Role of Glutamine-151 of Human Immunodeficiency Virus Type-1 Reverse Transcriptase in RNA-Directed DNA Synthesis†

Neerja Kaushik, Dylan Harris, Nisha Rege, Mukund J. Modak, Prem N. S. Yadav, and Virendra N. Pandey*

Department of Biochemistry and Molecular Biology, UMD−New Jersey Medical School, Newark, New Jersey 07103

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ABSTRACT: Glutamine-151 of HIV-1 RT has been shown to be a catalytically important residue through the characterization of its mutant phenotype Glu151Ala (Sarafianos et al., 1995a). To further understand the role of this residue, we have extended this analysis to include polymerization on natural RNA template in addition to DNA template. We find that Q151A mutant exhibited a severe reduction in the polymerase activity without any significant effect on the affinity for dNTP substrate. Unlike DNA-directed reactions, the rate-limiting step for RNA-directed reactions does not appear to be either at the dNTP binding step or the chemical step. Analysis of the products formed on natural heteromeric HIV-genomic RNA template annealed with an 18-mer DNA primer with a sequence complementary to the primer binding site (PBS) has shown that addition of nucleotides is nonlinear with time since the enzyme appears to stall on the RNA template following the incorporation of the first nucleotide. The Q151A mutant was found to be nearly devoid of pyrophosphorolytic activity on a RNA−PBS template-primer. Similar properties have been previously reported for a mutant of R72 (R72A) of HIV-1 RT (Sarafianos et al., 1995b). However, R72 was implicated in stabilizing the transition state ternary complex before and after the phosphodiester bond formation (Kaushik et al., 1996; Sarafianos et al., 1995b). Our results with Q151A suggest that the side chain of Q151 may help stabilize the side chain of R72, and the loss of pyrophosphorolysis activity observed with the Q151 mutant may be the indirect manifestation of this stabilizing effect on R72. These observations point to the functional interdependence of residues Q151 and R72 in the polymerase function of the enzyme. An analysis of the 3D model structure of HIV-1 RT bound to DNA−DNA and RNA−DNA template-primer reveals that the guanidine hydrogen of R72 seems to stabilize Q151 by hydrogen bonding with its amide oxygen. A systematic conformational search of the side chain of Q151 also suggests a stable orientation where its specific interaction with the base of the RNA template may aid in stabilizing it.

Retroviruses containing a single-stranded RNA genome have been of long-standing biological and pharmacological interest. They propagate in the host cells without interrupting the growth of the host and are transmitted as a provirus from one cell generation to the next. They cause a variety of diseases including cancer (leukemia, sarcoma), anemia, arthritis, and immunodeficiency states. Among these, the retrovirus causing immunodeficiency in humans (HIV retrovirus) has been the target of massive crusade in modern biology. A unique feature of their replication is the reverse transcription of their single-stranded RNA genome and its integration as duplex DNA into the host chromosome (Gilboa et al., 1979). Reverse transcription is carried out at an early stage after infection by the virion-encapsidated reverse transcriptase. The enzyme is multifunctional, exhibiting both RNA- and DNA-dependent polymerase activities as well as an RNase-H activity that is both exo- and endonucleolytic (Collett et al., 1978; Gilboa et al., 1979; Smith et al., 1984; Varmus & Swanstrom, 1985). These features, combined with the apparent lack of reverse transcription in the normal metabolism of eukaryotic cells, have made the reverse transcriptase an attractive target in the search for chemotherapeutic agents to combat the spread of retroviral infections. A computer-assisted comparison of the amino acid sequences of the various reverse transcriptases and also the sequences of other enzymes led to the proposal for a specific functional organization of the viral enzymes (Johnson et al., 1986; Delarue et al., 1990). Positions of similarities among various polymerases suggested that the N-terminal part of the reverse transcriptase should contain the DNA polymerase activity whereas the C-terminal part should contain the RNase-H activity. These domain assignments were confirmed upon the availability of the 3D crystal structure of HIV-1 RT[1] (Kohlstaedt et al., 1992; Jacobo-Molina et al.,

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* Address correspondence to this author. Tel: 973-972-0660. Fax: 973-972-5594.

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[1] Abbreviations: A, Q, N, and R represent single letter codes for alanine, glutamine, asparagine, arginine amino acids, respectively; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; IPTG, isopropyl-β-thiogalactopyranoside; poly(rA)·$(dT)_{18}$, polyriboadenylic acid annealed with $(oligodeoxythymidylic\ acid)_{18}$; poly(rC)·$(dG)_{18}$, polyribocytidylic acid annealed with $(oligodeoxyguanylic\ acid)_{18}$; poly(dC)·$(dG)_{18}$, polydeoxycytidylic acid annealed with $(oligodeoxyguanylic\ acid)_{18}$; dNTP, deoxyribonucleoside triphosphate; dATP, dGTP, dCTP, and dTTP represent nucleoside triphosphates of deoxyadenosine, deoxyguanosine, deoxycytidine, and thymidine, respectively; HIV-1 RT, human immunodeficiency virus type-1 reverse transcriptase; IMAC, immobilized metal affinity chromatography; IDA−Sepharose, iminodiacetic acid−Sepharose; MuLV, murine leukemia virus; HIV PBS-RNA template, HIV-1 genomic RNA template corresponding to primer binding sequence region; PBS-DNA template, HIV-1 genomic DNA template corresponding to the RNA-PBS sequence.

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1993). The availability of the crystal structure of HIV-1 RT further stimulated interest in the structure−activity analyses of RT, particularly the mechanism of drug resistance caused through mutations. The viral enzyme is a 117-kDa heterodimeric protein (p66/p51); the smaller p51 subunit is derived from the larger p66 subunit by the proteolytic cleavage within the COOH-terminal and removal of the 15-kDa RNase-H domain (Dimarzo-Veronese et al., 1986; Lightfoote et al., 1986). The polymerase domain of the two subunits is folded differently in spite of having the same amino acid sequence (Kohlstaedt et al., 1992). The N-terminal region of the larger subunit is folded into an open structure containing the polymerase active site cleft while the smaller subunit is closed and compact (Wang et al., 1994). The polymerase cleft is further folded into three distinct subdomains, which have been referred to as the palm, finger, and thumb of a right hand (Kohlstaedt et al., 1992). Although a large number of amino acid residues on these subdomains have been subjected to extensive site-directed mutagenesis, the functional role of most of them is not yet known (Larder et al., 1987, 1989; Boyer et al., 1992, 1994a; Wakefield et al., 1992; Chao et al., 1995; Tisdale et al., 1993). However, mutant derivatives of some of the residues on the finger and palm subdomain have been extensively characterized and shown to play some distinct role in the polymerase function. For example, Met184 residing at the hairpin loop of $\beta9-\beta10$ has been implicated in the error-prone DNA synthesis by the WT enzyme whereas mutation of Met→Val was found to enhance the fidelity of the enzyme with increased catalytic efficiency (Pandey et al., 1996; Wainberg et al., 1996). Kinetics of utilization of phosphorothioate analogs of dNTP have implicated Asp 186 in stabilizing the transition state ternary complex by coordinating with the α-phosphate group of dNTP while Asp185 was postulated to interact with 3′O of the primer terminus (Kaushik et al., 1996). Photoaffinity labeling with dTTP in the binary complex identified Lys73 on $\beta4$ as the site for dNTP cross-linking (Cheng et al., 1993) while truncation of αE was shown to modulate RNase-H function and also impair DNA strand transfer (Ghosh et al., 1995). Mutation of Arg78 to Ala on the αB helix has been shown to reduce the DNA binding ability of the enzyme (Sarafianos et al., 1995c). Gln151 located at the joining loop of $\beta8-\alpha E$ and Arg72 on $\beta4$ in the finger subdomain were shown to be involved in the formation of the dNTP binding pocket of the enzyme (Sarafianos et al., 1995a,b). We have further investigated the role of Gln151 and have shown that, with RNA-directed DNA synthesis, the rate-limiting step occurs after the phosphodiester bond formation while with DNA template it occurs at the dNTP binding step.

MATERIALS AND METHODS

Materials

Restriction endonucleases and DNA-modifying enzymes were from Promega or Boehringer Mannheim; Sequenase and DNA sequencing reagents were from U.S. Biochemicals. HPLC-purified dNTPs were obtained from Boehringer Mannheim. Mutagen-M13 *in vitro* mutagenesis kit was purchased from Bio-Rad Laboratories. Expression vector pET-28a and *Escherichia coli* expression strain BL21 (DE3) were obtained from Novagen. All other reagents were of the highest purity grade and were purchased from Fisher, Millipore Corp., Boehringer Mannheim, and Bio-Rad. Fast-flow chelating Sepharose (iminodiacetic acid−Sepharose) for immobilized metal affinity chromatography (IMAC) and synthetic template-primers were purchased from Pharmacia, and ^{32}P-labeled dNTPs and ATP were the products of Dupont/New England Nuclear Corp. Sequencing primers and oligonucleotides containing the desired mutational changes were purchased from Midland Certified Reagent, Dallas, TX.

An HIV RNA expression clone pHIV-PBS was a generous gift from Dr. M. A. Wainberg (Arts et al., 1994). This clone contains the 947-bp fragment of HIV-1 genome (+473 to +1420) that supplies RNA corresponding to the PBS region.

Methods

Construction of Expression Plasmids and in Vitro Mutagenesis. For obtaining appropriate 66/51 heterodimers of HIV-1 RT free from contaminating 66/66 homodimers, we constructed two recombinant plasmids (pET-28a-RT66 and pET-3a-RT51 containing P66 and P51 encoding regions, respectively) with metal binding hexahistidine (His-Tag) sequences at the N-terminal region of only the P66 subunit (Pandey et al., 1996). The *Xba*I and *Sac*I fragment (1.432 kb) of pET-3a-RT_{51} encoding the polymerase domain of HIV-1 RT was subcloned in bacteriophage M13 mp18 and used as the template for site-directed mutagenesis. The mutagenesis protocol using uracil-containing DNA template was essentially as described by Kunkel et al. (1987). After ascertaining the mutation in M13 by DNA sequencing, the desired mutation was introduced in both the subunits as follows: *Nde*I and *Kpn*1 fragment from M13 mp18 was cloned in RT_{66} expression cassette, and *Nhe*I and *Sac*I fragment was cloned in RT51 expression cassette to introduce the desired mutation in both the subunits (Sarafianos et al., 1995a,b; Pandey et al., 1996).

Expression and Isolation of 66/51 Heterodimeric HIV-1 RT and Its Mutant Derivatives. The growth of *E. coli* BL-21 containing pET-28a-RT66 and pET-3a-RT51 clones carrying the WT or mutant subunits and the induction of the enzyme protein were carried out as described before (Sarafianos et al., 1995a,b; Pandey et al., 1996). The heterodimers were prepared by mixing the cell pellets of p66 and p51 clones at the appropriate ratios as described by Le Grice et al. (1991) with slight modification (Pandey et al., 1996). The protein preparations were stable for months at −20 °C. The protein concentrations were determined by using the Bio-Rad calorimetric kit as well as by spectrophotometric measurements using $\epsilon_{280} = 2.6 \times 10^5\ M^{-1}\ cm^{-1}$ (Kati et al., 1992).

DNA Polymerase Assay. The reaction conditions and composition of the reaction mixtures were the same as described before (Pandey et al., 1996); any variation used in a specific experiment is as described in the corresponding figure legend. Unless otherwise indicated, all reactions were carried out at 25 °C. RNAse-H activity assays were performed essentially as described elsewhere (Basu et al., 1989).

Reverse Transcription of HIV-1 PBS RNA Template. An HIV RNA expression clone pHIV-PBS (Arts et al., 1994) was used for the preparation of HIV-1 genomic RNA. This clone contains a 947-bp fragment of HIV-1 genome (+473 to +1420) that supplies RNA corresponding to the PBS region. The plasmid pHIV-PBS was linearized with *Acc*I and transcribed using T7 RNA polymerase. The enzyme,

Chart 1: Oligomeric DNA Template-Primers Used

37 meric self-annealing TP

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   A  C
C          GCAGTCTTCTCC -3'
 T         CGTCAGAAGAGGATCCCTC  -5'
   C  A
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47/18 mer TP

5' CTTCCATTCACACACTGC-3'
3'-GAAGGTAAGTGTGTGACGATGTCTGACCTTGTTTTTGTGACATTGAG-5'

U5-PBS-DNA template annealed with PBS DNA primer

5'-**GTCCCTGTTCGGGCGCCA-3'**
3'-**CAGGGACAAGCCCGCGGT** GAC GAT CTC TAA AAG GTG TGA CTG
ATT TTC CCA GAC TCC CTA GAG ATC AAT GGT CTC AGT GTG TTG
TCT GCC-5'

HIV-1 U5-PBS RNA template annealed with 18 mer PBS DNA primer

5'-***GTCCCTGTTCGGGCGCCA***-3'
3'-GAAAGCGAAAGUC***CAGGGACAAGCCCGCGGU*** GACGAUCUCUAAAAG
GUGUGACUGAUUUUCCCAGACUCCCUAGAGAUCAAUGGUCUCAGUGUGUUGU
CUGCCCGUGUGUGAUGAACUUCUGAGUUCCGUUCGAAAUAACUCCGAAUUCG
UCACCCAAGGGAUCAUCGGUCUCUCGAUGGUCCGAGUCUAGA-5'-RNA sequence of HIV

buffer, and rNTP solutions were from Boehringer Mannheim; the transcription reaction was carried out according to the manufacturer's protocol.

An aliquot of the transcribed primer binding sequence RNA was annealed with 5′-^{32}P-18mer primer complementary to the PBS (see Chart 1). The molar ratio of RNA template to 18mer DNA primer was approximately 3:1. The molarity of the 3′-primer termini was considered as the final molarity of the annealed template-primer. Reverse transcription reactions were carried out by incubating 2.5 nM HIV PBS RNA/18mer template-primer with 50 nM WT enzyme or 100 nM Q151A mutant in a total reaction volume of 5 μL containing 25 mM Tris-HCl, pH 7.5, 1 mM DTT, 100 μg/mL BSA, 1 mM $MgCl_2$, and 100 μM of each dNTP. Reactions were initiated by the addition of enzyme and terminated by the addition of an equal volume of Sanger's gel loading dye at different time intervals (Sanger et al., 1977). The reverse transcription products were resolved on 16% polyacrylamide−urea gel.

DNA Polymerase Reaction on HIV-1 U5-PBS-DNA Template. A 90-base DNA (PBS-DNA) corresponding to the RNA sequences at the PBS binding region was synthesized and purified by reverse-phase HPLC column. The sequence of the U5-PBS-DNA template annealed with 5′-^{32}P-labeled 18mer PBS−DNA primer (see Chart 1) at a molar ratio of 2:1 was used as the template-primer for DNA-directed polymerase reaction under similar conditions as described for the reverse transcription reactions.

Pyrophosphorolysis Activity. Pyrophosphorolysis activity was estimated by analyzing the products of the reaction on a denaturing polyacrylamide gel. The DNA substrate was prepared by annealing 5′-^{32}P-18mer PBS primer with U5-PBS HIV-1 RNA template or DNA template at 1:2 molar ratio of primer versus template. The reaction mixture contained 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 100 μg BSA/mL, 5 mM $MgCl_2$, 5 nM labeled template-primer (0.5×10^6 Cerenkov CPM/pmol of 3′-primer termini), 1 mM pyrophosphate, and 200 nM mutant enzyme in a final volume of 5 μL. The reactions were carried out at 25 °C for 60 min and quenched with equal volume of Sanger's gel loading dye. The samples were heated at 90 °C for 3 min and analyzed by electrophoresis on a 16% denaturing polyacrylamide−urea gel followed by autoradiography.

Cross-Linking of Enzyme to Template-Primer. We have used the self-annealing 37meric template-primer as the DNA template-primer and poly(rA)·$(dT)_{18}$ as the RNA−DNA template-primer for the binding studies (see Chart 1). The 37mer TP as well as dT_{18} primer was 5′-labeled using [γ-^{32}P]-ATP and T_4 polynucleotide kinase according to the standard protocol (Ausubel et al, 1987). The labeled oligomers were purified on a NAP-10 column (Pharmacia) and adjusted to the required specific activity with the unlabeled oligomer. Poly(rA)·(5′-^{32}P-$dT)_{18}$ was prepared by mixing equimolar aliquots of [^{32}P]dT_{18}poly(rA) in an annealing mixture containing 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 100 mM NaCl followed by heating the mixture at 65°C for 10 min and then slow cooling to room temperature. For cross-linking, 512 nM enzyme and 50 nM labeled TP (4×10^4 Cerenkov cpm/pmol) were incubated on ice for 10 min in a reaction mixture containing 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 2 mM $MgCl_2$, and 5% glycerol in a final volume of 50 μL. The mixture was exposed to UV irradiation, and the TP cross-linked enzyme species were resolved by electrophoresis on SDS−polyacrylamide gel, and the extent of cross-linking was quantitated by excising the radioactive bands and measuring the Cerenkov counts associated with the gel piece.

Nucleotidyltransferase Activity of E−TP Covalent Complex. Nucleotidyltransferase activity of the enzyme containing covalently cross-linked template-primer was carried out as described previously (Pandey et al., 1994). Fifteen picomole of the enzyme was cross-linked with 25 pmol of the unlabeled TP under standard conditions as described above in a final volume of 50 μL. To the irradiated mixture, 100 μL of a solution containing 100 mg/mL Ni^{2+}−iminodiacetic acid−Sepharose suspension in buffer A was added, mixed, and incubated on ice for 10 min. The suspension was centrifuged at 1000*g* for 5 min, and the supernatant was discarded. The pellet was washed with 0.5 mL of buffer A and resuspended in 50 μL of elution buffer containing 20 mM Tris-HCl/200 mM imidazole at pH 8.0. The suspension was incubated on ice for 10 min and centrifuged. The supernatant was transferred to a separate tube, and the pellet was discarded. The nucleotidyltransferase reaction was initiated by the addition of 50 μL of solution containing 1 μM complementary [α-^{32}P]dNTP (5 μCi) and 5 mM $MgCl_2$. The reaction mixture was incubated for 30 min at room temperature and terminated by the addition of 1% SDS and 20 mM EDTA. An aliquot of the reaction mixture was subjected to SDS−PAGE followed by autoradiography. The radioactivity associated with the E−TP covalent complex was determined by Cerenkov counting after excising the radioactive band from the gel.

Effect of Thiophosphoryl Substitution at the α-Phosphoryl Group of dNTPs on the Polymerase Activity. The effects of the thiophosphoryl-substituted dNTP on the polymerase activity of the mutant enzyme were determined with [poly-(rA)·$(dT)_{18}$ together with dTTP and its phosphorothioate analog (dTTPαS). The dTTPαS analog was pure Sp diastereomers and was used at 50 μM concentration. The gel-purified oligomeric primers were 5′-^{32}P-labeled and annealed with a 2-fold excess of the respective templates. A typical reaction contained 2.5 nM labeled template-primer (10^6 Cerenkov cpm/pmol), 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 100 μg/mL BSA, 50 μM either normal dTTP or its thiophosphoryl (dTTPαS) analog, and 25 nM WT enzyme or 200 nM mutant enzyme in a final volume of 30 μL. All

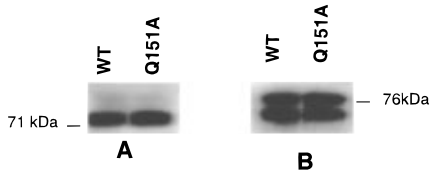

FIGURE 1: Photochemical cross-linking of RNA−DNA and DNA−DNA template-primers to the Q151A mutant and the wild-type HIV-1 RT. Wild-type HIV-1 RT and its mutant derivatives were covalently cross-linked with (A) poly(rA)·5′-^{32}P(dT)18 or (B) 5′-^{32}P-37mer self-annealing template-primer by UV irradiation as described in the Materials and Methods. The irradiated mixtures were subjected to SDS−PAGE and autoradiography.

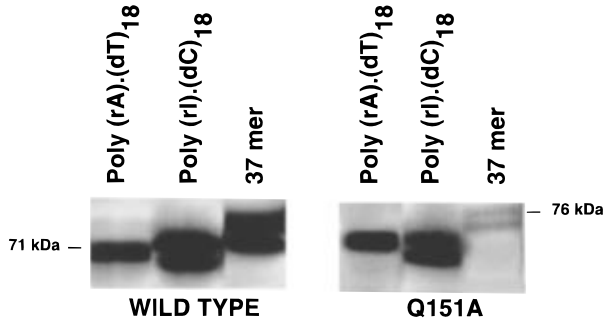

FIGURE 2: Catalytic competence of Q151A mutant enzyme cross-linked with RNA−DNA and DNA−DNA template-primers. The WT enzyme and its Q151A mutant derivative were individually cross-linked with poly(rA)·$(dT)_{18}$, poly(rC)·$(dG)_{18}$, and 37meric self-annealing DNA template-primer, and the E−TP covalent complexes were isolated as described in the Materials and Methods. The nucleotidyl transferase reaction catalyzed by the E−TP covalent complex was demonstrated by incubating the complex with α-^{32}P-labeled Mg·dTTP as described before. An aliquot of each reaction mixture was analyzed by SDS−PAGE and subjected to autoradiography.

reactions were carried out at 25 °C, and 5-μL aliquots were removed at different time intervals and quenched with an equal volume of the DNA gel loading dye. The samples were heated at 70 °C for 3 min, and the products were resolved by electrophoresis on a 16% denaturing polyacrylamide−urea gel. The labeled products were detected by autoradiography.

RESULTS

Biochemical Properties of Q151A Mutant Enzyme. Q151A mutant enzyme showed significantly reduced yet detectable activities with poly(rA)·$(dT)_{18}$ and poly (dC)·$(dG)_{15}$ template-primers. Steady-state kinetic parameters have shown that the replacement of Glu with alanine causes reduction in K_{cat} by 100-fold with RNA template-directed reactions while only a 5-fold reduction was seen with DNA-directed reactions (Sarafianos et al., 1995a). However, affinity for dNTP substrate exhibited by this mutant was exactly opposite to its catalytic activity seen with RNA- and DNA-directed reactions. For example, it showed drastically reduced catalytic activity on RNA template without exhibiting any apparent change in the affinity for dNTP substrate. In contrast, a drastic reduction in the dNTP binding affinity was seen with DNA template with only a moderate effect on the K_{cat} (Sarafiano et al., 1995a).

Effect of Q151A Mutation on the Formation of Enzyme−Template-Primer Binary Complex. The notable reduction in the K_{cat} for polymerase reaction of the mutant enzyme with RNA-directed reaction without any apparent change in the K_m for dNTP substrate suggested a possible defect in the TP binding function of the enzyme. To confirm this premise, the formation of E−TP complex was assessed by direct photochemical cross-linking of both RNA−DNA and DNA−DNA template-primers. We have used poly(rA)·$(dT)_{18}$ and a self-annealing 37mer oligomeric DNA as template-primers for the binding studies. The oligomeric primer was prelabeled with ^{32}P at 5′ position before binding and subsequent cross-linking to enzyme. As judged by the extent of cross-linking, both the mutant and the wild-type enzyme showed similar binding affinity for both DNA−DNA and RNA−DNA template-primers (Figure 1). The specificity of cross-linking of both the template-primers to the mutants and wild-type enzymes was determined by measuring the extent of the cross-linking in the presence of poly(dC), oligo$(dT)_{15}$, and poly(dC)·$(dG)_{18}$. The binding and cross-linking of labeled TP was not affected by either poly(dC) template or oligo(dT) primer alone when added to the pre-incubation mixture before or after the binding step. However, the cross-linking of the labeled TP was effectively competed out by poly(dC)·$(dG)_{18}$, suggesting that the binding of these TPs was specific and no change in the affinity for both the template-primers was observed (data not shown).

Catalytic Competence of Enzyme−DNA Covalent Complexes. Since the Q151 mutant exhibited no change in its affinity for binding to RNA−DNA and DNA−DNA template-primers, the possible defect was assumed to be at the dNTP binding step. Sarafianos et al. (1995a) have earlier shown that the Q151A mutant does not incorporate dNTP on the cross-linked TP and have concluded that the defect is due to impairment of dNTP binding. This assumption was consistent with high K_m for dNTP substrate observed with DNA-directed reactions. However, it is not known whether a similar dNTP binding defect occurs with RNA-directed reactions. Earlier, it has been shown that the wild-type Klenow fragment covalently linked with template-primer in its polymerase domain can catalyze a single nucleotide addition on to the 3′-OH terminus of the immobilized template-primer (Pandey et al., 1994). Since Q151A mutant enzyme showed equal affinity for both RNA−DNA and DNA−DNA template-primers, the ability of their E−TP covalent complex to bind dNTP in the ternary complex was assessed in the subsequent catalytic step. The nucleotidyltransferase activity of the complex on the immobilized template-primer was monitored by incorporation of ^{32}P-labeled deoxynucleotide complementary to the first template base. Results depicted in Figure2 clearly show that the enzyme−DNA:DNA−template-primer covalent complex of the Q151A mutant failed to catalyze nucleotide addition onto the immobilized template-primer when [α-^{32}P]dTTP was used as a substrate. In contrast, nucleotidyltransferase activity of the mutant enzyme−(RNA−DNA) covalent complex was found to be nearly equivalent to the WT enzyme−(RNA−DNA) covalent complex, suggesting that dNTP binding may not be the rate−limiting step with RNA-directed reaction (Figure 2). These results provide qualitative evidence suggesting plausibly different roles played by Q151 on RNA versus DNA template-directed reactions.

Incorporation of dTTPαS. The nucleotidyltransferase experiment suggests that the dNTP binding step could be the rate-limiting step with DNA-directed reactions but not with RNA directed reactions. The possibility of the chemical step (phosphodiester bond formation) being the rate-limiting step was therefore examined by testing the ability of Q151A

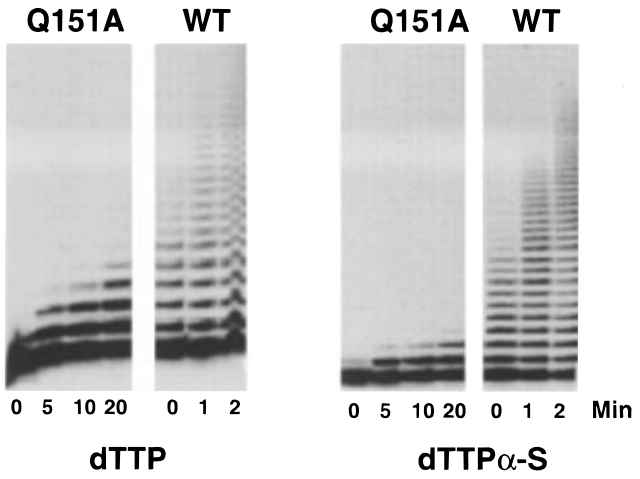

FIGURE 3: Incorporation of dNTP and dNTPαS on RNA−DNA template-primer by wild-type HIV-1 RT and its Q151A mutant derivative. Wild-type (25 nM) and mutant enzymes (200 nM) were incubated separately with 2.5 nM poly(rA)·5′-^{32}P-$(dT)_{18}$ in the presence of 50 μM dTTP or dTTPαS as described in Materials and Methods. Aliquots were withdrawn at indicated time points and analyzed on denaturing polyacrylamide gel as described above. As indicated, the left panel shows the incorporation of dTTP while the right panel indicates the incorporation of dTTPαS.

mutant enzyme to catalyze the incorporation of dTMP *versus* dTMPαS into poly(rA)·(dT)18. A significant difference in the utilization of normal versus phosphorothioate analog of dNTP, also known as sulfur elemental effect, would imply reactivity with α-phosphate of dNTP (Kaushik et al., 1996). Figure 3 shows the products formed with dTTP and dTTPαS as a function of time with the WT HIV-1 RT and its mutant derivative Q151A. As shown in the figure, no sulfur elemental effect was seen with the Q151A mutant as the extent of addition of dTTP and dTTPαS remained similar, implying that the chemical step is not affected by this mutation.

Time Course of Primer Extension Reaction Directed by RNA and DNA Templates. Since the Q151A mutant exhibited a substantial reduction in the catalytic efficiency with RNA template-directed reactions without any effect on the dNTP binding function, we carried out product analysis to identify whether the rate-limiting step of the reaction with these mutants precedes or follows the bond formation. Using 47/18mer as the DNA:DNA template-primer and HIV genomic RNA annealed with 18mer PBS primer as the RNA:DNA template-primer, a time course of nucleotide incorporation was performed. The reaction products were analyzed on polyacrylamide−urea gel. We expected that the time required to catalyze the addition of the first and second nucleotide would indicate whether the rate-limiting step for these mutants is before or after the bond formation. Figure 4, panels A and B, depicts the rate of incorporation of nucleotide as a function of time for the Q151A mutant and the WT enzyme. It was found that, for the WT enzyme, the rate of incorporation was linear with time with both DNA and RNA templates. Both the WT enzyme and the Q151A mutant exhibited linearity of primer extension on the DNA template-directed reactions, although the mutant exhibited a lower efficiency (Figure 4B). However, with RNA as the template, primer extension was found to be nonlinear with time (Figure 4A), suggesting that the reaction step following the chemical step might have been affected by this mutation.

Pyrophosphorolysis Activity of Q151A Mutant. The pyrophosphorolysis activity may be considered as a reversal

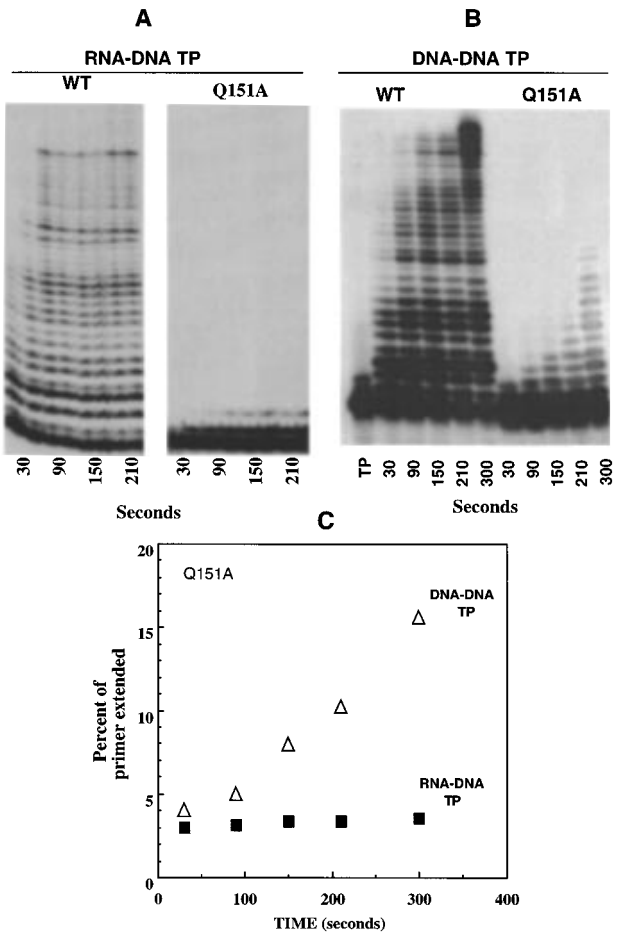

FIGURE 4: Time course of incorporation of dNTP by Q151A mutant and WT HIV-1 RT on RNA−DNA and DNA−DNA template-primers. The HIV−RNA template annealed with 5′-^{32}P-labeled heteromeric 18mer PBS DNA primer and 47mer/5′-^{32}P-18mer DNA−DNA template-primer were used to assess the time course of extension by the Q151A mutant enzyme. The wild-type (25 nM) and mutant enzyme (200 nM) were incubated with 2.5 nM labeled template-primer in the presence of 50 μM of each of the four dNTP (panel A with RNA template; panel B with DNA template) as described in Materials and Methods. Aliquots were withdrawn at indicated time points, and the reaction products were analyzed on a denaturing 16% polyacrylamide−urea gel followed by autoradiography on Kodak X-ray film. The RNA and DNA template-directed reaction products catalyzed by Q151A were quantitated by phosphorimager, and the percent of labeled primer extended was plotted as a function of time (panel C).

of the polymerase reaction and, therefore, would require the participation of the same residues involved in both the forward and reverse reactions. In the overall pyrophosphorolysis reaction, the primer is sequentially cleaved from the 3′ end in the presence of PP_i resulting in the generation of dNTP. Since polymerase activity of the Q151A mutant was severely impaired with RNA-directed reaction, effect of this mutation on the reverse reaction (pyrophosphorolysis) was assessed to confirm its participation in dNTP/PP_i binding. It was expected that the residue involved in the binding of the phosphoryl group of dNTP in the forward polymerase reaction should also be involved in the binding of PP_i in the reverse (pyrophosphorolysis) reaction. With this premise, we expected that the Q151A mutant exhibiting no change in the affinity for dNTP substrate with RNA template will be able to catalyze the reverse reaction more efficiently. We used 47/5′-^{32}P-18mer as well as 5′-^{32}P-PBS primer annealed with HIV-1 RNA template as the template-primers to assess the degradation of the labeled primer by these mutants in

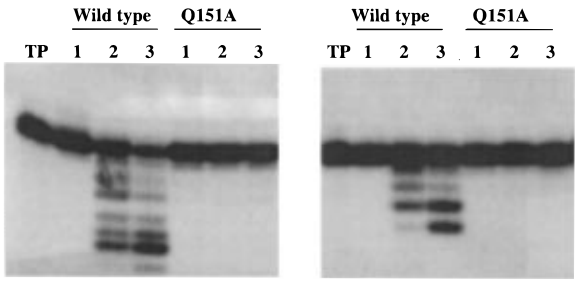

FIGURE 5: Pyrophosphorolysis reaction catalyzed by Q151A mutant and WT HIV-1 RT. Two different substrates representing RNA−DNA [poly(rA)·(dT)18 (panel A) and DNA−DNA (47/18mer) template-primer (panel B) were used for this experiment. The gel-purified 5′-^{32}P-labeled oligomeric primer annealed with the respective templates was incubated with the mutant derivatives in the presence of 1 mM sodium pyrophosphate as described in Materials and Methods. Each panel shows the electrophoretic separation of the reaction products after 60 min of incubation at 25 °C.

the presence of PP_i. Surprisingly, as shown in Figure 5, panels A and B, the Q151A mutant was nearly devoid of pyrophosphorolysis activity. Similar results have also been obtained with the R72A mutant (Sarafianos et al., 1995b), suggesting that residues Q151 and R72 may be sharing/interchanging their role in dNTP/PP_i binding in the forward and reverse reaction, respectively.

DISCUSSION

Glutamine-151 is the third residue of the highly conserved LPQG motif present among the RNA-dependent DNA polymerase class of enzymes (Johnson et al., 1986). Of the four residues in this motif, two residues preceding Q151, namely, Leu149 and Pro150, have been shown to be crucial for the polymerase function of the enzyme (Boyer et al., 1994). Glycine-152, the residue following Q151 in this motif, has also been found to be an important residue as G→A mutation completely incapacitates the polymerase function of the enzyme (Boyer et al., 1994). Probably Gly152 may be critical for positioning the side chain of Q151 in the cleft by maintaining the loop structure of LPQG motif. Substitutions of Q→E, Q→A at 151 position have been reported to inactivate 70−95% of the polymerase activity, whereas Q→N substitution retains WT activity (Boyer et al., 1994; Sarafianos et al., 1995a). In the 3D crystal structure of HIV-1 RT, Q151 is located on the β8−αE hairpin in the finger subdomain of the large catalytic cleft in the polymerase domain (Kohlstaedt et al., 1992; Jacobo-Molina et al., 1993). The structural data from the RT−DNA binary complex suggest that, although Q151 is close to the bound DNA, it is not within interacting distance and therefore may not be directly involved in DNA binding (Tantillo et al., 1994; Pandey et al., 1996; Kaushik et al., 1996). Our photoaffinity labeling experiments confirmed this premise as both DNA−DNA and RNA−DNA template-primers cross-link to the Q151A mutant with equal affinities as that of the WT enzyme. However, differential polymerase activity on RNA- versus DNA-directed templates was clearly seen with Q→A substitution at the 151 position. Its ability to copy RNA template was severely impaired as compared to the DNA template. Most interestingly, the affinity for dNTP substrate remained unchanged with RNA template while a 10-fold reduction in the affinity could be discerned when DNA templates were used in the reaction (Sarafianos et al., 1995a). This poses a pertinent question regarding the actual functional role of Q151. Does this residue change its functional role depending upon the type of template it encounters? To be more precise, does the Q151 residue have two different and independent roles to play during the first and second strand synthesis of the viral genome? Our studies with Q151A support this hypothesis. Q151 may be directly involved in the formation of the dNTP binding pocket when DNA is the template as indicated by the inability of the covalent complex of the enzyme−(DNA−DNA−template-primer) to catalyze the incorporation of the first nucleotide on the immobilized primer terminus. A recent report stating that substitution of Q→M at 151 position confers resistance to all the dideoxynucleoside inhibitors of HIV-1 RT also suggests the importance of this residue in dNTP binding (Shirasaka et al., 1996). However, the dNTP binding function is not apparent with RNA templates. Although the extent of polymerase reaction is drastically reduced with RNA templates, there is no change in the $K_{m_{dNTP}}$, suggesting that the reaction step affected by Q→A substitution could be other than the dNTP binding step. This premise is strengthened by the observation where Q151A mutant enzyme covalently linked with RNA−DNA template-primer could catalyze the incorporation of nucleotide on the cross-linked primer as efficiently as the WT enzyme. The two important steps before the bond formation involve DNA and dNTP bindings, and both these steps seem unaffected by Q→A substitution when RNA is a template. The possibility of the chemical step being the rate-limiting step is also ruled out as no significant difference in the rate of incorporation of dNTP and dNTPαS could be seen with this mutant. The alternate possibility was to examine if the reaction steps following the chemical step (phosphodiester bond formation) is affected by this mutation. A time course of the incorporation of the nucleotide on RNA−DNA template-primer is in agreement with this notion. It was observed that the Q151A mutant stalls on the template after the addition of the first nucleotide and the time taken for the second nucleotide addition was several fold higher than the first nucleotide. Such pause patterns were conspicuous by their absence on DNA-directed reactions. Similar results have been obtained with mutants of Arg72 (R72A), which also exhibited very low k_{cat} without any apparent change in the affinity for the dNTP substrate (Sarafianos et al., 1995b).

An analysis of several 3D crystal structures of HIV-1 RT (PDB file names 1HMI, 3HVT, 1HNI) revealed a variation of 2.8−7.1 Å distance between the position of side chains of Q151 and R72. In DNA-bound and nevirapine-bound crystal structure, the distance between the two side chains is 3.1 and 3.8 Å, respectively, while in TIBO-bound crystal structure, this distance is 6.6 Å. This would imply that the orientation of Q151 side chain is flexible depending upon the environment around it (i.e., bound inhibitor or template-primer). We therefore examined the possible orientation of the side chain of Q151 in the 3D molecular model of RT−DNA:DNA−dNTP and RT−RNA:DNA−dNTP ternary complexes (Figure 6 A,B). In the ternary complex with DNA template, the amide nitrogen of Q151 was found to be within interacting distance of 3.6 Å from the nucleotide base while with the RNA template the amide oxygen of Q151 was at a distance of 2.9 and 3.6 Å from the N3 atom of template nucleotide and guanidino nitrogen of Arg 72, respectively. The interaction between R72 and Q151, possibly through hydrogen bonding, may stabilize both the side chains, and

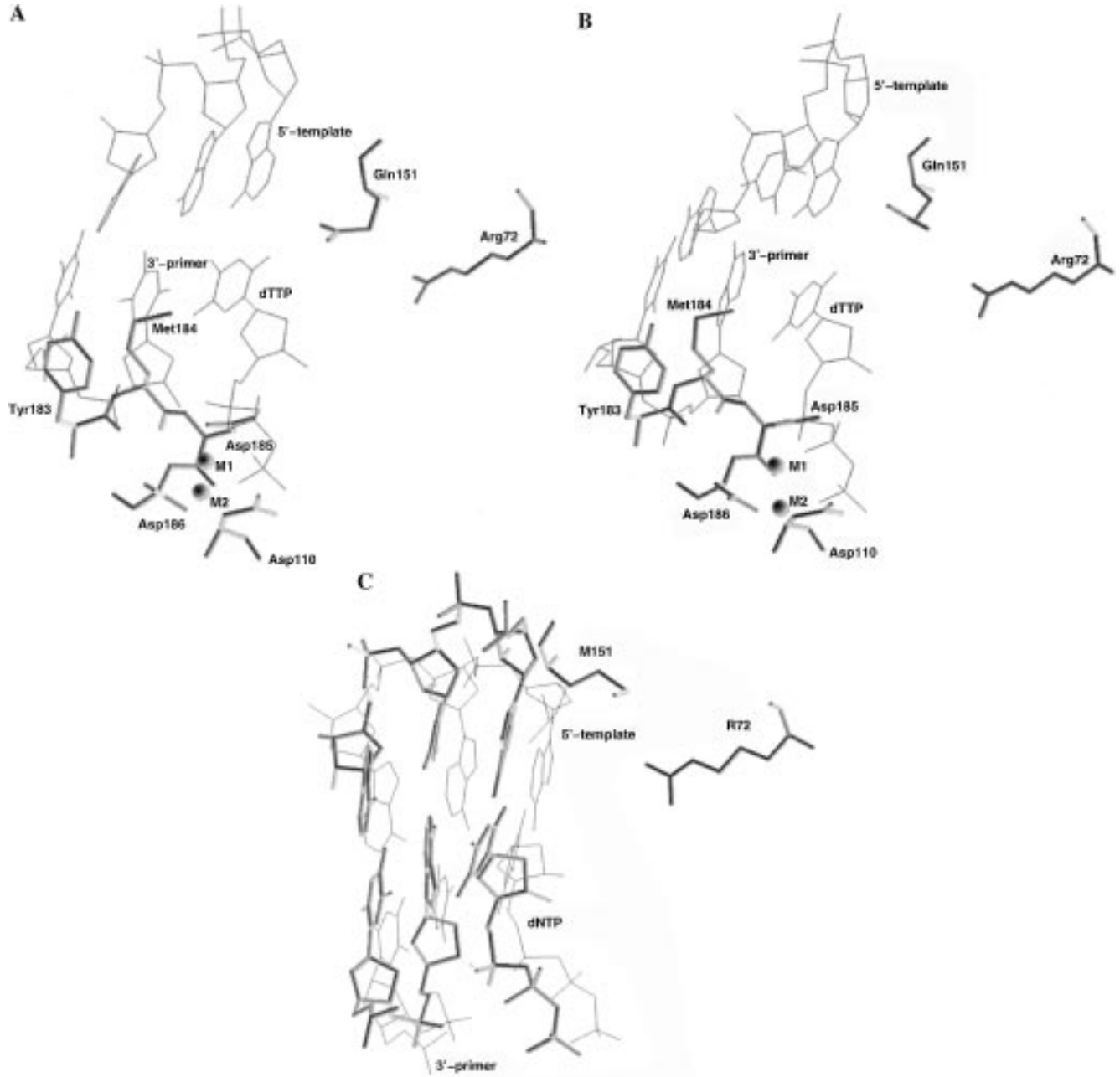

FIGURE 6: Molecular models of E−TP−dNTP ternary complex showing postulated interaction of (A) Q151 and R72 with dNTP and DNA:DNA template-primer, (B) Q151 and R72 with dNTP and RNA:DNA template-primer, (C) Q151M and R72 with dNTP and RNA:DNA template-primer. The complete 3D model structure of the pretransition state as well as transition state ternary complex based on the Cα coordinates of DNA-bound HIV-1 RT has been described before (Pandey et al., 1996; Kaushik et al., 1996). The crystal structure coordinates of RNA:DNA hybrid duplex were taken from the Protein Data Bank (file name 219D; Gonzalez et al., 1995). The prepolymerase ternary complex with RNA:DNA hybrid was made by replacing the DNA:DNA template-primer. A systematic conformation search for the side chain of amino acid residues along the RNA:DNA template-primer was performed. The orientation of the side chain of Q151 exhibiting favorable interactions and/or least steric hindrance with the surroundings were selected (panel B). In most of the cases, orientation of the side chains was similar along the primer DNA whereas a significant conformational change in the residues along the RNA template was noticed. For instance, the orientation of the side chain of Q151 in the enzyme−DNA:DNA−dNTP ternary complex is more inclined toward the dTTP substrate. The distance between O2 of dTTP and side chain amino (NE2) of Q151 is 3.6 Å while N3 of the template nucleotide is at a distance of 7.3 Å from the side chain (OE1) of Q151. The distance between R72 and Q151 is 7.0 Å. In the enzyme−RNA:DNA−dNTP ternary complex, the position of the side chain OE1 of Q151 was found to be within the interacting distance of 3.0 Å from the N3 atom of the template nucleotide while the position of the side chain of the Q151 is at a distance of 7.0 Å from dTTP. In this model, the distance between the side chain position of Q151 and R72 is 4.7 Å. Interestingly, in several other RT crystal structures [PDB file 1HMI (Jacobo-Molina et al., 1993), 3HVT (Kohlsteadt et al., 1992), and 1HNI (Ding et al., 1995)], significant variations (2.8−7.1 Å) between the position of side chain of Q151 and the neighboring side chain of R72 have been noticed. In one of the crystal structures, it has a favorable hydrogen bonding interaction with the neighboring side chain of R72. This suggests that the side chain position of Q151 is stabilized through its interaction with template and dTTP substrate or with the neighboring residue such as R72. In the presence of DNA template, Q151 seems to be interacting with dTTP. However, in the presence of RNA template it is relatively closer to the template nucleotide. To assess the relative interaction of the side chain of Q151M (a natural drug-resistant phenotype) with RNA and DNA templates, the two ternary complexes with RNA:DNA (thick line) and DNA:DNA (thin line) template-primers were superimposed (panel C). The mutant modeling of Q→M at position 151 was carried out using the LOOK program of Molecular Application Group, and the structure was energy refined. To define the orientation of M151, a systematic conformational search was carried out, and its possible interaction with dNTP and RNA template nucleotide were explored. During the search, we found that the methyl group of M151 interacts with the base moiety of the template nucleotide. It is also evident from the model that the sulfur atom of M151 may exhibit a weak electrostatic interaction with the guanidino nitrogen of R72.

thus removal of either of the side chains would affect the function of the other. This hypothesis was tested by examining the pyrophosphorolysis activity of the Q151 A mutant. Since Arg72 was found to be extremely crucial for the reverse reaction as judged by the complete loss of this activity of its mutant, the Q151 A substitution was expected to affect this function of R72 if the two side chains of these residues had a stabilizing effect on each other. As expected and is consistent with the model, Q151A was also found to be devoid of pyrophosphorolysis activity. This raised the question of the rate-limiting step for the Q151A mutant. According to the kinetic scheme investigated with the Klenow fragment, the two steps before the bond formation are (i) the formation of E·DNA·dNTP ternary complex and (ii) the subsequent conformational change of the ternary complex to a form (E*·TP·dNTP) that is poised for catalysis, (Similar to the 'two-step' binding mechanism for dNTP, the existence of two-step release mechanism for PP_i where the conformational change of $E^*{\cdot}TP_{n+1}{\cdot}PP_i$ species to $E{\cdot}TP_{n+1}{\cdot}PP_i$ complex has also been suggested (Dahlberg & Benkovic, 1991). Results of our mutant enzyme appear consistent with the above described kinetic scheme. For instance, mutants of the residue involved in the first step of binding should have impaired affinity for dNTP (increase in K_m). In contrast, mutation of the residue required for the second step, leading to the conformational change, may be expected to slow down this step without exhibiting any significant change in the K_m for dNTP. Since the rate-limiting step for HIV-1 RT has also been shown to be a nonchemical step involving a conformational change immediately preceding the bond formation (Hsieh et al., 1993), mutants of the residue involved in this step would drastically reduce the k_{cat} of the reaction without any significant affect on the K_m for dNTP. Thus, in the polymerase reaction, Q151 may be involved in the binding of dNTP in the first stage of the E·TP·dNTP ternary complex formation with DNA templates as judged by the unusually high K_m for dNTP. While in case of RNA template, the involvement of Q151 in the formation of dNTP binding pocket may be through interacting with the first template base and also stabilizing the side chain of Arg at the 72 position. The R72 has been postulated to play a key role in the second step leading to the conformational change of E·TP·dNTP ternary complex to E*·TP·dNTP complex (Kaushik et al., 1996). A drastic reduction in the k_{cat} seen by R→A substitution at the 72 position without any significant change in the K_m for dNTP is consistent with this postulation (Sarafianos et al., 1995b). Following the chemical step of the reaction, a second conformational change of $E^*{\cdot}TP_{n+1}{\cdot}PP_i$ to $E{\cdot}TP_{n+1}{\cdot}PP_i$ may also be facilitated by R72 preceding the PP_i release. Similar to the proposed role of R72, namely, its role after the chemical step, the Q151 may also coordinate in the binding and release of PP_i moiety by stabilizing the side chain of R72. The interdependence of Q151 and R72 in the two-step binding process of PP_i in the ternary complex in the reverse reaction is consistent with the observation that mutants of both the residues (Q151A, R72A) are nearly devoid of pyrophosphorolysis activity. Furthermore, the observed low rate of incorporation of the first nucleotide by the Q151A mutant and the unduly slower rate in the addition of the second nucleotide could be due to its direct effect of interaction/stabilization of Arg72 which, in turn, may slow down the conformational change preceding and following the chemical step of the reaction. Thus, Q151 may be suggested to be involved in coordinating the binding of dNTP in the DNA-directed reaction while with RNA template its involvement in the conformational change step of ternary complex via interaction with R72 seems likely. A natural mutation of Q→M at this position was shown to be resistant to most of the nucleoside analogs (Shafer et al., 1994; Shiraska et al., 1995). An examination of the 3D molecular model structure of E·TP·dNTP ternary complex with substitution of Q→M at position 151 revealed that the M151 side chain is in the vicinity of both RNA template and R72 (see Figure 6C). It may provide hydrophobic interaction (via methyl group) with the RNA template nucleotide and also a week interaction (via sulfur atom) with the guanidino group of R72. Recently, it has been established that binding of correct dNTP in the ternary complex is solely governed by the size and shape of the binding pocket rather than its hydrogen bonding ability with the template nucleotide (Moran et al., 1997). In HIV-1 RT, the putative dNTP binding pocket is generated by template and primer nucleotides along with the side chains of Q151, R72, Y115, F160, Y183, and M184 and three carboxylate residues D110, D185, and D186. Some of the amino acid residues of the putative dNTP binding pocket exhibit significant influence on the fidelity of DNA synthesis. For example, substitution of M184 or Y115 with alanine makes the enzyme more error prone (Pandey et al., 1996; Martin-Hernandez et al., 1996) while the Q151M mutant exhibits significantly higher fidelity as compared to the wild-type enzyme (Dylan Harris et al., unpublished results). The error prone DNA synthesis catalyzed by the wild-type HIV-1 RT is possibly due to multiprong interaction of glutamine at 151 with dNTP, template, and Arg 72 resulting in the formation of a more flexible binding pocket for the incoming dNTP substrate. A substitution at this position with a residue such as methionine with weak interactions without compromising the polymerase function of the enzyme is expected to generate a more rigid pocket shape and size that, in turn, may result in higher selectivity for incoming nucleotide. This premise is supported by the observation that HIV-1 RT with Q151M mutation discriminates well against most of the dideoxy nucleoside analogs. In view of the present data, it would be interesting to determine if the resistant phenotype conferred by Q151M mutation occurs during RNA-directed (−) or DNA-directed (+) strand DNA synthesis during the viral replication.

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