Elucidation of the Role of Arg 110 of Murine Leukemia Virus Reverse Transcriptase in the Catalytic Mechanism: Biochemical Characterization of Its Mutant Enzymes†

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ABSTRACT: Based on the projected three-dimensional equivalence of conserved amino acids in the catalytic domains of DNA polymerases, we propose Arg 110 of MuLV RT to be an important participant in the catalytic mechanism of MuLV RT. In order to obtain evidence to support this proposition and to assess the functional importance of Arg 110, we carried out site directed mutagenesis of Arg 110 and replaced it with Lys, Ala, and Glu. The mutant enzymes were characterized with respect to their kinetic parameters, ability to bind template-primers, and the mode of DNA synthesis. All the three substitutions at 110 position resulted in severe loss of polymerase activity without any significant effect on the RNase H function. In spite of an approximately 1000-fold reduction in k_{cat} of polymerase activity with three mutant enzymes, no significant reduction in the affinities for either template-primer or dNTP substrates was apparent. Mutant enzymes also did not exhibit significant sulfur elemental effect, implying that the chemical step, i.e., phosphodiester bond formation, was not defective. Examination of the mode of DNA synthesis by the mutant enzymes indicated a shift from processive to the distributive mode of synthesis. The mutants of R110 also displayed significant loss of pyrophosphorolysis activity. Furthermore, the time course of primer extension with mutant enzymes indicated severe reduction in the rates of addition of the first nucleotide and even further reduction in the addition of the second nucleotide. These results suggest that the rate limiting step for the mutant enzymes may be before and after the phosphodiester bond formation. Based on these results, we propose that Arg 110 of MuLV RT participates in the conformational change steps prior to and after the chemical step of polymerase reaction.

Murine leukemia virus (MuLV)[1] is a member of the retrovirus family, and its reverse transcriptase (RT) has been extensively characterized (Modak & Marcus 1977; Verma, 1975). MuLV RT is a single subunit enzyme that possesses both polymerase and RNase H activities. The gene coding for the RT region of the proviral DNA has been cloned in high expression vectors, which has permitted identification of domains responsible for the various catalytic functions (Roth et al., 1985). For example, using pyridoxal 5′-phosphate as a substrate binding site directed reagent (Modak, 1976; Basu, A., et al., 1988), Lys 103 was identified as a probable participant in the dNTP binding function (Basu, S., et al., 1988). Similarly, using a derivative of phenylglyoxal, Lys 329 was shown to be involved in the template-primer binding function of MuLV RT (Nanduri & Modak, 1990). Photoaffinity labeling of MuLV RT with oligo(dT) was successfully used in the identification of three regions, one in the polymerase domain and two in the RNase H domain (Tirumalai & Modak, 1991). This region has been suggested to participate in the binding of primer strand. Yet another study with ferrate ion mediated oxidation of MuLV RT was used to specifically detect the amino acid residues that are likely to interact with the template-primer nucleotides (Reddy et al., 1991). The successful cloning of the gene coding for MuLV RT has permitted a site directed mutagenesis approach to clarify the functional participation of desired residues. Using this approach, Lys 103 was mutagenized to Leu 103 with the result that the mutant enzyme exhibited no polymerase activity (Basu et al., 1990). An investigation of the role of Cys 90 by mutagenesis also revealed that it is a nonessential residue, in spite of the observation that its derivatization with bulky sulfhydryl reagent results in the loss of DNA binding and therefore the polymerase activity of the enzyme (Basu et al., 1993).

During the past five years, a significant shift in reverse transcriptase research has occurred in that the enzyme from HIV-1 has been the major target of extensive investigation for the obvious reason. A number of structures of HIV-1 RT apocrystal and cocrystal structure of DNA bound or inhibitor bound enzyme have also been resolved (Kohlsteadt

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[1] Abbreviations: A, E, K, and R represent single letter codes for Ala, Glu, Lys, and Arg, respectively; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; IPTG, isopropyl β-D-thiogalactopyranoside; poly(rA)·(dT)$_{18}$, poly(riboadenylic acid) annealed with (oligodeoxythymidylic acid)$_{18}$; poly(rC)·(dG)$_{18}$, poly(ribocytidylic acid) annealed with (oligodeoxyguanylic acid)$_{18}$; poly(dC)·(dG)$_{18}$, poly(deoxycytidylic acid) annealed with (oligodeoxyguanylic acid)$_{18}$; dNTP, deoxyribonucleoside triphosphate; dATP, dGTP, dCTP, and dTTP represent nucleoside triphosphate of deoxyadenosine, deoxyguanosine, deoxycytidine, and deoxythymidine, respectively; PP_i, pyrophosphate; RT, reverse transcriptase; HIV-1 RT, human immunodeficiency virus type 1 reverse transcriptase; IMAC, immobilized metal affinity chromatography; IDA-Sepharose, iminodiacetic acid−Sepharose; MuLV, murine leukemia virus; pol I, *Escherichia coli* DNA polymerase I; PBS, primer binding site; PSM, protein solubilizing medium; CD, circular dichroism; UV, ultraviolet.

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et al., 1992; Jacobo-Molina et al., 1993; Smerdon et al., 1994; Ding & Arnold 1995; Ding et al., 1995). Extensive mutagenesis analyses of HIV-1 RT have been carried out, and amino acids responsible for catalytic functions, as well as other properties, and those responsible for drug resistance have been identified (Boyer et al., 1994a,b; Sarafianos et al., 1995a−c; Pandey et al., 1996; Kaushik et al., 1996a). In spite of these extensive studies, the mechanism of drug resistance in HIV-1 RT remains to be clarified. Similarly, the relationship between the two subunits of HIV-1 RT, from the catalytic view point, needs to be resolved. As far as the mechanism of DNA synthesis is concerned, HIV-1 and MuLV RTs share many common properties. Yet, MuLV RT is a single subunit enzyme that appears to be able to carry out an identical biological function as that of HIV-1 RT. It is therefore an attractive enzyme system to elucidate the similarities and differences in the mechanistic aspect of the two enzymes. In order to get an insight into the mechanistic similarity, it was essential to identify the catalytically important amino acids in MuLV RT. One reasonable approach was to utilize known sequences of MuLV RT and HIV-1 RT and choose the target for mutagenesis based on the knowledge of equivalent residues in HIV-1 RT (Johnson et al., 1986; Delarue et al., 1990). Using the knowledge gained from the comparison of 3D structure of the Klenow fragment with that of HIV-1 RT (Yadav et al., 1994), we were able to postulate some of the functionally equivalent residues in MuLV RT. Thus, Arg 110 and Lys 103 were projected to be functionally equivalent to Lys 758 and Arg 754 of Klenow fragment or that of Arg 72 and Lys 65 of HIV-1 RT. Recent resolution of the crystal structure of a fragment of MuLV RT containing this region has confirmed our projection of spatial equivalence (Georgiadis et al., 1995). However, no experimental data exist on the role of Arg 110 in MuLV RT. In this paper, we describe the results of the site directed mutagenesis of Arg 110 and the enzymatic properties of the three mutant derivatives which permit deduction of a catalytic role for this residue. Our studies have shown that Arg 110 is crucial for the catalytic function of MuLV RT and is probably involved in the conformational change step in both the forward and the reverse reactions.

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 Biorad laboratories. Expression vector pET-28a and *E. 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 Biorad. 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.

Methods

Construction of Expression Plasmids. We constructed recombinant plasmid containing the entire MuLV RT encoding region with metal binding hexahistidine (His-Tag) sequences at the N-terminal region. The coding sequence was derived from pB6B15.23 which contains the full coding region for MuLV RT (Roth et al., 1985; a kind gift from S. P. Goff). The 2016 bp sequence coding for MuLV RT was amplified from pB6B15.23 using PCR (Saiki et al., 1985). In the same step, we introduced an *Nde*I restriction site at the 5′ end and *EcoR*1 and a TAA stop codon at the 3′ end of the RT coding sequence. Using these restriction sites, the amplified fragment was ligated with *Nde*I and *EcoR*1 digested pET-28a (His-Tag containing expression vector) to construct the pET-28a-MRT plasmid. Since cloned PCR product is known to contain nonspecific mutation, we replaced the polymerase domain of pET-28a-MRT by subcloning 847 bp *Kpn*1−*Sal*1 fragment from the original clone (pB6B15.23). Prior to the cloning, the *Sal*1 unique site in the vector was abolished by blunt end ligation. This construct contains T7 promoter and metal binding hexahistidine (His-Tag) sequences at the N-terminal region.

In Vitro Mutagenesis. The *Kpn*1 *and Sal*1 fragment (847 bp) of pB6B15.23 encoding the polymerase domain of MuLV RT was subcloned in bacteriophage M13 mp19 and used as the template for site directed mutagenesis. The protocol of Kunkel et al. (1987) utilizing deoxyuracil containing DNA template was used for mutagenesis. After ascertaining the mutation in M13 by DNA sequencing, the desired mutation was introduced into the MuLV RT expression cassette by subcloning the *Kpn*1−*Sal*1 fragment from M13 mp19. The recombinant plasmid (pET-28a-MRT) was then introduced into *E. coli* BL-21(DE3) for induction and isolation of the enzyme protein.

Expression and Isolation of MuLV RT and Its Mutant Derivatives. The growth of *E. coli* BL-21 containing pET-28a-MRT, carrying the wild-type or its mutant derivatives, and induction of the enzyme protein were carried out as described for Klenow fragment and HIV-1 RT with slight modification (Pandey et al., 1994a, 1996; Sarafianos et al., 1995a,b). In brief, the cells were grown in Luria broth containing kanamycin at 30 °C. The induction of the enzyme was carried out by the addition of 100 μM IPTG when the cell density reached an $OD_{595} = 0.3$. The incubation was continued at 30 °C for 5 h, and cells were harvested by centrifugation at 12000*g* for 20 min at 4 °C. The cell pellet obtained from a 400 mL batch was suspended in 8 mL of lysis buffer (40 mM Tris-HCl, pH 8.0, 0.1% Nonidet P40, 1 mM PMSF, 500 mM NaCl, and 3 mg/mL lysozyme) and incubated on ice for 60 min. The suspension was briefly sonicated and centrifuged at 28000*g* for 45 min. The cell-free extract was diluted 2.5-fold with buffer A (50 mM Tris-HCl, pH 8.0, 1 mM PMSF, and 10% sucrose) and applied to a 5 mL DEAE-cellulose column pre-equilibrated with 0.1 M NaCl in buffer A. The column was washed with 1 volume of the equilibration buffer. The flow through and the buffer wash containing RT protein were combined and applied to a Ni^{2+}−iminodiacetic acid−Sepharose (IDA-Sepharose) column (2.5 mL) pre-equilibrated with buffer B (20 mM Tris-HCl, pH 8, 500 mM NaCl, 5 mM imidazole) at a flow rate of 1.0 mL/min. The column was washed extensively with buffer B until the A_{280} was reduced to a negligible level. A

second wash (15 mL) with 75 mM imidazole in buffer B removed most of the weakly bound contaminating proteins, and finally MuLV RT was eluted from the column with 15 mL of 300 mM imidazole in buffer B. The individual fractions were analyzed for enzyme purity and assayed for RNA dependent DNA polymerase activity. The active peak fractions were pooled and dialyzed against buffer C (20 mM Tris-HCl, pH 7.0, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, and 50% glycerol). The isolated proteins were more than 98% pure, as judged by SDS−PAGE (Laemmli, 1970). The protein preparations were stable for months at −20 °C. The protein concentrations were determined by using the Biorad colorimetric kit and also by densitometric scanning of the Coomassie blue stained protein band on the gel and comparing the intensity with known amounts of BSA.

DNA Polymerase Assay. The composition of the reverse transcriptase reaction mixture is described below; any variation used in a specific experiment is as described in the appropriate figure legends. Unless otherwise indicated, all reactions were carried out at 25 °C. Reactions were quenched either by the addition of equal volume of Sanger's gel loading solution (Sanger et. al., 1977) (for gel analysis) or by the addition of ice cold 5% trichloroacetic acid. The quenched reaction products were analyzed in some cases by polyacrylamide−urea denaturing gel or quantitated by collecting the TCA-precipitable materials on Whatman GF/B filters and counting for radioactivity in a liquid scintillation counter, as described before (Pandey & Modak, 1987). The reactions were carried out in a final volume of 100 μL containing 50 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, 0.1 μg/mL bovine serum albumin, 200 nM of the desired template-primer, 5 mM $MgCl_2$ (or 1 mM $MnCl_2$ for poly(rA)·$(dT)_{18}$ extension reaction), and 40 μM tritiated dNTP substrate corresponding to the homopolymeric template-primers. With heteromeric template-primers 40 μM each of the four dNTPs were present, with one of them being radiolabeled. The molar ratio of the primer to template was 1:1 unless indicated otherwise. The concentrations of template-primers are expressed in terms of the 3′-hydroxyl primer termini. RNase H activity assays were performed essentially as described elsewhere (Basu et al., 1989).

Pyrophosphorolysis Activity. In the pyrophosphorolysis reaction, a terminal nucleotide from the primer terminus is cleaved in the presence of PP_i to generate dNTP. There are two procedures to determine this activity. The first one utilizes [^{32}P]PP_i and monitors the generation of [^{32}P]dNTP (Srivastava & Modak, 1980). The second assay and the one adopted in this work monitors the reduction in the length of 5′-end-labeled primer. Pyrophosphorolysis activity associated with MuLV RT was estimated by analyzing the products of the reaction on a 16% denaturing polyacrylamide gel. The substrate was prepared by annealing 5′-^{32}P-18 mer DNA primer with heteromeric RNA template at equimolar concentrations. The reaction mixture contained 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 100 μg of BSA/mL, 2.5 mM $MgCl_2$, 2 nM of heteromeric RNA−5′-^{32}P-18 mer template-primer (10^6 Cerenkov cpm/pmol), 500 μM pyrophosphate, and 100 nM wild-type or 1.0 μM mutant enzyme in a final volume of 6 μL. Since Mg·PP_i has a very high stability constant (250 000 M^{-1}; James & Morrison, 1966), we find that by increasing the concentration of PP_i, a substantial amount of Mg·PP_i is precipitated in the reaction. Furthermore, the higher concentration of Mg·PP_i (<2 mM) is inhibitory to the reverse reaction catalyzed by the WT enzyme. The extent of pyrophosphorolysis products generated with the HIV-1 RT and Klenow fragment was also reported to be significantly reduced at or above 2 mM Mg·PP_i (Kaushik et al., 1996a,b). The reactions were carried out at 25 °C for 20, 40, and 60 min and quenched with an equal volume of Sanger's gel loading solution (Sanger et al.,1977). The samples were heated at 100 °C for 3 min and resolved by electrophoresis on a 16% denaturing polyacrylamide−urea gel, and the labeled products were detected by autoradiography.

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
```

47/18 mer TP

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5'-CTTCCATTCACACACTGC-3'
3'-GAAGGTAAGTGTGTGACGATGTCTGACCTTGTTTTTGTGACATTGAG
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18/26 mer TP

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5'-CCGGCTAGTAGTACTAGT-3'
3'-GGCCGATCATCATGATCATTGTCATC-5'
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HIV primer binding sequence RNA template/DNA PBS primer

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5'-GTCCCTGTTCGGGCGCCA-3'
3'-CAGGGACAAGCCCGCGGUGACGAUCUCUAAAAGGUGUGACUGAUUUUCC
CAGACUCCCUAGAGAUCAAUGGUCUCAGUGUGUUGUCUGCCCGUGUGUGAUGAC
UUCUGAGUUCCGUUCGAAAUAACUCCGAAUUCGUCACCCAAGGGAUCAUCGGUU
CUCGAUGGUCCGAGUCUAGA----------5'  RNA sequence of HIV
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Cross-Linking of Enzyme to Template-Primer. We used a self-annealing 37 meric template-primer and (dT_{26})/(dA_{18}) as the DNA-DNA template-primer and poly(rA)·$(dT)_{18}$ as the RNA-DNA template-primer for the binding studies (see Chart 1). The 37 mer TP, dT_{18}, and dT_{26} oligomers were 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 desired specific activity with unlabeled oligomer. Poly(rA)·(5′^{32}P-$dT)_{18}$ was prepared by mixing equimolar concentrations of [^{32}P]dT_{18} and 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 at room temperature. In the cross-linking experiments, 1.2 μM enzyme and 50 nM labeled TP (2×10^4 cpm /pmol) were incubated on ice for 10 min in a reaction mixture containing 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 5 mM $MgCl_2$ (or 1 mM $MnCl_2$), and 5% glycerol in a final volume of 50 μL. The mixture was exposed to 254 nm UV irradiation at 375 mJ/cm^2 in a Spectrolinker (Spectronic Corp.); TP cross-linked enzyme species were resolved by electrophoresis on SDS−polyacrylamide gel; the extent of cross-linking was quantitated by excising the radioactive bands, and associated radioactivity was measured as Cerenkov radiation.

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., 1994b). MuLV RT was cross-linked to desired template-primer in a reaction mixture containing 15 pmol of the enzyme, 25 pmol of the unlabeled

TP, 50 mM Tris-HCl, pH 7.5, 1 mM DTT, and 5 mM $MgCl_2$ (or 1 mM $MnCl_2$) in a final volume of 50 μL as described above. The nucleotidyltransferase reactions were then initiated by the addition of 0.5 M NaCl together with 10 μCi of complementary [α-^{32}P]dTTP with a final concentration of 0.5 μM. The reaction mixture was incubated for 30 min at room temperature and terminated by the addition of protein solubilizing solution (Laemmli, 1970). An aliquot of the reaction mixture was subjected to SDS−polyacrylamide gel electrophoresis 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.

Reverse Transcription of HIV-1 PBS-RNA Template. An HIV-RNA expression clone pHIV-PBS was a generous gift from Dr. M. A. Wainberg (Arts et al., 1994). It was used for the preparation of HIV-1 genomic RNA. This clone contains 947 bp fragment of HIV-1 genome (from +473 to +1420) which includes the RNA in the PBS region. The plasmid pHIV-PBS was linearized with *ACC*I and was transcribed using T7 RNA polymerase. The enzyme, buffer, and rNTPs were from Boehringer Mannheim. In brief, the transcription reaction contained 1 μg of linearized pHIV-PBS, 1 mM of each of four rNTPs, 40 mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$, 10 mM DTT, 2 mM spermidine, 20 units of RNasin, and 40 units of T7 RNA polymerase in a final volume of 20 μL. The reaction mixture was incubated at 37 °C for 2 h. The reaction mixture was supplemented with 20 units of DNase I (RNase free) and incubated further for 15 min to remove the DNA. The reactions were then terminated by heating at 65 °C for 5 min.

An aliquot of the transcribed primer binding sequence RNA (496 base long) was annealed with 5′-^{32}P-18 mer primer complementary to the PBS (see Chart 1). The molar ratio of RNA template to 18 mer DNA primer was approximately 3:1. The molarity of 3′ primer termini was considered as the final molarity of annealed template-primer. Reverse transcription reaction was carried out by incubating 2.5 nM PBS-RNA/18 mer template-primer with 50 nM of WT enzyme or 200 nM of mutant enzyme in a reaction mixture containing 25 mM Tris-HCl, pH 7.5, 1 mM DTT, 100 μg/mL BSA, 5 mM $MgCl_2$, and 1 mM of each dNTP in a final volume of 5 μL. Reaction was initiated by the addition of enzyme and terminated at different time intervals by the addition of equal volume of Sanger's gel loading solution (Sanger et al., 1977). The reverse transcription products were resolved on 16% polyacrylamide−urea gel.

Effect of Thiophosphoryl Substitution at the α*-Phosphoryl Group of dNTPs on the Polymerase Activity.* The ability to utilize the thiophosphoryl substituted dNTP by Arg 110 mutants was determined with RNA-DNA [poly(rA)•$(dT)_{18}$] together with the deoxynucleoside 5′-*O*-(1-thiotriphosphate) (TTPαS) substrate. The TTPαS analogs were pure S_p diastereomer. The gel purified oligo$(dT)_{18}$ primer was 5′-^{32}P-labeled and annealed with a 5-fold excess of the poly(rA) template. A typical reaction mixture in a final volume of 30 μL 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, 200 μM of either TTP or its thiophosphoryl (TTPαS) analog, and 25 nM of the WT enzyme or 200 nM of the mutant enzyme. All reactions were carried out at 25 °C, and 5 μL samples were removed at different time intervals and quenched with an equal volume of Sanger's gel loading solution (Sanger et al., 1977). The samples were heated at 100 °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.

Processivity Measurement. We have used heteromeric RNA-DNA template-primer for the measurement of processivity of DNA synthesis by mutant and wild-type enzymes. Five picomoles of gel purified 18 mer primer complementary to PBS of HIV genome was 5′-labeled using [γ-^{32}P]ATP and polynucleotide kinase (Ausubel et al., 1987) and annealed with 5 pmol of 496 base RNA template containing the primer binding sequence of HIV genome. The enzyme was first incubated with the labeled TP in an incubation mixture containing 40 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 2 nM primer termini and 100 nM of wild-type enzyme or 400 nM of mutant enzyme in a total volume of 3 μL. After incubation for 1 min at room temperature, polymerase reaction was initiated by the addition of a 3 μL solution containing 1 mM each of the four dNTPs and a 500-fold excess of the nonradioactive heteromeric template-primer as the trap (which quantitatively traps all of the free enzyme or the enzyme dissociated from the template-primer during the reaction). The reaction was allowed to continue at 25 °C, and 2.5 μL sample was aliquoted at desired time points and mixed with 2 μL of Sanger's gel loading solution (Sanger et al., 1977). Samples were kept frozen in dry ice until gel analysis. The reaction products were analyzed on a denaturing 20% polyacrylamide gel containing 7 M urea followed by autoradiography of the gel.

RESULTS

Sequence Alignment To Locate Catalytically Important Amino Acids in MuLV RT. We had earlier identified spatially equivalent residues in the catalytic region of HIV-1 RT and *E. coli* DNA pol I (Yadav et al., 1994). Extension of that analysis to MuLV RT, in spite of the fact that no 3D structure for it was available at that time, seemed possible since direct comparison of selected region of primary sequence blocks could be carried out. Using aspartate triad and YXDD motif as a reference point, we were able to locate a number of conserved residues that would qualify to be catalytically important. In addition, secondary structure prediction of a region containing the suspected residues in MuLV RT and their similarity to the corresponding region in HIV-1 RT have enabled us to predict and tentatively propose the spatially equivalent residues in MuLV RT. Table 1 lists these residues together with their functional implication. Recently, a 3D structure of a fragment of MuLV RT containing the catalytic region has been resolved (Georgiadis et al., 1995); most of our predictions seem valid in the 3D context. In the present work, we are reporting the detailed analysis aimed at resolving the role of one of these residues, namely, Arg 110 of MuLV RT.

Mutagenesis and Purification of Mutant Enzyme. MuLV RT gene construct pET-28a-MRT encoding the wild-type MuLV RT which contains sequences for hexahistidine at the N-terminal region was constructed and used for the mutagenesis studies reported here. The WT clone was constructed by PCR by amplifying the MuLV RT coding region from pB6B15.23 clone encoding *trpE*−MuLV RT fusion protein (Roth et al., 1985). While amplifying the MuLV

Table 1: Conserved and Spatially Equivalent Residues in the Catalytic Domain of Klenow, HIV-1 RT, and MuLV RT[a]

Klenow fragment	HIV-1 RT	MuLV RT	proposed function
D705	D110	D150	metal binding
D710	D113	D153	dNTP binding
D882	D185	D224	α-P coordination
E883	D186	D225	metal binding
H881	M184	V223	fidelity, dNTP binding
N845	Q151	Q190	fildelity
K758	R72	R110	PP_i binding/conformational change
R841	R78	R116	template binding
R754		K103	dNTP binding
P680	P95	Y134	structural integrity
I679	I94	N135	structural integrity

[a] Using 3D structural equivalence, secondary structure prediction schemes, and sequence homology analyses, tentative equivalent residues in the catalytic domain of MuLV RT have been identified. The comparative analysis of the amino acid sequences around the individual residue listed above for HIV-1 RT and Klenow fragment with that of the appropriate primary sequence alignment for MuLV RT (Delarue et al., 1990; Boyer et al., 1992) has permitted the tentative postulation concerning the identity and function for spatially equivalent residues of MuLV RT listed above.

RT coding region, unique *Nde*1 and *Eco*R1 restriction sites were also introduced through upstream and downstream primers, respectively. The amplification was carried out for only 10 cycles in the presence of high concentration of template DNA (1 μg of pB6B15.23) and at low dNTP concentration (30 μM) to achieve high fidelity of amplification. After cloning the PCR product in pET-28a vector to construct the pET-28a-MRT, the 847 bp region (*Kpn*1 and *Sal*1 fragment) encoding polymerase domain was replaced from pB6B15.23 to ensure that the polymerase domain of MuLV RT is free from any other mutation which might have been introduced during the PCR amplification. The same 847 bp fragment was also subcloned in M13mp19 for mutagenesis. The oligonucleotide directed mutagenesis of R110 was carried out on this subclone as described by Kunkel et al. (1987). Three mutations were carried out in which Arg was replaced with Ala (neutral amino acid), with Lys (which displays similar charge as that of Arg), and with negatively charged Glu. Successful mutagenesis was confirmed by DNA sequence analysis of the coding region. The mutagenized 847 bp region was subcloned from M13 clone into its original cassette in pET-28a-MRT.

The recombinant mutant clones were introduced into an expression strain *E. coli* BL-21 (DE3), a λ DE3 lysogen containing T7 RNA polymerase gene under the control of the *lac* UV5 promoter (Rosenberg et al., 1987). The mutant enzymes were induced by IPTG and purified from the cell lysate as described in the Materials and Methods. The purified mutant enzymes were found to be homogeneous with a purity of greater than 95%. The level of their expression, solubility, and chromatographic characteristics were identical with those of the WT enzyme. All the mutants and the wild-type enzymes were found to contain nearly equivalent activity for RNase H (data not shown). The far-UV spectral analysis of the wild-type and the mutant enzyme suggests that there is no change in the 3D structure of the protein (Figure 1).

Characterization of R110A, R110K, and R110E Mutant Enzymes. During the early steps in purification, we noted that all the three mutant enzymes were severely impaired in both RNA dependent and DNA dependent polymerase activities but did retain RNase H activity. Using purified enzymes, we determined the steady state kinetic parameters for all the mutant species and compared these with the wild-type enzyme. As the enzyme utilizes two substrates, i.e., dNTP and template-primer, pseudo-first-order conditions were created for determining constants for one substrate, keeping the second substrate at saturating level. Poly(rA)·$(dT)_{18}$ or poly(dC)·$(dG)_{18}$ were used as template-primers along with dTTP or dGTP as the substrate dNTP. The initial velocity of the reaction was determined by measuring the rates of incorporation of α-^{32}P- or ^{3}H-labeled dNTP into the homopolymeric TP as a function of variable dNTP substrate concentrations. The K_m values for dNTP substrate and V_{max} for polymerase reaction catalyzed by the wild-type MuLV RT and the mutant enzymes were determined by Eadie−Hofstee plots of the initial velocity data (Figure 2). The results summarized in Table 2 show that the replacement of Arg with either Ala (R110A) or Lys (R110K) causes approximately 1000-fold reduction in k_{cat} when poly(rA)·$(dT)_{18}$ was used as TP. The enzyme was found to be nearly inactive when positively charged side chain of Arg was replaced with negatively charged side chain of Glu. Most interestingly, the affinity for dNTP substrate was only marginally reduced as only 2−3-fold increase in the $K_{m(dNTP)}$ was observed. Similar results were also obtained with poly-(dC)·$(dG)_{18}$ template-primer (Table 2).

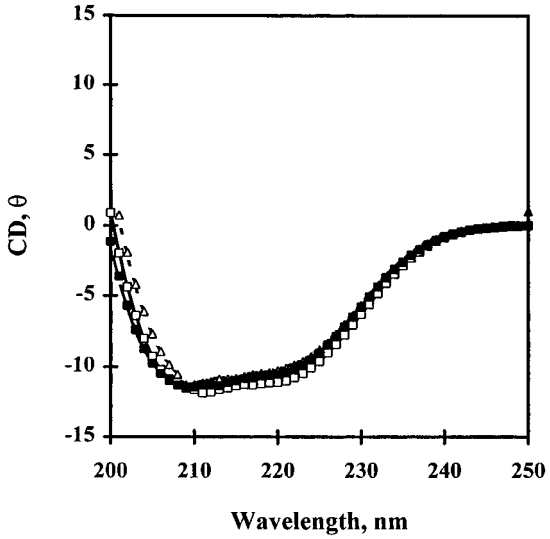

FIGURE 1: Far-UV circular dichroism spectra of MuLV RT. The CD spectra of wild-type (□), R110A (△), and R110E (■) were recorded on an Aviv 62DS spectropolarimeter at 0 °C coupled with a microprocessor and a temperature control device (±1 °C). The data were corrected for buffer background using the software provided by Aviv Associates (Lakewood, NJ). All the spectra were recorded in 20 mM Hepes buffer containing 100 mM NaCl with a path length of 0.1 cm. The final concentration of protein samples was 0.1 μg/μL. Each spectrum shown in this Figure is the average of three replicates.

Effect of R110 Mutation on the Formation of Enzyme−Template-Primer Binary Complex. The drastic reduction in the k_{cat} for polymerase reaction of all the three mutant enzymes, without significant 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 with individual mutant enzyme was assessed by their photochemical cross-linking with RNA-DNA and DNA-DNA template-primers. We used poly(rA)·

Table 2: Kinetic Parameters of the WT MuLV RT and Its Mutant Derivatives[a]

	poly(rA)·$(dT)_{18}$/TTP			poly(dC)·$(dG)_{18}$/dGTP		
enzyme	$K_{m(TTP)}$ (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})	$K_{m(dGTP)}$ (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
WT	15.2	3.3	2.16×10^5	14.6	2.9	1.9×10^5
R110A	49.3	3.5×10^{-3}	71	43.2	2.5×10^{-3}	58
R110K	46.0	6.8×10^{-3}	147	48.42	5.7×10^{-3}	117
R110E	52.9	9.9×10^{-4}	18	ND	ND	ND

[a] The steady state kinetic parameters for the WT MuLV RT and mutant derivatives of R110 were measured with the indicated template-primers and corresponding dNTP substrate as described in the Materials and Methods.

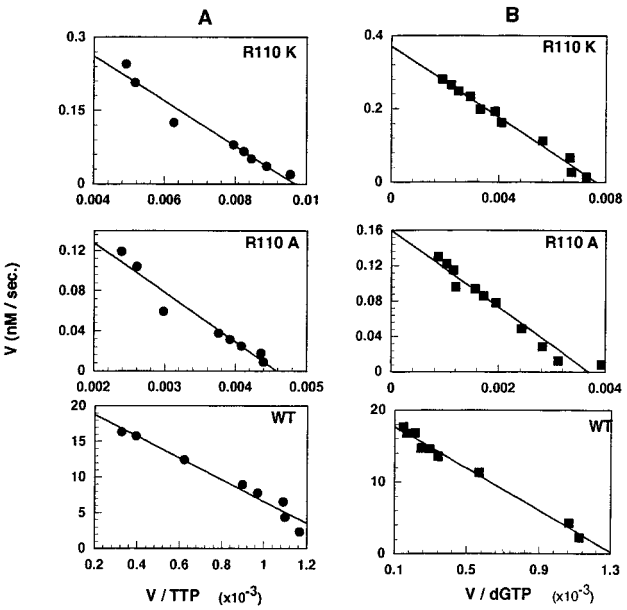

FIGURE 2: Eadie−Hofstee plots of steady state kinetic data for wild-type MuLV RT and its mutant derivatives. The measurement of initial velocity (V) of the reaction was carried out using poly(rA)·$(dT)_{18}$ (panel A) or poly(dC)·$(dG)_{18}$ (panel B) as the template-primer and varying concentrations of the respective dNTP (TTP or dGTP) substrates as described in Materials and Methods. Each point is an average of two sets of experiment.

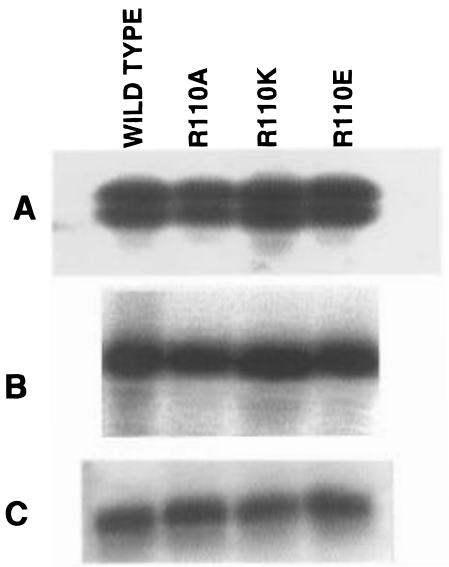

FIGURE 3: Effect of R → A, R → ,K and R → E substitution at the 110 position of MuLV RT on the formation of the E-TP complex. 1.2 μM of enzyme protein incubated with 50 nM of 5′-^{32}P-labeled template-primer was cross-linked in the UV Spectrolinker and subjected to SDS−PAGE and autoradiography as described in the text. The extent of E-TP covalent complex was estimated either by Cerenkov cpm of excised radioactive gel piece or by densitometric scanning of the autoradiogram. The E-TP complexes shown in various panels are as follows: (A) 5′-^{32}P-37 mer self-annealing template-primer; (B) poly(rA)·(5′-^{32}P-$dT)_{18}$; (C) (5′-^{32}P-$dA)_{26}$/$(dT)_{18}$.

$(dT)_{18}$, $(dT)_{26}$·$(dA)_{18}$, and a self-annealing 37 mer oligomeric DNA as the template-primers in the cross-linking studies. The oligomeric $(dT)_{18}$ primer, $(dT)_{26}$ template, and 37 mer self-annealing template-primer were prelabeled with ^{32}P at the 5′ position, which permitted detection of covalently cross-linked E-TP complexes on SDS−polyacrylamide gels. The concentration of TP used in these studies was subsaturating, which was expected to reveal changes in the DNA binding affinity of mutant enzymes, if any. Results show that the extent of cross-linking of all the mutant enzymes and that of the wild-type enzyme to various TPs are quite similar, implying mutation at R110 does not change the binding affinity for both DNA-DNA and RNA-DNA template-primers (Figure 3 A−C). Furthermore, even under saturating concentrations of TP, both the wild-type and the mutant enzymes exhibited similar extents of cross-linking (data not shown). The specificity of the cross-linking of the template-primers to the mutants and the wild-type enzymes was further 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 preincubation 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 various TPs was specific (data not shown).

Catalytic Competence of Enzyme−DNA Covalent Complexes. Since R110 mutants exhibited no significant change in their affinity for dNTP substrate as well as for RNA-DNA and DNA-DNA template-primers, the possible catalytic defect in these mutant enzymes was thought to be at the chemical step involving phosphodiester bond formation. This assumption seemed consistent with the extremely low k_{cat} observed for these mutants with no apparent defect in the dNTP and template-primer binding. In order to obtain some evidence in support of this notion, we used the E-TP covalent complex as the source of enzyme and determined the ability of this complex to catalyze the addition of dNTP onto the immobilized template-primer. Previously, we had shown that the wild-type Klenow fragment as well as wild-type HIV-1 RT covalently cross-linked with template-primer can catalyze an addition of single nucleotide onto the 3′-OH terminus of the immobilized template-primer (Pandey et al., 1994b, 1996; Sarafianos et al., 1995a,b). Since all three mutants of R110 showed similar affinity for both dNTP and template-primers, the ability of their E-TP covalent complexes to form the productive ternary complex would indicate if the catalytic step was indeed affected. Results depicted in Figure 4A,B show that the enzyme template-primer covalent complexes of R110 mutant enzymes are inefficient in catalyzing

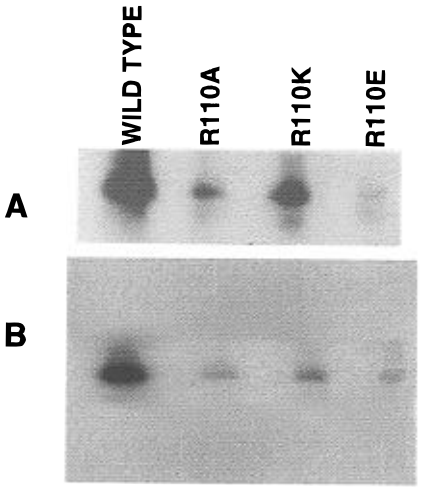

FIGURE 4: Catalytic competence of E−(template-primer) covalent complexes of R110 mutant enzymes. The WT MuLV RT and its R110 mutant derivatives were cross-linked with (A) unlabeled 37 meric self-annealing template-primer or (B) poly(rA)·$(dT)_{18}$ as described in the Materials and Methods. The irradiated mixture containing E-TP covalent complex was further incubated in 50 μL of reaction mixture containing 50 mM Tris-HCl, pH 7.8, 1 mM DTT, 500 mM NaCl, and 10 μCi of [α-^{32}P]dTTP. The presence of 0.5 M NaCl in the reaction mixture does not affect the catalytic competence of E-TP complexes (Pandey et al.. 1994a). The incubations were carried out at room temperature for 30 min, and the extent product synthesis was determined by SDS−polyacrylamide gel electrophoresis followed by autoradiography as described before.

nucleotide addition on both the immobilized template-primers with the exception of E-TP complex of R110K which appeared moderately active in catalyzing the addition of a nucleotide particularly with DNA-DNA template-primer. The fact that the k_{cat} of all mutants for polymerase reaction under processive condition is reduced by approximately 1000-fold, and yet the addition of the nucleotide (even though it is restricted to the first nucleotide) onto the covalently linked primer terminus is reduced only to a smaller extent, suggests that R110 may or may not be directly involved in the chemical step of the reaction.

Comparative Utilization of dTTP and Its α*-Thio Analog by the Mutant Enzymes*. Differential utilization of deoxynucleotide substrates with modification at the α-phosphate position has been used to assess the participation of a particular amino acid in the chemical step of the polymerase reaction (Polesky et al., 1992; Pelletier et al., 1994; Kaushik et al. 1996a). A phosphorothioate analog of dNTP readily provides such a reagent, and its utilization in the polymerase reaction has been termed as the sulfur elemental effect. Thus, a significant difference in the utilization of normal versus phosphorothioate analog of dNTP by the mutant enzymes would imply reactivity of R110 with α-phosphate of dNTP. We, therefore, examined the ability of R110A and R110K mutant enzymes to catalyze the incorporation of dTMP versus Sα-dTMP into poly(rA)·$(dT)_{18}$. Figure 5 shows the products formed with dTTP and Sα-dTTP as a function of time with MuLV RT mutant derivatives. As seen from the Figure, no sulfur elemental effect was evident with both R110A and R110K mutant enzymes, as the extent of addition of dTTP and dTTPαS remained the same, implying that the chemical step per se is not affected by this mutation.

Mode of DNA Synthesis of Mutant Enzymes. Extremely low k_{cat} of R110 mutants may result from the defect in the

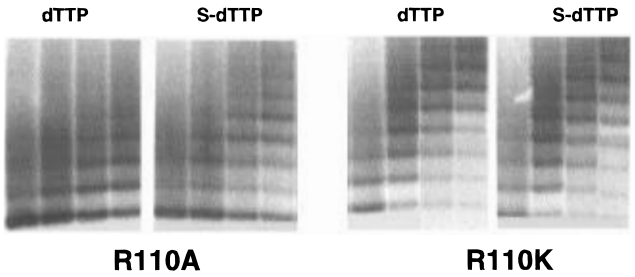

FIGURE 5: Incorporation of dNTP and dNTPαS on DNA-DNA template-primer by R110 mutant derivatives of MuLV RT. 200 nM of individual mutant derivatives was incubated with 2.5 nM poly(rA)·$(dT)_{18}$ template-primer in the presence of 200 μM dTTP or dTTPαS as described in the Materials and Methods. Aliquots were withdrawn at indicated time points and subjected to denaturing polyacrylamide−urea gel for product analysis. The left panels show the incorporation of dTTP while the incorporation of dTTPαS is shown in the right panels.

translocation to the next template base following the incorporation of the nucleotide. This would cause prolonged pausing of the mutant enzyme following each cycle of catalysis. To examine this possibility, an experiment was carried out to determine processivity of DNA synthesis by the wild-type enzyme and its mutant derivatives. The processivity is the probability of translocation of the polymerase enzyme along the template and represents the average number of cycles of nucleotide addition during a single enzyme DNA encounter. The template-primer used in the determination of processive mode of synthesis was a 496 base long heteromeric RNA template annealed with 5′-^{32}P-labeled 18 mer DNA primer. Wild-type MuLV RT and its mutant derivatives were incubated with the labeled template-primer to allow the formation of enzyme−TP complex. The polymerization reaction was initiated by the addition of all four dNTPs and 500-fold excess of the nonradioactive heteromeric RNA-DNA template-primer as the trap for free enzyme as well as enzyme dissociated from the labeled TP. The presence of the trap at the binding step effectively masks the processive reaction by trapping all the free enzyme molecules (Figure 6, lane B). In the absence of trap, the labeled product ranging from 19 mer to 90 mer resulting from both processive and nonprocessive synthesis could be seen (Figure 6, lane A).

As seen in Figure 6, the wild-type MuLV RT is highly processive as 18 mer primer is effectively extended, yielding labeled products ranging from 19−90 mer within 60 s of incubation at room temperature (Figure 6, lanes 1−3, WT). The intensity of each labeled product remained the same even upon 10 min incubation, suggesting that most of the enzyme molecules, after completing the synthesis ranging from 1 to 72 nucleotide incorporation per processive cycle, have dissociated from the labeled TP and are effectively tied to the trap. In contrast, all the mutant derivatives of R110 appeared to incorporate just one nucleotide onto the 18 mer primer, resulting in the accumulation of 19 mer product for up to 10 min (Figure 6, lanes 1−3, R110A, R110K). These results suggest that the mutant derivatives of R110 are unable to carry out processive synthesis of DNA and have become distributive. Alternatively, mutant enzymes may be defective in translocation and may therefore remain bound to the template-primer after the addition of the first nucleotide.

Time Course of Incorporation of dNTP on Heteromeric RNA-DNA Template-Primer. In order to further probe the

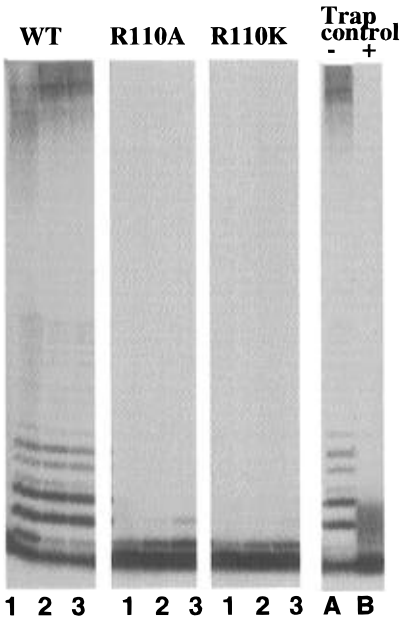

FIGURE 6: Effect of R110A and R110K mutations on the mode of DNA synthesis on heteromeric RNA-DNA template-primer. The 5′-^{32}P-labeled 18 mer DNA primer annealed with the 496 base long heteromeric RNA template containing the PBS region of HIV genome was used as the template-primer. Following incubation of the individual enzyme with the labeled TP, the reaction was initiated by the addition of 1 mM each of the four dNTPs and excess of the heteromeric RNA-DNA as the trap (see Materials and Methods). Each set represents experiments with the wild-type, R110A, or R110K mutant enzyme. In lanes 1, 2, and 3 of each set, the trap was added along with the dNTP substrates, and the product length generated represents a single processive synthesis event during 1, 5, and 10 min of the incubation, respectively. Lanes A and B show the effectiveness of the trap. Lane A represents DNA synthesis catalyzed by WT enzyme on the labeled template-primer for 10 min in the absence of trap. In lane B, the heteromeric RNA-DNA trap was added to the labeled template-primer before the binding step.

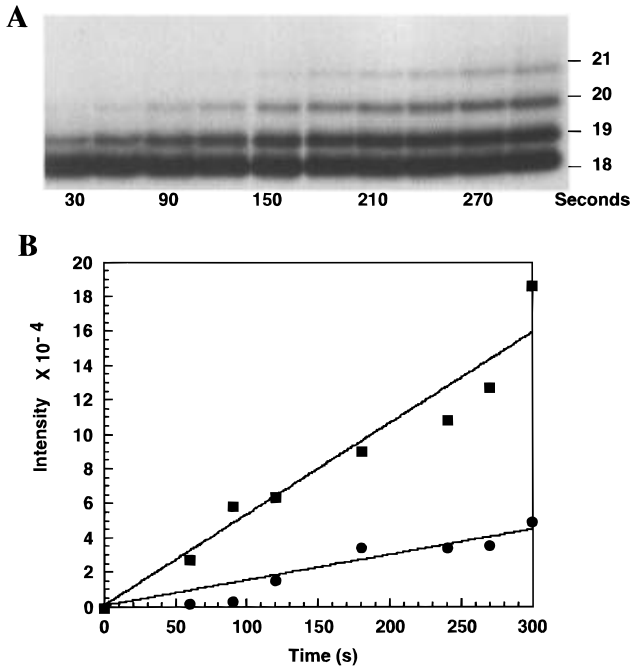

FIGURE 7: Time course of incorporation of dNTP with heteromeric RNA-DNA template-primer by R110A mutant. (A) The 5′-^{32}P-labeled 18 mer PBS primer annealed with 496 base RNA template was used to assess the time course of extension of 18 mer to 19 mer and 20 mer by R110A mutant enzyme. The mutant enzyme (200 nM) was incubated with 2.5 nM of the labeled template-primer in the presence of 1 mM of each of 4 dNTPs as described in the Materials and Methods. Aliquots were withdrawn at the indicated time points, and the reaction products were analyzed on denaturing 16% polyacrylamide−urea gel followed by autoradiography on Kodak X-ray film. (B) The amount of starting labeled 18 mer primer extended to 19 mer and above as seen in panel 7 A was quantitated by phosphoimager and plotted as a function of time.

defective stage in the catalytic abilities of the mutant enzymes, we carried out product analysis to identify whether the rate limiting step of the reaction with these mutant enzymes precedes or follows the bond formation. Using the RNA-DNA template-primer and excess of the mutant enzyme, a time course of nucleotide incorporation was performed and the reaction products were analyzed on polyacrylamide−urea gel. We expected that the estimation of time required to catalyze addition of first and second nucleotide would indicate if the rate limiting step for these mutants is before or after the bond formation. It was reasoned that if the rates of addition of the first and the second nucleotide differ significantly, then the rate limiting step should be following the bond formation. Figure 7A shows the time course of reaction products of R110A mutant enzyme. As can be seen from the figure, the extension of 18 mer primer to 19 mer and that of 19 meric product to 20 mer is linear with time. However, the rates of extension to 20 mer product are significantly reduced (Figure 7B). Similar results were obtained for R110K and R110E mutant enzymes (data not shown), which suggests that the major rate limiting steps with these mutant enzymes are before as well as after the point of phosphodiester bond formation.

Pyrophosphorolysis Activity of Mutant Derivatives of Arg 110. The pyrophosphorolysis activity may be considered as reversal of polymerase reaction and therefore would require the participation of the residues at the same step of (forward or reverse) reaction. 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 mutant derivatives of residue R110 was severely impaired, the effect of these mutations on the reverse reaction (pyrophosphorolysis) was assessed to confirm the suspected participation of this residue in PP_i binding or stabilizing the transition state complex of the reverse reaction. It was expected that the residue involved in the conformation change of E-TP-dNTP ternary complex to transition state complex in the forward polymerase reaction should also be involved in a similar step in the reverse (pyrophosphorolysis) reaction. With this premise, we expected that mutants of residue R110, exhibiting no change in the affinity for dNTP and template-primer substrates, should be defective in catalyzing the reverse reaction if R110 has a role in conformational change steps in both directions. We used 5′-^{32}P-heteromeric 18 mer DNA primer annealed with a 496 base long heteromeric RNA template to assess the degradation of 5′-^{32}P-18 mer by these mutants in the presence of PP_i. The results depicted in Figure 8 clearly show that all the three mutants of R110 are defective in pyrophosphorolysis activity as judged by the rather limited extent of pyrophosphorolysis reaction. In contrast, wild-type enzyme is capable of continued pyrophosphorolysis as evidenced by the production of 17, 16, 15, and 14 mer and lower products. Similar results were also obtained with R72A mutant of HIV-1 RT and K758A of Klenow fragment (Pandey et al., 1994a; Sarafianos et al., 1995b; Kaushik et

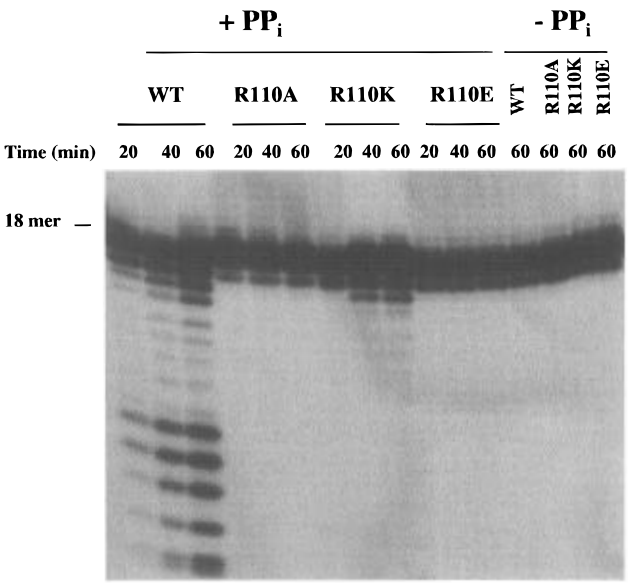

FIGURE 8: Pyrophosphorolysis activity of wild-type MuLV RT and its mutant derivatives. Heteromeric RNA-DNA was used as the template-primer for this experiment. The 5'-^{32}P-labeled 18 meric PBS primer annealed with the 496 base long heteromeric RNA template was incubated with 0.1 μM WT enzyme or 1 μM mutant enzymes in the presence or absence of PP_i under standard conditions as described in the Materials and Methods. Reactions were carried out for indicated time points at 25 °C, and the products were analyzed on 16% polyacrylamide−urea denaturing gel.

al., 1996b), suggesting a similar role for residue R110 in the polymerase function of MuLV RT.

DISCUSSION

The availability of the crystal structure HIV-1 RT has stimulated interest in structure/activity analyses of reverse transcriptases, and particularly the mechanism of drug resistance through the mutations. The knowledge available about the Klenow fragment and striking similarities of its 3D structure with HIV-1 RT has enabled this laboratory to locate some of the highly conserved residues in the catalytic domain of HIV-1 RT which are at spatially equivalent positions in the Klenow fragment (Yadav et al., 1994). Using 3D structural equivalence, secondary structure prediction schemes, and sequence homology analyses, we have tentatively identified equivalent residues in the catalytic domain of MuLV RT (Table 1). Thus, Arg 110 of MuLV RT was proposed to be analogous to Arg 72 of HIV-1 RT and Lys 758 of *E. coli* DNA polymerase I, which have been shown to be involved in the PP_i binding/removal function and possibly in crucial conformational change steps preceding and following phosphodiester bond formation in the catalysis of DNA synthesis (Sarafianos et al., 1995b; Kaushik et al., 1996a). To obtain evidence in support of the proposed functional role for Arg 110 and to probe the nature of its participation in the catalytic process, we carried out site directed mutagenesis at the 110 position. Three different amino acid substitutions were introduced; in the first, the positively charged Arg was replaced with uncharged alanine (R110A), the second was replaced with Lys which has similar charge (R110K), and the third replacement was with opposite charge residue (R110E). Biochemical analysis of these mutant enzymes indicated that most likely R110 is not required in the binding of either the template or the primer strand of DNA-DNA and RNA-DNA template-primers since all the three mutant enzymes exhibited near-identical binding affinity for both the template and primer strands as compared to the WT enzyme. Yet their k_{cat} was reduced by approximately 1000-fold. Such drastic reduction in k_{cat} is expected from mutants of those residue which are directly involved in a crucial step in the reaction or those which are essential for stabilizing the structural integrity of the enzyme. The latter possibility was ruled out by examining the patterns of heat inactivation, gel mobility, and the CD spectrum of WT enzyme and its mutant derivatives (Figure 1). All the parameters examined were nearly identical to the WT enzyme, suggesting no influence on the folding pattern and structural integrity of the enzyme by these mutations. Involvement of Arg 110 in the dNTP binding function was probed by measurement of K_m for dNTP using poly(rA)·$(dT)_{18}$ and poly(dC)·$(dG)_{18}$ as the template-primers (Figure 2). Results shown in Table 2 indicated only a moderate 2−3-fold increase in K_m for dTTP, suggesting no significant change in the affinity for the dNTP substrate. A defect in the binding of the template-primer by the mutant enzymes was also ruled out by assessing the extent of photoaffinity labeling of wild-type and mutant enzymes with various RNA-DNA and DNA-DNA template-primers (Figure 3). A qualitative indication of a defect in the phosphodiester bond formation step was indicated by limited ability of E-TP covalent complexes to catalyze the addition of a single nucleotide onto the immobilized primer (Figure 4). However, the participation of R110 in the chemical step was subsequently ruled out based on the fact that mutant derivatives of R110 do not exhibit any sulfur elemental effect as judged by the rate of primer extension with dNTPαS (Figure 5). This is consistent with other DNA polymerases where the sulfur elemental effect was seen only with mutants of those carboxylate residues which have been implicated in the chemical step, by virtue of their ability to effectively coordinate the divalent metal ions with dNTP and DNA substrates (Polesky et al., 1992; Pelletier et al., 1994; Kaushik et al., 1996a). Therefore, the possible defect observed with mutants of R110 appeared to be at a step where the conformational change of the ternary complex (E-DNA-dNTP) to a transition state complex (E*-DNA-dNTP) is necessary for the catalysis. Drastic reduction in k_{cat} without significant change in the affinity for dNTP and DNA and lack of sulfur elemental effect support this presumption. Processivity analysis indicated that the mutant enzymes have lost the ability to processively synthesize DNA (Figure 6). Time course experiments further suggest that the additions of the first and second nucleotide under nonprocessive conditions exhibit different rates of polymerization (Figure 7A,B). The second nucleotide addition is decreased by at least 3−5-fold. This decrease is in addition to the severe reduction seen in the first nucleotide. The delay in the addition of second nucleotide is indicative of a defect in release of the enzyme from the template-primer after the chemical step. Such a defect conceivably results in delay of the release of PP_i concomitant with translocation and release of enzyme from the template-primer. This is consistent with the proposed second conformational change of $E^*{\cdot}DNA_{n+1}{\cdot}PP_i$ to $E{\cdot}DNA_{n+1}.PP_i$ (Dahlberg & Benkovick, 1991). The significant loss of pyrophosphorolysis activity of mutant derivatives of R110 supports the above interpretation. Similar observations have been made with R72 of HIV-1 RT (Sarafianos et al., 1995b) and Lys 758 of Klenow

fragment (Kaushik et al., 1996b), which have been postulated as functionally analogous to R110 of MuLV RT (Table 1). It is also interesting to note that among the 3 mutants, Arg to Lys substitution shows some activity in both polymerase and pyrophosphorolysis reactions, whereas Arg to Glu substitution shows total lack of it, implying that the positive charge of guanidino group of Arg plays a minor role and that geometry of this group is of prime importance in the catalytic reaction.

Recently, a high resolution crystal structure of 32 kDa fragment of MuLV RT representing palm and finger subdomain of the enzyme has been reported (Georgiadis et al., 1995). This N-terminal fragment spanning residues 10−278 was derived from a limited proteolytic cleavage of MuLV RT of 74.8 kDa molecular mass. In this structure, Arg 110 is located on β5 sheet in the finger subdomain, and its side chain is oriented toward the cleft. The location of β4 and β5 sheets in the structure seems to be at a strategic position which may be able to influence binding of both dNTP and DNA in the cleft. Earlier, Lys 103 in the β4-β5 loop region of MuLV RT was identified as the reactive site for pyridoxal phosphate (PLP) and was concluded as an essential residue for dNTP binding function (Basu, A., et al., 1988). Subsequent mutagenesis studies have also established that substitution of Lys → Leu at position 103 results in the severe loss of polymerase function of the enzyme (Basu et al., 1990).

Georgiadis et al. (1995) have modeled a pre-polymerase ternary complex of MuLV RT consisting of enzyme-DNA-dNTP, and in this model Arg 110 has been proposed to interact with the sugar-phosphate backbone of the template strand of the modeled DNA. Our studies, as discussed above, suggest that the postulated role of Arg 110 based on the modeled ternary complex may be incorrect. The role of R110 in MuLV RT seems to be similar to R72 in HIV-1 RT and K758 in Klenow fragment, which we had projected as spatially equivalent residues (Table 1). Thus at least one positively charged residue in different polymerases appears to be involved in the conformational change step before and after the bond formation (Sarafianos et al., 1995b; Kaushik et al., 1996b). Our preliminary studies on some other residues in MuLV RT, based on the functional role of the analogous residue in HIV-1 RT, have also indicated their functional similarities. For example, substitution of Val → Met or Val → Ala at the 223 position in the YVDD motif of MuLV RT considerably reduces the fidelity of the mutant enzyme (Chowdhury et al., unpublished results), which is consistent with the results obtained with HIV-1 RT where substitution of Met → Val at the 184 position in the YMDD motif enhances the fidelity of the enzyme (Pandey et al., 1996). In contrast, wild-type MuLV RT (Val 223) is more sensitive to both ddA and ddG as compared to its V223M mutant derivative (Chowdhury et al., unpublished results), while the M184V mutation in HIV-1 RT results in a high level of resistance to both the drugs (Gu et al., 1992). The drug resistance/sensitivity properties of the two enzymes indicate that, in spite of catalytic similarities, the mechanism of drug resistance seen in HIV-1 RT may have additional effectors. It is quite likely that the subtle differences in the catalytic apparatus resulting from the dimeric nature of HIV-1 RT may be responsible for the drug resistance. Indeed a careful structural examination of the subunits of HIV-1 RT has shown that, in spite of the distinct folding pattern of individual subunit, the so-called catalytically inert p51 subunit contains an intact dNTP binding pocket (Yadav et al., 1995). A report on possible dimer formation by MuLV RT has also appeared (Telesnitsky and Goff, 1993). However, independent confirmation of that report is still lacking. Our own efforts to demonstrate dimerization of MuLV RT monomers, during RNA or DNA directed DNA synthesis, have not been successful (to be published elsewhere). In summary, in spite of many similarities in the catalytic domain, each polymerase class is likely to have some unique feature which may result in conferring some unique property. In the present study, we have identified and clarified the catalytic role of Arg 110 in the reverse transcriptase class belonging to mammalian type C retrovirus and implicated this residue in the catalytic process.

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