacavir, didanosine (ddI), or stavudine (2–4) and has recently been shown to have increased frequency in subtype C HIV-1 (5). Mutations M41L, D67N, K70R, L210W, T215F/Y, and K219Q/E/N (6), which are primary resistance mutations for AZT and stavudine, are called thymidine analog mutations (TAMs), AZT, or excision-enhancing mutations. TAMs cause cross-resistance to NRTIs by enhancing ATP-mediated excision (7–9) that we visualized in our recently determined crystal structures of RT excision product complexes. M184V/I is the primary mutation that causes resistance to lamivudine (3TC) and emtricitabine (10, 11). The Thr69 insertion occurs in combination with TAMs and allow RT to excise a broader range of NRTIs (12–15). Q151M causes NRTI multidrug resistance that is often accompanied by several secondary mutations and termed the Q151M complex. There are complex interactions between these mutations that have a broad range of effects on NRTI resistance that drive their relative frequencies of occurring together. Among the most frequent in treatment-experienced populations are M184V and

NRTI, nucleotide and nucleoside RT inhibitor; HIV-1, human immunodeficiency virus, type 1; TFV, tenofovir; ddI, didanosine; TAM, thymidine analog mutation; DP, diphosphate; TP, triphosphate; dATP, dideoxy-ATP; AZTpppA, AZT adenosine dinucleoside tetraphosphate; dATP-αS, 2′-deoxyadenosine-5′-O-(1-thiotriphosphate).

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substrate, and interact with other NRTI resistance mutations.

The most relevant crystal structures available for analyzing K65R are of wild-type HIV-1 RT-DsDNA complexes with dTTP (28) or TFV-DP (29). These structures provide the basic framework for understanding DNA polymerization and binding of TFV-DP to RT, but they do not explain the effects of the K65R mutation on polymerization, excision, or TFV resistance. Several modeling studies have proposed that the K65R mutation alters the positioning and binding of the NRTI-TP or surrounding amino acid residues or that the conformational mobility of the K65R-containing fingers loop is reduced (21, 24, 30–32), however, there is no universal agreement on the structural basis for the broad effects of the K65R mutation. Here we report the crystal structures of ternary complexes of K65R mutant RT-DsDNA with TFV-DP or the natural substrate dATP. The K65R crystal structures presented here show how this mutation restricts the structural adaptability of the enzyme by forming a molecular platform involving the conserved residue Arg72; the platform may discriminate against the incorporation of NRTIs, interfere with the binding of ATP as an excision substrate, and interact with other NRTI resistance mutations.

**EXPERIMENTAL PROCEDURES**

**Expression, Purification, and Crystallization of RT—**K65R HIV-1 RT was expressed in *Escherichia coli* and purified, as reported earlier (29). The K65R mutation is present on the p66 subunit only, which also contains a mutation Q258C for cross-linking with the nucleic acid. The p51 subunit is truncated at the C terminus residue 428, followed by Gly-Gly-His-7. The mutation C280S is present in both subunits. A 27-mer template DNA (5'-ATG GTC GGC GCC CGA ACA GGG ACT GTG-3') was custom synthesized by Integrated DNA Technologies (Coralville, IA). The 20-mer primer DNA (5'-ACA GTC CCT GTT CGG CGC CC-3') bearing a cross-linkable thioalkyl tether (on G in the sequence) was synthesized and annealed to the template. The 27-20-mer dsDNA was cross-linked to K65R RT at the p66 C258 site, the cross-linked primer was extended with a dideoxy-G at the 3'-end through RT polymerization (33), the cross-linked K65R RT-DsDNA complex was purified using Ni²⁺-nitrilotriacetic acid and heparin columns in tandem, and the purified complex was concentrated to ∼10 mg/ml. The K65R RT-DsDNA sample was co-crystallized with TFV-DP by hanging drop vapor diffusion against the well solution containing 50 mM Bistris propane, pH 6.4, 100 mM ammonium sulfate, 5% (v/v) glycerol, 5% (w/v) sucrose, 10% (w/v) polyethylene glycol 8000, and 20 mM MgCl₂ (or MnCl₂). The crystallization drops, containing 2 μl of RT-DsDNA-TFV-DP added to 2 μl of the well solution, produced crystals in 2–3 weeks at 4 °C. Crystals of K65R RT-DsDNA-TFV-DP were obtained by soaking the crystals of K65R RT-DsDNA-TFV-DP in the well solution containing 10 mM dATP for 10 min. The substitution of TFV-DP by dATP in the crystal by soaking was confirmed using a fluorescent dATP analog (supplemental Fig. 1).

**Crystallography—**Crystals of K65R RT-DsDNA-TFV-DP complex were stabilized in crystallization buffer containing 12% polyethylene glycol 8000 and then cryoprotected by dipping in the respective transfer solution containing 25% glycerol for ∼5 s and cryocooled in liquid N₂. The x-ray diffraction data sets were collected at Cornell High Energy Synchrotron Source beam line F1. Three different data sets, each to 3 Å resolution, were collected from three crystals of K65R RT-DsDNA-TFV-DP; two crystals grew in the presence of MgCl₂, and one grew in the presence of MnCl₂. The structure was solved by molecular replacement using the protein atoms from the wild-type HIV-1 RT-DsDNA-TFV-DP structure (29) as the starting model. The model was refined using the three individual data sets that were non-isomorphous. The crystallographic phases and figures of merit from individually refined structures were input to DMMULTI in CCP4 (34) for multiple crystal form averaging.

**Chemical structures of dATP, TFV-DP, AZT-TP, and 3TC-TP.**

**FIGURE 1.** Chemical structures of dATP, TFV-DP, AZT-TP, and 3TC-TP.
one metal at the polymerase active site in the current structures was confirmed by the electron density map calculated from the crystal that contained MnCl₂ during crystallization (supplementary Fig. 3).

The K65R RT-dsDNA-dATP complex was obtained by soaking the crystals of K65R RT-dsDNA-TFV-DP in crystallization solution containing 10 mM dATP for 10 min. The soaked crystals were stabilized at 12% polyethylene glycol 8000 and cryoprotected following the procedure described for TFV-DP-containing crystals. The 10 mM concentration of dATP was maintained in both the stabilization and cryoprotectant solutions. The dATP-soaked crystals were very sensitive to x-ray exposure; therefore, diffraction data from three crystals were merged to obtain an almost complete data set (Table 1) with high redundancy. High redundancy (~13.5) of data resulted in a relatively high \( R_{\text{merge}} \) value; however, the merged overall data set produced better quality electron density maps compared with the maps from less complete individual data sets. The K65R RT-dsDNA model from the K65R RT-dsDNA-TFV-DP structure was used to determine the structure of the K65R RT-dsDNA-dATP complex. Programs CNS 1.2 (35) and COOT (36) were used for refining and model building of both of the structures. The diffraction data and refinement statistics are listed in Table 1. The coordinates and structure factors for the crystal structures of K65R mutant RT-dsDNA-TFV-DP and K65R mutant RT-dsDNA-dATP complexes are available from the Protein Data Bank with accession codes 3JSM and 3JYT, respectively.

Pre-steady State Kinetics of Single Nucleotide Incorporation—For the biochemical studies, wild-type and K65R mutant heterodimeric p66/p51 HIV-1 RT were expressed and purified as described (27). The DNA 19-mer primer (5'-GTCCCTGTT-CGGGCGCCAC) and 36-mer template D36A (5'-TCTCTATGT-GTGGCGCCCGAACAGGGACC-TGAAAGC) were used in the study. Pre-steady state constants (\( k_{\text{pol}} \)) were determined using dATP (Roche Applied Science) or dATP-S (Sp isomer) (Biolog, Bremen, Germany) at 5 times the \( K_{d} \) value (200 \( \mu \)M for wild-type RT and 75 \( \mu \)M for K65R RT) and 100 nM active wild-type or K65R RT with a KinTek rapid quench-flow apparatus as described (37).

RESULTS

Ternary Complex of K65R Mutant RT-dsDNA-TFV-DP—The crystal structure of K65R RT-27:21-mer dsDNA-TFV-DP (supplemental Fig. 2) was determined at 3.0 Å resolution and refined to \( R_{\text{work}} \) and \( R_{\text{free}} \) of 0.252 and 0.285, respectively, using 49,494 reflections (Table 1). Multiple crystal form averaging
Structures of K65R RT

using three independent data sets yielded improved and unbiased electron density maps that helped to enhance the quality of this structure. The ternary complex of K65R RT crystallized in a crystal form similar to that of wild-type RT·dsDNA-TFV-DP (29). Comparison of the wild type and K65R mutant structures shows similar RT conformation, mode of dsDNA-binding, and crystal packing; the root mean square deviation for all Cα atoms is ∼0.5 Å when both structures are overlaid. The position and conformation of TFV-DP in the new structure could be determined by well defined electron density in difference maps calculated prior to the inclusion of the TFV-DP molecule in the refinement (Fig. 2A). The TFV-DP molecule in the structure unambiguously fits the electron density in a low energy conformation, base-pairs with the template and primer, and coordinates a catalytic Mg2⁺ ion via three phosphate oxygen atoms. As observed in the wild-type RT·dsDNA-TFV-DP crystal structure, only one Mg2⁺ ion (metal-B) is present in the polymerase active site that coordinates with the catalytic aspartates Asp110 and Asp185, the main-chain carbonyl of Val111, and one oxygen atom from each phosphate group of TFV-DP. The presence of only one metal ion at the active site of the current structure was confirmed by determining the crystal structure of K65R RT·dsDNA-TFV-DP with Mn2⁺ ions (supplementary Fig. 3) replacing Mg2⁺ ions, which is in agreement with our earlier analysis (38) indicating that the binding of the second Mg2⁺ ion at the polymerase active site requires coordination with Asp185. In the current structures, the side chain of D186 has turned away from the putative metal-A binding site.

Amino acid residues K65R, Arg72, and Tyr115, which interact with TFV-DP, are clearly defined in the electron density map (Fig. 2), and key interactions of RT with the bound nucleotide substrate are maintained. The mutated residue K65R has an orientation similar to Lys65 in the wild-type RT·dsDNA-TFV-DP (29) and wild-type RT·dsDNA·dTTP structures (28) where one of the guanidinium Nη nitrogens interacts with the γ-phosphate oxygen (N···O distance of 2.6 Å). The other guanidinium Nη nitrogen of K65R interacts with the oxygen atom linking the γ- and ϕ-phosphates of TFV-DP, which mimics the interaction of K65 with TFV-DP in the wild-type RT·dsDNA-TFV-DP structure (29).

The major difference between wild-type and the K65R RT structures is at the β3-β4 fingers loop. The Ca position of Arg65 on the flexible β3-β4 loop is displaced (“pushed back”) by about 0.5 Å compared with the Ca position of Lys65 in the dTTP-bound wild-type RT structure for accommodating 1-bond length longer arginine when substituted for lysine (supplementary Fig. 3). The rearrangement of the β3-β4 loop accommodates a sulfate ion that interacts with the side chain of Arg72 and the main chain amide group of Lys66. The guanidinium plane of K65R is stacked with the guanidinium plane of Arg72 with an approximate distance of 3.0 Å between the two guanidinium planes. The Arg72 guanidinium plane is also stacked on its other side with the adenine base of TFV-DP. Arg72 is a highly conserved amino acid residue that is critical for RT function. The side chain of Arg72 interacts with all three components (base, sugar, and α-phosphate) of an incoming dNTP (Fig. 2) and is expected to stabilize the transition state of the nucleophilic attack by the 3’-end of the primer on the α-phosphate of the dNTP. A comparison of the crystal structures of the RT ternary complexes suggests that the stacking of the guanidinium groups of K65R and the conserved Arg72 imposes a constraint on adaptability of both of the amino acid residues. To obtain a better understanding of how the K65R mutation helps RT discriminate between TFV-DP and dATP, we also determined the crystal structure of the K65R mutant RT·dsDNA·dATP complex.

Ternary Complex of K65R Mutant RT·dsDNA·dTTP—Our attempt to co-crystallize dATP with the 27:21-mer dsDNA cross-linked K65R mutant RT produced crystals that had a shape similar to the rodlike crystals of K65R RT·dsDNA-

TABLE 1

| Protein Data Bank code | K65R mutant RT·dsDNA·TFV-DP | K65R mutant RT·dsDNA·dATP |
|------------------------|-----------------------------|---------------------------|
| Protein Data Bank code | 3JSM                        | 3JYT                      |
| X-ray source           | CHESS F1                    | CHESS F1                  |
| No. of crystals used   | 1                           | 3                         |
| Wavelength (Å)         | 0.979                       | 0.9179                    |
| Space group            | P3,12                       | P3,12                     |
| Cell constants (a, b, c in Å; α, β, γ in degrees) | 170.31, 170.31, 155.44; 90, 90, 120 | 169.72, 169.72, 155.44; 90, 90, 120 |
| Resolution range (Å)   | 50.0–3.0                    | 50.0–3.3                  |
| No. of unique reflections (no. of observations) | 49,494 (164,942) | 37,809 (510,405) |
| Completeness (%)        | 95.9                        | 98.6                      |
| Rmerge (in last shell)  | 0.156 (0.563)               | 0.190 (0.724)             |
| Rmerge (averaged)       | 0.184 (0.708)               | 0.177 (0.611)             |
| Average IM (Å)          | 5.6                         | 8.6                       |
| σ cut-off               | −1.0                        | −1.0                      |

Reefinement statistics

| Total no. of atoms (DNA/inhibitor or substrate atoms) | K65R mutant RT·dsDNA·TFV-DP | K65R mutant RT·dsDNA·dATP |
|------------------------------------------------------|-----------------------------|---------------------------|
| Resolution (Å)                                       | 8,855 (901/27)              | 8,853 (901/30)            |
| No. of reflections (Rmerge set)                      | 49,494 (1,530)              | 37,789 (1,140)            |
| Completeness (%)                                     | 95.8                        | 98.5                      |
| Root mean square deviations                         | 0.009                       | 0.008                     |
| Bond lengths (Å)                                     | 1.69                        | 1.46                      |

Refinement statistics

| Refinement statistics | K65R mutant RT·dsDNA·TFV-DP | K65R mutant RT·dsDNA·dATP |
|-----------------------|-----------------------------|---------------------------|
| Bond angles (degrees) | 0.009                       | 0.008                     |
| Root mean square deviations | 1.69                        | 1.46                      |
TFV-DP complex; however, the crystals of the dATP complex did not grow to a size suitable for diffraction studies. Therefore, the feasibility of exchanging the incoming nucleotides was evaluated. Crystals of the RT·dsDNA·TFV-DP complex were soaked with 5 mM 2AP-TP for 5 min, and fluorescence of 2-aminopurine was measured. The resulting fluorescence demonstrated that 2AP-TP had replaced TFV-DP (supplemental Fig. 1). This approach was used to obtain the structure of the RT·dsDNA·dATP complex by soaking the natural substrate dATP into the crystals of RT·dsDNA·TFV-DP complex. The soaked crystals diffracted X-rays to 3.3 Å compared with the 3.0 Å resolution diffraction from the parent RT·dsDNA·TFV-DP crystals. The dATP-soaked crystals were relatively sensitive to radiation damage, and diffraction data from three crystals were merged to obtain the final data set. The structure solution revealed clear electron density for the dATP molecule and the interacting amino acid residues (Fig. 2). A total of 37,789 reflections (Table 1).

The overall structure of K65R RT·dsDNA·dATP complex is similar to that of K65R RT·dsDNA·TFV-DP complex. The adenine bases and the triphosphates of TFV-DP and dATP superimpose when the two ternary complex structures of K65R mutant RT are compared (Fig. 3A). TFV-DP has an acyclic phosphonomethoxypropyl group substituted for the ribose α-phosphate moiety of dATP (Fig. 1); this difference can be seen in the superimposed structures. In the dATP-bound structure, the ribose ring is stacked against the aromatic ring of Tyr115. The 3′-OH of dATP forms a hydrogen bond network with its β-phosphate oxygen (3.1 Å) and the main-chain amino group of Tyr115 (distance 3.1 Å). Because TFV-DP lacks a 3′-OH group, the interactions that involve the 3′-OH of dATP are not present, and the methyl group of TFV-DP stacks with Tyr115 in a way that is analogous to but less extensive than the stacking of the ribose ring of dATP and Tyr115. Superposition of the two structures shows that the oxygen atom in the phosphonomethoxypropyl group of TFV-DP is positioned ~1.7 Å away from the ribose C4′ atom of dATP (Fig. 3A), which reorients the side-chain rotameric conformation of Arg72. In the TFV-DP·bound K65R RT structure, Arg72 has a rotameric conformation (rotamer-2) that is analogous to the orientation of Arg72 in the wild-type RT·dsDNA·TFV-DP structure (29) but distinct from a common R72 rotamer (rotamer-1) in dATP·, dTTP· (28), and AZTppppA·bound RT·dsDNA structures; the two distinct rotameric conformations of Arg72 differ by ~1.6 Å at the position of Ne (Fig. 3); the distinct rotameric conformations of Arg72 in TFV-DP versus dATP-bound RT ternary complexes were further confirmed by our recent high resolution structures of RT·RNA·DNA·TFV-DP (or dATP) complexes determined at 2.57 (or 2.6) Å. The rotamer-2 conformation of Arg72 allows both Nη atoms to have polar interactions with TFV-DP, whereas only one Nη atom of Arg72 (in the rotamer-1 conformation) can have such interactions with dATP. This difference in these interactions might partly compensate for the complete loss of the 3′-OH hydrogen-bonding network and partial loss of stacking with Tyr115 for TFV-DP compared with dATP. The side chain of K65R also has two rotameric conformations, depending on whether dATP or TFV-DP is bound (Fig. 3, B and C). These different rotamers facilitate better stacking of the guanidinium planes of K65R and Arg72 in the two structures.

Incorporation Kinetics of dNTP and NRTIs—Biochemical studies showed that the K65R mutation can cause drug resistance to NRTIs and reduce both the nucleotide incorporation and excision by RT. Three independent pre-steady state analyses of K65R incorporation of dATP versus the NRTIs TFV-DP and ddATP (the active metabolite of ddI) are summarized in Table 2 (23, 25, 39). In all studies, the K65R mutant shows a modest decrease in the incorporation rate (kpol) for dATP (4.5-fold) without a significant change in the binding affinity (Kd). K65R RT is resistant to TFV-DP and ddATP because the mutation dramatically decreases the incorporation of these analogs (≥20-fold).

The kinetic steps of dNTP incorporation (kpol) for HIV-1 RT consist of the conformational step of the closing down of the fingers loop (β3·β4) that contains residue 65 that leads to the

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positioning of dNTP for nucleophilic attack and the chemical step of phosphodiester bond formation (40–43). Evidence for the conformational change of the fingers loop as the rate-limiting step during polymerization versus the chemistry step has been generated by studying the incorporation of modified dNTPs that contain a phosphorothioate group at the $\alpha$-phosphate (dNTP-$\alpha$-S). The difference in the rate of incorporation of a natural dNTP and the dNTP-$\alpha$-S is termed the “elemental effect” (44). The rate of the chemical step is estimated to be decreased 30–100-fold by the presence of the thio group; thus, an elemental effect close to 1 suggests that the incorporation rate ($k_{pol}$) is determined primarily by a rate-limiting step other than the chemistry step. The elemental effect for wild-type and K65R RT was investigated (Table 3). K65R showed a comparably slower $k_{pol}$ for incorporation of dATP and dATP-$\alpha$-S by 4.2- and 4.9-fold, respectively. Thus, the elemental effect observed for wild-type and K65R mutant HIV-1 RT was 1.0 and 1.1, respectively (Table 3), supporting the hypothesis that the K65R mutation slows the rate-limiting conformational step for nucleotide incorporation, probably by conformational restriction by the K65R/Arg$^{72}$ molecular platform rather than the chemical step of incorporation.

**DISCUSSION**

**Significance of the K65R Mutation for RT Function**—Biochemical and clinical data have shown that K65R has broad phenotypic effects on NRTIs. Clinically, the K65R resistance mutation develops after treatment with abacavir, ddI, stavudine, and TFV disoproxil fumarate and causes reduced susceptibility to all approved NRTIs with the exception of AZT, where full susceptibility is retained (20). Biochemically, K65R RT decreases the rate of incorporation ($k_{pol}$) of all natural substrates and approved NRTI drugs; the incorporation kinetics of the adenosine analogs dATP, TFV-DP, and ddATP (the active metabolite of ddI) determined by several groups are shown in Table 2. An $\alpha$-boranophosphate-modified ddATP, however, incorporates slightly more efficiently than dATP for K65R RT (45). Interestingly, any other substitution at position 65 also significantly decreases both the binding and the incorporation of dNTPs and catalytic efficiency of polymerization (31, 46). The rate of reverse reaction of pyrophosphorolysis and ATP-mediated excision is also decreased. This is most notable for incorporated AZT, where the decreased incorporation of AZT-TP is counteracted by significantly decreased excision after its incorporation that results in full susceptibility of K65R viruses to this NRTI (14, 26, 27).

Previous crystal structures of wild-type HIV-1 RT ternary complexes show interaction between the $\text{N}3\text{r}$ atom of Lys$^{65}$ and the $\gamma$-phosphate of the dNTP. In the current K65R structures, one of the guanidinium nitrogens of Arg$^{65}$ functionally replaces the $\text{N}3\text{r}$ atom of Lys$^{65}$ by interacting with a $\gamma$-phosphate/$\beta$-$\gamma$-linker oxygen of dNTP (or TFV-DP) (i.e. in general, the mutation does not alter the hydrogen-bonding interactions of the bound dNTP (or an analog)). In fact, the bound dATP has only one polar interaction with Arg$^{65}$, and the interaction is analogous to the interaction between dTTP and Lys$^{65}$ (28). The K65R substitution adds a bulky guanidinium group, which can form additional hydrogen bonds with restricted geometry (47) and can participate in hydrophobic stacking. The guanidinium groups of Arg$^{65}$ and Arg$^{72}$ are stacked in both K65R mutant structures (Fig. 3, B and C); the Arg$^{72}$ guanidinium plane is also stacked with the adenine base of dATP (or TFV-DP). Two guanidinium nitrogens of Arg$^{72}$ form hydrogen bonds with the $\alpha$-phosphate (or phosphonate) of the dNTP (or TFV-DP) and the side chain of Gln$^{151}$. Residue Arg$^{72}$ would be required to adapt to subtle changes in dNTP conformation in the steps of binding, incorporation, or excision. As is evident from the current structures, the K65R mutation restricts the adaptability of both the Arg$^{65}$ and Arg$^{72}$ side chains via the stacking of guanidinium planes. In fact, Arg$^{65}$ and Arg$^{72}$ stack to form a stable platform that interacts with all three parts (base, deoxyribose/acrylic linker, and $\alpha$-phosphates/phosphonate) of dATP or TFV-DP (Fig. 3), which might explain the reduced rate of nucleotide incorporation by K65R mutant RT compared with wild-type RT (Table 2). The structural constraint by the K65R/Arg$^{72}$ platform may also act like a “checkpoint” that helps discriminate among dNTPs for correct base pairing with the template, which thereby provides a possible explanation for the reported increase in fidelity of the K65R mutant (30). The lack of a significant elemental effect suggests that the mutation primarily affects the conformational change rather than the phosphodiester bond formation (Table 3). These kinetic data correlate with the structural information indicating that the K65R/Arg$^{72}$ platform would restrain the movements of these residues and hinder the efficiency of nucleotide incorporation/excision. The decreased rates of the incorporation of dNTPs appear to account for decreased viral replication capacity of the K65R mutant (30).

**K65R Mutation and Resistance to TFV**—The major chemical difference between TFV-DP and dATP is the substitution of an
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FIGURE 4. Three distinct mechanisms of NRTI resistance through mutations at three distinct sites. Superposition of excision-enhancing mutation or TAM (M41L, D67N, K70R, T215Y, and K219Q) RT-dsDNA-AZTppppA structure4 on K65R RT-dsDNA-dATP structure at their dNTP-binding sites; AZTppppA is the product of AZT monophosphate by ATP-mediated excision. Although the two structures contained two distinct sites of mutations and crystallized in two distinct space groups, they superimpose very well at the active site region. The K65R mutation has a more significant effect on incorporation of ddATP than on the incorporation of dATP (Table 2), although the only difference between the two is that ddATP lacks the 3' OH. As seen in the dATP-bound K65R RT structure (Fig. 2B), the 3' OH helps define the relative positioning of the deoxyribose and the phosphates through its interaction with the β-phosphate oxygen and the main-chain amino group of Tyr115. The loss of the interactions with the 3' OH might also account for the observation that ddATP is incorporated more slowly by wild-type RT than is dATP. The absence of this interaction network, added to the constraint imposed by the K65R/Arg72 platform, may prevent ddATP from attaining the positioning of the phosphates and the deoxyribose ring required for optimal incorporation.

Implications for K65R Interactions with Other Resistance Mutations—K65R is surrounded by other residues associated with NRTI resistance and is commonly associated with M184V or Q151M but is negatively associated with others, such as L74V or most TAMs (20). The K65R/M184V double mutant has increased resistance to the NRTIs ddI, abacavir, 3TC, and emtricitabine but improved susceptibility to TFV, stavudine, and AZT compared with K65R alone (23, 48). The mutations
K65R and M184V impose structural restraints on the two sides of the deoxyribose binding site (supplemental Fig. 4) to cause reduced incorporation efficiency of natural nucleotides (23). The M184V-mediated positional constraints and narrowing at the polymerase active site are consistent with the observed increase in $K_\text{d}$ for dNTP binding (49). The incorporation of TFV-DP, which has a flexible acyclic oxypropyl linker substituted for the ribose ring, is minimally affected by the addition of the $\beta$-branched M184V mutation and K65R-induced platform (Fig. 4 and supplemental Fig. 4), whereas the natural substrate dATP is more affected by the smaller and less adaptable binding pocket.

Mutations that are negatively associated with K65R show unfavorable complementarity with the K65R/Arg$^{72}$ platform. L74V is negatively associated with K65R (20, 50) and is positioned underneath the template base that complements the base of the incoming dNTP (supplemental Fig. 5). Leu$^{74}$ is surrounded by the side chains of Phe$^{61}$, Ile$^{63}$, Arg$^{72}$, and Gln$^{151}$, which provide it a compact hydrophobic environment to support the template base that is analogous to the Arg$^{72}$ support of the base of the incoming dNTP. Both residues appear critical for maintaining the base pairing between the dNTP and the template. Our modeling (supplemental Fig. 5) suggests that the constraint on Arg$^{72}$ caused by the K65R mutation (via the K65R/Arg$^{72}$ platform) and the alteration of the adjacent molecular surface by the L74V mutation removes the support for the template base proximal to where it is base-paired with the dNTP (supplemental Fig. 5), causing drastically reduced substrate incorporation kinetics (21).

TAMs (M41L, D67N, K70R, T215Y/F, and K219Q/E/N) cause enhanced NRTI excision, a major mechanism of resistance where the excision of incorporated nucleotides is mediated by ATP. Our recent crystal structure of a TAM RT bound to primer-template and AZTppppA (the ATP-mediated excision product of AZT monophosphate; Fig. 4) has shown that T215Y significantly contributes to the binding of ATP to the mutant RT for excision by stacking with its adenine base and T215Y significantly contributes to the binding of ATP to the precatalytic complexes with RT; rather, the side chain of K65R has an enhanced interaction with the side chain of Arg$^{72}$ to form a molecular platform that restricts adaptability of the polymerase active site and causes both a decreased rate of substrate incorporation and NRTI excision (23, 25). The effect of K65R appears to be on the rate-limiting conformational step of nucleotide incorporation; this idea is supported by the lack of an elemental effect that would also affect the excision reaction. An analogous platform with alternate rotameric conformations for both Arg$^{72}$ and Arg$^{65}$ is created upon binding of TFV-DP that enhances the ability of RT to discriminate between TFV-DP and dATP.

**CONCLUSIONS**

Discrimination between a nucleotide analog and a natural nucleotide can occur at the steps of binding to RT, catalytic reaction of polymerization, and/or enhanced excision. Different mutations (or sets of mutations) generate resistance to NRTIs through distinct mechanisms. Biochemical and structural data have elucidated two distinct mechanisms of NRTI resistance of 1) discrimination due to steric hindrance by M184V/I mutation to 3TC-TP and emtricitabine-TP (49, 54) and 2) the ATP-mediated excision of incorporated NRTIs exemplified by the TAMs (7–9), which is the primary mechanism of resistance to AZT. Our current structures demonstrate a third “conformational restriction” mechanism of NRTI resistance that cross-talks with the previous two NRTI resistance mechanisms. The K65R mutation neither enhances nor reduces the interaction of dATP or TFV-DP in the precatalytic complexes with RT; rather, the side chain of K65R has an enhanced interaction with the side chain of Arg$^{72}$ to form a molecular platform that restricts adaptability of the polymerase active site and causes both a decreased rate of substrate incorporation and NRTI excision (23, 25). The effect of K65R appears to be on the rate-limiting conformational step of nucleotide incorporation; this idea is supported by the lack of an elemental effect that would also affect the excision reaction. An analogous platform with alternate rotameric conformations for both Arg$^{72}$ and Arg$^{65}$ is created upon binding of TFV-DP that enhances the ability of RT to discriminate between TFV-DP and dATP.

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